GPVI and CLEC-2 Regulation and Signal Transduction: From Development Through to Adulthood

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Abstract

The platelet collagen receptor, GPVI, and podoplanin receptor, CLEC-2, have been shown to share a common tyrosine-kinase linked signalling pathway. GPVI contains a full ITAM ($YxxL_{n6-12}YxxL$) within its intracellular tail, whilst CLEC-2 contains a hemITAM (YXXL). Both receptors rely on common, key signalling proteins including Src, Syk, LAT, Btk and PLCγ2, however the biological implications of GPVI and CLEC-2 stimulation are significantly different; GPVI is implicated in haemostasis and thrombosis responses whilst CLEC-2 appears to be involved in the newer roles of platelets, such as their roles in infection, immunity, and development. It has also been shown that the organisation and importance of the signalling proteins in the pathway differ between GPVI and CLEC-2. The aims of this thesis are to explore the regulation and function of the tyrosine kinase-linked signalling pathway downstream of the two (hem)ITAM receptors in response to different stimuli at different stages of development.

In this thesis, I show that both GPVI and CLEC-2 require an intact, functional Syk kinase domain of Syk to support signalling, regardless of the maintained phosphorylation of certain adapter docking sites. I also show that both GPVI and CLEC-2 are hyporeactive and expressed at lower levels on the platelet surface throughout development, and that these defects appear to be tyrosine kinase-linked pathway specific. Lastly, using samples from homozygous GPVI deficient patients, I have shown that both fibrin and fibrinogen appear to be agonists for GPVI. Overall, I have shown that are likely subtle differences in the regulation and downstream

signalling of GPVI and CLEC-2 throughout development and to several different stimuli – including the novel agonists fibrin and fibrinogen.

Publications arising from this thesis

Journal Articles

Hardy AT, Palma-Barqueros V, Watson SK, Malcor JD, Eble JA, Gardiner EE, et al. Significant Hypo-Responsiveness to GPVI and CLEC-2 Agonists in Pre-Term and Full-Term Neonatal Platelets and following Immune Thrombocytopenia. Thromb Haemost. 2018;118(6):1009-20.

Onselaer MB, **Hardy AT**, Wilson C, Sanchez X, Babar AK, Miller JLC, et al. Fibrin and D-dimer bind to monomeric GPVI. Blood Adv. 2017;1(19):1495-504.

Mangin PH, Onselaer MB, Receveur N, Le Lay N, **Hardy AT**, Wilson C, et al. Immobilized fibrinogen activates human platelets through glycoprotein VI. Haematologica. 2018;103(5):898-907

Book Chapters

Rayes J, **Hardy AT**, Lombard SE, Montague SJ, Watson SP, Lowe KL. The Role of CLEC-2 in and Beyond the Vasculature. 2017:129-38.

In dedication to the ever-loving memory of

JOHN HENRY GEORGE ARTHUR HARDY

who always encouraged my pursuit of knowledge

and is dearly missed every single day

and in dedication to

LAYLA DEBORAH ELISE HARDY

for showing me the meaning of unconditional love

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Abbreviations

AA	Arachidonic Acid
ACD	Acid Citrate Dextrose
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
APC	Allophycocyanin
ATP	Adenosine Triphosphate
bFGF	Basic Fibroblast Growth Factor
BSA	Bovine Serum Albumin
Btk	Bruton's Tyrosine Kinase
CALDAG-GEFI	Calcium and Diacylglycerol-regulated Guanine Nucelotide Exchange Factor I
cAMP	Cyclic Adenosine Monophosphate
CD41	Cluster of Differentiation 40
cGMP	Cyclic Gunaosine Monophosphate
CLEC-2	C-Type Lectin-Like Receptor 2
COX	Cyclooxygenase
CRP	Collagen Related Peptide
CTC	Circulating Tumour Cell
DAG	Diacylglycerol
DC	Dendritic Cells
DVT	Deep Vein Thrombosis
ECL	Enchanced Chemiluminescence
FcRy	Fc Receptor Gamma Chain

FITC	Fluroescein
Fxa	Factor 10a
GDP	Guanosine Diphosphate
GEF	Guanine Nucelotide Exchange Factor
GPCR	G Protein Coupled Receptor
GPVI	Glycoprotein 6
GTP	Guanosine Triphosphate
hemITAM	Hemi Immunoreceptor Tyrosine-Based Activation Motif
IgG	Immunoglobulin G
IL	Interleukin
IP	Prostacyclin Receptor
IP3	Inositol Triphosphate
IP3R	Inositol Triphosphate Receptor
ITAM	Immunoreceptor Tyrosine-Based Activation Motif
ITP	Immune Thrombocytopenic Purpura
KO	Knock Out
LAT	Linker of Activated T-Cells
LEC	Lympathic Endothelial Cell
mAb	Monoclonal Antibody
MI	Myocardial Infarction
MK	Megakaryocyte
Mpl	Myeloproliferative Leukemia Protein
MPV	Mean Platelet Volume
NK	Natural Killer

NO	Nitric Oxide
P2Y	Purinergic Receptor
PAR	Protease-Activated Receptor
PE	Pulmonary Embolism
PE	Phycoerythrin
PEG	Polyethylene Glycol
PFA	Paraformaldeyde
PGI2	Prostaglandin I2
PH	Pleckstrin Homology
PI3K	Phosphoinositide 3-Kinase
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
PLA	Phospholipase A
PLC	Phospholipase C
PLCβ	Phospholipase C beta
PLCγ2	Phospholipase C gamma 2
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PVDF	Polyvinylidene Diflouride
RA	Rheumatoid Arthritis
RBC	Red Blood Cell
sCD40L	Soluble Cluster of Differentiation 40 Ligand
SD	Standard Deviation

SDS	Sodium Dodecyl Sulphate
SEM	Sub-Endothelial Matric
SEM	Standard Error of the Mean
SEM	Scanning Electron Microscopy
SFK	Src Family Kinase
SH2	Src Homology 2
SH3	Src Homology 3
SLE	Systemic Lupus Erythematosus
Syk	Spleen Tyrosine Kinase
TF	Tissue Factor
TMD	Transmembrane Domain
TNF	Tumour Necrosis Factor
TP	Thromboxane A2 Receptor
tPA	Tissue Plasminogen Activator
TPO	Thrombopoietin
TTP	Thrombotic Thrombocytopenic Purpura
Tx	Thromboxane
TxA2	Thromboxane A2
VEGF	Vascular Endothelial Growth Factor
vWF	Von Willebrand Factor
WBC	White Blood Cell
WT	Wild Type
ZAP-70	Zeta Chain Associated Protein Kinase 70

Chapter 1

General Introduction

1.1 Introduction to platelets

1.1.1 A brief history of platelets

Platelets are anucleate cells of the haematopoietic system, the first functional assessment and description of which is attributed to the Italian physician Giulio Bizzozero in the early 1880's (14). Platelets are around three micrometres in diameter, have volumes of around ten femtolitres and have been found to circulate at concentrations of between 150-400,000 platelets per microlitre of blood; this small size and high abundancy originally led many to disregard platelets as unimportant 'cellular dust', or even to confuse them with fibrin clots or degenerated leukocytes (15, 16). However once Bizzozero performed his pioneering experiments – applying light pressure with a needle to arteries in the mesentery of anaesthetised animals – he observed platelets tethering at the site of damage. Initially only a few platelets arrived, however before long hundreds of platelets had aggregated and formed a thrombus which impaired the blood flow through the section of artery. This was the first time that this had been observed, and the first description of platelets playing an integral role in haemostasis (14, 16).

Platelets were originally thought of solely as regulators of haemostasis and thrombosis for many years, and much of the initial research performed in the field focussed on this important function (17). However, Bizzozero himself hypothesised that cells with such a high blood concentration and ubiquitous expression must be involved not just in haemostasis and pathological thrombotic processes, but also in other physiological processes (14). Indeed, recent studies have shown that platelets play roles in

maintaining vascular integrity as well as in a number of developmental processes (18-23); there is also evidence that platelets may play roles in a much wider range of pathophysiological processes such as inflammation, infection and immunity, and cancer metastasis (24-30). As platelets are second only to red blood cells in abundance, these newly uncovered roles may be extremely important in the pathophysiology of many diseases both inside and out of the cardiovascular system, just as Bizzozero hypothesised nearly 150 years ago.

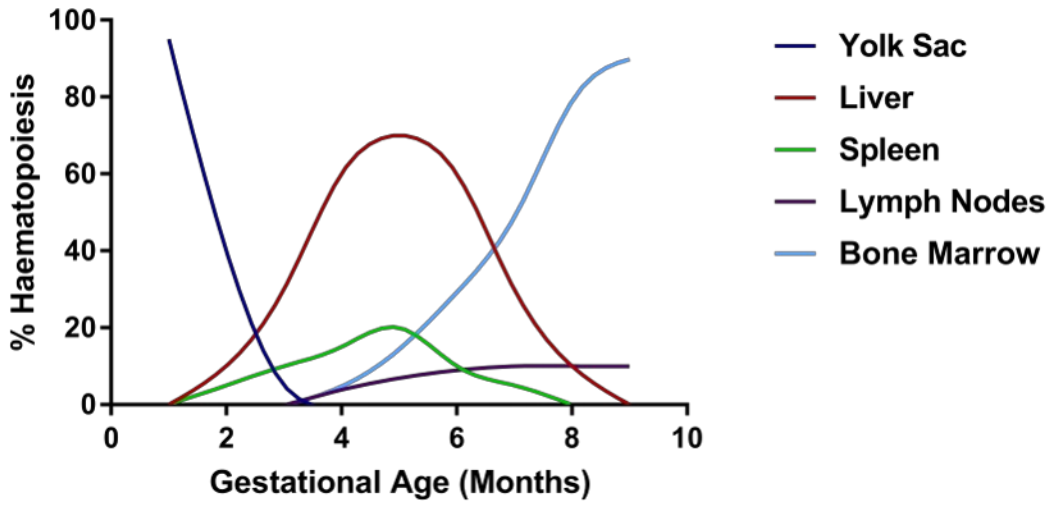
1.1.2 Platelet production

In adults, platelets are produced from the cytoplasm of megakaryocytes (MKs) contained within the bone marrow (31, 32). MKs are polyploid cells around 50-100μm in size, around thirty times the size of the platelets that they produce, and are extremely rare; indeed most sources agree that they comprise less than 1% of all nucleated cells contained within the bone marrow (15, 33). As platelets have a lifespan of only around ten days, and during haemostatic challenge large numbers of platelets are consumed, MKs are required to be able to produce large numbers of platelets in extremely short periods of time (34). To produce platelets MKs respond to a plethora of stimuli, including thrombopoietin and sphingosine-1-phosphate, to initiate a process of megakaryocyte development and differentiation, subsequently resulting in a process of proplatelet formation, extension, release and eventual platelet release (15, 35, 36). During proplatelet formation, MKs form a number of elongated cytoplasm-containing membrane protrusions which migrate from the bone marrow into the lumen of nearby blood vessels (15). Once in the lumen, mature platelets begin budding from the tips of the proplatelet protrusions and enter the circulatory system (15). Each MK can form

between ten and twenty of these proplatelet protrusions, and thus each individual MK is capable of producing around 10^3 platelets (37).

Interestingly, during embryogenesis and neonatal development, the sites and processes of thrombopoiesis are vastly different to those observed in adults (2-4). Initially platelet production occurs in the yolk sac of developing embryos, before moving to the liver and eventually to the bone marrow and spleen, which are the sites associated with adult thrombopoiesis; figure 1.1 details the timescales of thrombopoiesis migration throughout development (2-5). In mice, it has been shown that platelets can be first detected in the peripheral blood from embryonic (E) day 9.5 and their numbers expand rapidly by E10.5 (5). Remarkably, a previously uncharacterised population of cells present in the yolk sac around these time points were discovered to share many MK-associated proteins, such as the thrombopoietin receptor Mpl, and were able to produce proplatelets (38). However, this newly discovered cell population appeared to be predominately diploid, compared with the polyploid MK cells found in adult bone marrow (38). This raises the possibility that platelet production may be inherently different during development, and also that the platelets produced by these MK-like cells in the yolk sac may differ from those derived from bone marrow MKs, as present in adults. It is also possible that platelet production at the other stages of development may be subtly different from that occurring in adults, and this may play a role in the previously observed altered functionality of embryonic and neonatal platelets (39-45). This is interesting to consider, particularly in light of more recent evidence which challenges the long-held belief that the bone marrow is responsible for the majority of platelet production in adults; for example the recent

paper by Lefrancais *et al*, building on previous work such as the study of Zucker-Franklin and Phillip, proposes that MKs contained within the lung microcirculation are responsible for the production of around 50% of the circulating concentration of platelets (46, 47). Whilst more work is needed to replicate this finding, the idea that platelets may be derived from different locations in adults – similarly to that observed throughout development – raises interesting questions about the roles of each site and the similarity of platelets from each; a comparison of the composition and reactivity of platelets derived from different locations – such as bone-marrow MKs and lung microvasculature MKs – would therefore be extremely welcome.



Main site of thrombopoiesis	Time during development	
	Human (Weeks)	Mouse (Days)
Yolk Sac	5-10	E7-E10
Liver	10-26	E10-P0
Bone Marrow	26+	P0+

Figure 1.1 – Haematopoiesis and thrombopoiesis through development. Thrombopoiesis begins in the yolk sac, before moving to the liver and spleen, and finally localising to the bone marrow and lymph nodes. Sites of haematopoiesis throughout development graph adapted from Rodak's Hematology: Clinical Principles and Applications (1-5)

1.1.3 Platelets in haemostasis

The prototypical role of platelets, as mentioned, is in haemostasis and thrombosis. In their role as mediators of haemostasis, platelets circulate in a quiescent state in close proximity to the vascular endothelium; platelets are kept close to the vessel wall by the concurrent circulation of larger red blood cells, which force the platelets to the edges of the vessel (48). In healthy blood vessels, the endothelial cells lining the vessel are constantly releasing soluble mediators such as nitric oxide (NO) and prostaglandin I_2 (PGI_2), also known as prostacyclin (49-51). These chemicals help to keep platelets in their quiescent circulating state by elevating intracellular levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) (49-51). Elevation of cAMP and cGMP causes activation of protein kinase A (PKA) and G (PKG), respectively. PKA and PKG have a number of substrates within platelets that, upon becoming phosphorylated, dampen or downregulate a number of key regulators of platelet activation. For example, PKA has been shown to directly phosphorylate GP1bβ, causing a reduction in the ability of the GP1b-V-IX complex to bind to its substrate, von Willebrand factor (vWF), whilst PKG has been shown to phosphorylate the inositol triphosphate (IP_3) receptor (IP_3R), causing decreased Ca^{2+} release from IP_3 sensitive stores (52-54). The above actions of PKA and PKG, alongside many more, result in platelets adopting an inactive, dormant state (54).

Upon injury to the vasculature, endothelial cells coating the blood vessels are damaged, causing both a reduction in the production of PGI_2 and NO and allowing platelets to come into contact with the sub-endothelial matrix (SEM). The SEM is a

mesh like structure containing a number of different pro-aggregatory proteins such as collagen(s), fibronectin, laminin and vWF (55). Platelets recognise and bind to a variety of these proteins via surface receptors such as GPVI and GP1b-IX-V – often in collaboration with adhesive receptors and integrins such as integrin $\alpha 2\beta 1$ – allowing slowing, tethering and recruitment to the breach in the vessel wall, where they become activated. Initial platelet activation leads to the transduction of a variety of molecular signals, discussed in more detail below, culminating in the mobilisation of Ca^{2+} ions from intracellular stores, degranulation of intracellular vesicles and morphological changes (56). The shape change allows platelets to increase their surface area, spreading out over the damaged section of the vessel and forming the beginnings of a vascular plug, or thrombus (56). The release of granular contents and other platelet-derived soluble mediators, such as ADP, thromboxane A_2 and fibrinogen, plays an important positive feedback role, further strengthening the activation of the already adherent platelets alongside recruiting and activating other circulating platelets (56). Fibrinogen plays a particularly important role as it forms bridges between platelets through the integrin $\alpha IIb\beta 3$, which holds the budding thrombus together at the site of injury (56); $\alpha IIb\beta 3$ is present on platelets in a low-affinity conformation but upon Ca^{2+} mobilisation, undergoes a conformational change to a high-affinity state – this process is termed inside-out signalling. It is also a point of overlap between platelets and the coagulation system as the $\alpha IIb\beta 3$ ligand, fibrinogen, is a key target for thrombin, which is a key player in both systems. Upon exposure to thrombin, fibrinogen is cleaved which allows it to be converted to fibrin (57), which is very important for clot retraction and full blockade of the vascular breach (58). This entire process occurs rapidly in response to vessel damage to arrest blood loss, and in the longer term to promote endothelial

growth and repair. Once the platelets have plugged the site of damage, they release a number of key angiogenic, inflammatory and tissue repair promoting factors (24, 59, 60). Key mediators such as vascular endothelial growth factor (VEGF), sCD40L and basic fibroblastic growth factor (bFGF) are all secreted from activated platelets (61-64), aiding in immune function, vascularisation and endothelial cell proliferation at the site of damage.

Due to the rapid initiation and propagation of platelet recruitment and activation, alongside the range of processes that platelets participate in, an active fibrinolytic system is required to prevent unwanted, pathological thrombosis. Without this system, inappropriate thrombosis would be able to occur unchecked, leading to serious and potentially catastrophic events such as pulmonary embolism (PE), myocardial infarction (MI), or stroke. To ensure effective prevention and speedy degradation of inappropriate thrombi, proteins and enzymes such as plasminogen/plasmin are actively released into the circulation and, upon contact with thrombi, perform functions such as the cleavage of fibrin molecules; plasmin is responsible for the cleavage of fibrin and is converted from plasminogen via enzymes such as urokinase and tissue plasminogen activator (tPA) (65). Processes such as fibrin cleavage prevent the growth and stabilisation of thrombi, releasing degradation products such as D-dimers (65, 66); D-dimer measurements are often used clinically as measures of fibrinolytic activity (65, 66). These degradation products, alongside the associated platelets, are then targeted for further degradation and clearance from the body. These processes are tightly controlled to prevent unwanted thrombosis whilst also allowing for physiological thrombus formation under haemostatic challenge (65). They are also the

same systems that are involved in the clearance of thrombi that have fulfilled their physiological purpose, however under these circumstances enzymes such as plasmin are activated in a highly localised response. This highly specific localisation is made possible due to the induced release of tPA and urokinase from the vascular endothelium via platelet-centred mechanisms such as induction of occlusive stress, thrombin stimulation and adrenaline release (65). Under physiological conditions, once the occlusive thrombus has been cleared and the vascular breach has been repaired, endothelial cells again release mediators such as NO and PGI_2, restoring the equilibrium and once again ensuring platelets remain in a dormant, quiescent state.

1.1.4 Platelets in thrombosis

Although platelets are integral for arresting bleeding after trauma, the processes involved in prevention of blood loss can become abnormally activated in several pathophysiological conditions. Indeed, in 2015, ischemic heart disease and stroke accounted for 15 million, or 26%, of the 56.4 million deaths recorded world-wide throughout the year; these two conditions are consistently within the top 10 causes of death (67, 68). Virchow's triad (figure 1.2) lists the three main interlinking causes of thrombosis: hypercoagulability, endothelial injury and blood stasis. When these conditions arise, aberrant platelet activation can occur, leading to the initiation of platelet aggregation and thrombus formation in the absence of any significant vessel damage; some examples of these conditions are obesity, atherosclerosis, cancer and even pregnancy (69-72). There are also numerous conditions whereby platelets behave normally in the initiation and retraction of thrombi, but where the machinery

used to breakdown and clear these clots once they have served their purpose is impaired; some examples of this are genetic mutations causing loss of function in proteins such as plasminogen, and acquired fibrinolytic disorders such as the development of auto-antibodies against key fibrinolytic proteins, including the pro-fibrinolytic endothelial receptor, annexin A2 (73).

Although thrombotic complications such as ischemic heart disease and stroke are consistently within the top ten causes of death worldwide, the true burden of cardiovascular disease can be seen in those that survive the initial cardiovascular events. For example, patients who survive initial ischemic strokes often develop severe life changing co-morbidities such as paralysis, vision and speech impairments, and memory loss. Not only is this a major burden on the patient and their families, in the long term, the specialist care required can put a large strain on local and national healthcare services. As mentioned above, aberrant thrombosis can lead to the development of both of these very severe, life-threatening conditions, clearly presenting a huge socio-economic burden (74-76).

Although a number of the risk factors for aberrant thrombosis can be addressed by changes in lifestyle, for example diet changes to prevent atherosclerosis and changes in activity to prevent deep vein thrombosis (DVT) (77, 78), there are still factors that cannot be addressed by lifestyle choices. There are also points at which lifestyle choices alone cannot alleviate the risk of a severe cardiovascular event in patients who have developed cardiovascular diseases. This is where most research in the platelet field has been focussed to date, discovering new treatments and interventions to

prevent strokes and heart disease once risk factors become unmanageable by changes in lifestyle alone, or if the patient does not make the necessary changes to lifestyle (79). However, as has been found time and time again, it is extremely difficult to distinguish between pathological thrombosis and physiological haemostasis; most current treatment regimes, such as warfarin, aspirin or clopidogrel treatment, are a balancing act of preventing thrombotic complications whilst maintaining adequate haemostatic functionality to prevent catastrophic blood loss after trauma or injury. This ideal of being able to completely separate the physiological and pathophysiological functions of platelets has often been referred to as the ‘holy grail’ of platelet research and, though many have tried, this magic bullet continues to remain elusive (80).

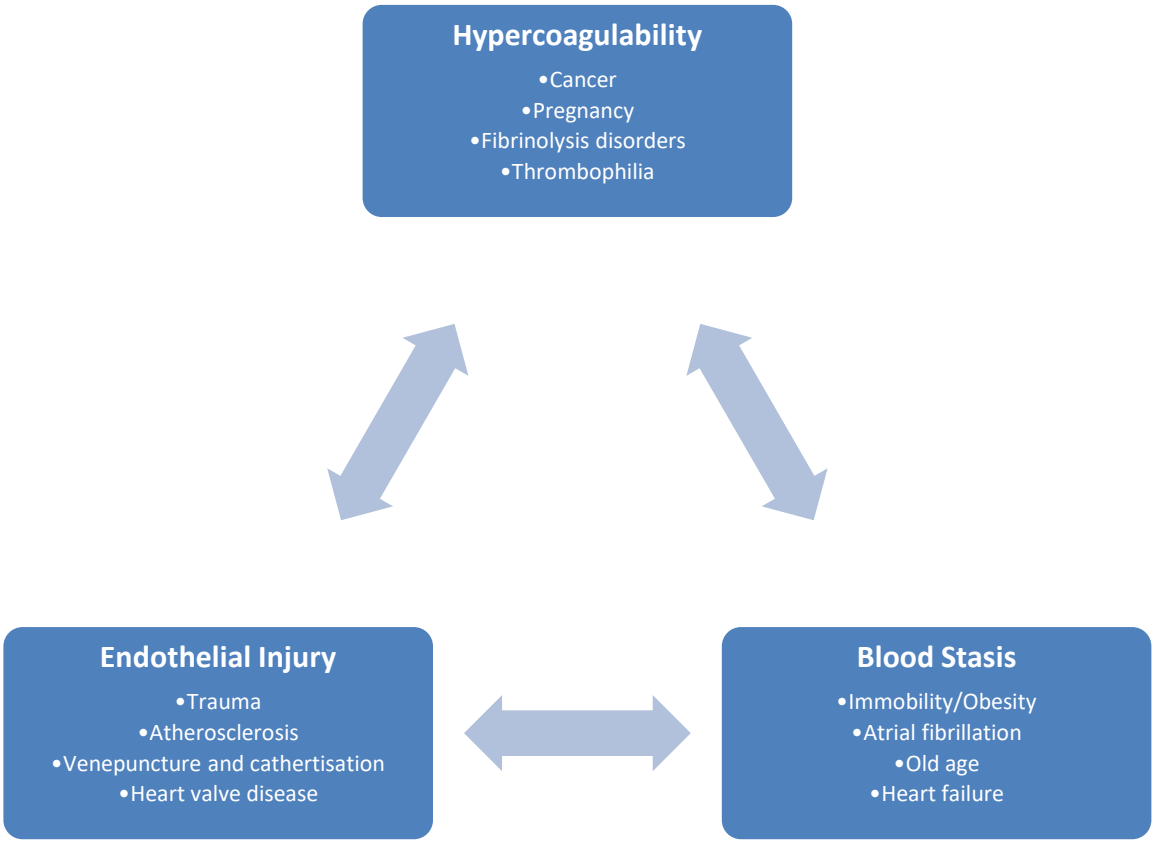


Figure 1.2 - Virchow's Triad. Graphic shows the interplay between the three main overarching causes of both physiological and pathological thrombosis. Endothelial injury is traditionally thought of in terms of haemostasis, however diseases such as atherosclerosis can also cause endothelial damage and dysfunction, leading to unwanted thrombus formation. A number of conditions can induce a hypercoagulable state, where platelets are kept in a pre-activated state which increases the risk of pathological thrombosis. Blood stasis is the final member of the triad and is usually, but not solely, a result of a sedentary lifestyle. This immobility may be due to a number of factors such as age, obesity or even enforced bed rest after surgery. Usually, one of these risk factors alone is not enough to cause thrombotic disease, however when there is a large level of interplay between these risk factors, the risk of developing a thrombotic complication jumps dramatically (9-13).

1.1.5 Thrombocytopenia

Thrombocytopenia is defined as having a platelet count of ≤150x10^9 per litre of blood and can lead to various complications, the most severe being pathological haemorrhage. Thrombocytopenia can be thought of in rough 'severity' bands:

- ≥150x10^9/l – Non-thrombocytopenic, non-symptomatic
- 100-150x10^9/l – Mild thrombocytopenia, symptoms such as excessive bruising
- 50-100x10^9/l – Moderate thrombocytopenia, symptoms such as excessive bleeding after traumatic events
- ≤50x10^9/l – Severe thrombocytopenia, symptoms such as spontaneous internal and external bleeding (81)

However, this classification is based solely on platelet count: qualitative changes in platelet reactivity can also influence the degree of bleeding.

As can be seen from the information listed above, as platelet counts drop the ability of the haemostatic system to function falls in parallel. Patients with a severe thrombocytopenia presenting with spontaneous blood loss are considered a haematological emergency and often require intensive hospital treatment. There are several possible causes of thrombocytopenia and, other than in patients with genetic platelet defects or specific immune-mediated thrombocytopenias, is often detected as a secondary symptom of a separate pathology (82, 83). Conditions such as leukaemia, autoimmune disease and sepsis may all contribute to the development of thrombocytopenia (84-86), as may some of the drugs used to combat these disorders (87-89).

Typically, only patients presenting with severe thrombocytopenia will require treatment and currently the gold standard treatment is either platelet or whole blood transfusion (90), alongside treatment for the underlying cause of the low platelet count (91). However, in the last ten years, breakthroughs in the development of thrombopoietin (TPO) mimetics has opened the door for completely new treatment regimes. First generation TPO mimetics were unsuccessful in the clinic, not due to lack of efficacy, but due to a subset of patients developing a paradoxical thrombocytopenia in response to treatment with a PEGylated recombinant form of TPO (92). It was later discovered that these patients had generated IgGs against the recombinant protein which could cross-react with endogenous TPO and inhibit megakaryocyte development and differentiation (93). Although the safety profiles of first generation TPO mimetics were poor, the efficacy and potential applications of TPO mimetics were too good to pass up completely. Second generation TPO-based therapeutics aimed to maintain the high efficacy of first generations whilst improving their safety and side effect profiles. Several different avenues were explored to enable this leading to the development of three new classes of TPO-mimetic drugs: TPO peptide mimetics; TPO non-peptide mimetics; and TPO antibody mimetics (92). The two most commonly used TPO mimetics are romiplostim, a peptide mimetic given as a subcutaneous injection (94), and eltrombopag, a non-peptide mimetic given as an oral tablet (95). Romiplostim causes an increase in platelet count after single injections (94), whereas eltrombopag requires daily dosing for at least ten days to induce an increase in platelet count (95). TPO mimetics are becoming more common in the clinic, especially in the treatment of

immune thrombocytopenic purpura (ITP), however they have not yet become accepted as first line treatments for any diseases (92, 96, 97).

1.1.6 Initial platelet activation in haemostasis and thrombosis

As stated, during the haemostatic response, there is an initial wave of platelet adhesion and activation at the site of vascular damage, followed by a secondary wave of platelet recruitment, thrombus growth and eventual vascular plug formation. It has been determined that to perform these key functions, platelets have a number of key mechanisms controlling the instigation and subsequent reinforcement of their activation. Whilst the majority of the reinforcement of platelet signalling and activity is mediated by GPCR agonists, the initial adhesion and induction of platelet activation at sites of vascular damage is typically mediated by adhesion receptors and receptors that signal via tyrosine kinases. These GPCR and tyrosine kinase-linked pathways are discussed in more detail below.

1.2 Platelet receptors in haemostasis and thrombosis

1.2.1 Integrins

There are five major integrins contained on the surface of platelets: $\alpha 2\beta 1$ (collagen), $\alpha 5\beta 1$ (fibronectin), $\alpha 6\beta 1$ (laminin), $\alpha v\beta 3$ (vitronectin, fibrinogen, vWF, prothrombin, thrombospondin) and $\alpha IIb\beta 3$ (fibrinogen, fibrin, vWF, fibronectin, vitronectin, thrombospondin) (98); there is also some evidence of ICAM-1 expression on platelets, which may be important in platelet-leukocyte interactions (98). $\alpha IIb\beta 3$ is the most intensely studied integrin on platelets, likely due to its high levels of

expression – it is the most abundant integrin on platelets – alongside its important role in thrombus formation as the major fibrinogen binding receptor on platelets (98).

Under resting conditions, platelet integrins are expressed on the cell surface in a low-affinity conformational state, rendering them unable to bind to their substrates (99). However, upon platelet activation and intracellular calcium flux, integrins undergo what is known as inside-out activation. This inside-out activation of integrins, especially αIIbβ3, relies upon activation of a number of proteins, including PKC and CALDAG-GEFI, to induce recruitment of proteins such as Talin-1 and Kindlin-3 to the intracellular tails of the integrins (99). This recruitment induces a conformational change in the integrin, allowing it to adopt a high affinity conformation and bind its substrate molecules. This whole process is dependent upon initial platelet signalling and subsequent Ca^{2+} mobilisation (99). There are only a few known integrin-based platelet disorders, with the most prominent being Glanzmann's thrombasthenia. Glanzmann's is characterised by defects in integrin αIIbβ3 expression or function and typically presents as a moderate to severe increase in mucosal bleeding (menorrhagia, epistaxis, gingival) and prolonged bleeding times, again highlighting the key role that αIIbβ3 plays in platelet function (100).

1.2.2 G protein-coupled receptors

G protein-coupled receptors are a large family of signalling proteins found on the membranes of a huge variety of cell types. They are characterised by seven trans-membrane helices, an extracellular N-terminus and an intracellular C-terminus; the intracellular C-terminal tail is associated with the Gα and Gβγ protein subunits involved

in signal transduction. Under resting conditions, the Gα subunit is bound to GDP, causing association with the Gβγ subunit and the receptor itself. Upon agonist binding, a conformational change occurs which allows disassociation of the Gα and Gβγ subunits, both from the receptor and each other, alongside exchange of the GDP molecule for a molecule of GTP. Once this has occurred, both the Gα and Gβγ subunits move away from the receptor and can elicit their intracellular effects. The intracellular effects of Gα signalling are dependent upon the isoform of the protein; four key Gα isoforms are: $G\alpha_i$ (▼cAMP), $G\alpha_s$ (▲cAMP), $G\alpha_q$ (PLC activation) and $G\alpha_{12/13}$ (Rho GTPase signalling). Gβγ subunits also appear to signal and whilst a large body of work suggests they appear to play important roles in the negative regulation and desensitisation of GPCRs, there is also some evidence that they may interact with phospholipases and phosphoinositide 3-kinase (PI3K). Some of the key GPCRs present on platelets – and their respective agonists – are:

- Protease-activated receptors (PAR) [Thrombin]
- Purinergic receptors ($P2Y_{(n)}$) [ADP]
- Prostacyclin receptors (IP) [PGI_2]
- Thromboxane A2 receptors (TP) [Thromboxane A2]

A full list of all GPCR's conclusively shown to be present upon the platelet surface can be found in table 1.1; GPCR's for which mRNA has been identified but for which protein has not been subsequently confirmed to be present are omitted.

Table 1.1 – Table listing all G protein-coupled receptors (GPCRs) confirmed as present on the platelet surface and their main physiological agonists. Receptors with mRNA-level evidence but without confirmatory protein level analysis is are not listed (101).

G-Protein Coupled Receptor	Main Physiological Agonist(s)
5-HT_{2a} receptor	Serotonin
Adenosine A_{2a} receptor	Adenosine
$\alpha 2_a$-adrenoceptor	Adrenaline, Noradrenaline
Proteinase-activated receptor (PAR) 1	Thrombin
Proteinase-activated receptor (PAR) 4	Thrombin
Prostanoid IP1 receptor	Prostacyclin
Prostanoid EP3 receptor	Prostaglandin
Prostanoid TP-α receptor	Thromboxane A2
Purinergic $P2Y_1$ receptor	ADP
Purinergic $P2Y_{12}$ receptor	ADP

1.2.2.1 PAR receptors

PAR receptors are the key signalling receptors for thrombin contained on the surface of platelets (102). There are four isoforms of PAR receptors which may form both hetero- and homo-dimers; PAR1, PAR2, PAR3 and PAR4. All of these receptors isoforms have slightly different affinities, activation and signalling kinetics, and expression profiles, and the ability to form dimers is often important in their roles in platelet activation (103). The PAR family are present in an inhibited state on the surface of platelets but, upon contact with thrombin, the N-terminus is proteolytically cleaved. This cleavage causes the formation of a new N-terminus with an activatory sequence for the receptor which acts as a tethered ligand for PAR receptors and, once exposed, induces irreversible receptor activation (103).

PAR1 is only expressed in human platelets and is not present in rodent platelets (104). PAR1 has a high affinity for thrombin and is responsible for fast but transient signal transduction. PAR3 appears to be only expressed in rodent platelets, and seems to act solely as a co-factor for PAR4 activation by thrombin, with no intrinsic G protein signalling capability (103-105); PAR1 also seems to be able to act as a PAR4 co-factor alongside its functional capability (103). PAR4 is expressed in both human and murine platelets, has a lower affinity for thrombin than PAR1 and PAR3, and is responsible for activation in response to high concentrations of thrombin (104); it accounts for slower but prolonged signal transduction and appears to play an important role in irreversible platelet aggregation.

All PAR isoforms play important roles in the activation of platelets, and as stated there is a complex level of interplay between the different receptor isoforms which can influence the signalling kinetics and specific transduction pathways involved in platelet function. For example, the formation of heterodimers of PAR1 and PAR4 on human platelets, and the observation that PAR4 is often cleaved secondarily to PAR1 via PAR1 bound thrombin, allows platelets to effectively respond to both low and high concentrations of thrombin (104). PARs appear to mainly signal through either the $G\alpha q$ or $G\alpha 12/13$ isoforms, activating either PLCβ – leading to PKC activation and IP_3 /DAG signalling – or Rho-GEF, both of which culminate in regulation of the platelet cytoskeleton and induction of platelet shape change (104). PAR antagonists have been developed and the PAR1 inhibitor vorapaxar has entered clinical trials. Vorapaxar has been shown to significantly reduce the risk of ischemic heart disease, however a significant increase in the rate of moderate-to-severe bleeding was observed when compared with standard care (106). Platelet PARs are extremely important receptors, and very attractive drug targets, however care must be taken as severe bleeding side effects may well present in patients treated with PAR inhibitors.

1.2.2.2 $P2Y_{(n)}$ receptors

The purinergic receptors contained on the platelet surface are a family of ADP and ATP-sensitive nucleotide receptors (107). The two-main platelet ADP-sensitive purinergic receptors which signal via G-proteins are $P2Y_1$ and $P2Y_{12}$; platelets also contain the $P2X_1$ receptor which is a ligand-gated ion channel that responds to ATP (107). ADP and P2Y receptors have been shown to be extremely important in the haemostatic process; patients with defects in the storage or release of ADP, or in the

expression or function of P2Y receptors present with a mild-to-moderate bleeding diathesis (108, 109).

$P2Y_1$ is present on the platelet surface with a copy number of around 150 per platelet (104). It is coupled to the $G\alpha q$ subunit and stimulates PKC and IP_3/DAG downstream signalling via activation of $PLC\beta$, culminating in shape change and Ca^{2+} mobilisation. $P2Y_1$ signalling is also responsible for the downstream activation of PLA_2 – which is a key step in the synthesis of thromboxane A2 from membrane-derived arachidonic acid (AA) – alongside activation of RhoA, providing a secondary mechanism by which it can drive platelet shape change (104, 107). $P2Y_1$ appears to be most important in initial platelet activation, inducing shape change but only transient platelet aggregation (104). This, alongside its wide tissue distribution and low platelet-surface copy number, make $P2Y_1$ a less appealing target for development of novel antithrombotic treatments than other platelet surface receptors. Indeed, whilst $P2Y_1$ knock-out mice and pharmacological platelet $P2Y_1$ inhibition experiments demonstrate impaired ADP-induced platelet activation and reductions in thromboembolism, there do not currently appear to be any clinical studies exploring $P2Y_1$ inhibitors as potential anti-thrombotic agents (110).

$P2Y_{12}$ is present at a copy number of around 600 per platelet, making it around four times more abundant at the platelet surface than $P2Y_1$ (104). $P2Y_{12}$ is coupled to the $G\alpha i$ subunit, and thus stimulation of this receptor leads to the inhibition of adenylyl cyclase, leading to a decrease in the intracellular concentration of cAMP (102, 104, 107). A reduction in the intracellular concentration of cAMP and subsequent inhibitory

pathways is associated with a pro-activatory state (102). Interestingly, the Gβγ subunit associated with the $P2Y_{12}$ receptor also appears to be capable of activating PLCβ and PI3K, causing Ca^{2+} release and integrin activation (104, 107). These two complementary mechanisms may explain why $P2Y_{12}$ stimulation contributes to a much stronger, irreversible platelet activation and aggregation than that seen with $P2Y_1$ stimulation. This fact, alongside the much more restricted expression profile of $P2Y_{12}$, has made it a very attractive antithrombotic target, and has spawned potent platelet inhibitors such as cangrelor and ticagrelor which have proven successful in the clinic (104).

1.2.2.3 Prostacyclin receptor

The prostacyclin receptor has only a single isoform expressed on the surface of platelets, and is responsible for the inhibitory effects of PGI_2 on platelets (102). The IP receptor is coupled to a Gαs subunit, causing an induction of adenylyl cyclase and subsequent increase in cAMP concentration upon PGI_2 binding (102). Increased levels of cAMP in the platelet cytosol elicits a variety of pro-inhibitory effects, as previously described, and ensures that in the general circulation platelets are kept in a quiescent state (52, 53, 102). Platelets from mice deficient in the IP receptor have lower basal cAMP levels than WT controls and show a propensity to develop thrombotic complications *in vivo*. Interestingly, no differences in *ex vivo* reactivity were observed in the IP deficient mice, suggesting that cAMP levels are important for maintaining the resting state of platelets *in vivo* but does not predispose platelets to spontaneous activation (104, 111). Several IP receptor agonists have been developed and some,

such as iloprost, are currently used clinically for conditions such as pulmonary arterial hypertension (112).

1.2.2.4 Thromboxane A2 receptors (TP)

TP receptors are widely distributed throughout the body and respond to the chemical mediator thromboxane (Tx) A2 (113). In the context of platelets, upon activation TxA2 is formed *de novo* from AA and released. AA, released from phospholipids in the plasma membrane via the activity of PLA_2, is converted into TxA2 via the action of platelet cyclooxygenase (COX) enzymes (114). There is only one thromboxane A2 receptor present on the platelet surface membrane – although two major splice variants have been proposed – which are coupled to both Gαq and Gα12/13 G-protein isoforms (102). There is also evidence that the two splice variants can have opposing effects, with the TPα variant causing stimulation of adenylyl cyclase and the TPβ variant inhibiting the same enzyme; most of this work has been performed in cell lines and expression systems and remains to be confirmed (102, 113).

Upon binding to the TP receptor, TxA2 induces signalling through the PLCβ pathway (Gαq) and the Rho-ROCK signalling pathway (Gα12/13). As previously stated, activation of the PLCβ signalling pathway causes IP_3 & DAG release, subsequent Ca^{2+} mobilisation and platelet responses including shape change and degranulation (113). Activation of the Rho-ROCK signalling pathways is responsible for downstream phosphorylation of myosin light chain, an important player in platelet membrane organisation. Phosphorylation of the myosin light chain can induce functional consequences such as platelet cytoskeleton reorganisation, shape change and

integrin activation (113). Although there is some interest in targeting the TP, the availability and efficacy of aspirin (a COX inhibitor preventing the conversion of AA to TxA2) as a platelet inhibitor potentially hampers the development of novel TxA2 or TP antagonists (115, 116).

1.2.3 ITAM and hemITAM receptors

1.2.3.1 ITAM and hemITAM motifs

ITAMs are a highly conserved signalling motif of YxxI/L_{6-12}YxxI/L present in many immune and antigen receptor signalling pathways (117). The tyrosine residues present in the ITAM domain become phosphorylated upon receptor activation and allow recruitment of Src-homology 2 (SH2) domain containing proteins, allowing the initiation of downstream signal transduction and cellular activation (117). A hemITAM motif is so named as it contains only one of the aforementioned YxxI/L amino acid sequences, in comparison to the full YxxI/L_{6-12}YxxI/L contained in the traditional ITAM motif (118). HemITAM containing receptors typically function as dimers, which allows the formation of a 'pseudo ITAM' between the two monomeric receptor subunits (119).

As suggested by the name, (hem)ITAMs are typically found within immune receptors located on the surface of immune cells such as B- and T-cells, neutrophils and platelets (120, 121). Although the motif is common between numerous receptors on many immune cell types, the functional responses downstream of these receptors can vary greatly, from induction of platelet activation and aggregation to playing an integral role in the maturation of B-cells (122, 123). Human platelets possess three major (hem)ITAM containing receptors and these are explored in more details below (124).

1.2.3.2 GPVI

GPVI is a 63 kDa immunoglobulin receptor, comprising 319 amino acids and expressed solely in the platelet and megakaryocytic cell lineage (125). It is the major signalling receptor for collagen contained on platelets (126) and is comprised of two linked extracellular domains, a highly glycosylated connector region (contributing largely to the molecular weight of the receptor), a trans-membrane domain and a relatively short intracellular tail. Linked to this intracellular tail is a dimer of the Fc receptor gamma chain (FcRγ) which contains the ITAM domains important for downstream signal transduction (126). GPVI contains a number of important motifs throughout that are involved in protein-protein interactions; for example the intracellular tail contains a motif allowing binding of Src family kinases (SFKs) whilst the trans-membrane domain contains an arginine that is integral in linking GPVI to the FcRγ chain (127). A schematic of GPVI, alongside the other major platelet (hem)ITAM receptors, is present in figure 1.3.

GPVI is mainly present on resting platelets as a monomer but upon activation forms dimers and begins to cluster in the membrane (128-130). The affinity of collagen for the monomeric form of GPVI is quite poor and it is unlikely that the levels of collagen present following the exposure of the sub-endothelial matrix – or the typically very low circulating concentrations (ng/ml) of serum collagen(s) – would be able to induce platelet activation solely via monomeric GPVI on the platelet surface (129, 131-134). This suggests an extremely important role for the dimerization and clustering of GPVI in the full and sustained activation of platelets in response to collagen (129-131).

Indeed more recent studies, including those from our group, have shown that a percentage of GPVI on resting platelets is present as the high affinity dimeric form, suggesting that dimer formation may be important for initiation of platelet activation via collagen with subsequent dimer clustering further increasing the signal (129, 130).

When binding to collagen, an intracellular signalling cascade is initiated which culminates in platelet activation – including aggregation – granule release and shape change (135). The first step in this pathway is the activation of Src family kinases (SFKs), particularly (but not limited to) Lyn and Fyn, which are a family of membrane bound proteins responsible for the phosphorylation of the tyrosine residues contained within the FcRγ-chain (126). This phosphorylation event allows the recruitment of spleen tyrosine kinase (Syk), a cytoplasmic kinase which plays an integral role in platelet signal transduction (126). Syk contains two Src-homology two domains (SH2), which allows it to bind to the phosphorylated tyrosine residues of the FcRγ-chain (136). This binding localises Syk to the plasma membrane and induces a conformational change, revealing a number of key residues within the molecule, whilst also exposing its kinase domain (137). Syk then undergoes a series of auto- and trans-phosphorylation events (mediated by SFKs), resulting in full activation and further signal transduction (126, 137). Once activated, Syk phosphorylates a range of downstream proteins including the adapter protein linker of activation of T cells (LAT) which contains numerous tyrosine residues (126, 138). Phosphorylation of these residues by Syk provides docking sites for other important signalling proteins in the pathway, such as Gads, Grb2, SLP-76, Vav1/3, Btk, and PLCγ2; upon docking of these proteins the superstructure is often referred to as the LAT signalosome (126, 138).

One of the key end effectors of this signalling pathway is phospholipase C γ2 (PLCγ2), a lipase responsible for the formation of IP_3 and DAG from the plasma membrane (126). A key responsibility for these two proteins is the mobilisation of intracellular Ca^{2+}, which induces processes such as granule release, platelet shape change, and integrin activation (139). Platelet activation by collagen leads to spreading and coverage of the site of damage as well as forming a base for a developing thrombus. This signalling pathway is summarised in figure 1.4.

GPVI has recently been shown to be a key signalling receptor for fibrin (140, 141). Mammadova-Bach, et al first noticed that patients with a deficiency in GPVI had a reduced peak in thrombin generation not only in response to collagen, but also in response to TF. This collagen-independent effect on thrombin generation was also observed in control platelets treated with a GPVI-blocking antibody, suggesting the possibility of a novel agonist for GPVI. This study also assessed spreading and thrombus formation; murine platelets deficient in GPVI and human platelets treated with a GPVI-blocking antibody fragment were assessed and both showed reductions in spreading on fibrin surfaces (141).

At the same time, Alesheri, et al also published a study describing GPVI as a novel signalling receptor for fibrin (140). In their study, Alsheri, et al stimulated platelets with thrombin and fibrinogen in the presence and absence of GPRP (an inhibitor of fibrin polymerisation), functional αIIbβ3, and GPVI; αIIbβ3 was inhibited by both integrilin and genetic ablation, whereas GPVI functionality was removed via use of a deficient mouse model. It was shown that thrombin stimulation of platelets induces increased

platelet protein phosphorylation, but fibrinogen stimulation does not. Increases in tyrosine phosphorylation over that induced by thrombin alone were observed however, when thrombin and fibrinogen were co-administered to untreated platelets; the pattern of phosphorylation following co-stimulation mirrored that following GPVI stimulation. Addition of GPRP alongside thrombin and fibrinogen abolished the observed increase in phosphorylation. When αIIbβ3 was blocked or removed, the phosphorylation response to thrombin was abolished, whereas the response to thrombin and fibrinogen remained; in GPVI deficient mice, the increased phosphorylation in response to thrombin and fibrinogen was lost. These experiments were used to show that fibrin – formed by the co-administration of thrombin and fibrinogen – stimulates platelets in a pattern similar to that observed following GPVI stimulation. Following on from this, platelet spreading was assessed, and seen to be impaired in response to a fibrin coated surface in GPVI deficient mouse platelets; similar spreading results were observed in murine platelets treated with the SFK inhibitor dasatinib. Interestingly, no difference in spreading was observed on monomeric (+GPRP) vs polymeric fibrin (-GPRP) coated surfaces, suggesting a common binding motif. They concluded their study using a $FeCl_3$-induced thrombotic injury model, where impaired thrombus stability and increased time-to-occlusion was observed in GPVI deficient mice (140); both groups agreed that thrombus growth and stability is dependent upon the expression and function of GPVI on platelets (140, 141).

These two landmark papers have opened up an entirely new avenue of research, and highlighted the importance of GPVI not just in the initiation but also in the propagation of thrombosis.

1.2.3.3 CLEC-2

CLEC-2, or the C-type lectin-like receptor 2, is a hemITAM containing glycoprotein also highly expressed on the surface of platelets (142, 143). This receptor does not appear to be restricted solely to the platelet and megakaryocytic lineage and has been identified on a small proportion of other immune cell types, however often at very low levels of expression (144, 145). There is some evidence that CLEC-2 expression may be induced at higher levels under specific conditions in some of these cell types, for example on dendritic cells stimulated with PDGF (146).

CLEC-2 was initially identified in a bioinformatics screen performed by Colonna *et al*. in the year 2000 (147). This study identified CLEC-2 at the transcript level in dendritic cells (DC) and in the liver but it was not until 2006 that Suzuki-Inoue *et al* and Chaipan *et al* identified high levels of CLEC-2 protein in the platelet and megakaryocytic lineage (142, 147, 148). This was quickly followed up by evidence of CLEC-2 at both the transcript and protein level in the platelet and megakaryocyte lineage, in the study by Senis *et al* in 2007 (148).

CLEC-2 belongs to the family of type II membrane proteins and comprises a carbohydrate-like recognition domain, trans-membrane domain and intracellular tail, the latter of which contains the hemITAM motif (Figure 2) (142, 148). Interestingly – although CLEC-2 contains a hemITAM – the receptor is active as a dimer, suggesting that SH2 containing proteins may be able to form a ‘pseudo’ ITAM by binding one YxxL repeat from each receptor (149); however, little work has been performed exploring this as a potential signalling mechanism. Another important difference between CLEC-

2 and GPVI is the importance of a tri-acidic amino acid sequence directly upstream of the hemITAM; these three acidic amino acid residues are present upstream of the YxxL repeat in several other hemITAM containing receptors (150). Currently, it appears that no tri-acidic amino acid sequences are found proximally in ITAM receptors in mammals. These amino acids are required for phosphorylation of the YxxL sequence present in hemITAM receptors (150).

CLEC-2 signals via the same set of signalling proteins as the ITAM receptor GPVI (figure 1.4), however as with the motif itself there are a number of key differences in the signal transduction. Initially, whereas GPVI is reliant upon SFKs for the initial phosphorylation of the ITAM, the hemITAM contained within CLEC-2 seems to rely much more heavily upon Syk for initial phosphorylation (151, 152). Severin *et al*, building upon the work of Spalton *et al*, showed that in the absence of the major platelet SFKs – Lyn, Fyn and Src – CLEC-2 phosphorylation still occurred, but was abolished in the absence of Syk (151, 152). However, SFKs are required for the downstream signal transduction as, although CLEC-2 phosphorylation is unaffected, PLCγ2 and platelet aggregation is impaired in the absence of SFKs (151). In an alternative model, Manne *et al* reported that inhibition of PI3K and/or Tec family kinases abolishes CLEC-2, but not GPVI, mediated Syk phosphorylation and platelet activation (153) and proposed that Tec/Btk and PI3K lie upstream of Syk and directly regulate its function in the CLEC-2 signalling pathway (153). Interestingly, these studies seem to suggest that, while GPVI and CLEC-2 utilise the same signalling proteins and pathway, there appear to be subtle yet significant differences between the exact organisation and contribution of each signalling protein between GPVI and CLEC-2.

The endogenous ligand for CLEC-2 has been identified as podoplanin. A 43-kDa protein was discovered in a number of different cell types throughout the 1990s, including lymphatic endothelial cells, fibroblastic reticular cells, and thymic epithelial cells, amongst others (154). However as many different groups were working on the protein at the same time it has had many different names such as GP38, E11 antigen and OTS-8 amongst others (154). The name podoplanin was coined in 1997 by Breiteneder-Geleff *et al*, due to its expression on kidney podocytes and possible role in podocyte foot flattening processes (155). As mentioned, podoplanin was found to be expressed in a number of different cell types and tissues, and one area where podoplanin is particularly highly expressed is on lymphatic endothelial cells (LECs) (154, 156). Expression of podoplanin on LECs, and its interaction with CLEC-2 on the surface of platelets, is especially important during development, where it has been shown to aid in the separation of the lymphatic and vascular systems (157). It has been shown in several studies that mice lacking CLEC-2, proteins involved in the CLEC-2 signalling pathway, or podoplanin have a number of severe phenotypes associated with defective lymphatic development (21, 157-160). In utero, the embryonic mice are oedematous and present with bleeds in the brain, developing lymphatic system and sometimes in the skin (159, 161). Those mice that survive go on to develop numerous physiological defects such as blood filled lymph nodes and vessels (especially noticeable in the mesentery) and chylous ascities (an accumulation of milky chyle in the peritoneum associated with defective lymphatic function) (159). The fact that this phenotype is present in mice lacking platelet CLEC-2, alongside evidence such as phenotype reconstitution in embryos treated with platelet inhibitors or with genetic

ablation of CLEC-2 signalling proteins, is indicative of an integral role for platelets in lymphatic development and subsequent separation from the vasculature (157, 159). It also provided one of the first important pieces of evidence supporting the role of platelets as important mediators of processes outside of haemostasis and thrombosis. This is the description of the role of CLEC-2 beyond haemostasis, not how CLEC-2 signals.

1.2.3.4 FcγRIIa

FcγRIIa is the final (hem)ITAM receptor present on human, but not mouse platelets (162, 163). FcγRIIa belongs to the Fc superfamily of receptors found on many different cells types, especially cells involved in infection and immunity, that recognise the constant Fc region of antibodies when bound to their target antigens (162, 164). FcγRIIa specifically binds to antibodies expressing an IgG Fc region and appears to be restricted in evolutionary expression to higher primates alone (162, 164).

The first mention of FcγRIIa in the literature was in the 1980's, and it was in 1985 that the receptor was first identified on human platelets (165). It is a type I trans-membrane glycoprotein with a general structure that is unique only to FcγRIIa and FcγRIIc (164, 166). Most Fc receptors are multimeric whereas FcγRIIa is a single chain receptor comprising two IgG-like extracellular domains, a trans-membrane domain and a cytoplasmic tail (164, 166). The cytoplasmic tail contains the ITAM motif enabling signal transduction downstream of the receptor (Figure 2).

The presence of FcγRIIa on human platelets supports the idea that platelets may be important mediators of immunity however, as the protein is not expressed in the rodent genome, this receptor was not assessed as part of this thesis (164, 166).

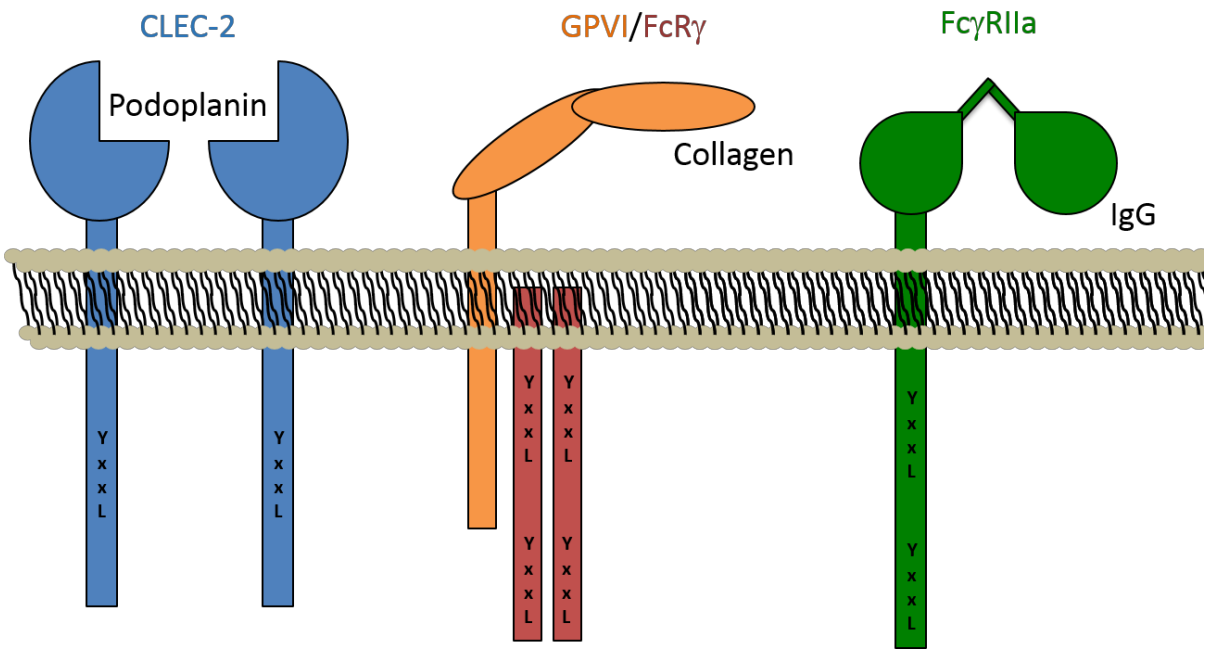


Figure 1.3 - (hem)ITAM receptors present on the surface of platelets. CLEC-2 amd GPVI are expressed in both human and murine platelets, whereas FcγRIIa is only expressed in human platelets. All three receptors contain at least one YxxL repeat in their intracellular tail which is the key motif for downstream signal transduction.

1.3 Key GPVI and CLEC-2 signalling proteins

1.3.1 Key proteins overview

As stated above, there are numerous key signalling proteins involved in (hem)ITAM signal transduction. Proteins such as SFKs and Syk are integral for initiation and proximal signalling events, as evidenced by the lack of signal transduction and platelet aggregation in the absence or inhibition of these proteins (151, 152). Surprisingly, although LAT would appear to play an essential adapter role, it is actually dispensable for platelet activation, with the caveat that loss of LAT dampens platelet responses and impairs thrombus formation (167). This is in comparison to the almost complete loss of GPVI signal transduction in the absence of the adapter SLP-76 and loss of both VAV1 and VAV3 (168, 169); interestingly, mice deficient in SLP-76 show defective lymphatic development, reminiscent of that seen in mice with severe deficits in the CLEC-2 signalling pathway (160). Other adapter proteins such as Gads appear to play less important roles in (hem)ITAM signal transduction (167). Downstream kinases, such as Btk and PI3K, also play particularly important roles in (hem)ITAM signal transduction, although their signalling contributions may be different depending on the receptor engaged (153); remarkably, mice deficient in the Tec family kinases Btk and Tec again recapitulate the developmental defects of mice deficient in CLEC-2, suggesting a particularly important role for both of these kinases downstream of the hemITAM receptor (153). Lastly, as stated above, the end effector of these pathways is PLCγ2, which is responsible for IP_3 and DAG formation and subsequent Ca^{2+} mobilisation. This protein is almost entirely indispensable for both GPVI and CLEC-2 induced platelet activation, as seen in mice deficient in PLCγ2 (142, 170).

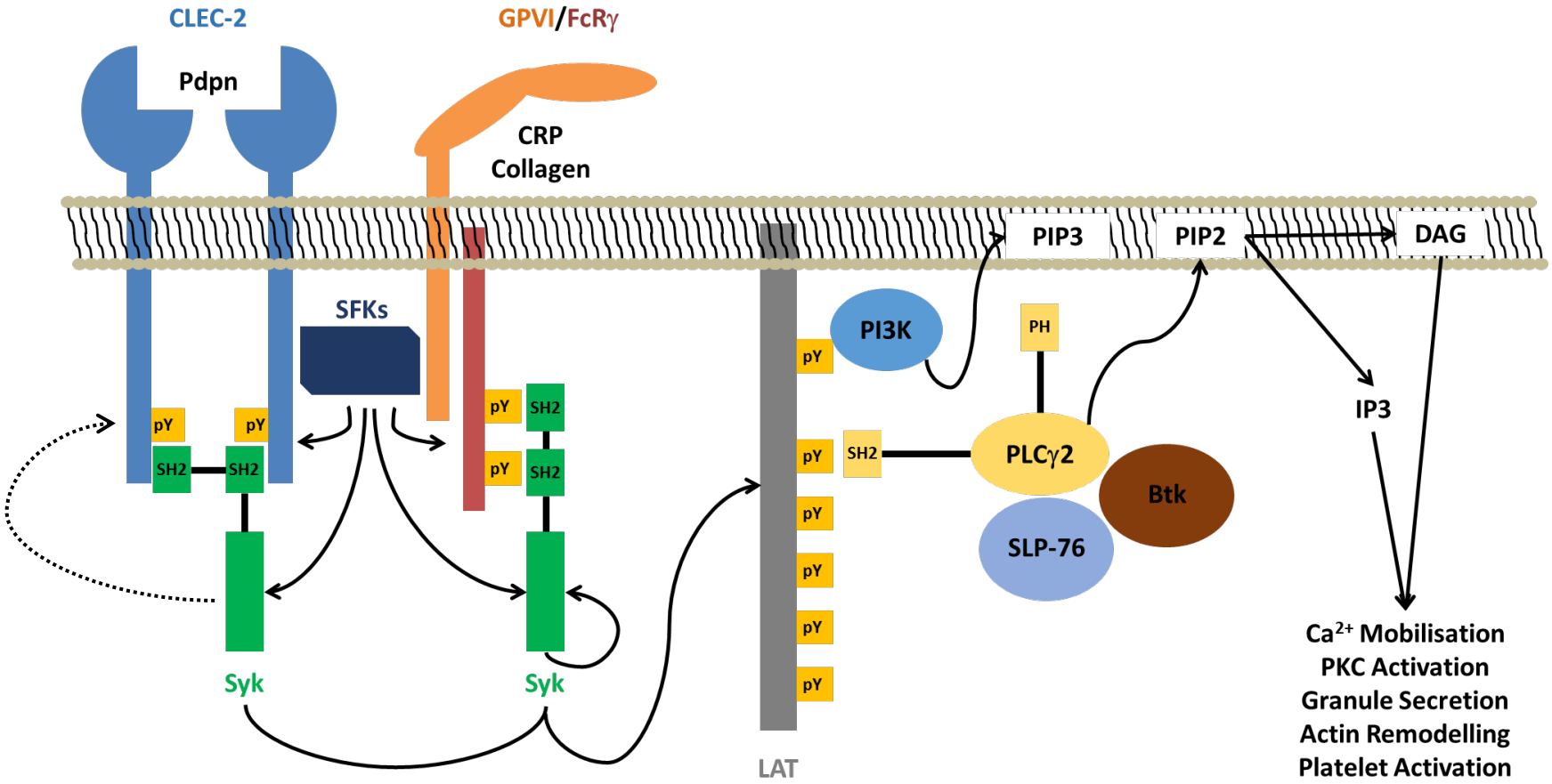


Figure 1.4 – Key signalling proteins and events downstream of the platelet (hem)ITAM receptors GPVI and CLEC-2. Upon receptor engagement via endogenous or exogenous ligands, SFKs are activated and phosphorylate tyrosine residues contained within the (hem)ITAM, allowing recruitment of Syk. Upon recruitment, Syk undergoes both trans- and auto-phosphorylation events, allowing full activation of the protein and phosphorylation of downstream proteins such as LAT; Syk can also positively feedback and phosphorylate the hemITAM contained within CLEC-2. Once LAT is phosphorylated, other proteins such as PI3K, SLP-76, Btk and PLCg2 are recruited and docked to LAT to form the 'LAT signalosome'. These proteins undergo several subsequent phosphorylation events, culminating in the activation of PLCg2. Activation of PLCg2 results in the lipolysis of PIP3 to PIP2, releasing IP_3 and DAG which induces Ca^{2+} mobilisation and PKC activation, causing full platelet activation.

1.3.2 Syk

Syk is a 72kDa cytoplasmic protein that is ubiquitously expressed in cells of the haematopoietic system; the only haematopoietic cells not expressing Syk, namely T-cells and natural killer (NK) cells, express a homologous protein known as ZAP-70 (8, 137, 171). Syk expression is highly conserved between almost all species of vertebrate with a decidedly similar structure, for example the main isoforms of Syk expressed in *Homo sapiens* and *Mus musculus* (common house mouse) differ by only six amino acids (172). Syk is comprised of two SH2 domains (N-SH2 and C-SH2) linked together by an amino acid linker region (linker A) (8). The C-terminal SH2 domain is then linked to a kinase domain via a second amino acid linker region (linker B) (8). There are also a number of serine, threonine and tyrosine amino acid residues dispersed throughout the protein which are extremely important in the regulation of Syk activity (173). Some residues, such as the dual tyrosine residues Y525/526 (murine Y519/520) located within the Syk kinase domain, are important for regulating the activity of Syk as a functional kinase (174, 175). Other residues, including Y323 (murine Y317) and Y352 (murine Y346), play important positive and negative adapter roles. For example, phosphorylation of Y323 has been shown to provide a docking site for the negative regulatory proteins c-Cbl (a ubiquitin ligase) and TULA-2 (a phosphatase); docking of these proteins to Syk has been shown to regulate the magnitude of Syk responsiveness to stimulation (176-180). Phosphorylation of Y352 on the other hand has been hypothesised to be an important docking site for many other proteins involved in signal transduction through the Syk pathway (179-183). Schematics representing the regulation of Syk

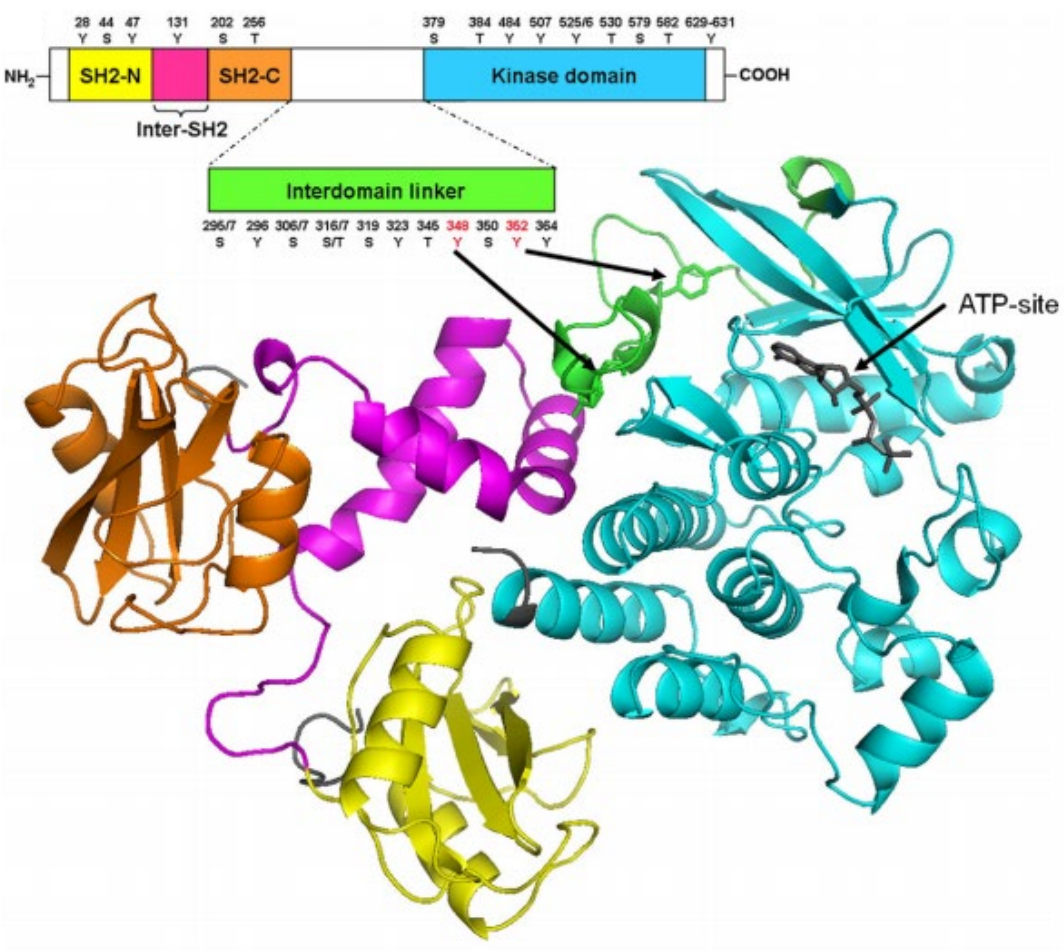
and detailing the key regulatory domains as well as several key tyrosine residues, can be seen in figures 1.5 and 1.6.

Under resting conditions, Syk is held in an autoinhibited state which restricts its ability to function. Although the exact mechanism by which Syk is autoinhibited has not been fully elucidated, the crystal structure of an autoinhibited Zap-70 molecule has been generated (184). As Syk and Zap-70 have very similar structures and functions, it is believed that the mechanism of autoinhibition is likely to be conserved (182). In this model, several intra-molecular bonds between linker A, linker B and the kinase domain of Syk form an interface which has been termed the 'linker-kinase sandwich' (184). This 'sandwich' holds the protein in a conformation which has a three-fold mechanism of inhibition. Firstly, the SH2 domains are pushed out of the alignment which allows them to efficiently bind to a phosphorylated ITAM domain; secondly, the kinase domain is forced out of its optimal arrangement resulting in a decreased ability to catalyse the phospho-transfer reaction; and finally, many of the important tyrosine residues mentioned above reside within, or extremely close to, the regions involved in the formation of the 'linker-kinase sandwich' and, as a result, are unable to become phosphorylated prior to conformational change (184-186). It is thought that the mechanism of autoinhibition of Syk is identical to that of Zap-70, as many of the residues that have been identified to be important in autoinhibition of Zap-70 are in the same relative locations within the Syk molecule, and the two proteins are structurally very similar (6, 184). However, without resources such as crystal structures this hypothesis remains to be definitively proven.

Upon coming into contact with a fully phosphorylated (hem)ITAM domain, it becomes energetically favourable for Syk to become stabilised in an active conformation (8, 186). Once this occurs, the protein induces calcium mobilisation and subsequent platelet activation as described above.

With regards to (hem)ITAM signalling, it is interesting to consider Syk as a potential point of divergence in the signal transduction machinery between GPVI and CLEC-2. It has been shown that CLEC-2 is much more dependent upon Syk for initial hemITAM phosphorylation, compared to SFKs filling this role downstream of GPVI (151, 152). As stated above, there is also evidence that proteins such as Btk and PI3K may impact on Syk signalling at different points within GPVI and CLEC-2 the signalling cascades (153). Lastly, due to the presence of potentially differentially phosphorylatable tyrosine residues distributed throughout the protein, this raises a possible mechanistic role for Syk in the differential effects of GPVI and CLEC-2 receptor engagement (180).

(a)


28 44 47 131 202 256
Y S Y Y S T
379 384 484 507 525/6 530 579 582 629-631
S T Y Y Y T S T Y
NH2
SH2-N
SH2-C
Kinase domain
COOH
Inter-SH2
Interdomain linker
295/7 296 306/7 316/7 319 323 345 348 350 362 364
S Y S S/T S Y T Y S Y Y
ATP-site

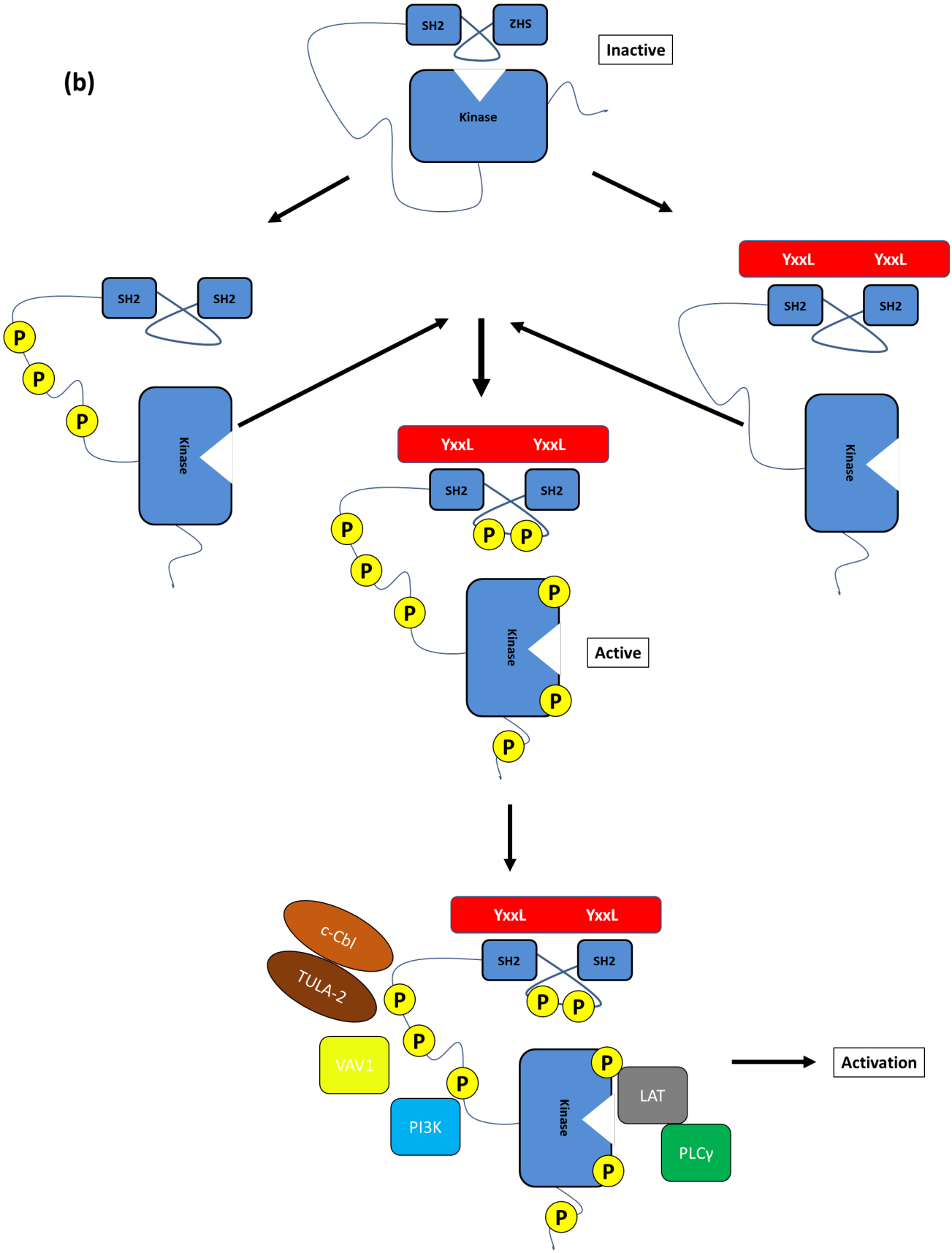


Figure 1.5 – Regulation of Syk activity via changes in conformation. Ths figure highlights (a) the crystal structure of Syk in complex with its non-hydrolysable substrate AMP-PNP, adapted from Gradler *et al*. (b) Mechanisms of Syk activation from an auto-inhibited, inactive state to an open, active, signalling structure via auto- and trans-phosphorylation and binding to phosphorylated SH2 domain-containing proteins, adapted from Kulathu *et al* and Mocsai *et al* (6-8).

1.3.3 LAT

LAT, or linker of activation of T-cells, is a ~36 kDa adapter protein involved in the platelet (hem)ITAM signalling pathway. As its name implies LAT was first discovered in T-cells in the early 90's, however it was only in 1998 the protein was cloned from stimulated Jurkat cells expressing the TCR (187). Initially, this 36-38 kDa protein was of interest as it provided a link between initiation of T-cell receptor signalling and T-cell activation – it was shown to be one of the most prominently phosphorylated proteins present after T-cell receptor stimulation (187). The protein was also known to be membrane bound and associated with other key signalling proteins in the TCR pathway, such as Grb2 and PLCγ1 (187). It was these characteristics that allowed Zhang et al to be confident of the identity of this newly cloned protein. Once cloned, the protein was named after its key signalling role – linking TCR engagement to T-cell activation (187).

The main human isoform of LAT contains 262 amino acids, and the sequence is highly conserved between species. The domain composition of the protein is also identical between species, containing a very short extracellular domain, a transmembrane region and a large intracellular domain (188). Within the intracellular domain there are a number of phosphorylatable amino acids, of which 10 are tyrosine residues, 31 are serine residues, and 11 are threonine residues. This high percentage of phosphorylatable amino acids highlights the key adaptor role that LAT plays in the (hem)ITAM signalling pathways – indeed many publications cite the 'LAT signalosome', reiterating the key structural nature of the protein (167).

Phosphorylation of these amino acids, particularly the tyrosine residues, by proteins such as SFKs and Syk allows the docking of various other key SH2-containing signalling molecules, such as Grb2, Vav1 and PLCγ1 & 2, and facilitates their localisation to the plasma membrane (167, 188, 189). This ability to localise proteins to the membrane is particularly important as it allows effectors such as PLCγ2 to elicit their effects, typically resulting in cell activation (188). It has also been shown that each of the tyrosine residues within the molecule appears to provide a distinct docking site allowing for recruitment of specific proteins; for example, phosphorylation of Y136 appears to allow recruitment and docking of PLC (189). A schematic showing the structure and position of a number of these key tyrosine residues can be seen in figure 1.6.

After the cloning of LAT, it was discovered that it may be involved in signal transduction in other haematopoietic cells containing tyrosine-kinase based signalling cascades, such as platelets (190). Indeed, phosphorylation of LAT was observed in platelets, and was much more potent following stimulation via GPVI compared with the GPCR-mediated response to thrombin (191). Since these initial descriptions of LAT and its role in platelet function much work has shown that LAT plays an important role in full platelet activation, particularly downstream of (hem)ITAM receptor induction (167, 192). For example LAT plays a key role in maximal platelet activation downstream of GPVI and CLEC-2, although residual activatory responses are observed following genetic ablation of LAT (167).

1.3.4 PLCγ2

Phospholipase C gamma 2 (PLCγ2) is a ~148kDa protein with particularly high expression in the haematopoietic lineage and the liver; in contrast the other PLCγ isoform, PLCγ1, has a much more ubiquitous expression profile (193). PLC enzymes have been mentioned in the literature as early as the 1960's. PLCγ2 is a member of the phospholipase family of enzymes, which catalyse the cleavage of phospholipids from the plasma membrane (193). The products of this phospholipid cleavage include bioactive products which are responsible for Ca^{2+} mobilisation and PKC activation (193-195). Ca^{2+} mobilisation and PKC activation result in the activation of cellular responses; in the context of platelets these two events are responsible for responses such as vesicle secretion, actin polymerisation and platelet spreading (196-198). For these reasons, PLCγ2 is often referred to as an end effector of these signalling pathways.

Similarly to Syk, LAT and other proteins in this signalling pathway, PLCγ2 has a number of phosphorylatable residues contained throughout the molecule, including several tyrosine amino acids. These tyrosine residues appear to be phosphorylated by Bruton's tyrosine kinase (Btk), another key component of the LAT signalosome, and SFKs (199-201). It has also been suggested that, similarly to the other proteins already discussed, each tyrosine residue may play an individual functional role upon phosphorylation. Two key tyrosine residues that have been characterised to date are Y759 and Y1217 (199, 202). Phosphorylation of these tyrosine residues appears to be important for the interaction of PLCγ2 with its activatory proteins, as well as correlated with full activation of its catalytic activity (202, 203). Again, a schematic

showing the structure of PLCγ2, alongside the location of several key domains and tyrosine residues, can be seen in figure 1.6.

As with the majority of important signalling proteins, a number of different genetically modified mouse models have been generated and used to explore the function and distribution of PLCγ2. Using different genetic mutations and specific targeting techniques PLCγ2 has been shown to play a vital role, not just in platelets, but in cells such as B- and T-cells as well (204); there is also some evidence that PLCγ2 may also be important in other cell types, such as osteoclasts, and processes such as maintenance of basal bone mass (205, 206). Platelets from mice lacking PLCγ2 show a significant reduction in response to collagen, and no response to CRP or convulxin stimulation. However, these PLCγ2 deficient platelets respond normally to stimuli that do not signal via tyrosine-kinase linked signalling pathways, for example ADP and thrombin (170). These mice are also seen to exhibit defects in physiological haemostasis, as measured by a prolonged tail bleeding time, and often present with other physiological defects such as retarded growth; these mice also exhibit a degree of perinatal lethality, possibly linked to the deficits in physiological haemostasis during development (204). All of the studies performed using these transgenic mice have highlighted an extremely important role for PLCγ2, not just in platelet function, but also in critical processes such as immune system function and bone metabolism homeostasis.

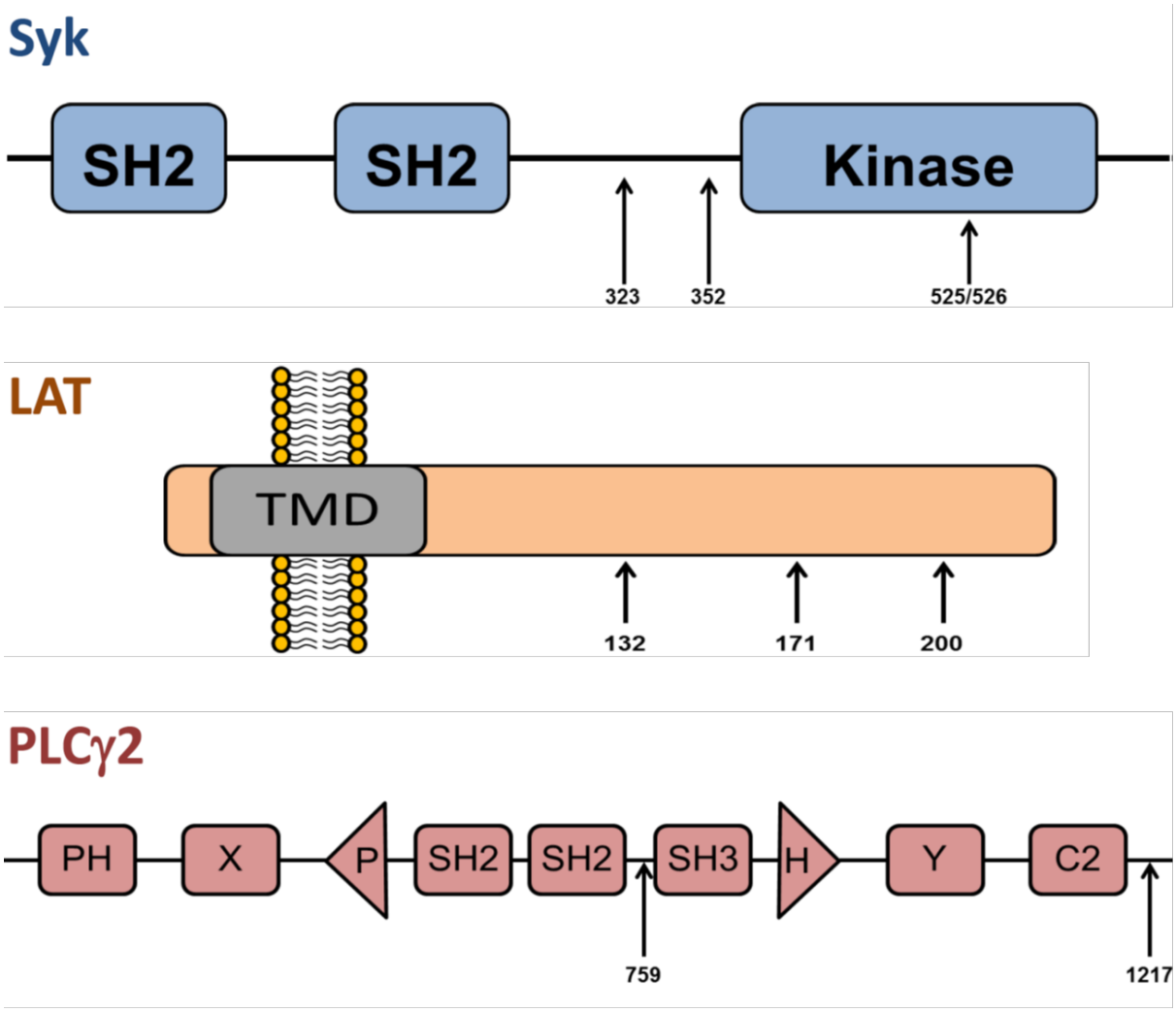


Figure 1.6 - Schematics showing the structure of Syk, LAT and PLCγ2. All domains have been listed, alongside a selection of the most important tyrosine residues involved in signal transduction.

1.4 Platelets outside of thrombosis and haemostasis

As shown above platelets play an indispensable role in the physiology and pathophysiology of haemostasis and thrombosis, however there is an increasing recognition of the role of platelets beyond these processes. Platelets have recently been shown to be important in a diverse range of processes and diseases, including inflammation, infection, immunity, cancer and development.

In cancer, platelets have been shown to play a number of different pro-cancerous roles (30, 207-210). Platelets contain large stores of pro-angiogenic factors, and the endothelium of cancerous cells is phenotypically more favourable for platelet adhesion and activation – for example increased tissue factor (TF), a potent platelet activator, has been observed in numerous types of cancer (207). This increased recruitment and activation of platelets in at cancerous sites allows local release of factors such as vascular endothelial growth factor (VEGF) which may help promote vascularisation of tumours (207).

Platelets have also been implicated in almost all stages of cancer metastasis. Platelets can come into contact with metastatic circulating tumour cells (CTCs) in the vasculature and become activated (207). Upon activation, platelets adhere to the CTCs via mechanisms such as fibrinogen-αIIbβ3 interactions and P-selectin binding. This coating of platelets is thought to protect the CTCs from destruction by immune cells through multiple mechanisms; for example by providing a mechanical 'shield' for CTCs and preventing direct contact the CTCs and cells such as natural

killer (NK) cells (207, 209, 211). Platelets may also aid CTC extravasation via a number of mechanisms including the harnessing of platelet-derived selectins, such as P-selectin (209).

The above pro-cancerous mechanisms of platelets suggest that, under certain circumstances, modulation of platelets and their reactivity may be beneficial in the treatment of certain cancers (212). Indeed, whilst there is a small level of disagreement in the literature, several meta-analyses and systematic reviews have highlighted a potential benefit of anti-platelet therapies – such as aspirin and/or clopidogrel – in both the treatment and prevention of cancer; the level of benefit does appear to depend upon the type of cancer, with the incidence of cancers such as colorectal and breast cancer appearing to react strongly to treatment with anti-platelet drugs (213-216). Whilst several of these retrospective studies have assessed clinical studies of anti-platelet therapies in cancer, the majority of these seem to have been performed with aspirin and, although the benefits seen with aspirin appear to be shared by other anti-platelet therapeutics such as clopidogrel, further clinical assessments are still required; there are currently a number of ongoing clinical studies exploring the use of anti-platelet therapies in cancer, some of which are listed on clinicaltrials.gov.

Platelets have also been shown to be important mediators of inflammation and inflammatory diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (217). The role of platelets in RA was not initially expected, however the presence of platelet-derived microparticles in the synovial fluid of RA

patients alongside the finding that depletion of platelets prevents increases in synovial endothelium permeability present the possibility that they may be important in the pathophysiology of this disease (217, 218). Furthermore, it was shown that serotonin released from platelets stimulated with intravenously injected collagen related peptide (CRP) was responsible for driving increased vascular permeability in RA patients (217-219). These findings suggest an important role for platelets in the progression of arthritic disease.

More recently, it has been shown that other cell types, particularly those involved in the immune system, can up-regulate the expression of podoplanin under certain stimuli (148, 220). One example of this is the finding that platelets can interact with agonistic molecules present on the surface of M1 polarised, inflammatory macrophages to become activated; the majority of these interactions are mediated via the podoplanin-CLEC-2 axis. One result of these interactions is an increase in the secretion of many pro-inflammatory cytokines such as TNFα and IL-23 from macrophages (221). This is particularly interesting to consider in the context of atherosclerotic plaque formation, where macrophages play a particularly important role, and where they are extremely likely to encounter platelets (222). The possibility of platelets inducing macrophages to release cytokines which will further reinforce the recruitment of M1 macrophages which then take up oxidised low-density lipoproteins (oxLDL) and become foam cells – these foam cells make up the majority of the atherosclerotic plaque (222-224). This interaction is also important as when atherosclerotic plaques rupture, they release numerous pro-thrombotic molecules, alongside exposing sub-endothelial proteins such as collagen (225); atherosclerotic

plaque rupture is a major risk factor for the development of thrombotic complications such as MI or stroke (226, 227).

Possibly the most surprising discovery was the importance of platelets throughout development, particularly in the development and separation of the blood and lymphatic circulatory systems as described above (21, 157-161). This may be one of the most novel and exciting fields of platelet research, especially when considered alongside observations such as a marked increase in the incidence rate of intraventricular haemorrhage in pre-term neonates (228).

It is of particular interest in the field that many of these novel functions of platelets outside of traditional haemostasis and thrombosis appear to be strongly mediated by the hemITAM receptors, GPVI and CLEC-2.

1.5 Aims

Whilst the proteins involved in the GPVI and CLEC-2 signalling pathways are well established; the above information highlights significant differences between both the initiation of signalling and the functional consequences following stimulation of these two receptors. This raises the possibility that there may be differences within the signalling pathways themselves which can partially account for the functional differences. Considering this, there are three main inter-related aims of this thesis:

1. Investigation of a possible adapter role for Syk, and exploration of specific tyrosine phosphorylation events on key signalling proteins, using a novel transgenic mouse model
    1.1 Hypothesis – Syk has a role as an adapter protein independent of its role as a kinase, and this adapter function can support a level of signal transduction and platelet activity in the absence of any kinase functionality
2. Exploration of neonatal platelet hyporeactivity, and determination of whether known hyporesponsiveness to collagen extends to platelet stimulation with CLEC-2 agonists
    2.1 Hypothesis – Neonatal platelets are hyporeactive to both GPVI and CLEC-2 stimulation due to an underlying, common signalling defect
3. Investigation of fibrin as a novel ligand for GPVI
    3.1 Hypothesis – Fibrin causes activation of platelets via an interaction with GPVI.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Reagents

Details of the reagents used throughout the course of this thesis are listed in the tables below. Table 1 lists the platelet agonists used, table 2 the platelet antagonists, and table 3 any antibodies and fluorescent stains used. If not otherwise stated, reagents were obtained from Sigma, or another such reputable commercial source.

Table 2.1 - Agonists

Agonist	Target/s	Source
α-CLEC-2 mAb (17D9)	CLEC-2	Bio-Rad (Kidlington, UK)
ADP	$P2Y_1$ $P2Y_{12}$	Sigma (Poole, UK)
Collagen (Horm)	GPVI α2β1	Nicomed (Linz, Austria)
Collagen Related Peptide (CRP)	GPVI	Dr R. W. Farndale (Cambridge University, UK)
Fibrin	GPVI αIIbβ3	Made in-house
Fibrinogen	GPVI αIIbβ3	Enzyme Research Laboratories (Swansea, UK)
Human PAR-4 Peptide (Sequence: AYPGKF)	PAR-4	AltaBioscience (Birmingham, UK)
Thrombin	PAR isoforms	Sigma (Poole, UK)

Table 2.2 - Antagonists

Antagonist	Target/s	Source
Dasatinib	Src BCR/Abl	Sigma (Poole, UK)
Forskolin	cAMP	Sigma (Poole, UK)
Heparin	Thrombin FXa	Sigma (Poole, UK)
PP2	Src	Sigma (Poole, UK)
PRT-060318	Syk	Portola Pharmaceuticals Inc (San Francisco, CA)
Prostacyclin (PGI_2)	PGI_2 receptor	Cayman Chemicals (Cambridge, UK)

Table 2.3 - Antibodies and fluorescent stains

Antibody/Stain	Target/s	Working Concentration	Source
α-βTubulin	Beta Tubulin	10µg/ml	Sigma (Poole, UK)
α-CLEC-2*FITC	CLEC-2	3 µg/ml	AbD Serotec (Kidlington, UK)
α-CLEC-2 (17D9)	CLEC-2	1µg/ml	Bio-Rad (Kidlington, UK)
Fibrinogen*Alexa 488	Fibrinogen binding (activated aIIbb3)	50µg/ml	Life Technologies (Madrid, Spain)
α-CD42b-FITC	GP1bα	1/30 dil (Cat No. M042-1)	Emfret (Eibelstadt, Germany)
α-GP1bα	GP1bα (Immune Depletion)	1.5µg/g	Emfret (Eibelstadt, Germany)
α-GPVI	GPVI	1µg/ml	Dr E. Gardiner (Canberra, Australia)
α-GPVI*FITC	GPVI	1/30 dil (Cat No. M011-1)	Emfret (Eibelstadt, Germany)
α-IgG*FITC	IgG	1/30 dil (Cat No. P190-1)	Emfret (Eibelstadt, Germany)
α-IgG	IgG (Immune Depletion Control)	1.5 µg/g	Emfret (Eibelstadt, Germany)
α-IgG2b*FITC	IgG2b	1/30 dil (Cat No. STAR134)	Bio-Rad (Kidlington, UK)
α-LAT Y171	LAT Y171 (human no.)	1/1000 dil (Cat No. 3581)	Cell Signalling Technology (Leiden, The Netherlands)
α-LAT Y191	LAT Y191 (human no.)	1/1000 dil (Cat No. 3548)	Cell Signalling Technology (Leiden, The Netherlands)
α-LAT Y132	LAT Y132 (human no.)	1/1000 dil (Cat No. ab4476)	Abcam (Cambridge, UK)
α-LAT Y200	LAT Y200 (human no.)	1/1000 dil (Cat No. ab68139)	Abcam (Cambridge, UK)
α-Phosphotyrosine (4G10)	Phosphotyrosine Residues	1/1000 dil (Cat No. 05-321)	Millipore (Buckinghamshire, UK)
α-PLCγ2 (B-10, mouse mAb)	PLCγ2	1/1000 dil (Cat No. sc-5283)	Santa Cruz (Heidelberg, Germany)
α-PLCγ2 (Q-20, rabbit mAb)	PLCγ2	1/1000 dil (Cat No. sc-407)	Santa Cruz (Heidelberg, Germany)

α-PLCγ2 Y1217	PLCγ2 Y1217 (human no.)	1/1000 dil (Cat No. 3871)	Cell Signalling Technology (Leiden, The Netherlands)
α-PLCγ2 Y759	PLCγ2 Y759 (human no.)	1/1000 dil (Cat No. 3874)	Cell Signalling Technology (Leiden, The Netherlands)
α-CD62p*PE	P-Selectin	50µg/ml	BD Biosciences (Madrid, Spain)
α-Rat*488 (2° antibody)	Rat IgG	20µg/ml	Fischer Scientific (Loughborough, UK)
α-Syk (4D10)	Syk	1/1000 dil (Cat No. sc-1240)	Santa Cruz (Heidelberg, Germany)
α-Syk Y323	Syk Y323 (human no.)	1/1000 dil (Cat No. 2715)	Cell Signalling Technology (Leiden, The Netherlands)
α-Syk Y352	Syk Y352 (human no.)	1/1000 dil (Cat No. 2701)	Cell Signalling Technology (Leiden, The Netherlands)
α-Syk Y525/526	Syk Y525/526 (human no.)	1/1000 dil (Cat No. 2711)	Cell Signalling Technology (Leiden, The Netherlands)
α-CD49b*FITC	α2β1	1/5 dil (Cat No. M070-1)	Emfret (Eibelstadt, Germany)
α-CD41a*PE	αIIbβ3	1/30 dil (Cat No. 561850)	BD Pharmigen (Oxford, UK)
α-CD41a*APC	αIIbβ3	1/120 dil (Cat No. 17-0411-82)	eBioscience (Hatfield, UK)
α-CD41/61-FITC	αIIbβ3	1/30 dil (Cat No. M025-1)	Emfret (Eibelstadt, Germany)

2.1.2 Mice

Syk K396R ($Syk^{K396R,fl/fl}$) mice were generated by and obtained from Taconic Biosciences (Cologne, Germany), using the targeting strategy outlined in figure 2.1. Syk KD mice were then crossed with PF4-Cre expressing mice (generated as previously described). Syk K396R; PF4-Cre ($Syk^{K396R,fl,fl;PF4\text{-}Cre}$) mice were bred as heterozygotes to allow use of litter matched wild type controls. Syk K396R radiation chimeric mice were generated as previously described (136); briefly, foetal liver cells were isolated from Syk K396R embryos and transplanted via tail vein injection into irradiated wild type C57Bl/6 mice. Wild type C57Bl/6 mice were obtained from Harlan laboratories (Envigo, UK). For neonatal mice studies, wild type mice underwent timed-matings to allow exact gestational age determination. Mice were obtained and assessed from embryonic day (E)17.5 through to post-natal day (P)14.5.

2.2 Methods

2.2.1 Human Blood Collection and Platelet Preparation

Blood from informed and consenting, drug-free adults was obtained on the day of experimentation by venepuncture into an anticoagulant solution of 10% (w/v) sodium citrate; once blood was obtained, 10% (w/v) acid citrate dextrose (ACD) was added to provide additional anticoagulation (ACD: 120mM sodium citrate, 110mM glucose, and 80mM citric acid). Consent complied with all local guidelines both in the UK and Chile and the guidelines set out in the Declaration of Helsinki. Washed platelets were obtained from whole blood samples via multiple centrifugation steps.

Firstly, whole blood was centrifuged for 20 minutes at 200g and 37°C to separate the platelet rich plasma (PRP) and platelet poor plasma (PPP); PRP was retained and PPP was discarded. PRP was then incubated with prostacyclin (10 µg/ml) to prevent unwanted platelet activation, before centrifugation for 10 minutes at 1000g and 37°C, causing deposition of a platelet pellet. Residual plasma was then discarded before the pellet was washed and resuspended in 3mls of ACD and 25mls of modified Tyrode's solution (134mM NaCl, 2.9mM KCl, 0.34mM Na_2HPO_4, 12mM $NaHCO_3$ 1mM $MgCl_2$, 5mM glucose, and 20mM HEPES; pH adjusted to 7.3). Once resuspended, prostacyclin (10 µg/ml) was again added before centrifugation was repeated for 10 mins at 1000g and 37°C. Platelet pellet was resuspended in modified Tyrode's buffer at a concentration of $2x10^7$ /ml for spreading analysis, $2x10^8$/ml for platelet aggregation assays and $4x10^7$ – $1x10^9$/ml for biochemical assays. Once resuspended, platelets were left for a minimum of 30 mins before experimental use to allow the effects of prostacyclin to wear off.

2.2.2 Murine Blood Collection and Platelet Preparation

Blood samples were obtained from mice via several different methods. For embryonic mice, pregnant females were culled at known gestational age via cervical dislocation and embryos were dissected out. Once isolated, embryos were decapitated and allowed to bleed into 10Units/ml of heparin in phosphate buffered saline (PBS). For post-natal juvenile mice and adult mice used as controls in the experiments exploring GPVI and CLEC-2 reactivity throughout development, mice were first culled via intraperitoneal (IP) injection of 50-100µl of Euthatal. Immediately

following confirmation of death, mice were decapitated and allowed to bleed into 10Units/ml heparin in PBS.

For all other terminal adult mice experiments, initial administration of an isoflurane/oxygen mix (5% (v/v) isoflurane, ~1.5 l/min O_2) was performed to anaesthetise the mouse prior to CO_2 narcosis (CO_2; 3-5 l/min). Once heartbeat cessation was observed, the peritoneum was opened, and the descending vena cava was isolated. Once isolated, the descending vena cava was punctured using a needle containing 100µl of ACD at 37°C and blood was withdrawn; once blood had been withdrawn, cervical dislocation was performed to confirm death. Blood was then diluted into 200µl of modified Tyrode's buffer before centrifugation in a benchtop centrifuge for 5 mins at 200g and room temperature. Once spun, the PRP and top 1/3 of the red blood cell (RBC) fraction were transferred to a fresh Eppendorf and centrifuged again for 6 mins at 200g and 37°C. Once spun, the separated PRP was transferred to a separate Eppendorf and then another 200µl of modified Tyrode's buffer was added to the remaining plasma and red blood cell mix, which was spun again for 6 mins at 200g and 37°C; this was performed to maximise the total platelet yield. Once this final spin was performed, the two sets of PRP were combined and spun in the presence of prostacyclin (10µg/ml) for 6 minutes at 1000g and 37°C to pellet the platelets. Platelet pellet was resuspended in modified Tyrode's buffer at a concentration of $2x10^7$ /ml for spreading analysis, $2x10^8$/ml for platelet aggregation assays and $4x10^7$ – $1x10^9$/ml for biochemical assays. Once resuspended, platelets were left for a minimum of 30 mins before experimental use to allow the effects of prostacyclin to wear off.

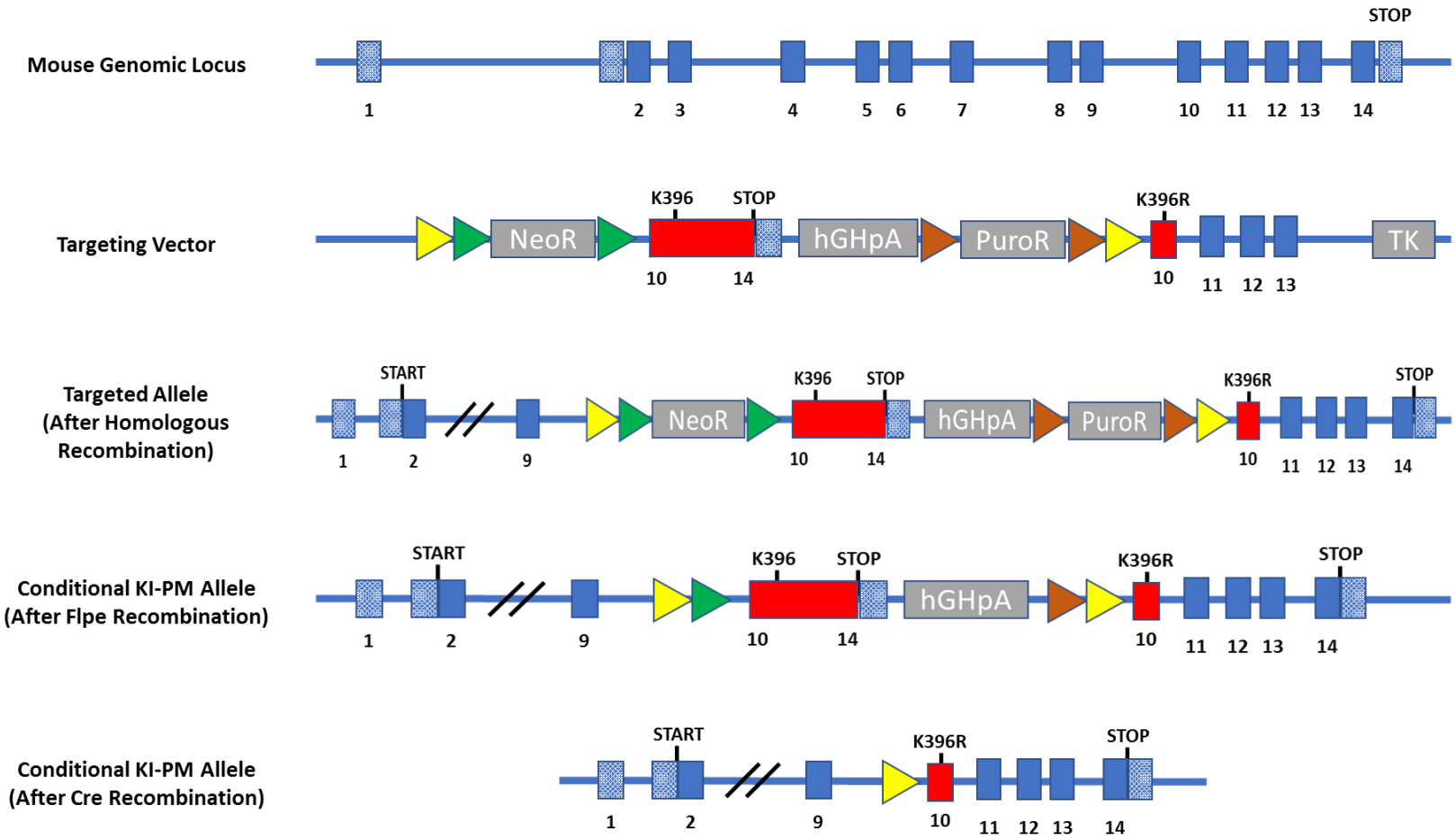


Figure 2.1 - Targeting strategy for generation of Syk K396R mice. A point mutation was introduced to induce an amino acid substitution in the kinase domain of Syk – the mutation resulted in a lysine to arginine substitution at site 396. The introduced allele was designed to allow expression of full length, wild-type Syk until mice were crossed with a Cre expressing mouse strain. Upon co-expression of the $Syk^{K396R,fl/fl}$ transgene and a Cre – such as a PF4-Cre transgene – the Syk point-mutated protein is expressed. The point mutation in Syk induces a lysine to arginine substitution, maintaining the overall structure of Syk but rendering it catalytically inactive.

2.3 Platelet Function Assays

2.3.1 Platelet Aggregometry

All platelet aggregometry experiments were performed in a dual-channel Born lumi-aggregometer (Model No. 460VS; Chronolog, Labmedics, Manchester, UK). Platelet aggregation was assessed in glass aggregometer cuvettes under stirring conditions (1200rpm) at 37°C; changes in optical density correlating to the magnitude of platelet aggregation were measured against a blank sample of modified Tyrode's buffer. Platelets were added to the glass cuvettes and allowed to rest for at least 1 minute before being transferred to the heating chamber within the aggregometer for 1 minute and finally into the heated, stirring assay chamber for 1 minute prior to the addition of any platelet stimuli. Changes in optical density were recorded on a manual chart recorder (Chronolog, Labmedics, Manchester, UK) for a minimum of five min post-stimulus addition. When the impact of platelet inhibitors or modulators of platelet aggregation was also being assessed, these inhibitors were allowed to incubate with the platelets inside the glass cuvettes for a minimum of 5 min prior to warming.

2.3.2 Platelet Biochemical Sample Generation

All platelet biochemical samples were generated in a dual-channel Born lumi-aggregometer as described above. However, prior to the warming and stirring of the cuvettes, platelets were incubated with the αIIbβ3 inhibitor, Integrilin (also known as

eptifibatide; 9μM) to prevent platelet aggregation from occurring. Platelets were then rested, warmed and stirred as above before the addition of a platelet stimuli. Stimulation was allowed to continue for a set time period before reactions were terminated with the addition of either ice-cold lysis buffer (2x: 300mM NaCl, 20mM Tris, 2mM EGTA, 2mM EDTA, 2% (v/v) IGEPAL CA-630, pH 7.4, plus the addition on the day of experimentation of 5μg/ml leupeptin, 5μg/ml aprontinin, 0.5 μg/ml pepstatin, 2.5mM Na_3VO_4, and 100μg/ml AEBSF) or Laemmli sample buffer (2x non-reducing: 20% (v/v) glycerol, 4% (w/v) SDS, 50mM Tris, and Brilliant Blue R to required colour; 2x Reducing: 20% (v/v) glycerol, 4% (w/v) SDS, 50mM Tris, 10% (v/v) β-mercaptoethanol, and Brilliant Blue R to required colour); 5x Laemmli sample buffers were also used for some experiments and made up following the same proportions as for the 2x solutions above. Lysis buffer was utilised for samples undergoing subsequent immunoprecipitation steps, whereas Laemmli sample buffer was used for samples used for direct whole cell protein analysis. Immediately following cell lysis, samples were stored on ice before being transferred to -20°C storage prior to use.

2.3.3 Platelet Spreading

All platelet spreading experiments were performed on coated glass coverslips contained within 24 well cell culture plates. Prior to the platelet spreading experiment, glass coverslips were coated overnight at 4°C with one of the following platelet agonists: collagen (100μg/ml); fibrinogen (100μg/ml); fibrinogen + thrombin (100μg/ml; 30 mins + 1Unit/ml); or fibrin (100μg/ml fibrinogen; 30 mins + 1Unit/ml thrombin; 30 mins + 20μM PPACK to neutralise residual thrombin). Once coated,

residual agonist solution was removed before coverslips were washed three times with PBS. Non-specific binding sites were then blocked via the addition of 5mg/ml of heat-denatures bovine serum albumin (BSA) for 1 hour at room temperature. Following three more PBS washes, platelets were added to the glass coverslips and incubated at 37°C and allowed to spread for 30 mins (human) or 45 mins (mice); if inhibitors or modulators of platelet function were used, these were incubated with the platelet suspension for a minimum of 10 minutes prior to their addition to the coverslips. Following their incubation period, coverslips were again washed three times with PBS before being fixed by the addition of paraformaldehyde (PFA; 3.7% (w/v)) for 10 minutes. For fluorescent actin staining, coverslips were then washed again before platelets were permeabilised via the addition of 0.1% (v/v) Triton X-100 for 5 minutes, followed by the addition of Alexa-488-phalloidin for 45 mins in the dark. Once stained, coverslips underwent a final wash step prior to mounting on glass microscope slides. Coverslips were imaged on a Zeiss Axiovert 200M microscope, and analysis of platelet spreading was performed using ImageJ (National Institutes of Health, Bethesda, MD); in each independent experiment, five images taken randomly throughout the coverslip were analysed (minimum 100 platelets per condition per experiment.)

2.3.4 Clot Retraction

Clot retraction experiments were performed in isolated PRP. PRP was transferred into an Eppendorf and 5-10µl of RBCs were added back into the PRP to allow for better resolution of the developing clot. To begin the clot retraction experiment, PRP

+ RBC were incubated with 2mM $CaCl_2$, 2mg/ml fibrinogen and 10Units/ml of thrombin. Images were taken at thirty-minute intervals for an hour and a half to document clot formation. Once clots had formed, they were washed in three changes of sodium cacodylate, fixed for 2 hours in 2% (w/v) glutaraldehyde, washed three more times in sodium cacodylate, and then dehydrated using increasing percentage acetone solutions in preparation for electron microscopy analysis. Electron microscopy was kindly performed by Robert Ariens from the University of Leeds.

2.3.5 Flow Cytometry

Flow cytometry was used to assess platelet surface receptor expression and platelet reactivity. All flow cytometry experiments were performed on a BD Accuri C6 flow cytometer (BD, Ann Arbor, Mi, USA). To assess platelet surface receptors, whole blood samples were diluted to give a concentration of ~10-20x10^9 platelets/L, before being dual stained for 30 mins under static, dark conditions at room temperature with an α-CD41*APC antibody – used to determine the platelet cell population – alongside a FITC conjugated antibody against the receptor of interest. After 30 mins reactions were terminated by the addition of a 4% (w/v) paraformaldehyde solution, followed by a 1/10 dilution of the sample with PBS. Once diluted, samples were then acquired on the flow cytometer.

To assess platelet reactivity, whole blood samples were diluted to give a concentration of ~10-20x10^9 platelets/L, before being triple-stained with an α-CD41*APC antibody, alongside an a-CD62*PE antibody and fibrinogen*Alexa 488.

Once antibodies and fluorescent fibrinogen had been mixed with the whole blood samples, this reaction mix was mixed 1:1 with 2x concentrated agonists and incubated at room temperature for 30 mins under static, dark conditions; PBS was used as a negative stimulation control in all reactivity experiments. After thirty minutes, reactions were terminated by the addition of a 4% (w/v) paraformaldehyde solution, followed by a 1/10 dilution of the sample with PBS. Once diluted, samples were then acquired on the flow cytometer. When run on the flow cytometer, 10,000 platelet positive events were acquired and analysed for each sample; platelet positivity was determined via CD41+ staining alongside gating on cell size.

2.4 Biochemical Analysis

2.4.1 Immunoprecipitation

Platelet lysates made using lysis buffer were defrosted before being pre-cleared by incubation with either Protein A or Protein G Sepharose beads (Pierce, Rockfield, IL) – dependent upon the species that the antibody of choice was raised in – under rolling conditions at 4°C for 1 hour; this was performed to reduce non-specific binding events in subsequent steps. After 1 hour, pre-clearing samples were spun in a benchtop centrifuge to pellet the beads and any non-specifically bound materials. Once pelleted, the supernatant was removed and transferred to a new Eppendorf, where the cleared supernatant was incubated with the specific antibody of choice and corresponding Sepharose beads at 4°C overnight with stirring. Following overnight incubation, samples were again spun to pellet the beads, before the remaining cleared supernatant was removed and stored for subsequent IP experiments. The pelleted beads were washed three times in lysis buffer, before

being eluted via addition of Laemmli sample buffer and subsequent boiling for 5 minutes at 100°C. Once boiled, samples were analysed via SDS-PAGE and western blotting analysis.

2.4.2 SDS-PAGE and Western Blotting Analysis

Platelet lysates or immunoprecipitated samples in sample buffer were boiled for 5 mins before being centrifuged at 8600g for 5 mins to pellet any insoluble cellular debris. Once centrifuged, samples were run on a pre-cast, gradient Bolt gel (4-12%) at 175V for 30-35 mins; alongside samples, a pre-stained molecular weight marker ladder was run to aid in determination of proteins of interest. Samples were separated via gel electrophoresis before samples were transferred from the gel to a polyvinylidene difluoride (PVDF) membrane using the trans-blot turbo electro-transfer system. Once proteins were transferred, membranes were blocked for one hour at room temperature in 5% (w/v) BSA dissolved in Tris-buffered saline with tween (TBS-T; 200mM Tris, 1.37M NaCl, 0.2% (v/v) Tween20, pH 7.6); 0.1% (w/v) sodium azide was added to the 5% (w/v) BSA in TBS-T to prevent bacterial contamination. Once blocked, BSA was removed from the membranes, which were then incubated overnight at 4°C under mixing conditions with primary antibodies diluted in 5% (w/v) BSA in TBS-T.

Once incubated overnight, membranes were washed three times for five minutes each in TBS-T. Once washed, membranes were then incubated with a secondary antibody specific to the species of the primary antibody, which was conjugated to a horseradish peroxidase enzyme; secondary antibodies were diluted in TBS-T and

membranes were incubated for 1 hour under mixing conditions at room temperature. After 1 hour, membranes were again washed three times for five minutes each in TBS-T, before being incubated with an enhanced chemiluminescence reagent (ECL, ThermoScientific, Paisley, UK) for a minimum of 1 minute before analysis. Membranes were either analysed via exposure to autoradiographic film or via the LiCor Odyssey-FC imaging system (LiCor, Cambridge, UK); autoradiographic film was used for generation of representative western blots and the LiCor system was used for protein band quantification.

Once imaged, membranes were then stripped of antibody via incubation with a stripping buffer (TBS-T + 2% (w/v) SDS), to which 1% (v/v) β-mercaptoethanol was added, before being heated to 80°C for 20 mins. After 20 mins, membranes were rinsed in TBS-T briefly, before stripping buffer without β-mercaptoethanol was added and heated to 80°C for 20 mins. Once heated for a final 20 mins, membranes were rinsed repeatedly in TBS-T before being re-blocked in 5% (w/v) BSA in TBS-T as above. Once blocked, the primary, secondary and ECL process was repeated utilising a loading control antibody, such as an antibody against βtubulin.

2.5 Statistical Analysis

All statistical analysis was performed using GraphPad Prism software (GraphPad, California, USA, various versions). Where graphically expressed, results are shown at arithmetic mean ± standard error of the mean, unless specifically stated otherwise. For parametric analysis, Student's t-test was performed when comparing between two groups, and an ANOVA (one- or two-way) with appropriate post-hoc

multiple comparisons test was selected for experiments with three or more groups of comparison. For non-parametric analysis, a Mann-Whitney U-test was performed when comparing between two groups, and a Kruskall-Wallis test with appropriate post-hoc multiple comparisons test was selected for experiments with three or more groups. Statistical significance was assumed with $p \leq 0.05$.

Chapter 3

Characterisation of a novel Syk kinase-dead mouse model

3.1 Introduction

As discussed in the introduction to this thesis, Syk is one of the most important proteins within the (hem)ITAM signalling pathway. It has been shown numerous times that mice genetically modified to have a complete loss of the Syk protein – or expression of a dysfunctional form of the protein – throughout all cell types display an embryonically lethal phenotype, possibly due to a failure of the lungs to inflate after birth (159, 229). These mice often display a number of severe physiological issues throughout development, such as defects in brain cerebrovasculture and lymphatic vascular formation (159, 161). It has also been shown that mice specifically lacking Syk, or again expressing a defective version of protein, specifically in the platelet/megakaryocytic cell lineage also display severe defects in the development of the brain and lymphatic vascular systems. Alongside the whole organism physiological defects, a dramatic impairment in the responses elicited following engagement of (hem)ITAM receptors on the platelet cell surface is also observed (136, 159). Interestingly, there is only a partial embryonic lethality phenotype observed in the platelet/megakaryocytic specific Syk deficient mice (136, 159).

Previous work from our group initially helped to identify that Syk was required for platelet (hem)ITAM function, and that it was platelets which appeared to be mediating the defects in lymphatic and brain vascular development – specifically via signalling through the CLEC-2 receptor (159). Building on these initial studies, work was recently undertaken to further explore the role that Syk and its regulation plays in platelet (hem)ITAM signalling and its functional consequences. Hughes *et al* fully

characterised a novel mouse model with a point mutation in the N-terminal SH2 domain of Syk, an important regulatory domain required for recruitment of Syk to phosphorylated tyrosine residues contained within proteins at the plasma membrane (136). The results from this characterisation showed that, even though Syk could still function as a kinase, without recruitment to the membrane signal transduction was abolished through the (hem)ITAM pathways. This was the first time that translocation of Syk to the plasma membrane was shown to be required for (hem)ITAM mediated platelet signalling, and the data also suggests that recruitment to the membrane appears to require functional forms of both SH2 domains (136).

Whilst the work discussed above has shown that the membrane localisation of Syk is integral for functional signal transduction – possibly due to its ability to act as a molecular scaffold following auto- and trans-phosphorylation events (180) – little work has been performed outside of cell lines to characterise how important the kinase domain of Syk is for signal transduction. It was hypothesised that the functionality of Syk may be mediated two-fold – via its kinase function and also via its adapter function – and that these two functions may be able to operate independently. To test this hypothesis, a novel mouse model was generated which carried a lysine-to-arginine substitution within the kinase domain of Syk (K396R), which has previously been shown to render kinases catalytically inactive due to the importance of this lysine residue in the phospho-transfer reaction; the Syk specific K396R mutation has also been used to study the effects of Syk kinase activity, mainly in cell lines (230-232). This mutant form of Syk was selectively expressed in

the platelet/megakaryocyte lineage and then the mice were fully characterised. The aims of this mouse model were to uncouple those effects of Syk mediated via its kinase function and those mediated by its adaptor capability. To further confirm that any functional differences in the Syk K396R mice were related to its adaptor function, Syk deficient ($Syk^{-/-}$) chimeric mice were generated and assessed as a negative control.

3.2 Results

3.2.1 Generation of a conditional Syk kinase-dead mouse model (Syk K396R)

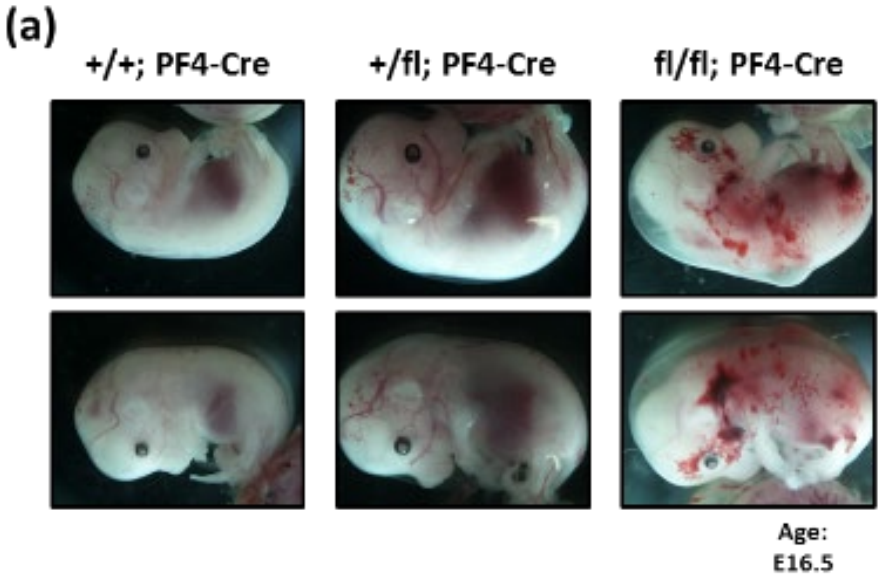
Mice were generated by Taconic, using the targeting strategy shown in figure 2.1 (Chapter 2), on a C57Bl/6 background. Once generated, $Syk^{K396R\ fl/fl}$ mice were crossed with PF4-Cre expressing mice, also on a C57Bl/6 background. Mendelian breeding ratios for all breeding strategies are detailed in table 3.1. Briefly, $Syk^{K396R\ fl/fl;PF4\text{-}Cre+}$ mice did not breed to calculated Mendelian ratios, and showed a very high incidence of embryonic lethality; litter sizes were consequently much smaller than expected. To counteract this, foetal liver cells were harvested from $Syk^{K396R\ fl/fl;PF4\text{-}Cre+}$ embryos and these cells were infused into bone marrow ablated adult mice to generate radiation chimeras. All subsequent assessments, unless otherwise stated, were conducted in the radiation chimeric mice.

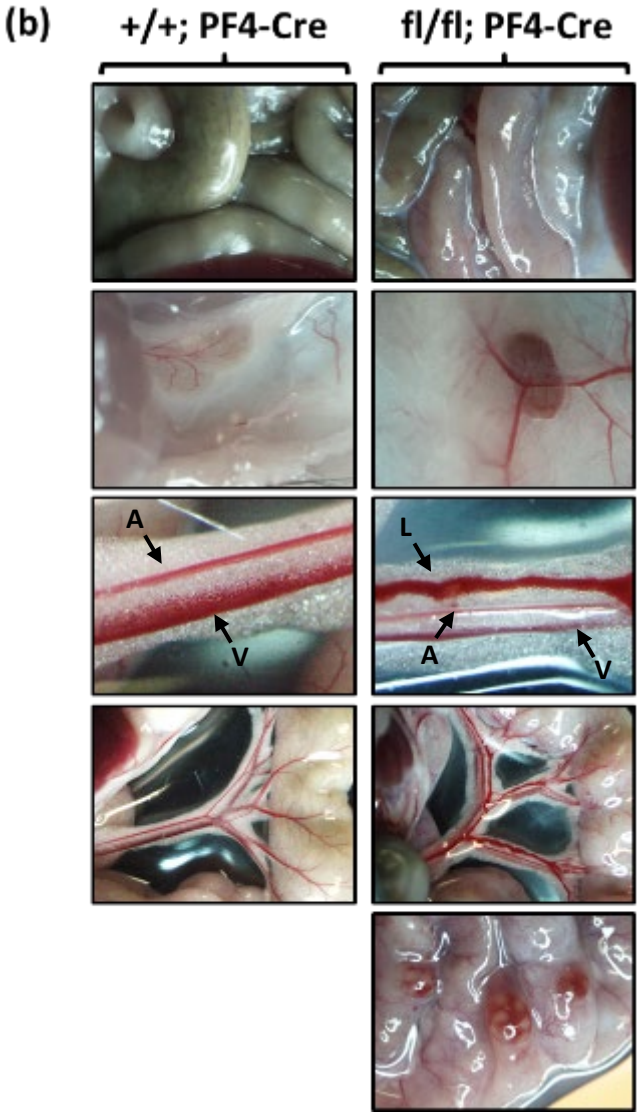
Table 3.1 – Mendelian breeding frequencies for all Syk^{k396R} breeding strategies. Syk^{K396R} mice were bred using two main strategies: +/fl; PF4-Cre x +/fl; PF4-Cre, and fl/fl x +/fl; PF4-Cre.Chi^2 analysis was performed to determine any differences between expected and observed breeding frequencies, p≤0.005.

	Genotype							
Breeding Strategy	**+/+**	**+/+; PF4-Cre**	**fl/+**	**fl/+; PF4-Cre**	**fl/fl**	**fl/fl; PF4-Cre**	**Total**	**Chi^2**
+/fl; PF4-Cre x +/fl; PF4-Cre								
Expected Frequency	6.25%	18.75%	12.50%	37.50%	6.25%	18.75%	100%	***
Expected Count	5.5625	16.6875	11.125	33.375	5.5625	16.6875	89	
Actual Count	10	26	12	35	4	2	89	
Actual Frequency	11%	29%	13%	39%	4%	2%	100%	
fl/fl x +/fl PF4-Cre								
Expected Frequency	0%	0%	25%	25%	25%	25%	100%	NS, n too small
Expected Count	0	0	2.25	2.25	2.25	2.25	9	
Actual Count	0	0	4	1	4	0	9	
Actual Frequency	0%	0%	44%	11%	44%	0%	100%	

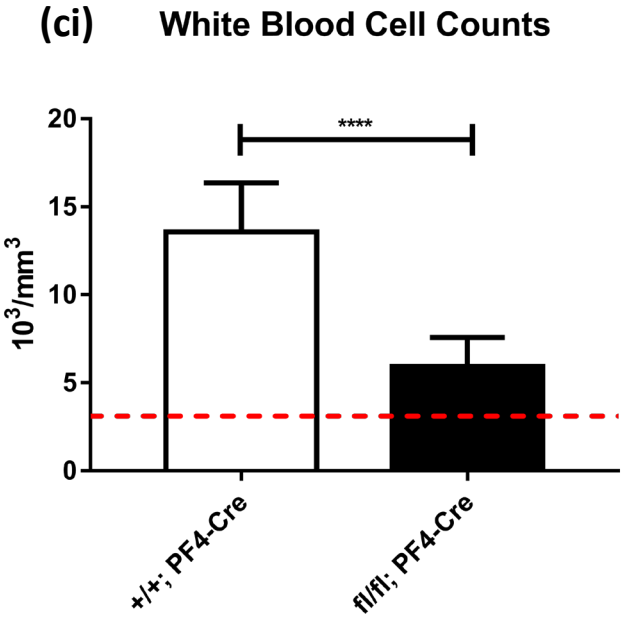
3.2.2 Syk K396R mice display developmental, lymphatic and haematological defects

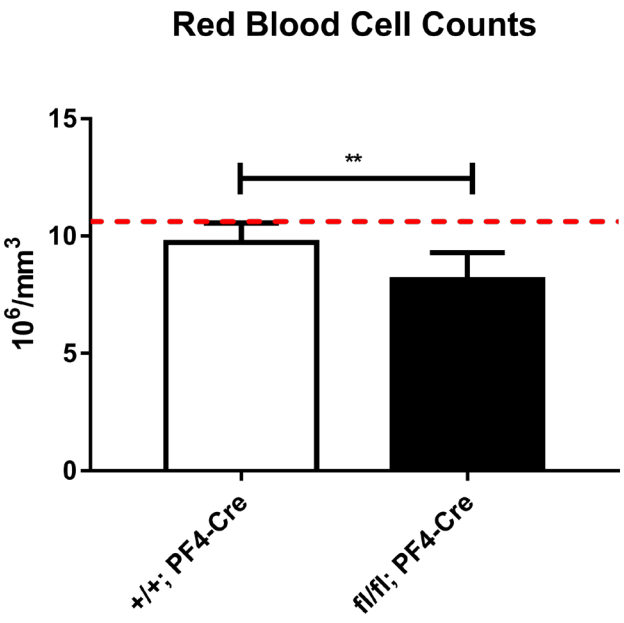
$Syk^{K396R\ fl/fl;\ PF4\text{-}Cre+}$ embryos assessed at embryonic day (E)16.5 displayed the hallmark phenotypes of mice deficient in Syk and/or CLEC-2 within the platelet/megakaryocytic lineage. Mice displayed significant haemorrhaging and blood spotting throughout the embryo alongside a large level of observable oedema, particularly surrounding the spine. The typical defects in lymphatic and blood vascular separation could also be seen in these embryos (figure 3.1a). In the irradiated mice infused with Syk K396R foetal liver cells, once the bone marrow was reconstituted mice began to display lymphatic defects. Chylous ascites was observable in these mice, alongside accumulation of red blood cells within both their mesenteric lymph vessels and lymph nodes (figure 3.1b). Mice also presented with raised, blood filled Peyer's patches throughout their intestines (figure 3.1b); Peyer's patches are secondary lymphatic organs involved in immune monitoring and are found throughout the ileum. Lastly, reconstituted mice displayed a significant drop in white and red blood cell counts (figure 3.1ci), a mild but significant increase in mean platelet volume (figure 3.3ci), and a significant change in immune system composition when compared with wild-type controls (figure 3.1cii). Interestingly, no changes in platelet count were observed between Syk K396R mice and wild-type controls (figure 3.1ci); red lines denote non-irradiated C57Bl/6 averages (233).

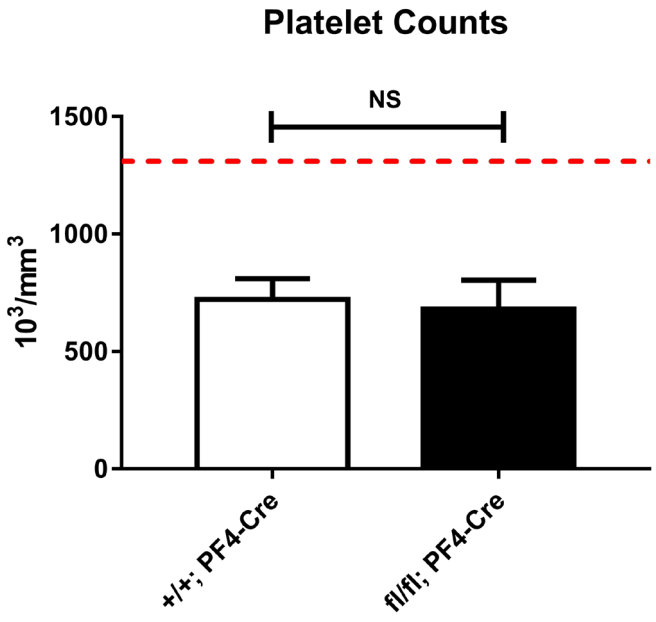

(a)
+/+; PF4-Cre
+/fl; PF4-Cre
fl/fl; PF4-Cre
Age:
E16.5

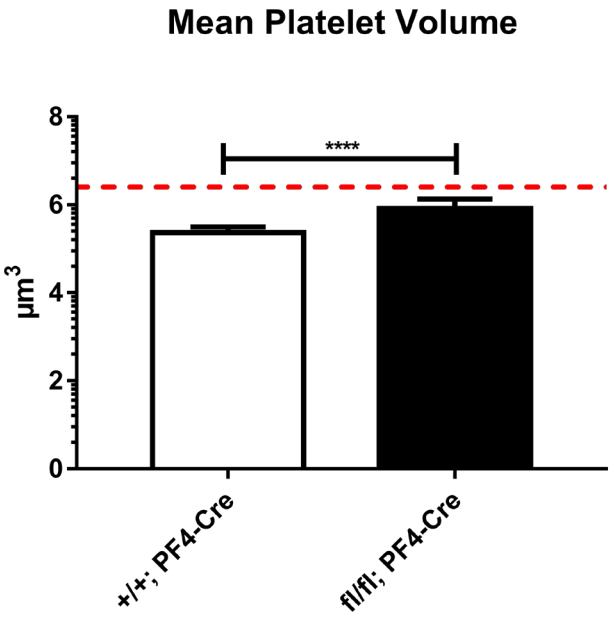

(b)
+/+; PF4-Cre
fl/fl; PF4-Cre
A
V
L
A
V

(ci)


White Blood Cell Counts
****
20
15
10
5
0
10^3/mm^3
+/+; PF4-Cre
fl/fl; PF4-Cre


Red Blood Cell Counts
**
15
10
5
0
10^6/mm^3
+/+; PF4-Cre
fl/fl; PF4-Cre


Platelet Counts
NS
1500
1000
500
0
10^3/mm^3
+/+; PF4-Cre
fl/fl; PF4-Cre


Mean Platelet Volume
****
8
6
4
2
0
µm^3
+/+; PF4-Cre
fl/fl; PF4-Cre

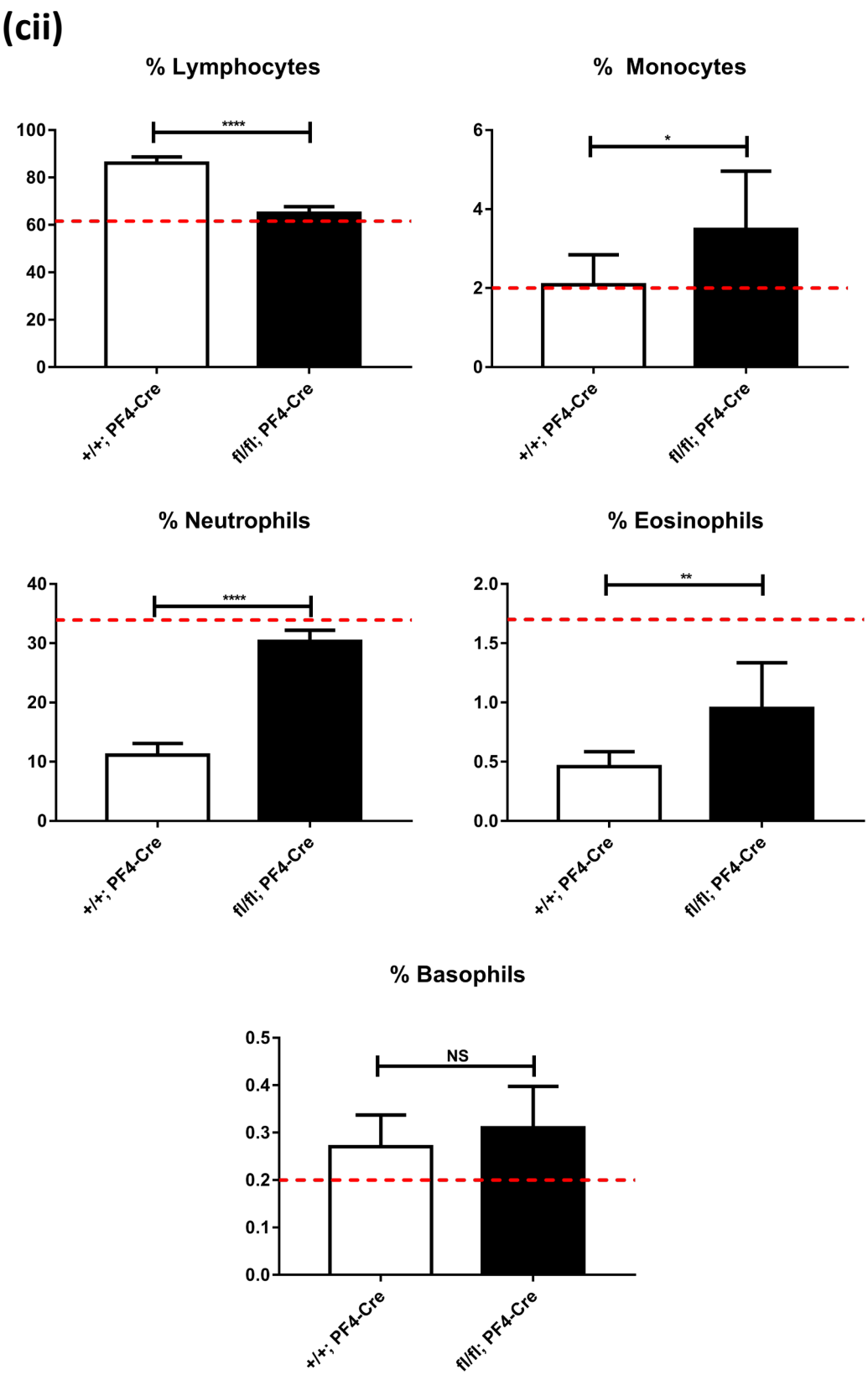
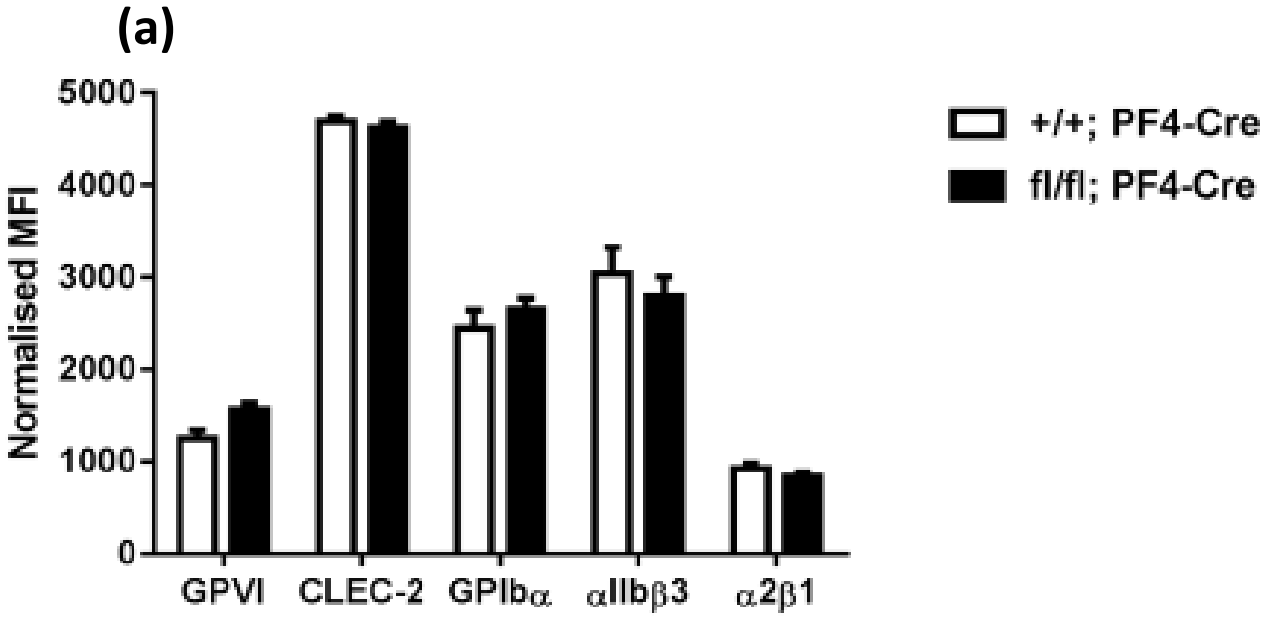


Figure 3.1 – Significant developmental, physiological and haematological defects are observed in Syk K396R embryos and irradiated/reconstituted chimeric adult mice. (a) Embryonic day (E) 16.5 mice were dissected and imaged; tail clippings were taken for genotyping (N≥3). (b) Adult Syk$^{K396Rfl/fl;PF4\text{-}Cre}$ and Syk$^{+/+;PF4\text{-}Cre}$ radiation chimeric mice were dissected and images were taken of the peritoneum, axillary lymph nodes, mesenteric vasculature and Peyer's patches; A = artery, V = vein, L = lymphatic vessel (N≥3). (c) White blood cell, red blood cell and platelet counts were assessed in whole blood samples, alongside mean platelet volume, using an ABX Pentra blood analyser (Horiba, Northampton, UK); white blood cell percentages were also measured; red lines denote non-irradiated C57Bl/6 averages (N=7). For blood counts, mean values are presented + standard deviation, differences were assessed using Student's t-test. *p ≤ 0.05, **p ≤ 0.01, *** p ≤ 0.005, ****p ≤ 0.001

3.2.3 Syk K396R mice express (hem)ITAM receptors and signalling proteins at the same level as wild-type controls

To ensure that all results observed were due to the loss of kinase activity of Syk, and not due to defects in (hem)ITAM receptor expression or trafficking, or in the expression of other key signalling molecules such as PLCγ2, receptor and signalling molecule expression profiles were assessed. Surface expression of GPVI, CLEC-2, GP1bα, α2bβ3 and α2β1 was not different between Syk K396R mice and wild-type controls (figure 3.2a). Similarly, the total cell expression levels of GPVI and CLEC-2 were the same in Syk K396R mice as in wild-type controls. Expression of Syk, LAT and PLCγ2 proteins were also consistent between Syk K396R and wild-type control animals (figure 3.2b). Overall, no differences were observed in receptor expression – both total and at the cell surface – or the expression of a number of key signalling molecules in Syk K396R mice.


(a)
Normalised MFI
5000
4000
3000
2000
1000
0
GPVI
CLEC-2
GPIbα
αIIbβ3
α2β1
+/+; PF4-Cre
fl/fl; PF4-Cre

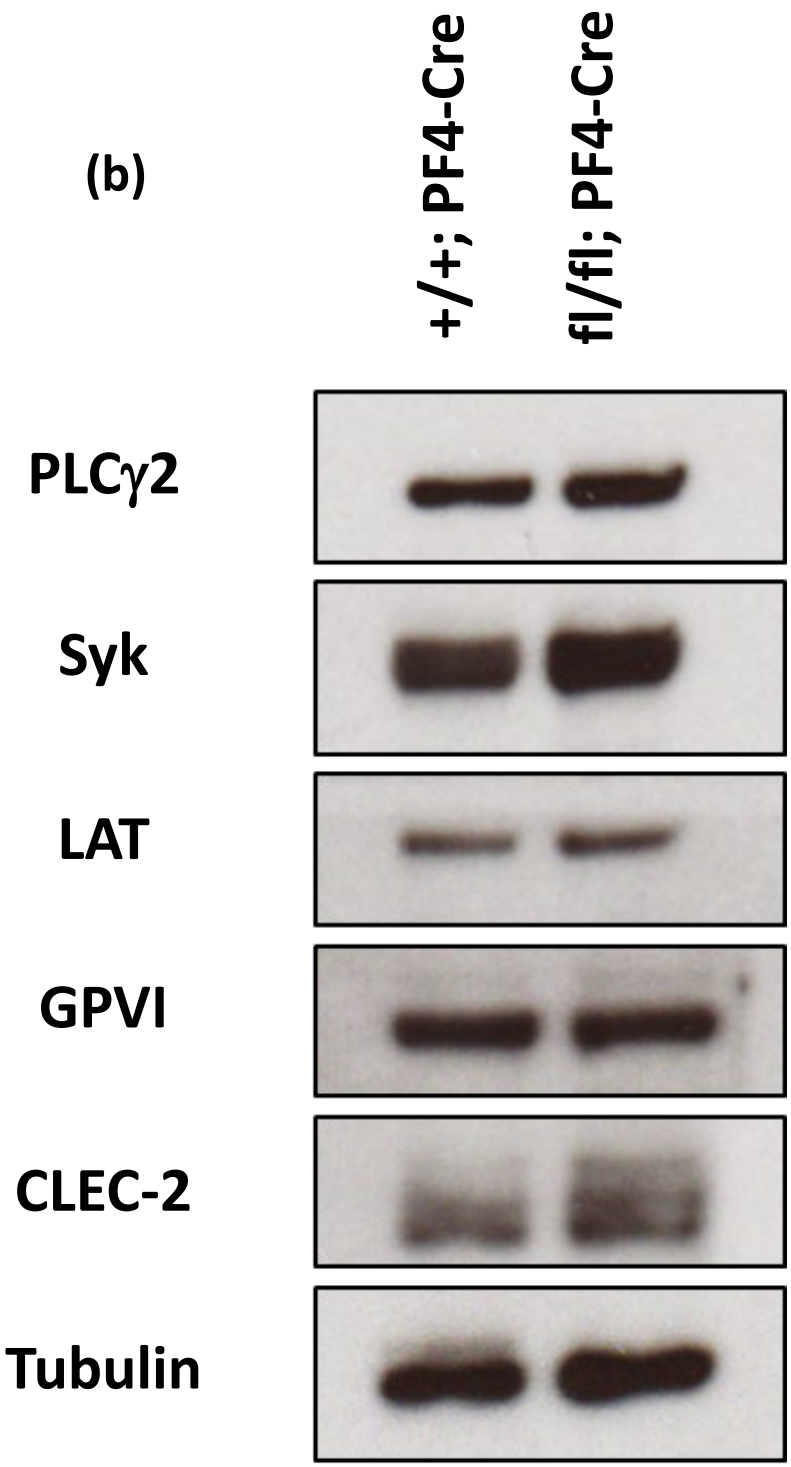
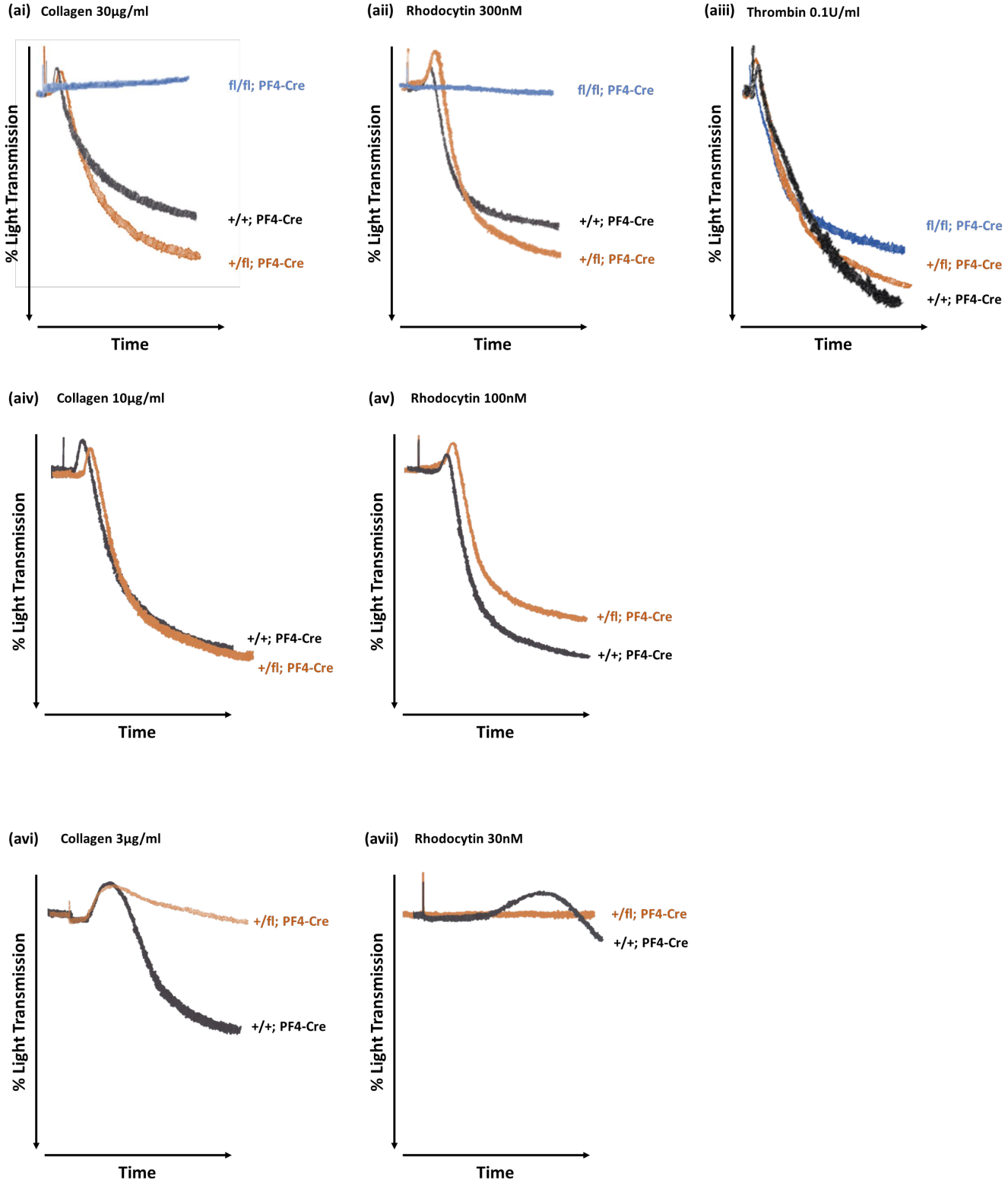


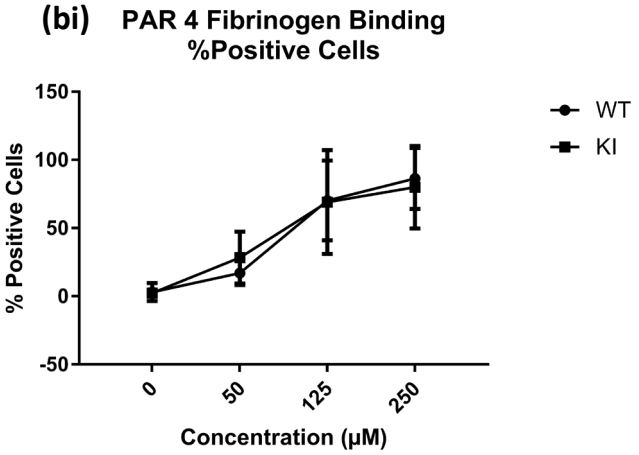
Figure 3.2 – Syk^{K396R} chimeras display no differences in surface or total expression of key platelet receptors and signalling proteins. (a) Surface expression of GPVI, CLEC-2, GP1bα, αIIbβ3 and α2β1 was assessed via flow cytometry (N=3). (b) Total expression of PLCγ2, Syk, LAT, GPVI and CLEC-2 was assessed via western blotting (N=3). For surface receptor expression analysis, data is presented as mean + standard deviation, differences were assessed via a one-way ANOVA.

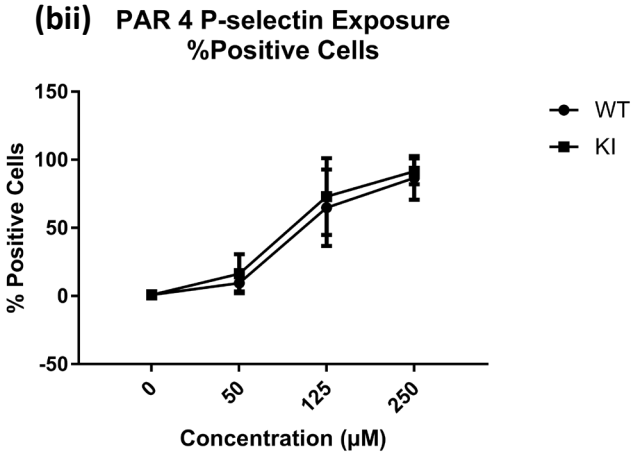
3.2.4 *Syk K396R mice display a significant impairment in (hem)ITAM – but not GPCR – signal transduction and responsiveness.*

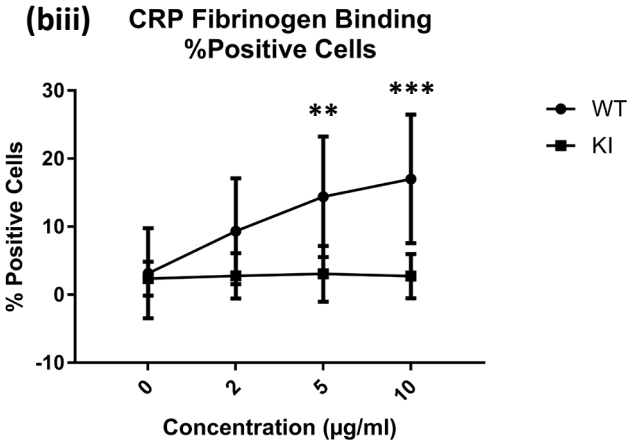
Once receptor and protein expression was confirmed to be similar between wild-type and Syk K396R mice, functional responses were assessed via lumi-aggregometry, flow cytometry and biochemical analysis in wild-type (+/+; PF4-Cre), heterozygous Syk K396R (+/fl; PF4-Cre) and homozygous Syk K396R (fl/fl; PF4-Cre). As measured by lumi-aggregometry, no differences were observed in response to 30µg/ml (figure 3.3ai) and 10µg/ml (figure 3.3aiv) collagen, 300nM (figure 3.3aii) and 100nM (figure 3.3av) rhodocytin or thrombin [0.1U/ml] (figure 3.3aiii) between wild type and heterozygous Syk K396R platelets. However, differences in aggregation were seen in heterozygous mice at 3µg/ml collagen (figure 3.3avi) and 30nM rhodocytin (figure 3.3avii). Furthermore, in homozygous K396R mice no response was observed in response to collagen (figure 3.3ai) or rhodocytin (figure 3.3aii), but no difference was observed in response to thrombin (figure 3.3aiii). Similar results were observed following flow cytometric analysis of platelet activation, as measured by both fibrinogen binding and P-selectin exposure. Wild-type platelets stimulated by increasing PAR-4 peptide, CRP or rhodocytin concentrations showed a typical dose response relationship for both fibrinogen binding and P-selectin exposure (figure 3.3bi-vi). However, homozygous Syk K396R mice showed no fibrinogen binding or P-selectin exposure in response to CRP (figure 3.3biii, 3.3biv) or rhodocytin (figure 3.3bv, 3.3bvi), but were indistinguishable from wild-types when stimulated with a PAR-4 peptide (figure 3.3bi, 3.3bii). Wild-type or homozygous K396R platelets were then stimulated with collagen [30µg/ml],

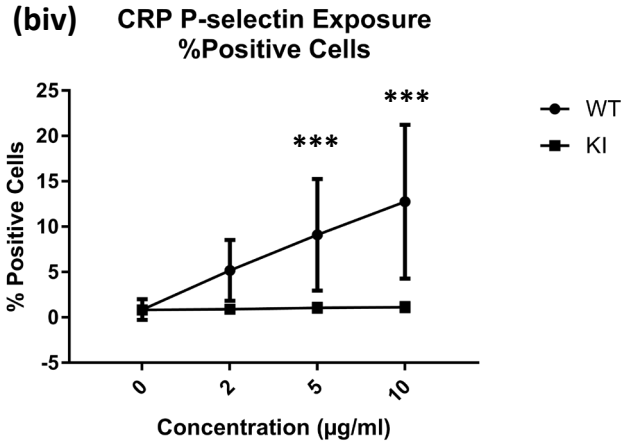
rhodocytin [300nM] or thrombin [0.1U/ml], and analysed via western blotting with a pan-phosphotyrosine antibody. Platelets from homozygous Syk K396R mice displayed a reduced level of phosphorylation of a protein ~72kDa and absent phosphorylation of proteins of ~135 kDa and ~32kDa in response to collagen and rhodocytin; these bands approximately correspond to the molecular weights of Syk, PLCγ2 and LAT, respectively (figure 3.3c).

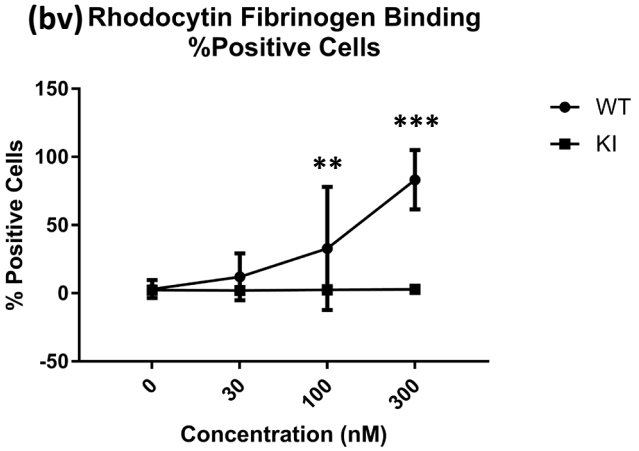

(ai) Collagen 30µg/ml
fl/fl; PF4-Cre
+/+; PF4-Cre
+/fl; PF4-Cre
% Light Transmission
Time
(aii) Rhodocytin 300nM
fl/fl; PF4-Cre
+/+; PF4-Cre
+/fl; PF4-Cre
% Light Transmission
Time
(aiii) Thrombin 0.1U/ml
fl/fl; PF4-Cre
+/fl; PF4-Cre
+/+; PF4-Cre
% Light Transmission
Time
(aiv) Collagen 10µg/ml
+/+; PF4-Cre
+/fl; PF4-Cre
% Light Transmission
Time
(av) Rhodocytin 100nM
+/fl; PF4-Cre
+/+; PF4-Cre
% Light Transmission
Time
(avi) Collagen 3µg/ml
+/fl; PF4-Cre
+/+; PF4-Cre
% Light Transmission
Time
(avii) Rhodocytin 30nM
+/fl; PF4-Cre
+/+; PF4-Cre
% Light Transmission
Time

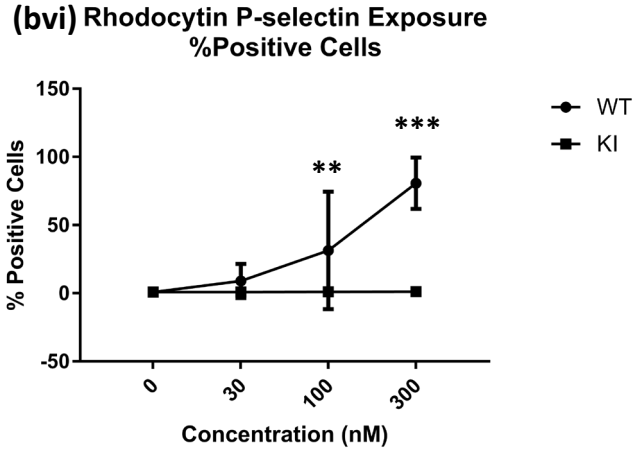

(bi) PAR 4 Fibrinogen Binding
%Positive Cells
% Positive Cells
150
100
50
0
-50
0
50
125
250
Concentration (μM)
WT
KI


(bii) PAR 4 P-selectin Exposure
%Positive Cells
% Positive Cells
150
100
50
0
-50
0
50
125
250
Concentration (μM)
WT
KI


(biii) CRP Fibrinogen Binding
%Positive Cells
% Positive Cells
30
20
10
0
-10
**
***
0
2
5
10
Concentration (μg/ml)
WT
KI


(biv) CRP P-selectin Exposure
%Positive Cells
% Positive Cells
25
20
15
10
5
0
-5
***
***
0
2
5
10
Concentration (μg/ml)
WT
KI


(bv) Rhodocytin Fibrinogen Binding
%Positive Cells
% Positive Cells
150
100
50
0
-50
**
***
0
30
100
300
Concentration (nM)
WT
KI


(bvi) Rhodocytin P-selectin Exposure
%Positive Cells
% Positive Cells
150
100
50
0
-50
**
***
0
30
100
300
Concentration (nM)
WT
KI

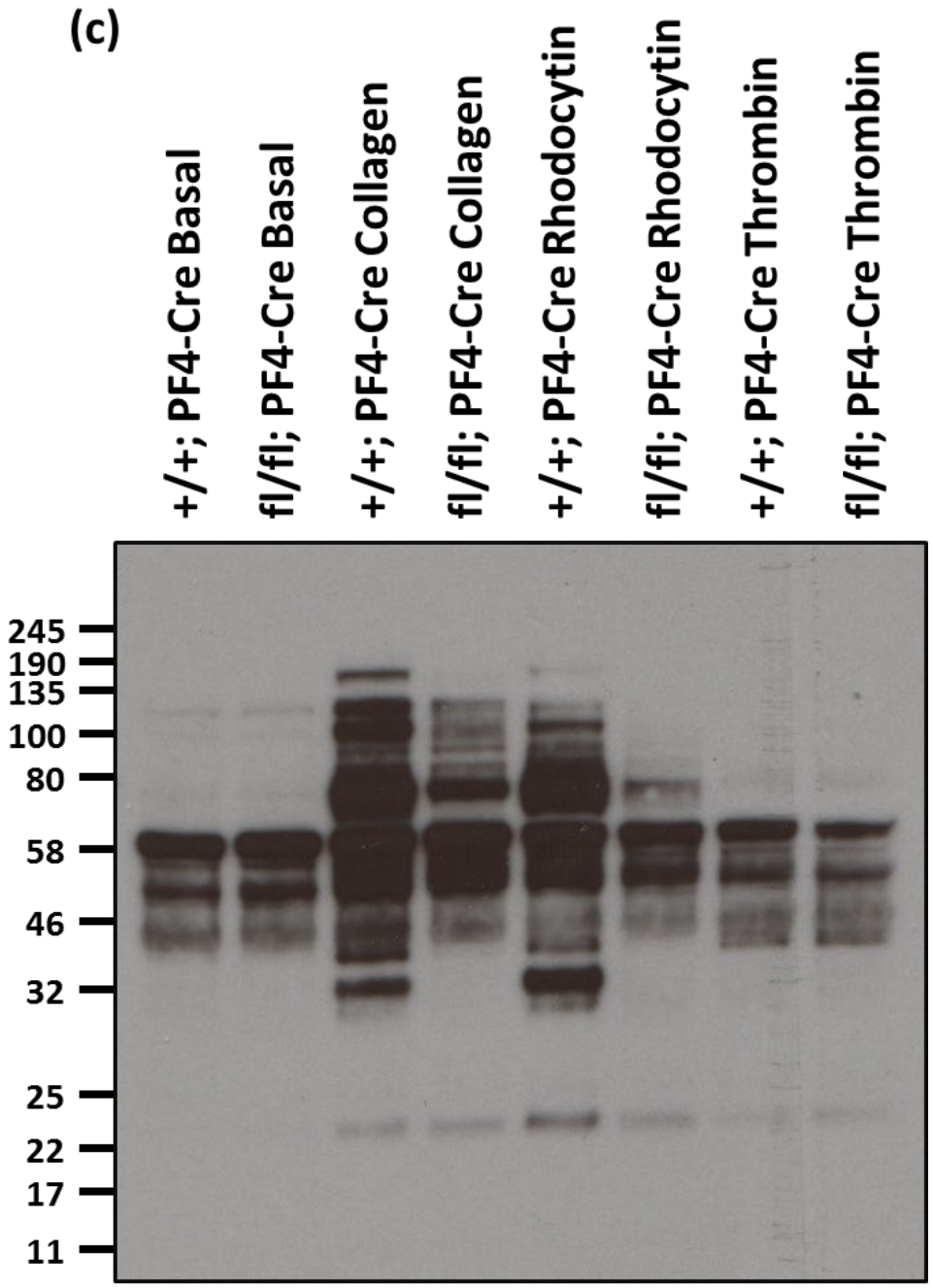
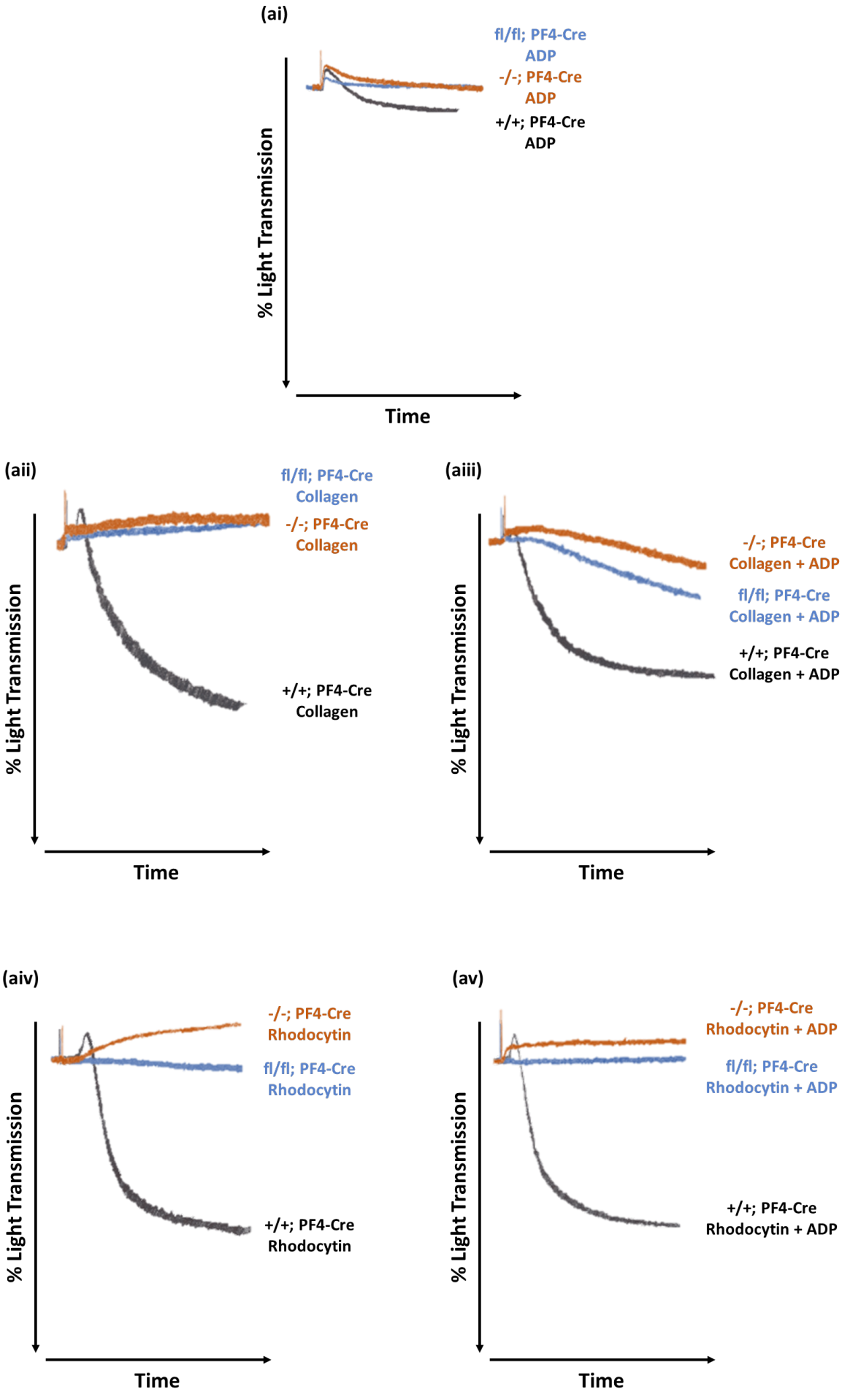


Figure 3.3 – Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$ but not Syk$^{K396R\ +/fl;\ PF4\text{-}Cre}$ mice display significant defects in aggregation, activation and intracellular signal transduction compared with Syk$^{K396R\ +/+;\ PF4\text{-}Cre}$ controls. (a) Aggregation responses were measured in platelets from homozygous, heterozygous and wild-type chimeras, in response to several doses of collagen, rhodocytin and single doses of thrombin (N=3). (b) Platelet activation was measured via flow cytometry by assessing fibrinogen binding and P-selectin exposure in response to increasing doses of a PAR-4 peptide, CRP and rhodocytin; data is presented as mean ± SD and statistical analysis was performed via use of t-tests performed individually at each dose for each agonist, **p ≤ 0.01, ***p≤ 0.005, ****p≤ 0.001 (N≥5) (c) Pan-phosphotyrosine western blots of homozygous and wild-type Syk^{K396R} chimeras following stimulation with collagen, rhodocytin and thrombin (N=3).

3.2.5 *A mild, partial potentiation effect is observed in Syk K396R and Syk KO platelets*

ADP synergises with other platelet agonists to induce powerful activation. Following the results observed for the homozygous Syk K396R mice, experiments were designed to test whether co-stimulation with ADP would synergise with collagen or rhodocytin to induce platelet activation. To test if this was the case, aggregations, flow cytometry activations and biochemical analysis were performed in wild-type (+/+; PF4-Cre), Syk K396R (fl/fl; PF4-Cre) and Syk KO (-/-) mice following co-stimulations. All mice displayed a minor shape change response when stimulated with ADP (figure 3.4ai), but neither Syk K396R or Syk KO mice underwent aggregation to collagen (figure 3.4aii) or rhodocytin (figure 3.4aiii); surprisingly there was a slow shape change response in Syk KO mice in response to both hemITAM agonists although this was not seen in all experiments. When co-stimulated with collagen and ADP, platelets from Syk K396R and Syk KO mice underwent weak aggregation (figure 3.4aiii). There was no change in response to ADP and rhodocytin in platelets from Syk K396R or Syk KO mice co-stimulated compared to rhodocytin alone (figure 3.4av). When assessed via flow cytometry by fibrinogen binding and P-selectin exposure, a small increase in fibrinogen binding was observed in Syk K396R platelets co-stimulated with CRP and ADP (figure 3.6bi, biii) or rhodocytin and ADP (figure 3.4bii, 3.4biv). A small increase was also observed in Syk KO mice, although there was a high level of variability in the responses for rhodocytin and ADP stimulation. Interestingly, when measure by P-selectin exposure, Syk K396R and Syk KO mice displayed no response irrespective of individual or co-stimulatory conditions (figure 3.4bii, 3.4biv).


(ai)
fl/fl; PF4-Cre
ADP
-/-; PF4-Cre
ADP
+/+; PF4-Cre
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Collagen
-/-; PF4-Cre
Collagen
+/+; PF4-Cre
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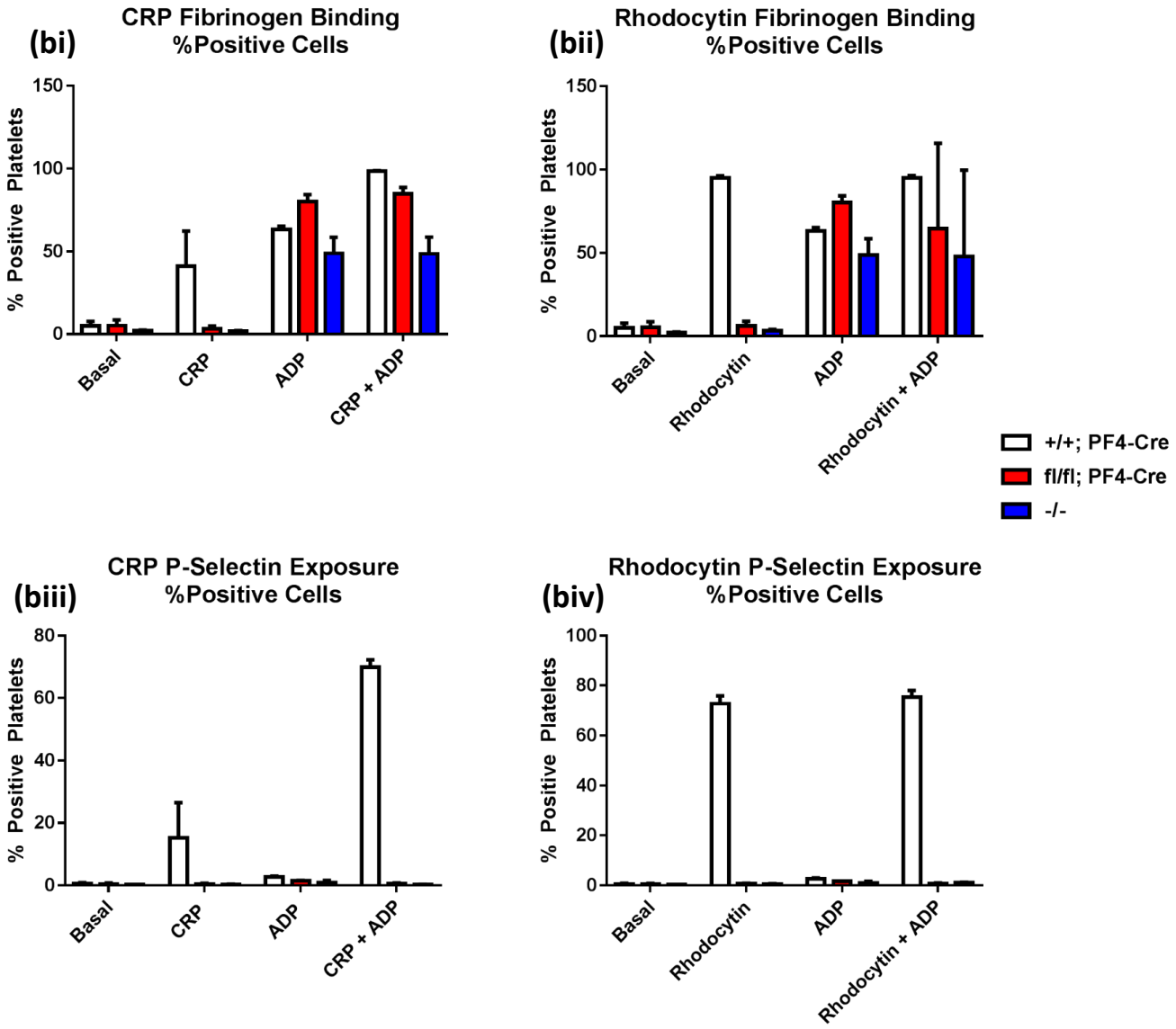


Figure 3.4 – Platelets from Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$ and Syk$^{-/-}$ chimeras display significant defects in aggregation and platelet activation compared with wild-type chimeras following individual and co-stimulation with collagen and rhodocytin ± ADP. (a) Aggregation was assessed in platelets from Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$, Syk$^{fl/fl}$ and wild-type chimeras following stimulation with collagen or rhodocytin ± ADP (N=3). (b) Platelet activation was measured via flow cytometry by assessing fibrinogen binding and P-selectin exposure in response to combinations of ADP, CRP and

3.2.6 Phosphorylation of Syk, but not the downstream targets LAT or PLCγ2, is maintained in Syk K396R mice.

Following the flow cytometry results, biochemical samples were generated and analysed via western blotting with both a pan-phosphotyrosine antibody (figure 3.5a) and a panel of phosphospecific antibodies against PLCγ2 (Y1217), Syk (Y525/526) and LAT (Y200) (figure 3.5b); these phosphospecific antibodies are all targeted against phosphotyrosine sites implicated in the activation state of their respective proteins. As seen in the previous western blotting experiments, when analysed using the pan-phosphotyrosine antibody a reduction in the level of phosphorylation of a band of around 72kDa and a complete loss of phosphorylation of proteins ~135kDa and ~32kDa was observed in Syk K396R mice compared with wild-type controls, even when platelets were co-stimulated with hemITAM agonists and ADP (figure 3.5a). When probed with phosphospecific antibodies, these results were confirmed; Syk K396R mice displayed a reduction in Syk Y525/526 phosphorylation and a complete loss of PLCγ2 Y1217 and LAT Y200 phosphorylation, both when stimulated solely with collagen or rhodocytin and also when co-stimulated with ADP (figure 3.5b). However, a lack of loading controls prevents full quantitation of these experiments, and not knowing whether there is equal protein loading in each lane does confound the interpretation of these results.

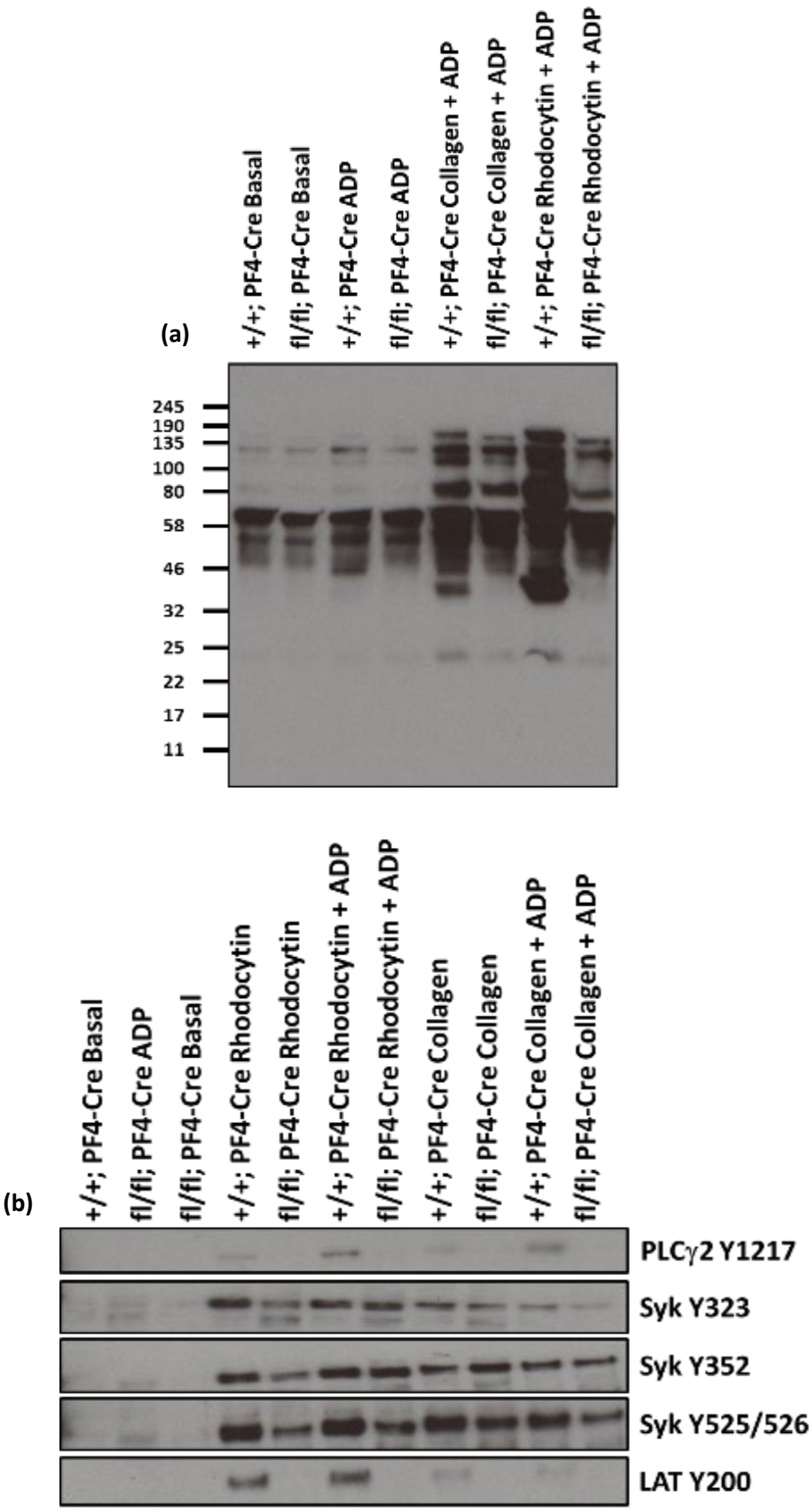
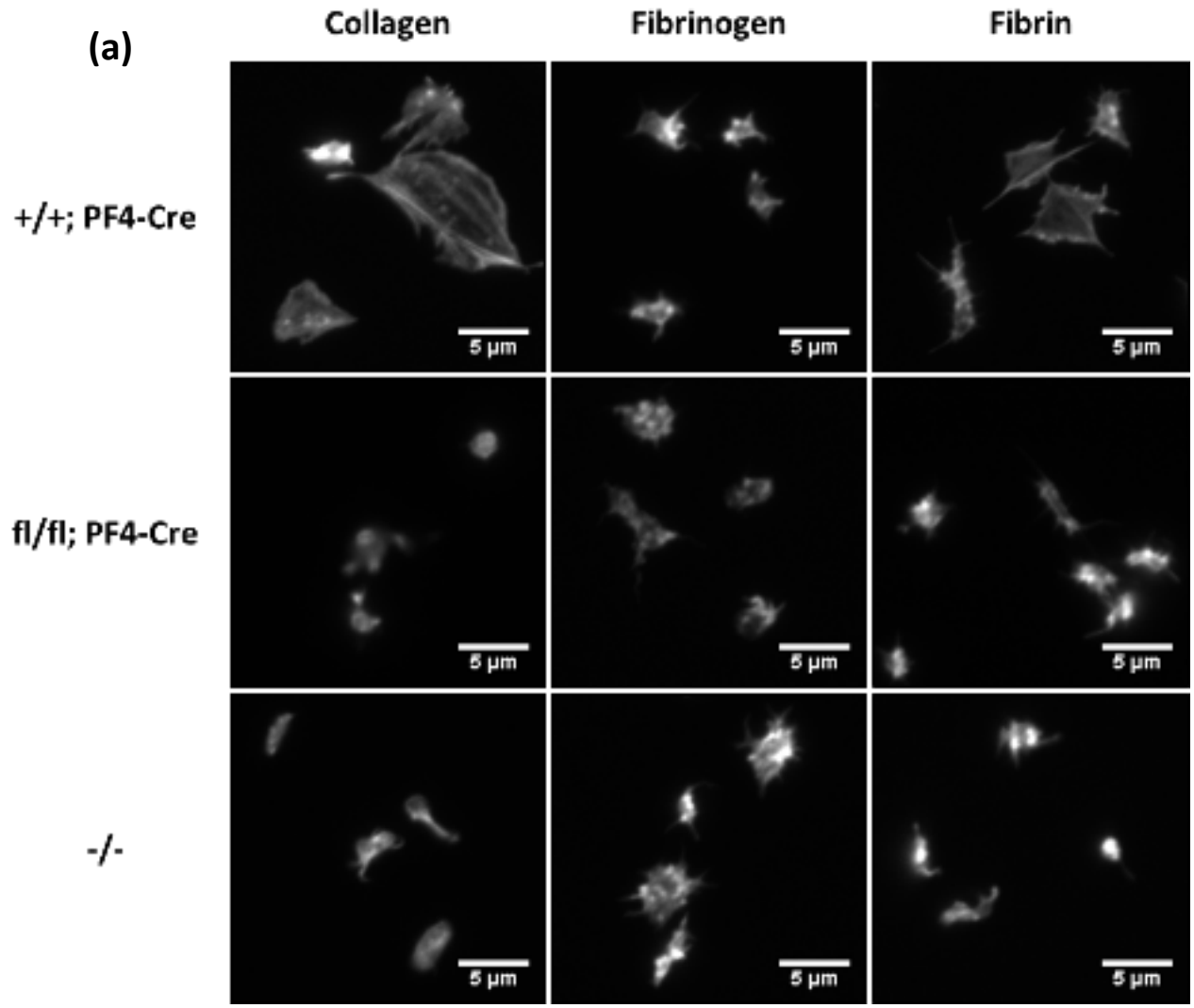


Figure 3.5 – Platelets from Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$ chimeras display a significant reduction in phosphorylation compared with wild-type chimeras following stimulation with collagen and rhodocytin ± ADP. (a) Pan phosphotyrosine western blots of platelets from Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$ and Syk$^{+/+;\ PF4\text{-}Cre}$ stimulated with collagen and rhodocytin ± ADP. (b) PLCγ2, Syk and LAT phosphospecific western blots of platelets from Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$ and Syk$^{+/+;\ PF4\text{-}Cre}$ stimulated with collagen and rhodocytin ± ADP (N=3).

3.2.7 Platelets from Syk K396R and Syk KO mice displayed a significant impairment in adhesion and spreading on a range of surfaces.

An assessment of platelet spreading was undertaken in wild-type, Syk K396R and Syk KO mice to probe a more physiological read-out of platelet function. Platelets from all three genotypes were spread on collagen, fibrinogen and fibrin – fibrin was included due to the novel finding that fibrin can activate platelets through GPVI and associated ITAM signalling. Representative pictures are contained in figure 3.6a, and full quantification of the spreading analysis is contained in figure 3.6bi-biii. As expected, spreading on collagen is significantly and largely impaired in Syk K396R and Syk KO mice compared to wild-type mice as measured by their area, perimeter and the total number of adherent platelets (figure 3.6bi). Surprisingly, when spreading on fibrin a larger impairment was observed in Syk K396R than in Syk KO mice compared with wild-type mice (figure 3.6bii). When spread on fibrinogen (figure 3.6biii), no significant impairments were observed for any genotypes.


(a)
Collagen
Fibrinogen
Fibrin
+/+; PF4-Cre
fl/fl; PF4-Cre
-/-
5 µm

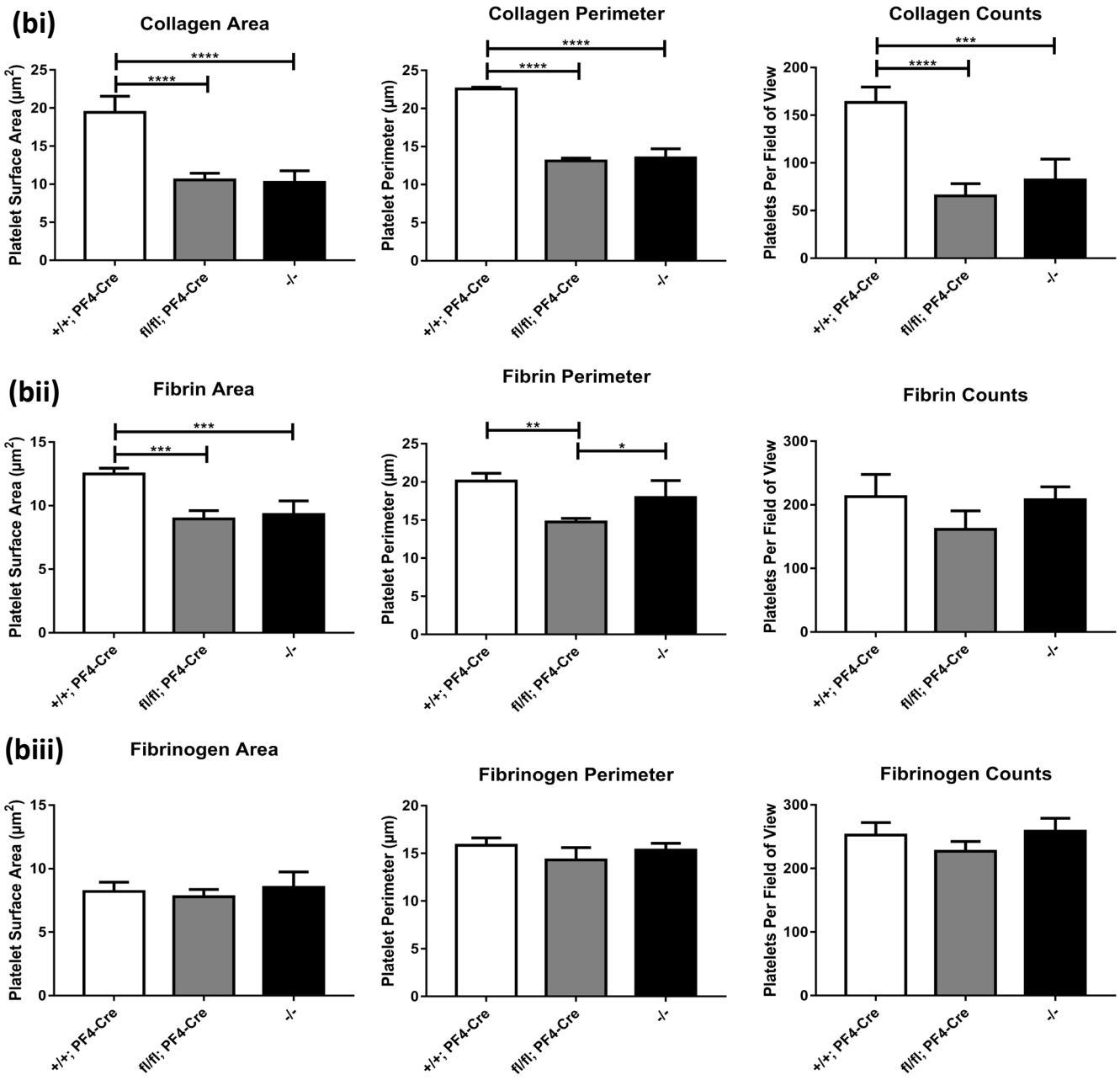


Figure 3.6 – Platelets from Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$ and Syk$^{-/-}$ chimeras present with deficits in platelet spreading and adherence on collagen and fibrin, but not fibrinogen, coated surfaces compared with Syk$^{+/+;\ PF4\text{-}Cre}$ chimeras. (a) Representative fluorescent images of platelets from Syk$^{fl/fl;\ PF4\text{-}Cre}$, Syk$^{fl/fl}$ and Syk$^{+/+;\ PF4\text{-}4Cre}$ chimeric mice spread on collagen, fibrinogen and fibrin coated surfaces, 5µm scale bars. (b) Graphs showing the area, perimeter and number of adherent platelets from Syk$^{K396R\ fl/fl;\ PF4\text{-}Cre}$, Syk$^{-/-}$ and Syk$^{+/+;\ PF4\text{-}Cre}$ spread on collagen, fibrin and fibrinogen coated surfaces ^{Spreading} of wild type (+/+; PF4-Cre), K396R (fl/fl; PF4-Cre) and Syk KO (-/-) platelets on collagen, fibrinogen and fibrin, data is presented as mean + SD and statistical analysis was performed using a one-way ANOVA with Tukey's multiple comparisons post hoc test (N=3).

3.3 Discussion

The experiments performed in this chapter have focussed around the characterisation of a novel Syk kinase-dead mouse model, and any potential adaptor functions of the protein unrelated to kinase function.

The breeding of the homozygous Syk K396R mice ($Syk^{K396R\ fl/fl;\ PF4\text{-}Cre}$) revealed an embryonically lethal phenotype, as confirmed via a subsequent Chi^2 test on the mendelian breeding frequencies; this phenotype appears in-line with complete systemic ablation of Syk (Syk KO)(159). This result was unexpected, due to survival of the platelet specific Syk knock-out mice ($Syk^{-/-;\ PF4\text{-}Cre}$ mice), albeit this too is non-Mendelian. It is likely that the expression of the dysfunctional protein is having an unknown and unexpected effect on cellular function, leading to an embryonically lethal phenotype. For example, complete loss of Syk may lead to the emergence of redundant pathways, which are not triggered when Syk is still expressed, albeit in a dysfunctional form; more work is required to determine the underlying cause of the embryonic lethality. It is also possible that, as shown in a recent paper by Calaminus et al (234), the PF4-Cre promoter is causing expression of the Syk K396R transgene in other myeloid and lymphoid cell types and inducing a worse phenotype; however the absence of a 100% embryonic lethality in the $Syk^{-/-;PF4\text{-}Cre}$ mice suggests this is unlikely to be the case (159). As with the Syk KO mice, one way around the embryonic lethality was the generation of radiation chimeric mice using foetal liver cells. All homozygous Syk K396R mice used in this chapter were radiation chimeras.

The first set of experiments performed were a series of physiological assessments of Syk K396R embryos (figure 3.1a) and also the adult Syk K396R chimeras (figure 3.1b). These experiments showed that only the homozygous Syk K396R embryos displayed the phenotype typically associated with mice deficient in CLEC-2 or proteins involved in its signalling pathway (21, 158); homozygous Syk K396R embryos displayed severe blood spotting, haemorrhages, oedema and evidence of lymphatic vasculature dysfunction. When adult radiation chimeric mice were assessed following bone marrow reconstitution, they displayed blood filled lymphatic vessels and nodes, chylous ascites and raised Peyer's patches as also seen in radiation chimeric mice reconstituted with CLEC-2-deficient bone marrow. This data suggests that a functional kinase domain is required for full signal transduction downstream of CLEC-2.

Following the physiological assessment of the Syk K396R chimeric mice, full blood counts were performed, including an assessment of the proportions of each white blood cell type. Interestingly, no difference in platelet count and only a mild, but significant, increase in MPV was observed in Syk K396R mice. This data is not in agreement with the data from Hughes *et al* using the Syk R41A mice, although the mice used in their study were not radiation chimeras and displayed systemic lymphatic defects which were not observed in the chimeric mice (136); the more severe blood-lymphatic mixing phenotype in the native $Syk^{R41A;PF4\text{-}Cre}$ mice is likely to result in a large circulating volume for platelets, leading to an apparent drop in platelet count. It was also interesting to note that there was a moderate, significant drop in red blood cell count and a large, significant drop in white blood cells – there

was also a significant difference in the composition of the leukocytes in the blood. Whilst the Syk K396R radiation chimeras were compared to irradiated and reconstituted wild-type controls, the complete bone marrow ablation and severe irradiation involved in the generation of these mice may affect these parameters, and they are not comparable to un-irradiated controls.

Once the radiation chimeras had been generated, the first set of experiments performed were an assessment of the levels of surface receptor expression alongside total receptor and signalling protein expression. These experiments were performed to ensure that any results that were observed in the subsequent experiments were a direct result of the Syk K396R mutation, and not a result of differential receptor trafficking or protein expression. Results from these experiments (figure 3.2) showed no difference in surface or total expression of receptors, and similarly no difference in signalling protein expression, attributing all subsequent experimental results to the effects of the mutant form of Syk.

Following on from the assessment of receptor and protein expression, a number of functional assays were performed on platelets from wild-type controls, heterozygous Syk K396R and homozygous Syk K396R mice. From these experiments it was seen that, in response to standard concentrations of collagen (30µg/ml) and rhodocytin (300nM), homozygous Syk K396R mice were completely unresponsive as measured by lumi-aggregometry (figure 3.3a). Interestingly, heterozygous Syk K396R mice displayed almost identical aggregation responses as wild-type mice, although some heterozygous mice did display a minor shift in dose response curve

at borderline inactive concentrations of collagen and rhodocytin (figure 3.3a). All three genotypes displayed indistinguishable responsiveness to the potent platelet agonist thrombin (figure 3.3a). Similar results were seen when platelet reactivity was assessed by flow cytometry in wild-type and radiation chimeric Syk K396R mice (figure 3.3b); radiation chimeric Syk K396R mice displayed no response to GPVI or CLEC-2 stimulation at any concentration but responded normally to an activating PAR-4 peptide. Syk K396R platelet samples were then stimulated with either collagen (30µg/ml) or rhodocytin (300nM) and taken for biochemistry. These samples showed reduced phosphorylation of a protein ~72kDa and absent phosphorylation of proteins ~135kDa and ~32kDa; as stated these molecular weights correspond roughly to the proteins Syk, PLCγ2 and LAT, respectively.

Taken together, the above results highlight that without a functional kinase domain, Syk cannot support the transduction of the activatory signal generated following engagement of GPVI or CLEC-2 into a functional response – neither aggregation nor degranulation responses are present in Syk K396R platelets. Interestingly, whilst a level of phosphorylation is maintained on a protein likely to be Syk – most likely the result of trans-phosphorylation mediated via SFKs – the absence of any phosphorylation of proteins likely to be PLCγ2 or LAT suggests that the kinase function of Syk is indispensable to the signal transduction downstream of GPVI and CLEC-2, and any adaptor function cannot bypass this key signalling function of Syk.

The fact that a level of phosphorylation was maintained on a ~72kDa protein likely to be Syk raised the possibility that co-stimulation of Syk K396R platelets may

overcome the signalling blockade induced via a lack of Syk kinase function. To assess this, platelets were co-stimulated with GPVI or CLEC-2 agonists in the presence and absence of ADP, a weak platelet agonist and mediator of 'second-wave' platelet activation. Syk KO chimeras were generated as a negative control for these experiments to confirm if any agonist potentiation effects observed were mediated via an independent adaptor function of Syk. Also, phosphospecific antibodies against key phosphotyrosine sites in PLCγ2, Syk and LAT were used in these experiments to confirm the results suggested using the pan-phosphotyrosine antibodies. Experiments performed in the previous section of this chapter – lumi-aggregometry, flow cytometry and western blotting – were repeated using wild-type, Syk K396R and Syk KO mice stimulated with hemITAM agonists alone or in combination with ADP. As per the previous results Syk K396R mice and, as expected, Syk KO mice did not undergo an aggregatory response to collagen or rhodocytin alone, whilst platelets from all three genotypes stimulated with ADP underwent a mild shape change response with no subsequent aggregatory response (figure 3.4).

Interestingly, some of the Syk K396R and Syk KO mice underwent a small, irregular shape change response following hemITAM stimulation alone, however these responses were not reproducible and seemingly random, suggesting a possible mechanism unrelated to GPVI or CLEC-2 receptor signalling, such as activation of the Rho/ROCK signalling pathway (235). Surprisingly, when co-stimulated with ADP, collagen but not rhodocytin induced a mild but reproducible aggregatory response which was not present when the agonists were used individually; although

there are possible explanations such as a possible divergence within the GPVI and CLEC-2 signalling pathways, or differences in receptor clustering characteristics, more work is needed to clarify this discrepancy fully. However, this potentiation effect was observed in both the Syk K396R mice and the Syk KO mice which is suggestive of a completely Syk-independent mechanism of platelet activation being responsible for the potentiation effects, rather than the ability of Syk to act as an adaptor protein; indeed Syk-independent potentiation effects have previously been described downstream of GPVI in platelets (236).

Flow cytometry stimulation experiments were repeated following co-stimulation. Surprisingly, Syk K396R platelets co-stimulated with CRP or rhodocytin and ADP did not appear to display the same level of potentiation as seen in the lumi-aggregometry experiments; indeed, no statistically significant differences were observed in Syk K396R mice when individual stimulation with ADP or CRP/rhodocytin was compared with co-stimulation. One possibility for this is the difference in agonist for GPVI, as induction of platelet activation following collagen and CRP stimulations are slightly different, although there are several other possibilities and as above more work is needed to confirm the true cause of this disparity. However as seen in the lumi-aggregometry experiments, the magnitude of any potentiation effects was similar between Syk K396R and Syk KO platelets, again suggestive of a completely Syk-independent mechanism. It is also interesting to note that the P-selectin exposure responses are drastically reduced in the Syk K396R and Syk KO mice following individual and co-stimulations, whereas the impairment in fibrinogen binding is milder. This is suggestive of a particularly severe

defect in platelet degranulation in the Syk-impaired mice, which may also partially explain the lower levels of aggregation observed in the lumi-aggregometry experiments.

Finally, biochemical analysis was performed using both pan-phosphotyrosine and phosphospecific antibodies following co-stimulation. Utilising the pan-phosphotyrosine antibody, very similar results were observed as per the individual agonist stimulations performed previously. Whilst stimulation with ADP on its own induced no phosphorylation events in either wild-type of Syk K396R platelets, it appeared that co-stimulation of platelets with collagen/rhodocytin and ADP induced greater phosphorylation of proteins within the hemITAM signalling cascade, although in Syk K396R mice the phosphorylation of the ~135kDa and ~32kDa proteins was still absent. Phosphospecific antibodies against key tyrosine residues contained within PLCγ2 (Y1217), Syk (Y323, Y352, Y525/526) and LAT (Y200) were used to assess the phosphorylation patterns of these proteins, and confirm results seen when using the pan-phosphotyrosine antibody. Use of these phosphospecific antibodies was also of particular interest due to the possibly distinct functional roles of these phosphorylation sites, particularly within Syk, as discussed in depth in the general introduction of this thesis. The results of the phosphospecific analysis confirmed that phosphorylation was maintained at a reduced level within Syk at all tyrosine sites,and was mildly potentiated in samples from co-stimulated platelets whereas PLCγ2 and LAT phosphorylation was completely abolished in Syk K396R platelets. Remarkably, the two phospho-sites within Syk where phosphorylation was most strongly maintained in Syk K396R mice appear to be involved in increasing

Syk activity; Y525/526 residues are located in the kinase domain of Syk and are required for full Syk activity, whilst Y352 is thought to provide a docking site for positive regulators of Syk activity. However the loss of downstream signal transduction does question the importance of these observations when considering potentiation effects in the Syk K396R mice.

The last set of experiments performed to characterise the Syk K396R mice were a set of platelet spreading assays. These assays were performed to explore platelet function in a slightly more physiological setting and to explore whether the activity of Syk K396R platelets differed when introduced to static agonists arranged on a surface. Both adherence and spreading of platelets on a collagen surface was significantly impaired in both Syk K396R and Syk KO mice when compared with wild-type controls, although surprisingly the severity of impairment seemed to be marginally higher in the Syk K396R mice compared with the Syk KO mice. Very similar results were observed when platelets were allowed to spread on a fibrin-coated surface, however, the severity of impairment in response to fibrin appeared to be less than that observed in platelets spreading on collagen, and no difference in adherence was observed. These results are in keeping with the recent findings that fibrin can act as a GPVI agonist in platelets, although with less potency than collagen, and that defects in GPVI and its signalling pathway impair this response (140, 141, 237). Again, Syk K396R mice showed a more severe impairment in adherence and spreading on fibrin than Syk KO mice. Lastly, platelets were allowed to spread on fibrinogen, which has typically been thought to interact with platelets via the integrin αIIbβ3. No significant differences in platelet spreading were

observed when platelets were incubated on a fibrinogen surface; this data does not support the recent findings that fibrinogen – particularly when immobilised on a surface – stimulates platelets through engagement of GPVI, however there is evidence that murine platelets are generally less sensitive to fibrinogen stimulation compared with human platelets (237, 238). These results also suggest that Syk K396R mice have a more severe deficit in spreading response than Syk KO mice. This is surprising, as in most cases a complete loss of protein would be expected to induce a more severe phenotype than partial inactivation, however possibilities such as an inability to utilise complementary pathways without a complete absence of protein may explain these results. However, these hypotheses are currently purely speculative, and more experiments would be required to confirm or deny them.

There are several limitations of the experiments performed within this chapter. Firstly, due to the embryonic lethality of this mutation, all functional experiments have been performed in reconstituted adult mice. Whilst the phenotypes are similar to those observed in other mouse strains with mutations in the GPVI and CLEC-2 signalling pathways, this model is not fully comparable to models such as the R41A mice (136). As similar mutations, such as the Syk R41A mutation, do not display the same levels of embryonic lethality, crossing of the Syk K396R mice onto a different background or placing it under the control of a different platelet-specific promoter – such as the newly developed GP1bα-Cre (239) – would be useful to determine if it is the mutation or a genetic interaction causing the high lethality. Other methodological limitations include the omission of loading controls for the western blots performed in this chapter, preventing quantitation of these biochemical assessments and potentially confounding the assessment of any differences

between the different genotypes. Also, whilst average haematological parameter values have been included on the graphs in this chapter, these values are liable to differ between labs due to differences in areas such as blood collection techniques and blood analysis machines. For more comparable results, blood counts of stock, un-irradiated mice could have been obtained at the same time as the counts for the irradiated mice as comparators.

Furthermore, whilst the functional tests performed in this chapter provide a good assessment of platelet reactivity, they are not true measures of *in vivo* haemostatic function, and so further assessments such as tail bleeding times or ferric chloride/laser injury models could be used in this model to assess the effects of this mutation on true haemostasis and thrombosis. Also, whilst co-stimulations have been used as a surrogate marker of Syk adapter function, experiments such as co-immunoprecipitations and pull-down assays could be performed to determine which proteins still interact with the Syk K396R protein. Lastly, whilst ADP was selected as a co-stimulator of platelets due to its ability to induce a mild, reversible platelet aggregation (240) – essentially 'priming' the platelets for further stimulation via GPVI and CLEC-2 agonists – it would also be of interest to use other co-stimulatory agonists and secondary mediators of platelet activation to assess potential cross-talk between the GPVI/CLEC-2 pathway and other platelet activation pathways.

Overall, this chapter has focused on the characterisation of a novel platelet specific Syk kinase dead mutant mouse model. These mice, with a lysine to arginine substitution at location 396, displayed an embryonically lethal phenotype, which was

unexpected when considered next to other Syk-impaired or Syk-deficient models – some of this may be explained by the apparently more severe functional phenotype of Syk K396R mice compared with Syk KO controls. Syk K396R embryos and radiation chimeric mice present with a typical defective lymphatic phenotype associated with a loss of CLEC-2 expression or signalling and display a complete loss of aggregatory, activatory and secretory responses to hemITAM agonist stimulation; full reactivity following GPCR activation is maintained in Syk K396R mice. This loss of response to hemITAM stimulation is accompanied by a loss of phosphorylation of a number of key signalling proteins in the hemITAM signalling pathway. Although Syk K396R mice can undergo a partial recovery in reactivity to hemITAM agonists when co-stimulated with ADP, these potentiation responses were mirrored in Syk KO controls and were also not accompanied by any recovery of key protein phosphorylation. This is suggestive of a Syk-independent mechanism which is responsible for the potentiation responses seen in Syk K396R and Syk KO mice. The minor shape change responses sometimes seen in response to individual hemITAM agonist stimulation in Syk K396R mice were also seen at a similar rate in Syk KO mice, again suggestive of a Syk-independent mechanism controlling this shape change response. Phosphospecific biochemistry performed on these samples showed that whilst phosphorylation of Syk at several key regulatory sites was maintained in Syk K396R platelets under both individual and co-stimulation conditions, no phosphorylation was observed on key activatory tyrosine residues contained within PLCγ2 or LAT irrespective of stimulus. Lastly, platelet adhesion and spreading on collagen, fibrin or fibrinogen was generally impaired in Syk K396R and Syk KO mice, although surprisingly the impairments were more severe under

all conditions in the Syk K396R mice. Taken together these results suggest that the kinase domain of Syk is integral to its function, and that whilst transphosphorylation events – likely mediated by SFKs – still occur on key regulatory and adaptor sites within Syk, these do not appear to be able to support signal transduction without a functional kinase domain.

Chapter 4

Murine neonatal platelets are hypo-responsive to GPVI and CLEC-2 agonists

4.1 Introduction

It has long been known that there are significant functional differences between neonatal and adult platelets; humans are typically defined as neonates for the first 28 days post-birth, irrespective of the length of gestation (241). Neonatal platelets, for example, have a defect in their responsiveness to many typical physiological platelet agonists such as collagen, ADP and thrombin (43, 242). What is also extremely interesting is that healthy neonates show no propensity to bleed, despite having defects in what have traditionally been seen as integral haemostatic pathways; some studies have even shown a mild increase in overall haemostatic capability (243, 244). Whilst some work has been performed in this area – aiming to understand the reasons why, despite presenting with often significant defects in platelet pathways that are extremely important in adult homeostasis, healthy neonates do not display any aberrant bleeding phenotypes – the molecular basis of these seemingly counterintuitive effects currently remains unknown.

It appears that the maintained haemostatic capability of neonates is mediated heavily by the differences in the GP1bα-vWF pathway of platelet activation. It has been shown that there are much larger vWF multimers in foetal and neonatal plasma, likened to those seen in adult patients presenting with thrombotic thrombocytopenic purpura (TTP) (245) – TTP is a condition characterised by widespread thrombosis occurring in the microvasculature, associated with haemolytic anaemia and severe thrombocytopenia (246, 247) – as well as a reported upregulation of GP1bα expression on the platelet surface. When taken

together, this provides a possible explanation for the maintenance of haemostasis; upregulation of the GP1bα signalling pathway seems to compensate for the hypo-reactivity of other pathways important in adult haemostasis. It is important to note, however, that there is still some controversy in the literature in this area, for example not all groups agree that neonates express higher levels of GP1bα compared with adults (248).

Regarding the functional deficits present in both murine and human neonatal platelets, many different explanations have been proposed, often individualised for each signalling pathway. However, there remains a level of controversy surrounding several of these proposals. A lack of response to adrenaline in humans, for example, seems to be due to a widely accepted decrease in the number of receptors expressed by human neonatal platelets (249). There appears to be less agreement, however, for the basis of poor responsiveness to other agonists, such as collagen. A number of possible mechanisms have been investigated and it has been shown that processes such as calcium mobilisation (242, 250) and phospholipase activity (43) are impaired in human neonatal platelets when compared with adult platelets, suggesting the presence of signalling defects. A major issue with many of these studies however is their age; very little work has been undertaken recently either refuting or confirming and building upon the initial findings in these original studies. Also, much of the methodology of these studies is dated – for example, only small panels of receptors have been assayed for expression – likely due to availability of reagents and instruments at the time. This is further emphasised by the fact that, to date, only one paper using murine neonates appears to look at the functional

capability of the CLEC-2 signalling pathway, which shares a common set of signalling proteins with the collagen receptor, GPVI (45). Also, very few studies have explored (hem)ITAM and ITIM receptor expression as a potential mechanism for collagen hypo-responsiveness in neonatal platelets.

One reason that it is particularly important to understand these functional differences between neonatal and adult platelets relates to the therapeutic options available for treating thrombocytopenia in neonates. The current treatment regime for neonatal thrombocytopenia is a platelet transfusion in conjunction with any appropriate treatment for the underlying cause, e.g. alloimmune thrombocytopenia, yet there does not appear to be any widely accepted and consistent guidelines for when and how neonatal platelet transfusion should be performed. Aside from the lack of clarity on neonatal platelet transfusion guidelines, no consideration is currently given to the aforementioned differences between neonatal and adult platelets.

The aims of the experiments presented in this chapter are to generate an exact time course of platelet function, alongside a time course of platelet surface receptor expression throughout gestation. We wanted to first confirm that platelets have a poor responsiveness to collagen before assessing if this extended to CLEC-2 agonists as well. Furthermore, we wanted to explore surface receptor expression as a potential mechanism for any lack of platelet response. There are some issues with the age stratification of neonates in a number of the studies; data is often not stratified via gestational ages but by birth weight and, although there does appear to be a correlation between birth weight and gestational age, this does not appear

to be absolute. We aimed to address this in the current study by using mice of precisely known gestational ages for platelet assessment, allowing us to build a much more detailed picture of the development of platelet function throughout development. Lastly, we utilised a model of platelet recovery after immune-thrombocytopenia to 1) assess the maturity of newly formed platelets and determine if they revert to a developmental-like phenotype and 2) explore the possibility of increased platelet production pressure as a mechanism controlling differential composition and impaired reactivity of neonatal platelets when compared with adults.

4.2 Results

4.2.1 Platelet size remains constant from late gestation through to adulthood

Platelet size was measured in all blood samples taken at all ages; size was assessed to determine whether any differences observed in subsequent receptor level assessments are due to differential expression or due to changes in platelet surface membrane surface area. No significant differences in platelet size were observed from late gestation through to adulthood (figure 4.1), with an average MPV of around 7 femtolitres.

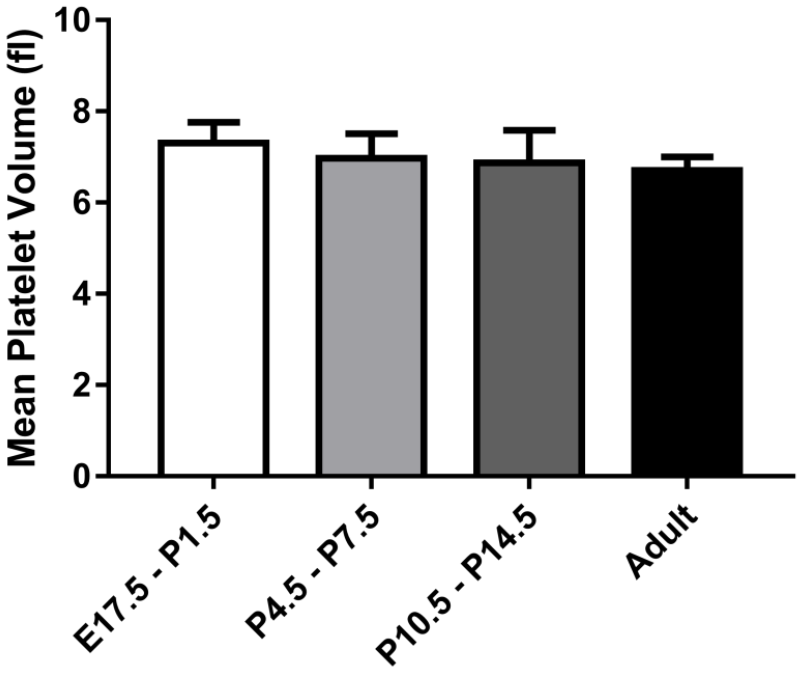


Figure 4.1 – No differences were observed in mean platelet volume (MPV) of platelets from embryonic, neonatal and adult mice. Whole blood samples were obtained from embryonic, neonatal and adult mice and mean platelet volumes were measured via use of an ABX Pentra blood analyser (Horiba, Northampton, UK). Data is presented as mean + SEM, and statistical analysis was performed using a one-way ANOVA (N≥6).

4.2.2 Neonatal platelets are hypo-reactive in response to GPVI and CLEC-2, but not PAR-4, stimulation

Fluorescent fibrinogen binding (fibrinogen*Alexa 488) and P-selectin exposure (CD62p*PE) were measured in unstimulated platelets and platelets stimulated with increasing doses of GPVI, CLEC-2 or PAR-4 agonists. The gating strategy is outlined in figure 2, but briefly consisted of collecting 10,000 events that were CD41+ (CD41*APC) and within the FSC-SSC size parameters corresponding to platelets (figure 4.2a). Activation was then measured as the percentage of cells that were positive for either fibrinogen (figure 4.2bi) or P-selectin (figure 4.2bii) after stimulation as compared to cells incubated with PBS.

Once appropriate gating strategies had been established, dose-response relationships were determined for collagen related peptide (CRP; GPVI), rhodocytin (CLEC-2) and an activatory PAR-4 peptide (PAR-4); CRP was chosen instead of collagen as it binds specifically to GPVI, allowing assessment of GPVI without activation of integrin $\alpha 2\beta 1$, as would be the case with collagen. The dose-response results are detailed below in figure 4.3. As seen in figure 4.3ai, fibrinogen binding in response to CRP is significantly impaired in neonatal mouse platelets up to day ten when compared with adult mouse platelets at all concentrations. Interestingly, as seen in figure 4.3bi, fibrinogen binding in response to rhodocytin is also significantly reduced in neonatal mouse platelets at all ages, but the impairment appears to be most prominent at low and intermediate concentrations. PAR-4 induced fibrinogen binding was unaffected in neonatal platelets, as seen in figure 4.3ci, with no

significant differences at any developmental ages when compared with adult platelets.

The exposure of P-selectin on the platelet surface following stimulation via GPVI, CLEC-2 and PAR-4 was also measured. Surprisingly, it was seen that P-selectin exposure was significantly reduced in response to all agonists at almost all gestational ages (figure 4.3). It also appeared that the pattern of response was very similar, at least with respect to stimulation with rhodocytin and a PAR-4 peptide,

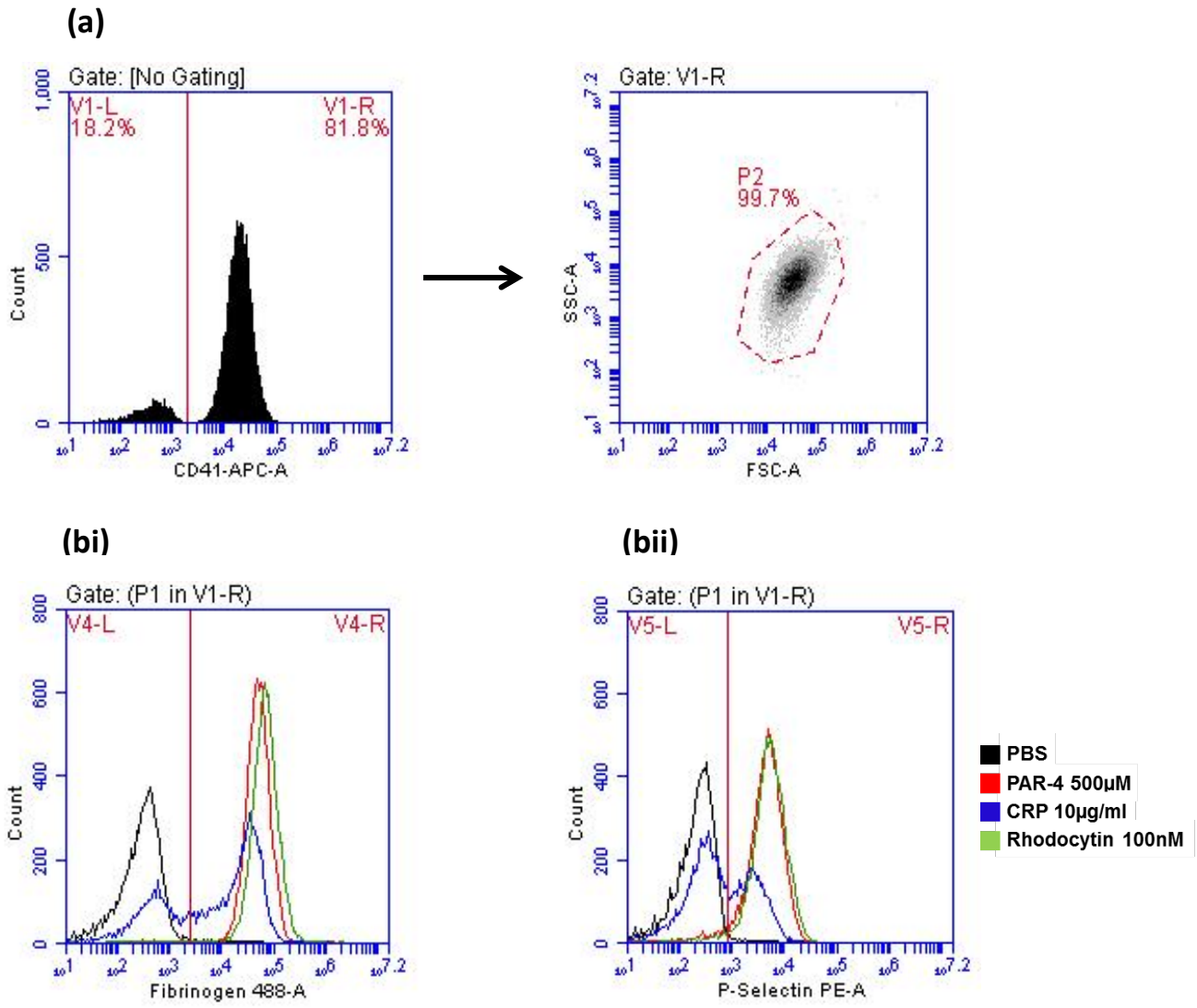


Figure 4.2 – Flow cytometry gating strategies used for determination of platelet populations and positive shifts denoting platelet activity; platelet populations were determined by CD41⁺ and FSC-SSC characteristics.

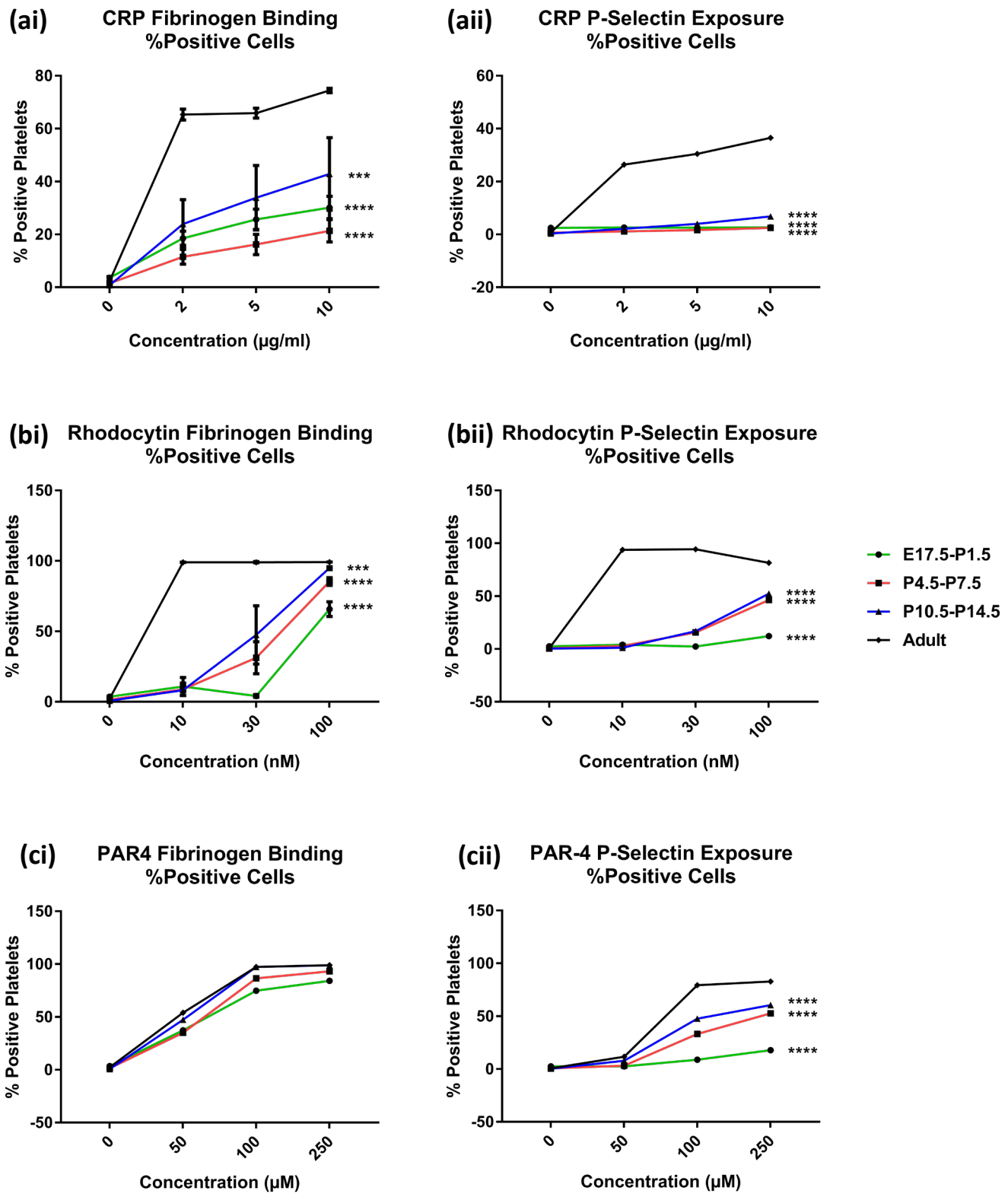


Figure 4.3 – Embryonic and neonatal murine platelets are hyporeactive to CRP and Rhodocytin, but not a PAR-4 peptide, up to day 14.5 post-birth. Platelet reactivity was assessed by fluorescent fibrinogen binding and P-selectin exposure in embryonic and neonatal mice, measured via flow cytometry. Whole blood samples were stimulated with increasing doses of CRP (a), Rhodocytin (b) or a PAR-4 peptide (c). Statistical analysis was performed using a two-way ANOVA with a Dunnett's multiple comparisons *post hoc* test comparing whole dose-response relationships, all groups were compared to adult dose-responses, ***p≤0.005, **** p≤0.001; data is shown as mean ± SEM, N≥6.

4.2.3 Platelet receptors are differentially expressed at the platelet surface throughout development

The surface expression of a panel of receptors containing αIIbβ3, α2β1, GP1bα, PECAM-1, GPVI, and CLEC-2 was assessed. As seen in figure 4, all of the receptors were expressed at different levels throughout development, with only PECAM-1 reaching levels comparable with adult levels by days 10.5-14.5 post-birth (figure 4.4d). PECAM-1 was expressed at significantly higher levels in neonates compared with adults before returning to normal levels, whilst αIIbβ3 was initially expressed at higher levels during gestation, before dropping below adult levels by days 10-14.5 post-birth. Interestingly, GPVI and CLEC-2 levels were consistently significantly lower in neonatal mice than in adult mice, as were α2β1 and GP1bα levels. However, the actual differences in surface receptor expression appear to be relatively mild, with a maximum drop in GPVI of around 27% and a maximum drop in CLEC-2 of around 40%.

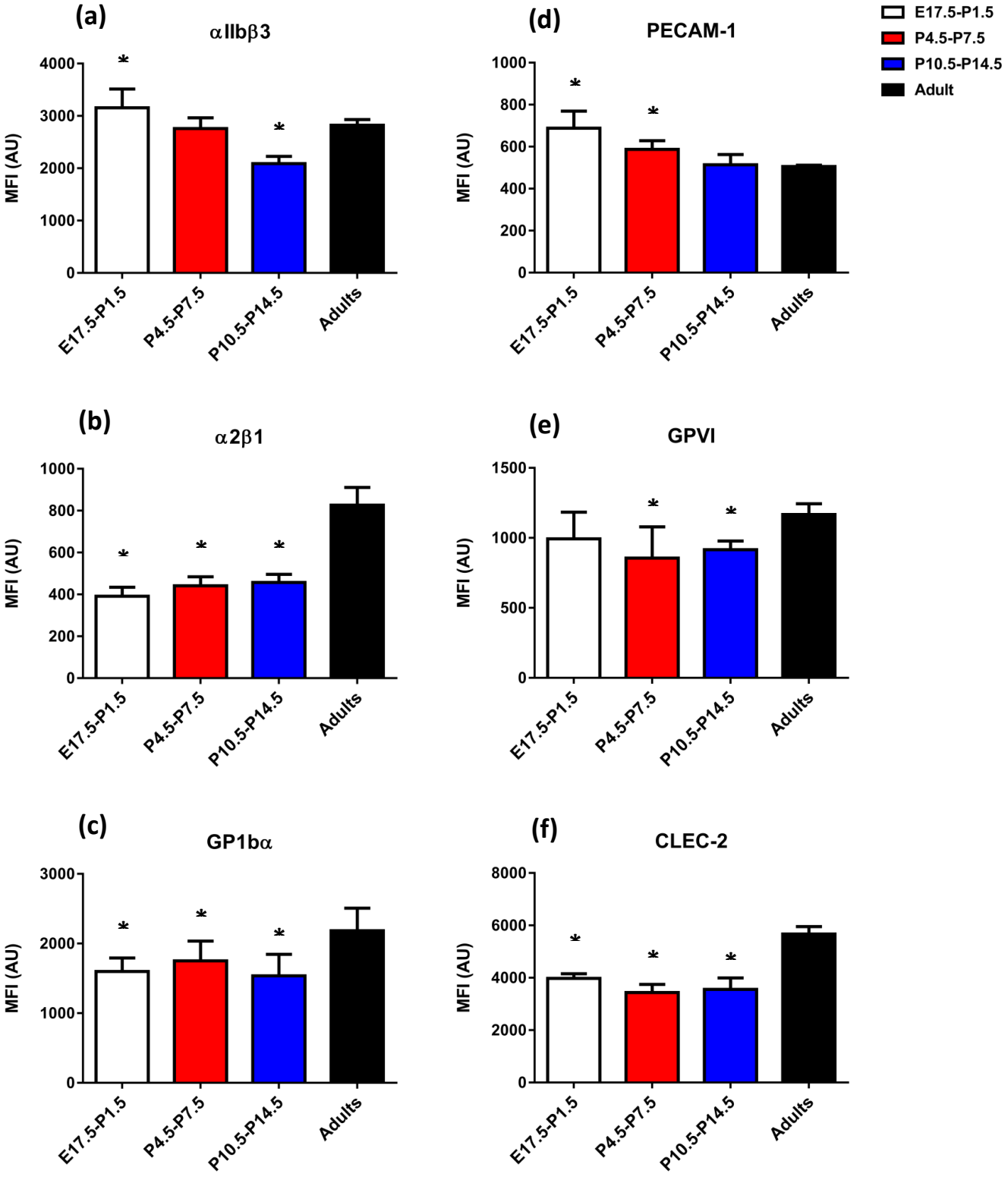


Figure 4.4 – Embryonic and neonatal murine platelets express different levels of surface receptors compared to adult mice. Whole blood samples obtained from embryonic, neonatal and adult mice were stained for the platelet surface receptors αIIbβ3 (a), α2β1 (b), GP1bα (c), PECAM-1 (d), GPVI (e) and CLEC-2 (f), before being analysed via flow cytometry. Statistical analysis was performed using a one-way ANOVA with Dunnett's multiple comparisons post-hoc test, *p≤0.05. Data is shown as mean ± SEM, N≥6

4.2.4 Murine platelets are hypo-reactive in response to GPVI and CLEC-2, but not PAR-4, stimulation following immune-induced thrombocytopenia

Although it was discovered that neonatal platelets are hypo-reactive to GPVI and CLEC-2 agonists, and several possible mechanisms controlling this were explored, the overarching reasons underlying the need for this lack of GPVI and CLEC-2 responsiveness remain to be elucidated. One hypothesis controlling this observed hypo-reactivity was that an increased stress on thrombopoietic mechanisms during periods requiring exponential platelet production – as would be observed during early development – may induce production of platelets from non-primary sites of thrombopoiesis and/or cause platelets to be released earlier in the thrombopoietic cycle to meet the increased demand. To model this situation, platelets generated in response to severe immune-induced thrombocytopenia were assessed for reactivity and composition.

The responsiveness of newly formed platelets in mice that had experienced an immune-induced thrombocytopenia was assessed via flow cytometry following stimulation with CRP, rhodocytin and an activatory PAR-4 peptide (figure 4.5), as for the neonatal mice experiments above. An impairment in reactivity was seen in response to CRP when measured by both fibrinogen binding and P-selectin exposure in newly formed platelets from immune-depleted mice and this hypo-reactivity was maintained up to days 7-9 post-depletion (figure 4.4a). A milder phenotype was seen in response to rhodocytin, where low to moderate doses of rhodocytin appeared to have less effect on the newly formed platelets, however this

impairment was no longer seen by days 4-5 post-depletion (figure 4.4b). PAR-4 stimulation was unaffected in the newly formed platelets, displaying a very similar phenotype to that observed in the neonatal platelets (figure 4.4c).

4.2.5 Platelet receptors are differentially expressed at the platelet surface following platelet depletion

The expression of platelet receptors on the cell surface was assessed via flow cytometry following immune depletion; The same panel of receptors (αIIbβ3, GP1bα, α2β1, PECAM-1, GPVI, and CLEC-2) were assessed in the newly formed platelets as those assessed in the neonatal platelets.

Interestingly, in opposition to the results observed in the neonatal platelets, all of the platelet receptors – with the exception of GPVI and CLEC-2 – were expressed at a higher level on the surface of platelets formed following depletion (figure 4.6). It was particularly of interest that not all of the receptors returned to non-depletion levels after 7-9 days post-injection, and also that the receptor expressed most highly following depletion was the inhibitory receptor, PECAM-1. It was also surprising that the (hem)ITAM receptors were not expressed at different levels following immune-induced thrombocytopenia.

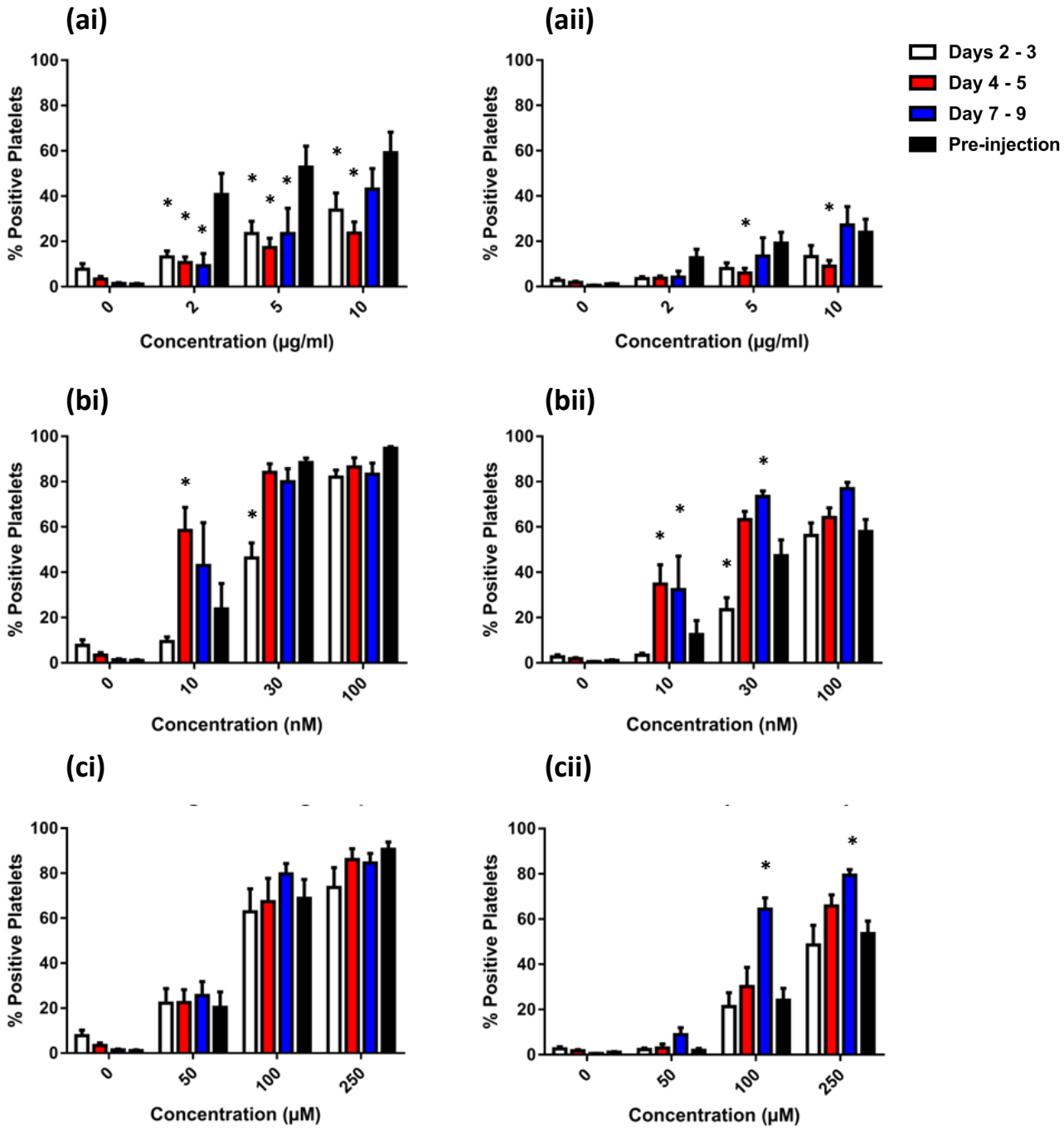


Figure 4.5 – Murine platelets are hyporeactive to CRP and Rhodocytin, but not a PAR-4 peptide, up to days 7-9 following immune-induced thrombocytopenia. Platelet reactivity was assessed by fluorescent fibrinogen binding and P-selectin exposure in response to increasing concentrations of CRP (a), Rhodocytin (b) and a PAR-4 peptide (c), in mice recovering from immune-induced thrombocytopenia. Statistical analysis was performed using a two-way ANOVA with a Dunnett's multiple comparisons *post hoc* test, *=p≤0.05. Data is shown as mean ± SEM, N≥6.

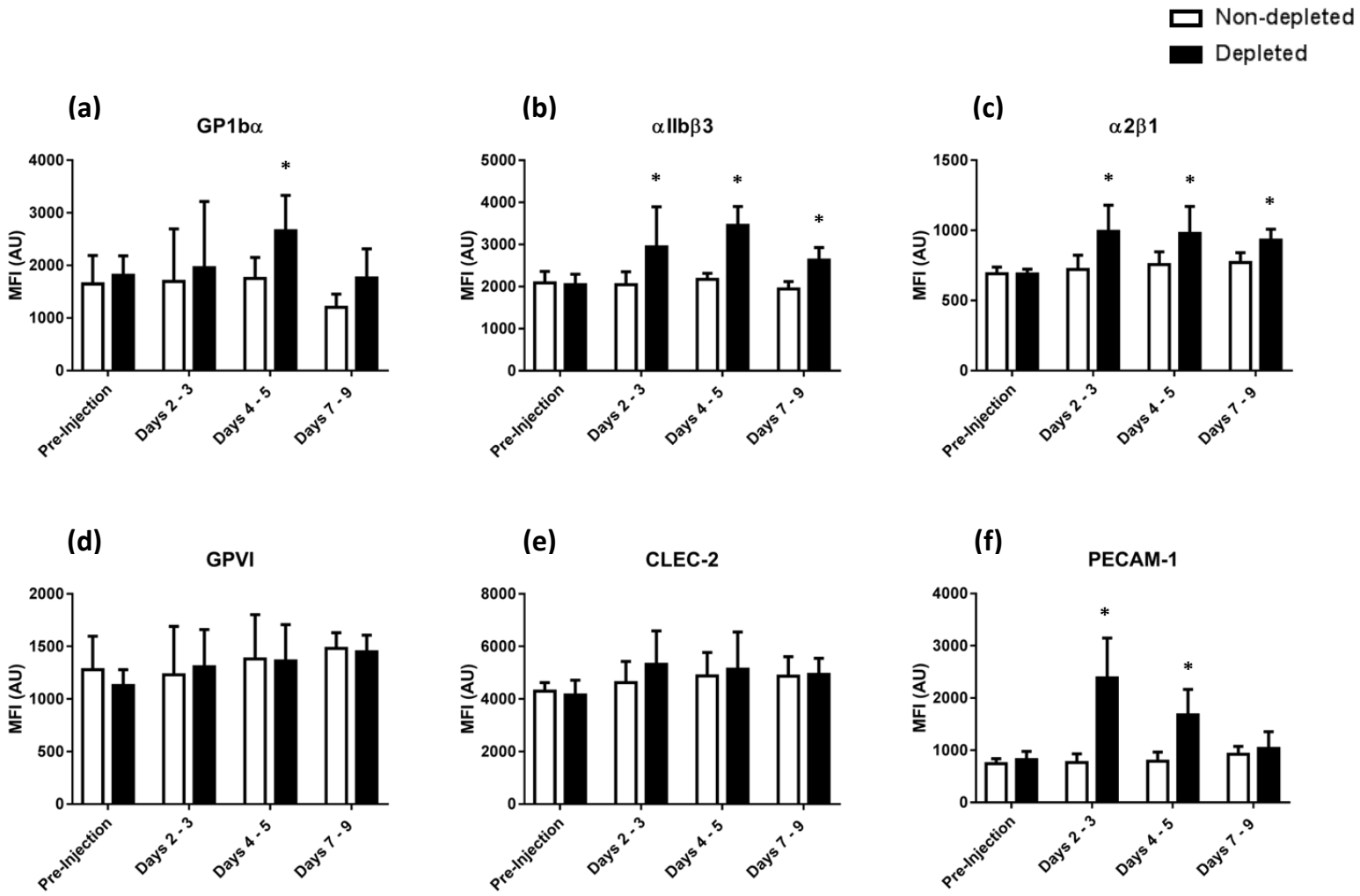


Figure 4.6 – Newly formed murine platelets express different levels of surface receptors following immune-depletion. Whole blood samples obtained from mice following immune-induced thrombocytopenia or IgG-injected controls were stained for the platelet surface receptors GP1bα (a), αIIbβ3 (b), α2β1 (c), PECAM-1 (d), GPVI (e) and CLEC-2 (f), before being analysed via flow cytometry. Statistical analysis was performed using a one-way ANOVA with Dunnett's multiple comparisons post-hoc test, *p≤0.05. Data is shown as mean ± SEM, N≥6.

4.2.6 Mean platelet volume is significantly increased following immune-depletion of platelets

Mean platelet volume was assessed in newly formed platelets following immune-depletion and compared with IgG injected controls. Size was again assessed to determine whether any differences observed in receptor levels are due to differential expression or due to changes in platelet surface membrane surface area. Following immune-depletion, a large increase in mean platelet volume was observed between days 2-3 and 4-5 post-depletion (figure 4.7); mean platelet volume appeared to normalise by days 7-9 post depletion. No significant differences were observed, however the variability of data was large and so a larger sample size may be needed.

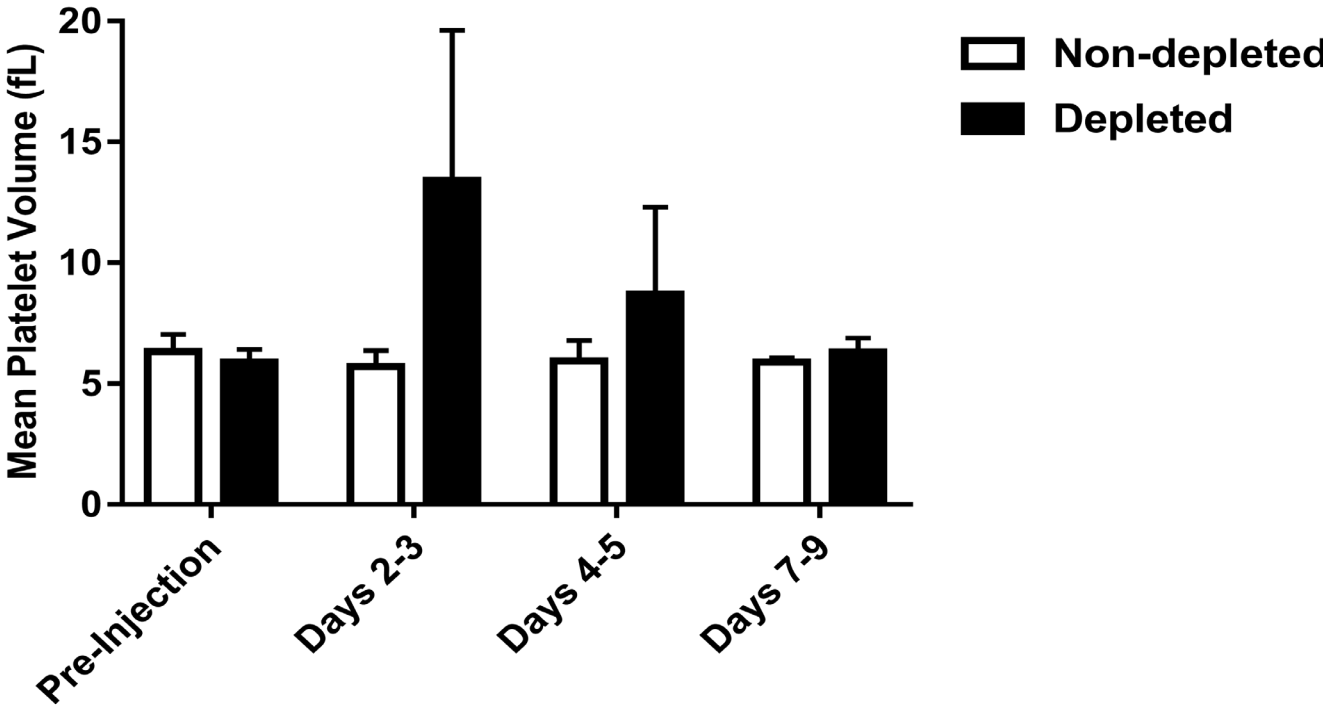


Figure 4.7 – Mean platelet volume is not significantly different following immune-induced thrombocytopenia. Mean platelet volume (MPV) was assessed in whole blood samples obtained following immune-induced thrombocytopenia or injection of control antibodies via use of an ABX Pentra blood analyser (Horiba, Northampton, UK). Data is shown as mean ± SEM, N≥6

4.3 Discussion

This chapter has explored the reactivity of embryonic and neonatal platelets as compared with adult platelets, as well as some potential mechanisms controlling the observed differences. A model system, involving the depletion of platelets from wild-type mice using a GP1bα antibody, was also used to try and recreate the conditions seen in developing murine platelets.

Firstly, the experiments in this chapter have shown an impairment in the platelet response to GPVI stimulation in neonatal platelets and, further, have shown a similar level of impairment in response to the CLEC-2 agonist, rhodocytin. It was also seen that there was a very mild impairment in low-dose PAR-4 reactivity (figure 4.3). The reduced response to CLEC-2 stimulation is interesting, as CLEC-2 has been shown to be integral for lymphatic and cerbrovascular development (159, 161). However we can see that high dose rhodocytin appears to stimulate similar responses to those observed in adults, suggesting that high concentrations of CLEC-2 agonists may be able to overcome the mechanisms controlling platelet hyporeactivity *in vivo*, whilst GPVI agonists may not; however more work is needed to establish whether this is the case.

Another interesting finding from the platelet activation studies was the finding that P-selectin exposure was significantly reduced in response to all agonists at most gestational ages (figure 4.3). It also appeared that the patterns of response were very similar, particularly in response to rhodocytin and an activating PAR-4 peptide.

Initially, this was thought to signal a defect in neonatal platelet granule secretion which was not present in the mecanisms controlling integrin activation. However, another possible explanation is that P-selectin protein levels are themselves developmentally regulated, and thus the differences observed were due to a combination of reduced platelet reactivity and a reduced pool of intracellular P-selectin. Indeed, the developmental regulation of P-selectin has been described by others, such as the group of James Palis (251), many of whom have seen similar patterns of P-selectin exposure in response to platelet stimulation as those described in this chapter. Whilst this observation is undoubtedly interesting, it confounds the use of this protein as a marker of neonatal platelet reactivity.

Once it was seen that the hypo-reactivity of platelets to GPVI agonists was replicable, experiments were performed to assess the differential expression of key signalling receptors on the platelet surface membrane as a potential mechanism. To explore this, the expression of several key platelet receptors on the surface membrane was assessed in embryonic and neontal platelets using flow cytometry. These results showed that, in general, the activatory receptors contained on the platelet surface are expressed at a lower level throughout embryogenesis and neonatal development when compared with adults (figure 4.4). It was interesting to note that, although the reduced receptor expression partially matches the time-course of platelet hypo-reactivity, the reductions in platelet surface protein expression were generally mild, and thus unlikely to fully mediate the large impairment in platelet reactivity observed. When taken together, these results

suggest that their may be another common mechanism controlling the hypo-reactivity seen to GPVI and CLEC-2 stimulation.

It should also be noted that the ITIM receptor, PECAM-1, appears to be expressed at a significantly higher level in gestational and early neonatal platelets compared with adult platelets (figure 4.4). There is some evidence that increased PECAM-1 signalling may impair collagen induced platelet activation (252). This proposes the distinct possibility that increased levels of PECAM-1 may be having an inhibitory effect on GPVI, and likely CLEC-2, induced signal transduction, leading to reduced platelet activity. More work would be required to definitively prove this as a mechanism however.

It is also intriguing that integrin αIIbβ3 levels appear to be elevated in gestational and newborn mice compared with adults but drop steadily throughout early development and fall below adult levels between days 10.5-14.5 post-birth. This is interesting when we consider that neonatal platelet function appears, at least with respect to GPVI and CLEC-2 stimulation, to be inversely proportional to age. There are a number of potential explanations for this, such as the possibility that αIIbβ3 levels are elevated as a compensatory mechanism for the poor responses elicited through other receptors, although from the dose-response assay (figure 4.3) the biggest impairment in fibrinogen binding occurs at the ages where the receptor levels are highest. This suggests that the elevated receptor levels do not appear to be compensatory and raises the possibility that there may be underlying issues with the activation of the integrin downstream of GPVI and CLEC-2 activation. This

hypothesis seems to be in agreement with other previous data, where PAC-1 staining of neonatal platelets was lower than that observed in adult platelets (253); PAC-1 is an antibody which binds to the active form of the αIIbβ3 integrin.

Following the observations that embryonic and neonatal platelets were hyporeactive in response to (hem)ITAM agonists, and that the receptor expression profiles partly mirrored this, a model system was trialled to two ends: firstly, a model system would allow more in-depth analysis of potential mechanisms controlling platelet hypo-reactivity in the longer-term, and secondly it could allow exploration of the overarching rationale for platelet hypo-reactivity throughout development. A model of high-stress platelet production was utilised to try and mirror the conditions present within the developing neonatal thrombopoietic system; mice were injected with a platelet depleting antibody and the reactivity and composition of the newly formed platelets were assessed. When reactivity was measured in these new platelets, a very similar phenotype to that present in the embryos and neonates was observed (figure 4.5); GPVI and, to a lesser extent, CLEC-2 reactivity was reduced in the newly formed platelets but no real effects on PAR-4 reactivity were observed.

Following the identification of a similar platelet reactivity phenotype, assessment of platelet receptors was then undertaken. Suprisingly, the opposite results were seen in the newly formed platelets following immune-depletion compared with the neonatal experiments (figure 6). Platelets from mice that underwent immune-depletion had much higher levels of all platelet receptors, with the exception of GPVI and CLEC-2 which were unchanged compared with non-depleted platelet controls.

However, a trend towards increased mean platelet volume was also seen in the platelets from mice undergoing depletion. This observation may suggests that due to a concurrent increase in platelet surface membrane area, the proportional changes in receptor expression would result in the only receptors with a different expression being GPVI and CLEC-2; this increase in platelet surface membrane would correspond to a decrease in the relative number of (hem)ITAM receptor molecules on the platelet surface. This decrease in the relative number of (hem)ITAM receptors on the platelet surface could partially account for the reduced response seen in these newly formed platelets. However, as previously seen, these differences are unlikely to fully account for the hyporeactivity in both the neonatal and platelet-depleted mice. The observation that the developing mice and the mice recovering from immune-depletion of platelets have similar phenotypes suggests that neonatal hypo-reactivity may be a byproduct of a high pressure, high volume thrombopoietic state, as seen in stages of exponential platelet production; it is also important to remember that neonatal platelets are produced in different locations than in adults, which may also impact their compositon and reactivity.

This becomes even more interesting when considering work which has been performed recently, where adult platelets were infused into embryonic mice vessles and rapid, spontaneous thrombosis was observed (254). This result suggests that developing embryos, and particularly their blood vessels, may express a higher proportion of platelet activating molecules than typically found within adult vessels.. However, this field of research is in its infancy and so more work is required to

support either hypothesis, or potentially link them together throughout the complex stages of development.

Further experiments that would be of interest would be to determine the expression levels and reactivity of a number of key signalling proteins in the (hem)ITAM signalling pathway, such as Syk, LAT and PLCγ2. Assessment of these proteins could help to determine if there is a common underlying signalling defect, which could act in conjunction with the reduced level of receptor expression to cause reduced platelet reactivity. Whilst work on the human arm of this study, performed by our collaborators in the group of Jose Rivera-Pozo, provides some evidence to suggest that this may be the case, more work is currently required – particularly in mice – and so this would be an area of particular interest (255). It would also be of interest to further utilise the platelet depletion model, as the greater blood volumes in adult mice treated with a platelet depleting antibody will allow for much more in-depth assessments of platelet function, platelet biochemistry experiments – as per those described above – and even *in vivo* assessments of platelet function.

There are a number of limitations of the experiments conducted within this chapter. Firstly, all of the experiments performed in this body of work have used inbred mice from the C57Bl/6 strain. Whilst the use of inbred mice is beneficial in some settings – such as the assessment of specific genetic mutations – it limits the generalisability of the results of these experiments; for example, it has been shown that the average baseline haematological parameters of different murine strains can vary significantly, potentially meaning that there may be issues such as different

pressures on thrombopoiesis in different strains (256). It would be of interest to repeat these experiments in either a different inbred mouse strain or in outbred mice to ensure that the developmental roles we have identified are consistent, and to generate more generalisable results. Furthermore, whilst the experiments here show some differences in the mean flourescent intensity of surface receptors, changes in these measurements do not necessarily correlate to absolute changes in receptor number. Therefore, it would be of interest to use tools such as flow cytometry kits allowing determination of absolute receptor counts, or western blotting for total protein levels if the issues surrounding minute blood volumes could be overcome.

Overall, this chapter has replicated the hypo-reactivty of neonatal platelets to GPVI stimulation as previously shown, and expanded this to the signalling pathway-sharing receptor CLEC-2. It has also shown that GPVI and CLEC-2 expression in developing mice is reduced, but not to an extent consistent with the strong impairment in reactivity. It has also shown a very similar pattern of reactivity in newly formed platelets following immune depletion, however the receptor expression results in this model are potentially ambiguous. Taken together, these results suggest that newly formed platelets are hypo-responsive to (hem)ITAM agonists, and that this hypo-reactivity may be a combined result of the location and high pressure, high volume nature of thrombopoiesis during development.

Chapter 5

Platelets from patients deficient in GPVI do not respond to fibrin or fibrinogen

5.1 Introduction

As described in the general introduction to this thesis, it has recently been shown that fibrin can activate platelets through GPVI (140, 141). In this chapter, I extend this work to patients with a homozygous mutation resulting in loss of expression of GPVI on the platelet surface. A novel, homozygous mutation in the extracellular domain of GPVI was described in four unrelated families in Chile (257). This mutation introduces an adenine nucleotide into exon six of the GPVI gene, resulting in the generation of a premature stop codon prior to the membrane domain (c.711_712insA). This stop codon results in translation of a truncated form of the protein which is absent from the platelet surface. The four unrelated families were spread throughout the breadth of Chile but share a Spanish familial name, suggesting a possible founder mutation. Platelets from these patients do not respond to the typical GPVI agonists collagen, convulxin or CRP (257).

In the present chapter, platelets from control, heterozygous GPVI c.711_712insA ($GPVI^{+/-}$) and homozygous c.711_712insA ($GPVI^{-/-}$) patients were assessed via aggregatory, spreading and biochemical analysis in response to collagen (positive control), fibrinogen (negative control) and fibrin. The $GPVI^{+/-}$ and $GPVI^{-/-}$ were from two separate families, both containing one heterozygote and one homozygote. This is the first time that platelets from patients with a congenital homozygous mutation in GPVI have been assessed for their responsiveness to the novel GPVI agonist, fibrin.

5.2 Results

5.2.1 Platelets from GPVI$^{-/-}$ patients do not aggregate in suspension following stimulation with collagen or fibrin

Platelet aggregometry was performed to measure the response to a homogenised suspension of cross-linked fibrin, fibrinogen, and collagen. The fibrin was made by incubating platelet poor plasma (PPP) with thrombin for 1 hour. Following formation and precipitation of the cross-linked fibrin mesh, the thrombin inhibitor PPACK was added to the fibrin suspension and mixed, before the fibrin-mesh precipitate was washed in PBS and sonicated on ice to form a homogenous solution of polymerised fibrin fibres.

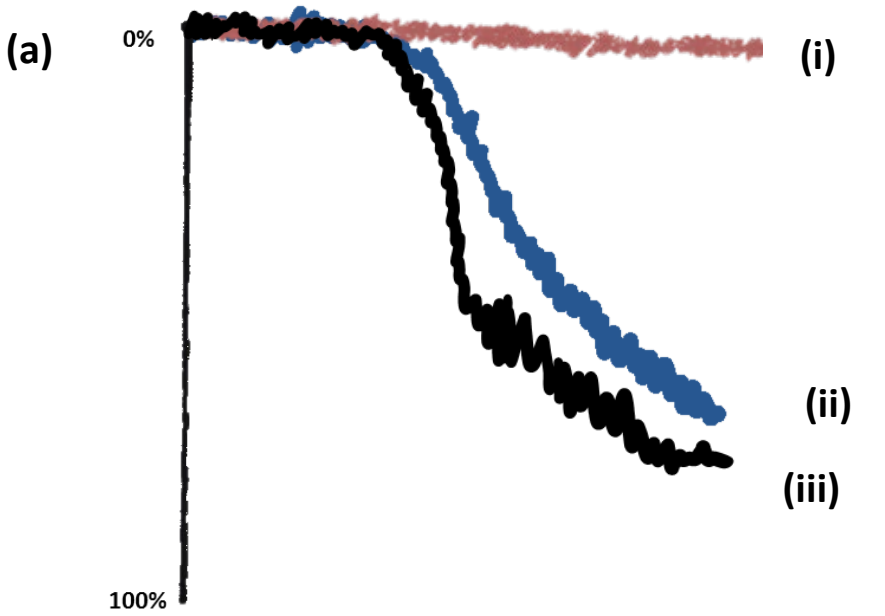
As seen in figure 5.1a, when stimulated with 30µg/ml collagen, platelets from a healthy control aggregated as expected and, interestingly, platelets from GPVI$^{+/-}$ patients aggregated almost normally, with only a very mild impairment in aggregation compared with controls. In contrast, platelets from GPVI$^{-/-}$ platelets displayed a complete lack of aggregatory response to collagen.

When stimulated with the fibrin suspension (100µg/ml fibrinogen + 1 U/ml thrombin; figure 5.1b), platelets from a healthy control displayed a very strong aggregatory response, similar to that observed for healthy control platelets stimulated with collagen. Whilst platelets from GPVI$^{+/-}$ patients did respond to suspended fibrin, in contrast to the almost normal reactivity observed in response to collagen they displayed a marked reduction in aggregatory response compared to healthy control

platelets. Remarkably, platelets from GPVI$^{-/-}$ patients displayed no aggregatory response to the fibrin suspension.

When 100µg/ml fibrinogen was added to the platelet suspensions, no aggregatory response was observed for any genotype. Reactivity was confirmed for all platelet preparations via stimulation with thrombin (figure 5.1c).

For all of the aggregatory stimulations, lysates were prepared and analysed via western blotting following immunoprecipitation for Syk. Non-stimulation and fibrinogen stimulation did not induce Syk phosphorylation – a marker of GPVI pathway activation – in platelets from controls, GPVI$^{+/-}$ patients or GPVI$^{-/-}$ patients. Syk phosphorylation was observable in healthy control samples as expected, as well as in GPVI$^{+/-}$ patient samples. No phosphorylation was observable following collagen stimulation of GPVI$^{-/-}$ platelets. Unfortunately, due to the addition of BSA to the platelet preparations and the relative insolubility of the cross-linked fibrin fibres used in the stimulations, no assessment of the phosphorylation status of Syk – nor global platelet protein phosphorylation status – following fibrin stimulations were possible.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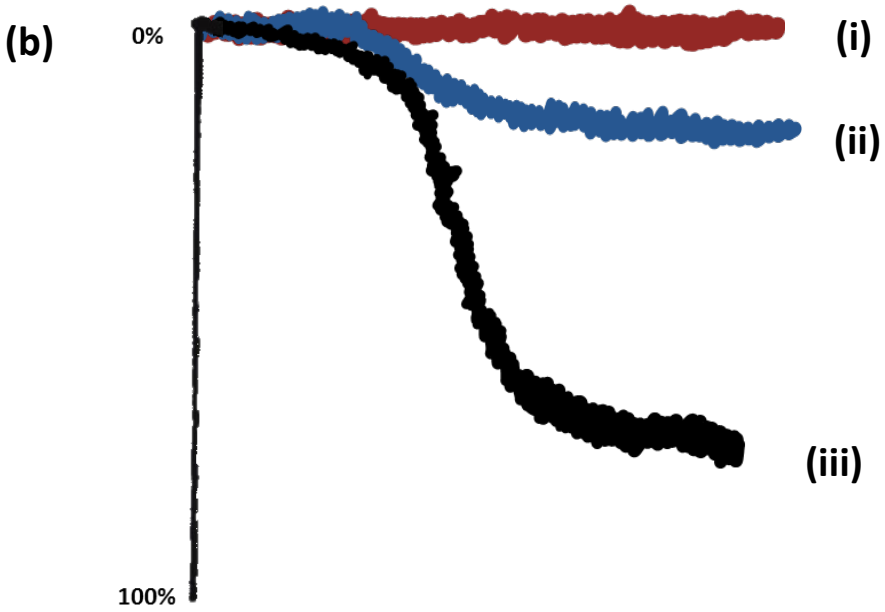
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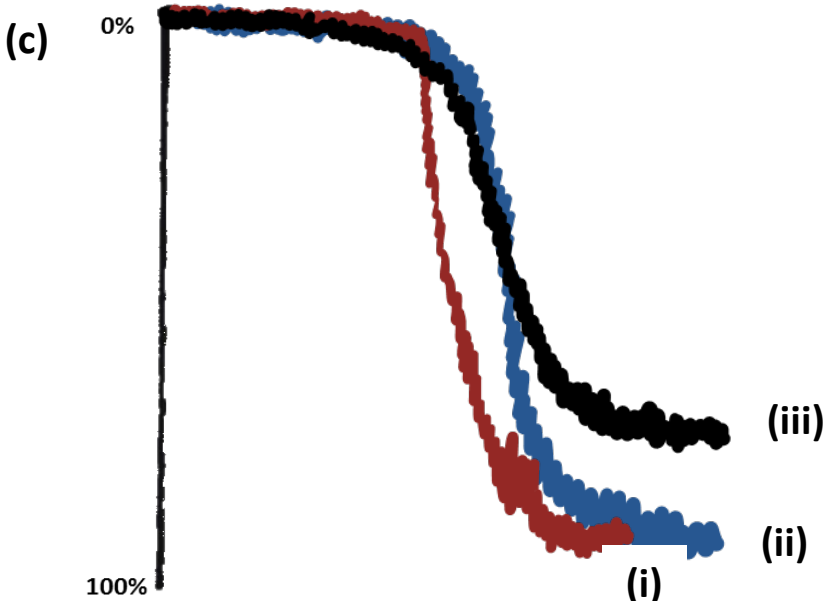
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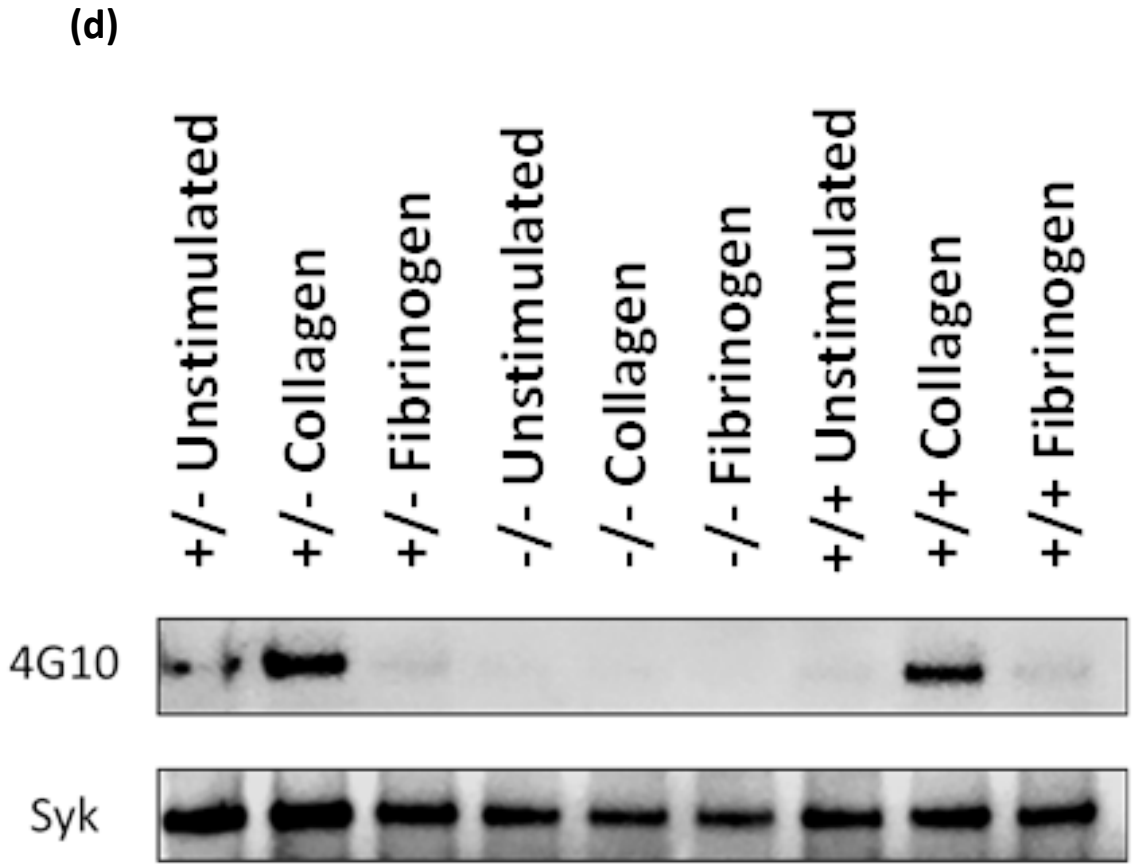


Figure 5.1 – Platelets in suspension from GPVI$^{-/-}$, but not GPVI$^{+/-}$ or GPVI$^{+/+}$ patients, display a complete loss of response to collagen and fibrin, but not thrombin. Aggregation responses were measured in washed platelets obtained from GPVI$^{-/-}$, GPVI$^{+/-}$ and GPVI$^{+/+}$ patients in response to collagen (a), fibrin (b) and thrombin (c) (GPVI$^{-/-}$, N=2; GPVI$^{+/-}$, N=2; GPVI$^{+/+}$, N=1). Phosphorylation of Syk was measured in platelets obtained from GPVI$^{-/-}$, GPVI$^{+/-}$ and GPVI$^{+/+}$ patients, and stimulated with collagen, fibrin and fibrinogen, via immunoprecipitation and subsequent pan-phosphotyrosine staining, figure is representative of N=2 (GPVI$^{-/-}$, GPVI$^{+/-}$) and N=1 (GPVI$^{+/+}$).

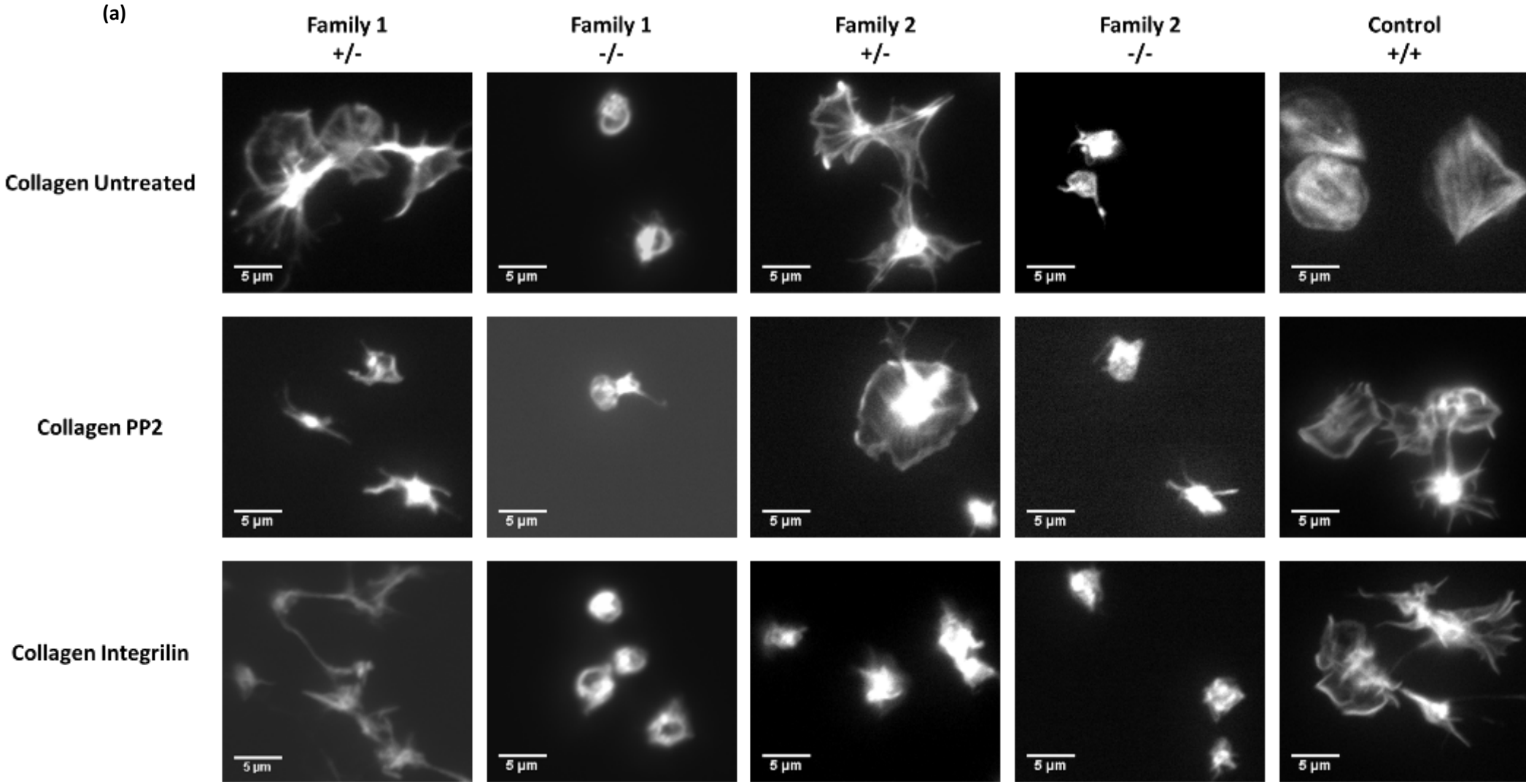
5.2.2 *Platelets from GPVI$^{-/-}$ patients display impaired spreading on collagen coated surfaces*

Platelet spreading on collagen was assessed in this study as it is the prototypical, physiological agonist for GPVI, and also acts a positive control for loss of function. Following incubation of untreated platelets from GPVI$^{+/-}$, GPVI$^{-/-}$ and control platelets on a collagen coated surface, a numerical reduction in the number of spread platelets and spread platelet surface area was observed in platelets from GPVI$^{-/-}$ patients when compared with control platelets, whilst untreated GPVI$^{+/-}$ platelets were indistinguishable from controls (figure 5.2).

Platelets were then pre-incubated with the αIIbβ3 inhibitor integrilin to isolate any GPVI specific spreading defects; αIIbβ3 is the most highly expressed platelet integrin and has been shown to play a key role in platelet spreading. When platelets were pre-incubated with integrilin and spread on collagen (figure 5.2), a marked reduction in spread platelet area was observed for all genotypes compared with untreated platelets. Interestingly, when the level of platelet adherence was assessed under these conditions, a numerical difference was observed in GPVI$^{-/-}$ samples compared with controls as expected, however less adherence was also observed in the GPVI$^{+/-}$ platelets from the patient in family 1 but not family 2.

Platelets were also pre-treated with the SFK inhibitor PP2, to ensure that tyrosine kinase signal transduction, and GPVI function, was completely inhibited in these samples, and also to determine if any spreading responses might be due to fibrin interacting with any of the other hemITAM receptors expressed on human platelets.

When platelets were pre-incubated PP2 and spread on collagen (figure 5.2), there was a mild reduction in spread area for all genotypes compared with untreated samples, however the largest differences again remained between GPVI$^{-/-}$ patients and controls (figure 5.2). Interestingly, in terms of platelet adherence to a collagen surface, PP2 induced similar changes to those observed in platelet preparations pre-treated with integrilin, with the largest differences observed between both GPVI$^{-/-}$ patients, alongside the GPVI$^{+/-}$ patient from family 1, and controls (figure 5.2).


(a)
Family 1
+/-
Family 1
-/-
Family 2
+/-
Family 2
-/-
Control
+/+
Collagen Untreated
Collagen PP2
Collagen Integrilin
5 µm

(b)

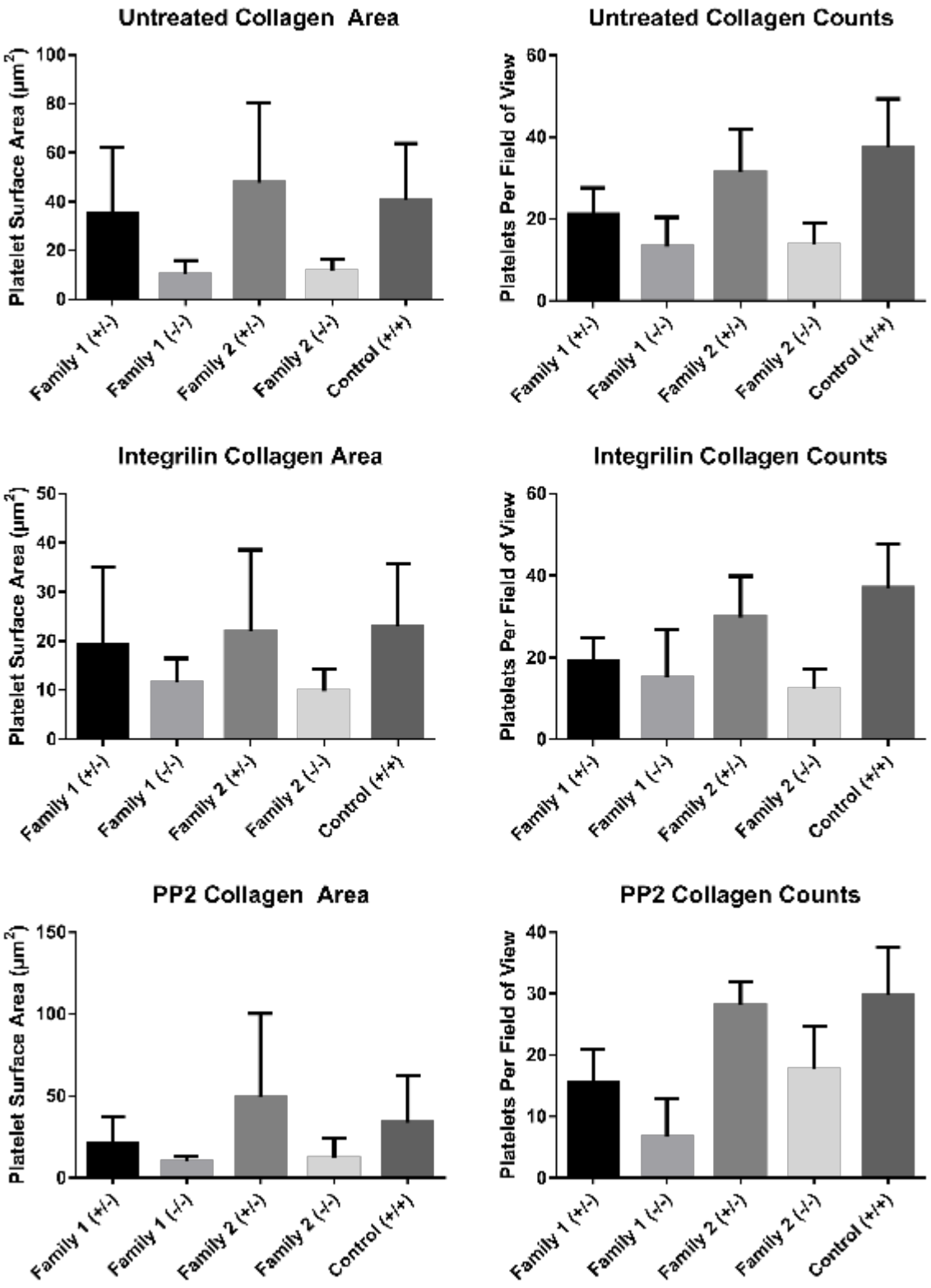


Figure 5.2 – Platelets from GPVI$^{-/-}$ and, to a lesser extent GPVI$^{+/-}$, patients display impaired spreading on collagen coated surfaces compared with a GPVI$^{+/+}$ control. (a) Representative microscopy figures of platelets in the presence and absence of PP2 and integrilin, obtained from GPVI$^{-/-}$, GPVI$^{+/-}$ and GPVI$^{+/+}$ subjects, when spread on collagen coated surfaces, scale bar = 5µM. (b) Quantitation of spread platelet area, perimeter and number of adherent platelets obtained from GPVI$^{-/-}$, GPVI$^{+/-}$ and GPVI$^{+/+}$ patients, in the presence and absence of PP2 and integrilin, when spread on collagen coated surfaces (GPVI$^{-/-}$, GPVI$^{+/-}$, N=2; GPVI$^{+/+}$, N=1).

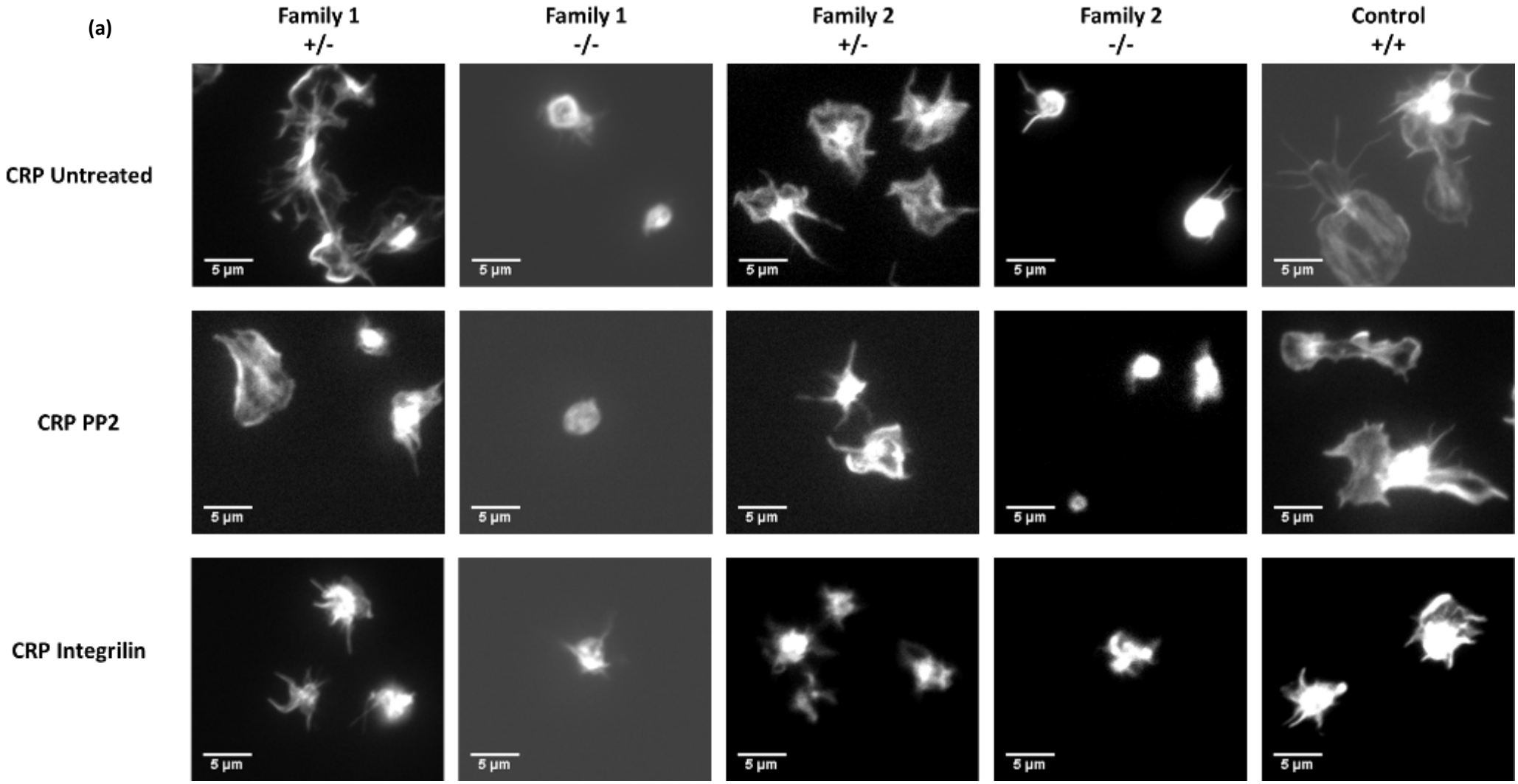
5.2.3 *Platelets from GPVI$^{-/-}$ patients display impaired spreading on CRP coated surfaces*

Platelet spreading on CRP was assessed in this study as whilst it is similar to collagen, it activates GPVI in the absence of concurrent stimulation of the integrin $\alpha 2\beta 1$; collagen induces activation of both receptors. The use of CRP allows for direct assessment of the impact of loss of GPVI on functional responses. Following incubation of untreated platelets from GPVI$^{+/-}$, GPVI$^{-/-}$ and control platelets, a large reduction in spread platelet surface area was observed in platelets from GPVI$^{-/-}$ patients when spread on CRP (figure 5.3); surprisingly, the GPVI$^{-/-}$ patient from family 1 displayed a larger impairment in platelet adherence on CRP than the GPVI$^{-/-}$ patient from family 2 (figure 5.3). Untreated GPVI$^{+/-}$ platelets were indistinguishable from controls in all readouts of platelet spreading (figure 5.3).

When pre-treated with integrilin, a global reduction in spread area compared with untreated samples was observed in response to CRP – similar to that seen for collagen. However, a surprising difference was observed in the GPVI$^{+/-}$ patient from family 2, alongside the expected differences in the GPVI$^{-/-}$ patients (figure 5.3) when compared with controls. As expected, GPVI$^{-/-}$ patients displayed a large reduction in platelet adherence compared to controls following integrilin pre-treatment (figure 5.3).

When platelets were pre-treated with PP2, a reduction in spread platelet area was observed in all genotypes – similar to that observed for PP2-treated samples spread on collagen – compared with untreated platelets; the GPVI$^{-/-}$ patients again

displayed the largest differences in spreading compared with controls (figure 5.3). Interestingly, when the number of adherent platelets was assessed, both $GPVI^{-/-}$ patients as well as the $GPVI^{+/-}$ patient from family 1 showed similar reductions compared to controls (figure 5.3).


(a)
Family 1
+/-
Family 1
-/-
Family 2
+/-
Family 2
-/-
Control
+/+
CRP Untreated
CRP PP2
CRP Integrilin
5 µm

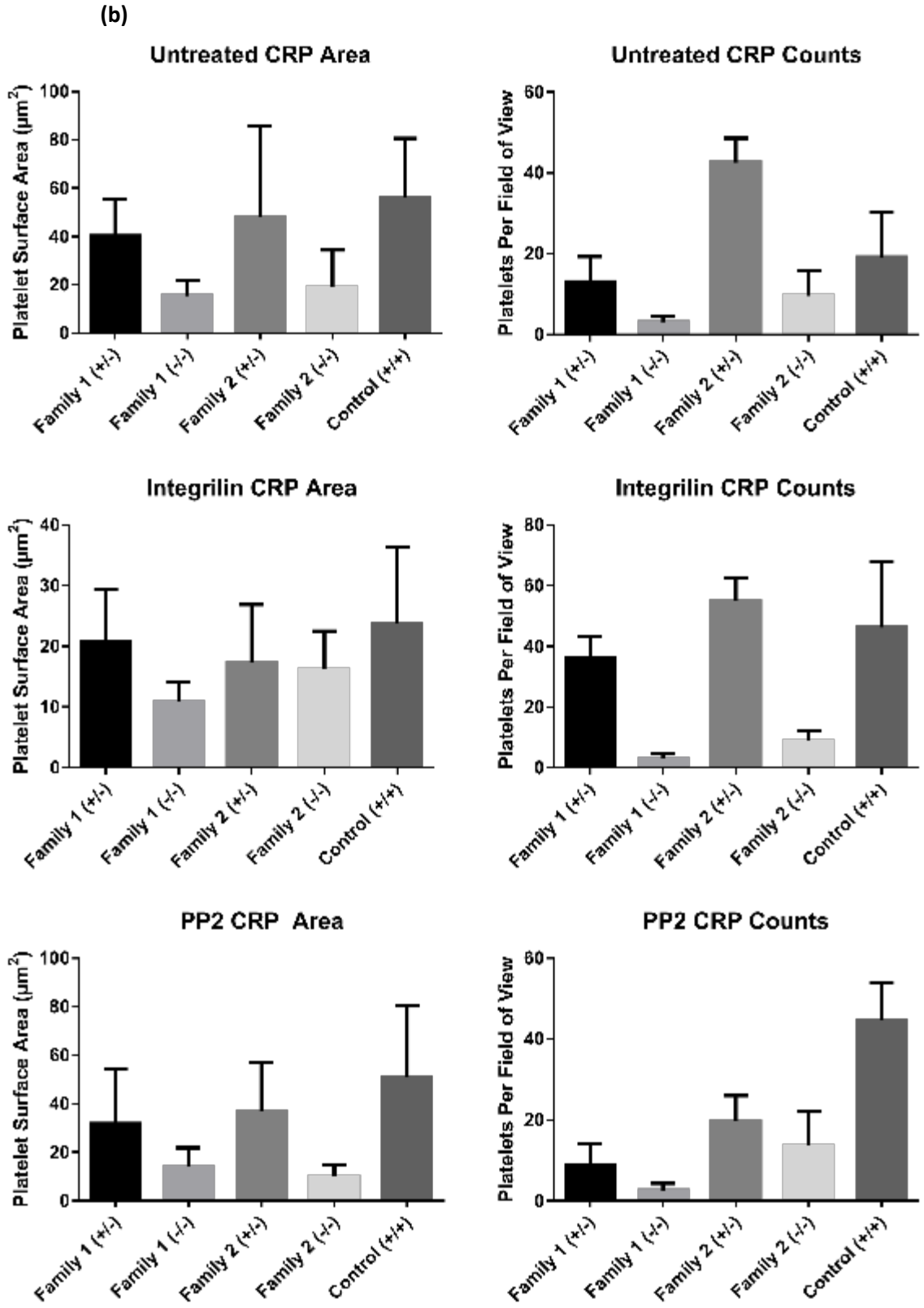


Figure 5.3 – Platelets from GPVI-/- and, to a lesser extent GPVI+/-, patients display impaired spreading on CRP coated surfaces compared with a GPVI+/+ control. (a) Representative microscopy figures of platelets in the presence and absence of PP2 and integrilin, obtained from GPVI-/-, GPVI+/- and GPVI+/+ subjects, when spread on CRP coated surfaces, scale bar = 5µM. (b) Quantitation of spread platelet area, perimeter and number of adherent platelets obtained from GPVI-/-, GPVI+/- and GPVI+/+ patients, in the presence and absence of PP2 and integrilin, when spread on CRP coated surfaces (GPVI-/-, GPVI+/-, N=2; GPVI+/+, N=1).

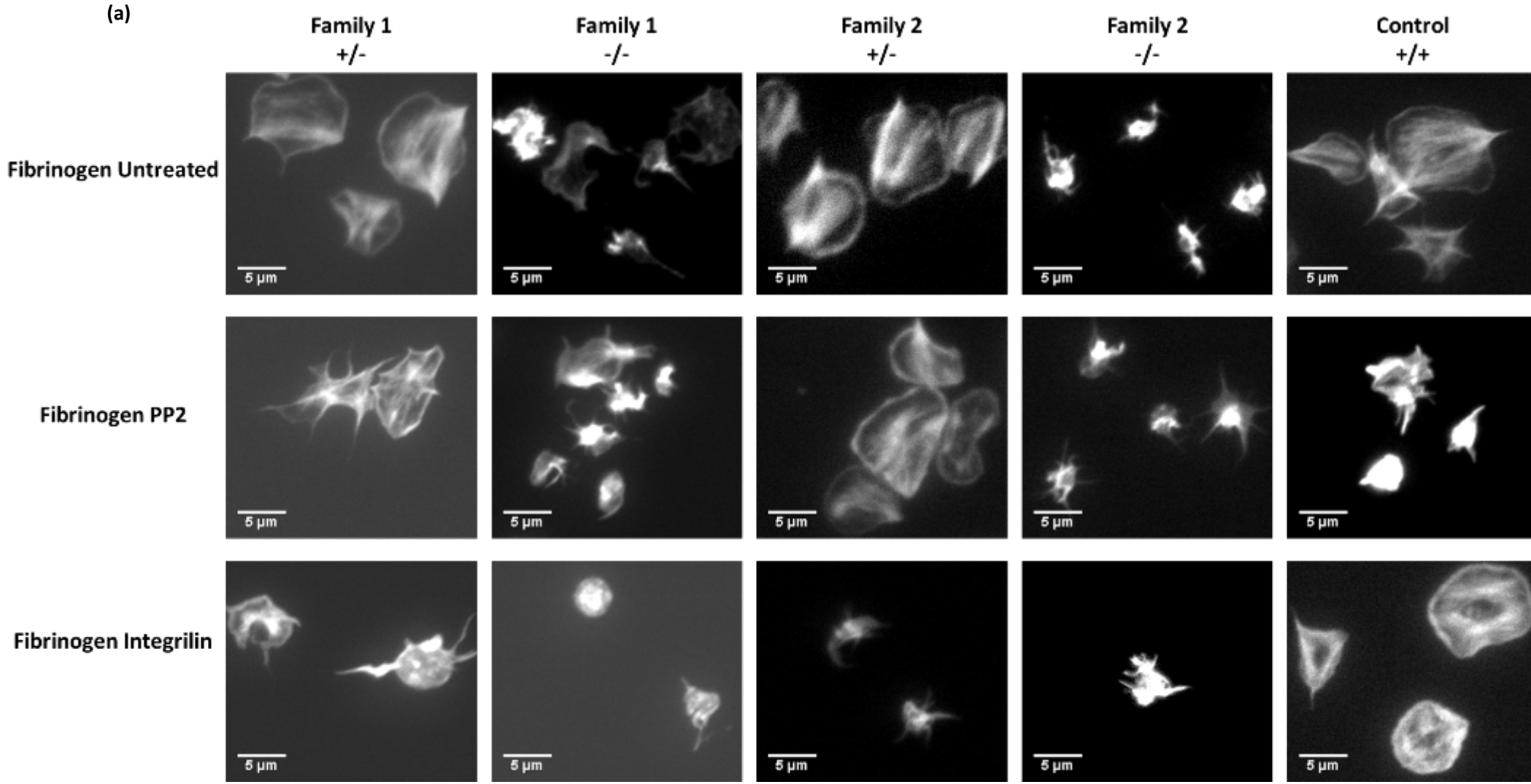
5.2.4 *Platelets from GPVI$^{-/-}$ patients display impaired spreading on fibrinogen coated surfaces*

Platelet spreading on fibrinogen was assessed in this study as a control, as it was not believed that fibrinogen interacts with GPVI to induce platelet spreading. Following incubation of untreated platelets from GPVI$^{+/-}$, GPVI$^{-/-}$ and control platelets, an entirely unexpected reduction in the number of adherent platelets and the spread platelet surface area was observed in platelets from GPVI$^{-/-}$ patients when spread on fibrinogen (figure 5.4). Again, untreated GPVI$^{+/-}$ platelets were indistinguishable from controls in all readouts of platelet spreading (figure 5.4).

When pre-treated with integrilin a large reduction in platelet surface area was observed for all GPVI$^{+/-}$ and GPVI$^{-/-}$ patient samples compared with controls (figure 5.4); integrilin appeared to have little effect on the control samples compared to untreated control platelet samples. Interestingly, integrilin pre-treatment induced the largest reduction in platelet adherence on fibrinogen in GPVI patients from family 1; both GPVI$^{+/-}$ and GPVI$^{-/-}$ patients from family 2 displayed much smaller differences in adherence compared (figure 5.4).

When pre-treated with PP2, no real differences in platelet spreading in GPVI$^{+/-}$ patients were observed compared with untreated samples; both GPVI$^{-/-}$ patients again displayed large reductions in platelet surface area compared with controls (figure 5.4). However, another extremely surprising result was observed in platelet adherence, as PP2 pre-treatment appears to potentiate the adherence of all patient

samples to a fibrinogen surface; the GPVI$^{+/-}$ patient from family 2 displayed an increase in platelet adherence compared with controls (figure 5.4).


(a)
Family 1
+/-
Family 1
-/-
Family 2
+/-
Family 2
-/-
Control
+/+
Fibrinogen Untreated
Fibrinogen PP2
Fibrinogen Integrilin
5 µm

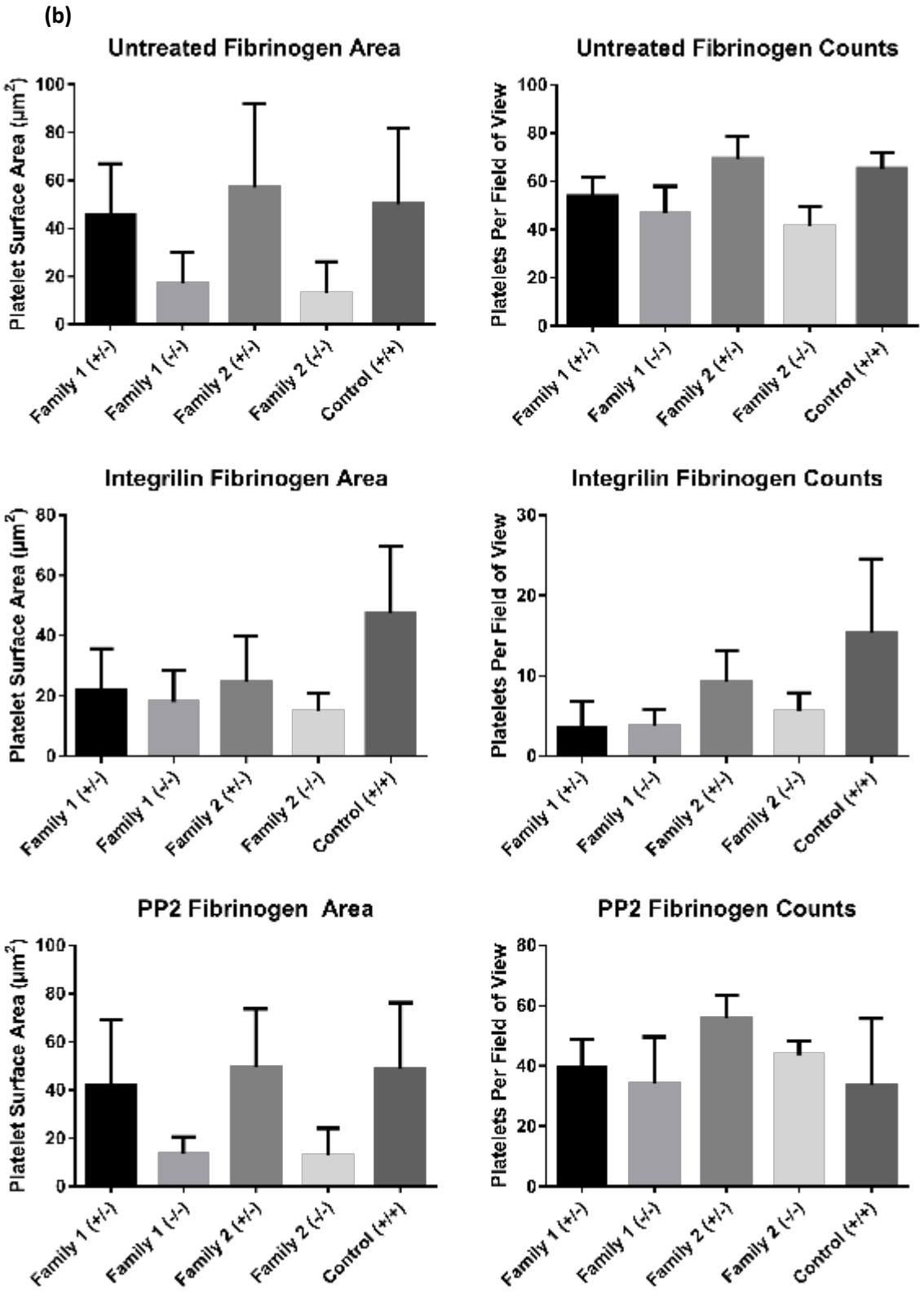


Figure 5.4 - Platelets from GPVI-/- and, to a lesser extent GPVI+/-, patients display impaired spreading on fibrinogen coated surfaces compared with a GPVI+/+ control. (a) Representative microscopy figures of platelets in the presence and absence of PP2 and integrilin, obtained from GPVI-/-, GPVI+/- and GPVI+/+ subjects, when spread on fibrinogen coated surfaces, scale bar = 5µM. (b) Quantitation of spread platelet area, perimeter and number of adherent platelets obtained from GPVI-/-, GPVI+/- and GPVI+/+ patients, in the presence and absence of PP2 and integrilin, when spread on fibrinogen coated surfaces (GPVI-/-, GPVI+/-, N=2; GPVI+/+, N=1).

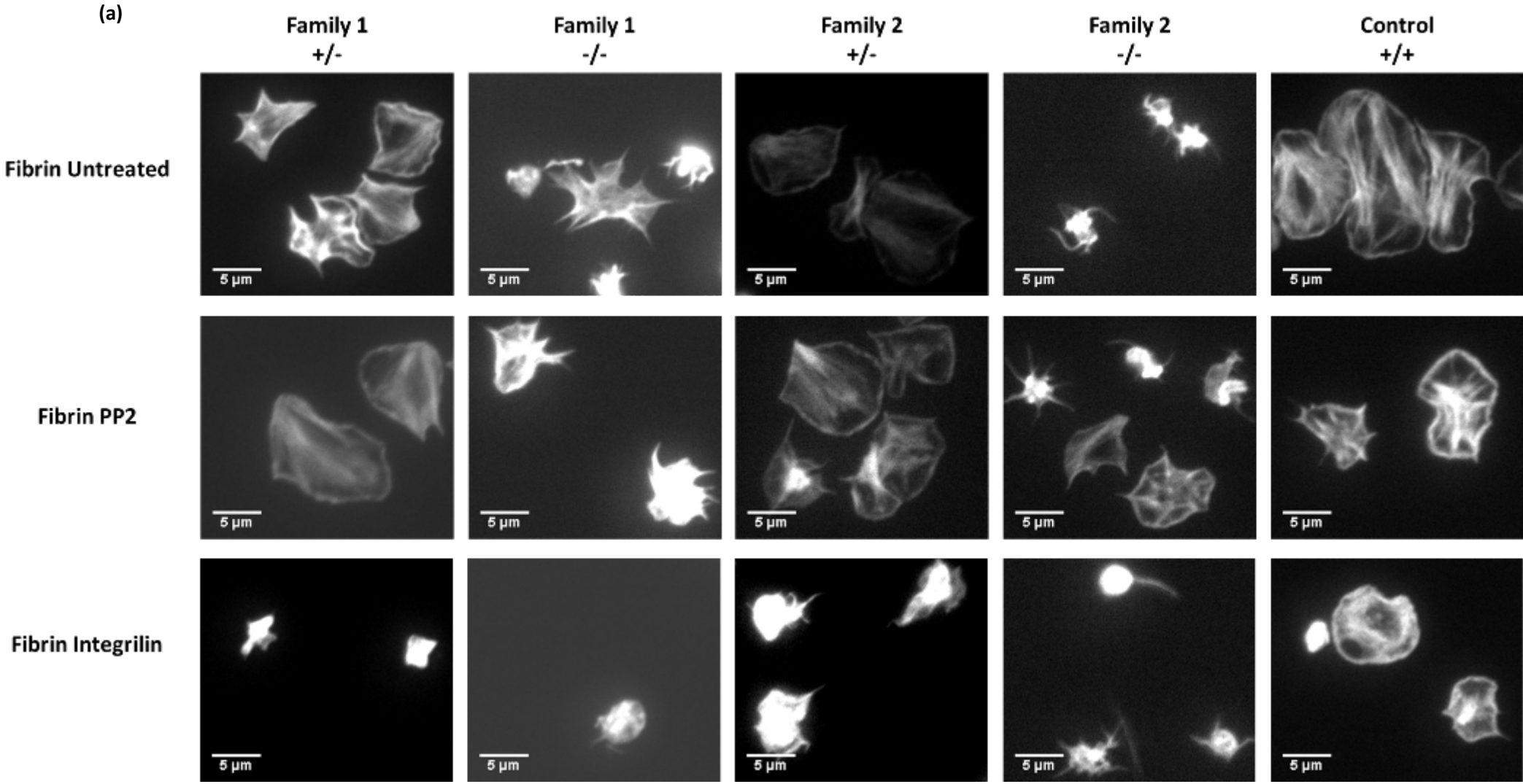
5.2.5 *Platelets from GPVI$^{-/-}$ patients display impaired spreading on fibrin coated surfaces*

Platelet spreading on fibrin was assessed in this study as it was recently described to be a novel agonist for GPVI. Following incubation of untreated platelets from GPVI$^{+/-}$, GPVI$^{-/-}$ and control platelets, a large reduction in spread platelet surface area was observed in platelets from GPVI$^{-/-}$ patients when spread on fibrin (figure 5.5). Interestingly, impairments in platelet adherence were observed in both GPVI$^{-/-}$ patients, alongside the GPVI$^{+/-}$ patient from family 1; the GPVI$^{+/-}$ patient from family 2 was much more similar to controls (figure 5.5). Untreated GPVI$^{+/-}$ platelets were indistinguishable from controls when measured via platelet surface area (figure 5.5).

When pre-treated with integrilin, whilst a reduction in platelet surface area was observed for all genotypes, the largest impairments were observed in the GPVI$^{+/-}$ patient from family 1; surprisingly smaller differences were observed in GPVI$^{-/-}$ patients compared with the heterozygous patient from family 1 (figure 5.5). When platelet adherence was assessed, both GPVI$^{-/-}$ patients displayed a substantial impairment compared with controls, as did the GPVI$^{+/-}$ patient from family 1 (figure 5.5).

When pre-treated with PP2, again a mild difference in platelet spreading was observed compared with untreated samples for all genotypes; again the GPVI$^{-/-}$ patients displayed the largest reduction in platelet surface area compared with controls (figure 5.5). However, PP2 pre-treatment again appears to potentiate the adherence of patient samples to a fibrin coated surface; the GPVI$^{+/-}$ patient from

family 2 again displayed an increase in platelet adherence compared with controls (figure 5.5).


(a)
Family 1
+/-
Family 1
-/-
Family 2
+/-
Family 2
-/-
Control
+/+
Fibrin Untreated
Fibrin PP2
Fibrin Integrilin
5 µm

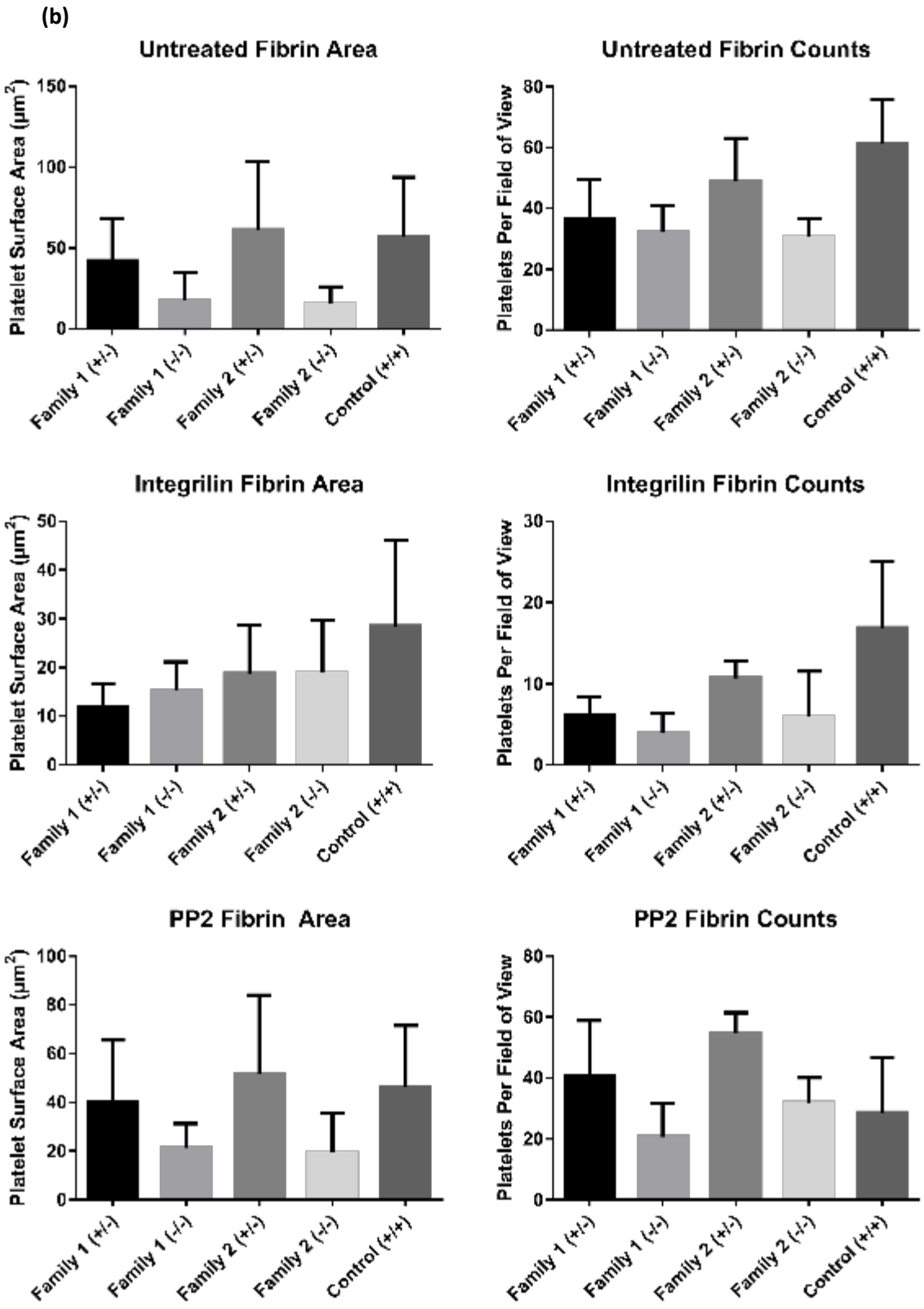


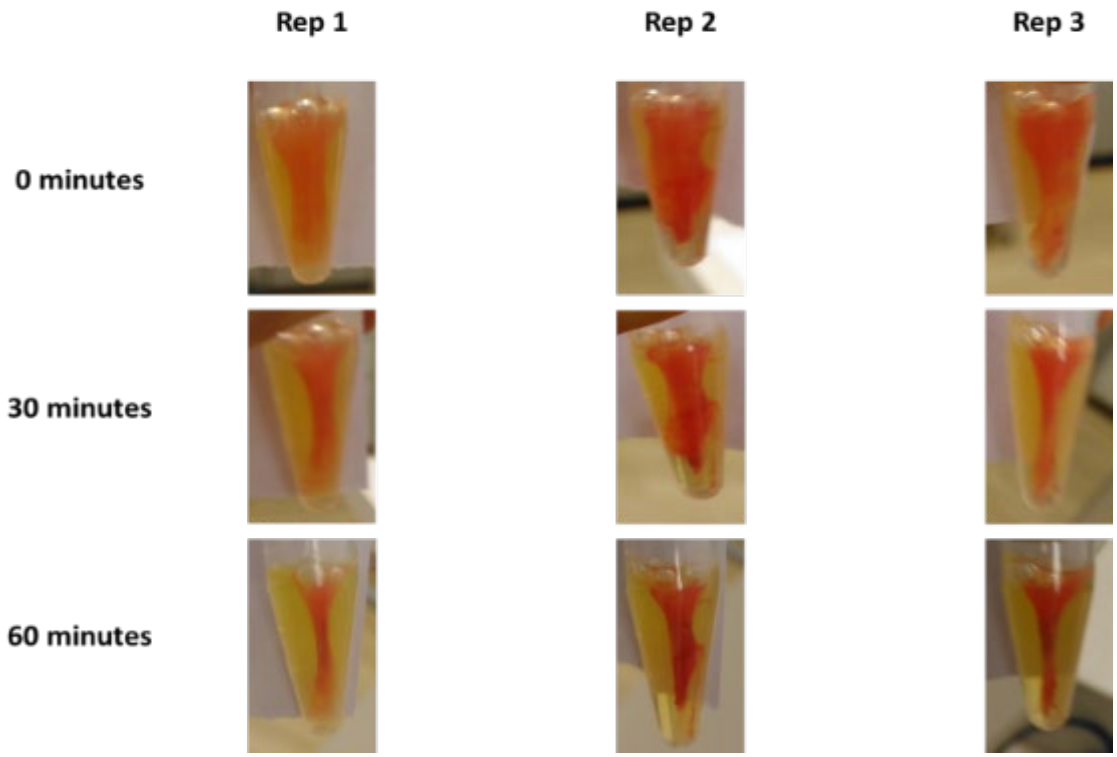
Figure 5.5 - Platelets from GPVI-/- and, to a lesser extent GPVI+/-, patients display impaired spreading on fibrin coated surfaces compared with a GPVI+/+ control. (a) Representative microscopy figures of platelets in the presence and absence of PP2 and integrilin, obtained from GPVI-/-, GPVI+/- and GPVI+/+ subjects, when spread on fibrin coated surfaces, scale bar = 5µM. (b) Quantitation of spread platelet area, perimeter and number of adherent platelets obtained from GPVI-/-, GPVI+/- and GPVI+/+ patients, in the presence and absence of PP2 and integrilin, when spread on fibrin coated surfaces (GPVI-/-, GPVI+/-, N=2; GPVI+/+, N=1).

5.2.6 *There appear to be no differences in the kinetics of thrombus formation between GPVI$^{-/-}$, GPVI$^{+/-}$ and controls.*

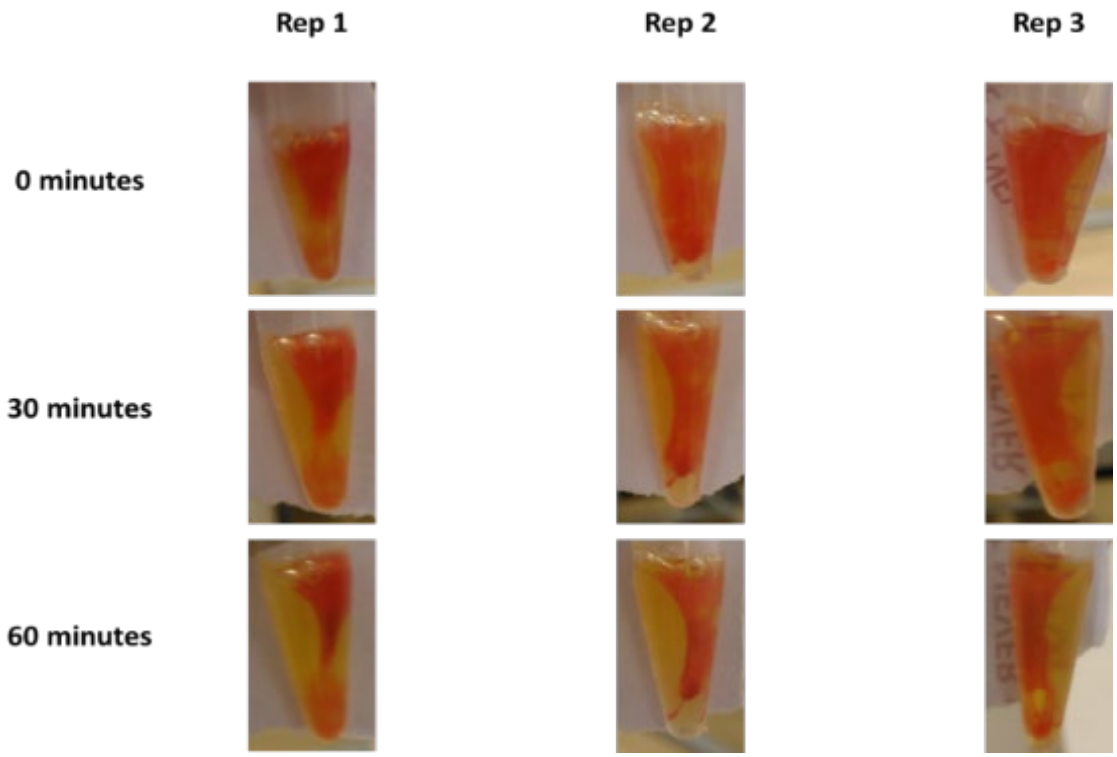
One of the key roles of fibrin is in the stabilisation and retraction of thrombi, and so it was hypothesised that patients deficient in GPVI may have impaired functional responses due to diminished fibrin signal transduction. To assess this, a modified clot retraction assay was performed, and the time-course of clot formation was measured.

As can be seen from the temporal analysis of thrombus formation (figure 5.6), thrombi formed over equal timescales regardless of genotype, and gross thrombi morphology was broadly similar across genotypes and timepoints (figure 5.6). Analysis of the kinetics of thrombus formation was performed visually, and as such further clot retraction experiments should be performed to assess the kinetics in more detail.

(a) **Control**



(b) **Heterozygote**



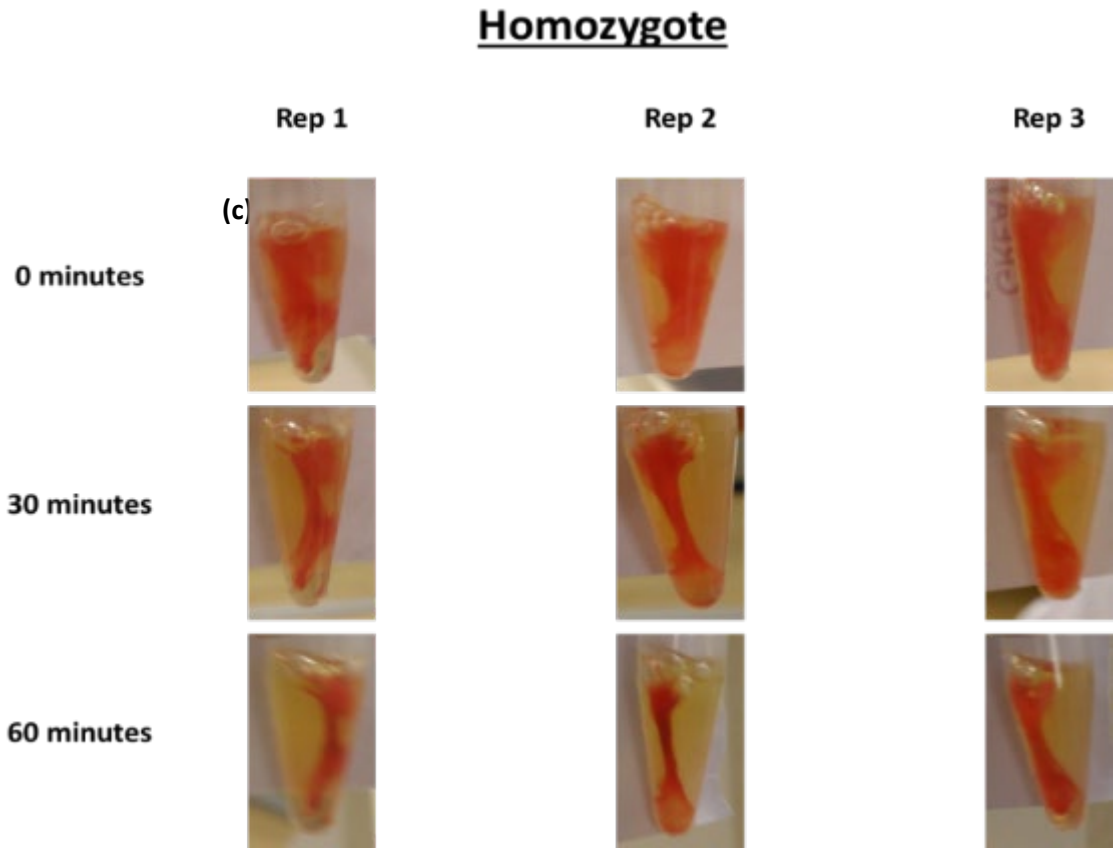


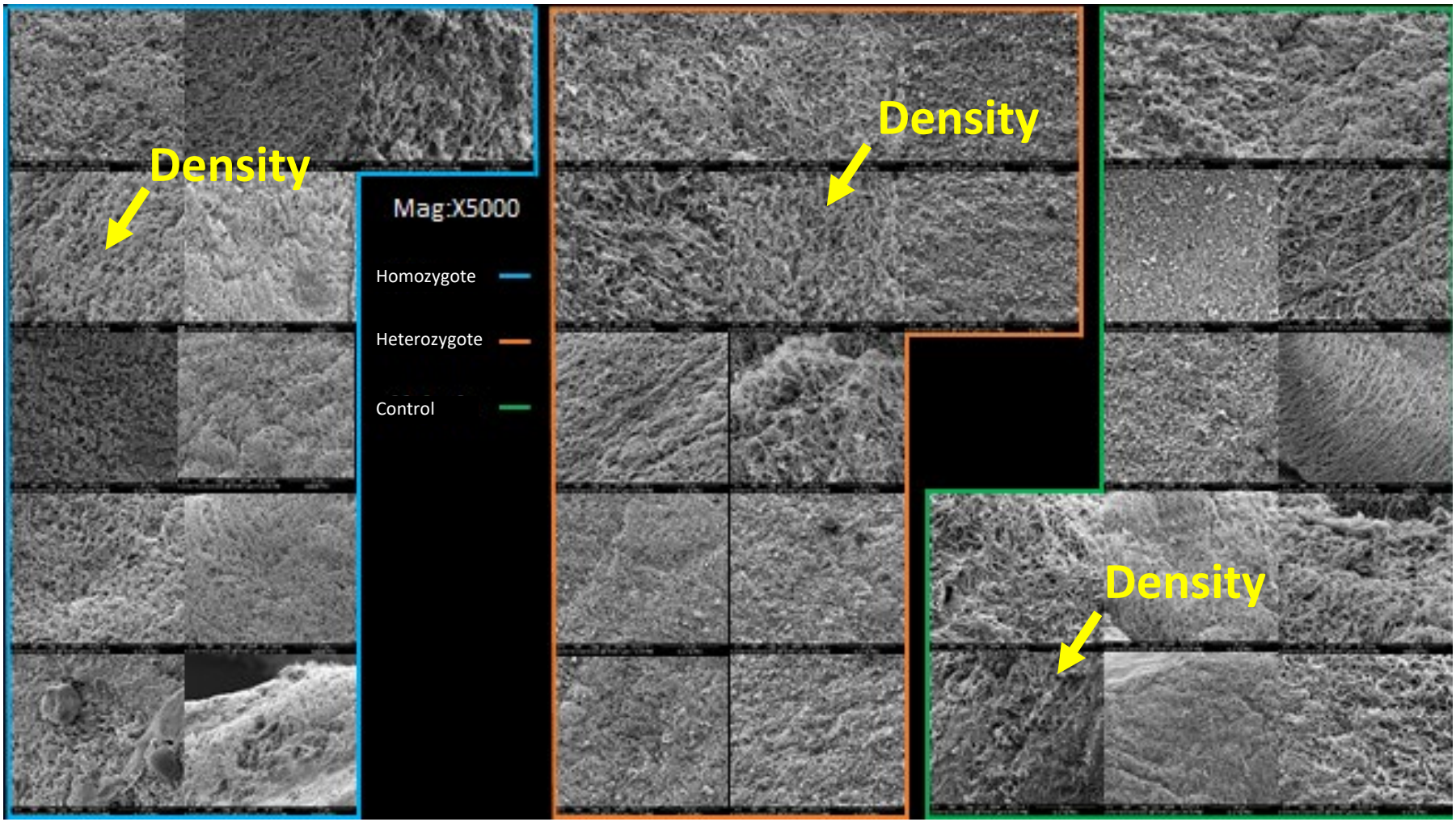
Figure 5.6 – GPVI$^{-/-}$ and GPVI$^{+/-}$ patients display no visual differences in the kinetics of thrombus formation compared with a GPVI$^{+/+}$ control. Platelet rich plasma was obtained from GPVI$^{-/-}$, GPVI$^{+/-}$ and GPVI$^{+/+}$ subjects and a small volume of red blood cells was re-introduced, before $CaCl_2$, fibrinogen and thrombin were added. Pictures were taken every 30 mins and after 60 mins, clots were dehydrated and fixed in glutaraldehyde for scanning electron microscopy analysis, N=3.

5.2.7 There appear to be no differences in the macro- or microscopic thrombus ultrastructure between GPVI$^{-/-}$, GPVI$^{+/-}$ and controls.

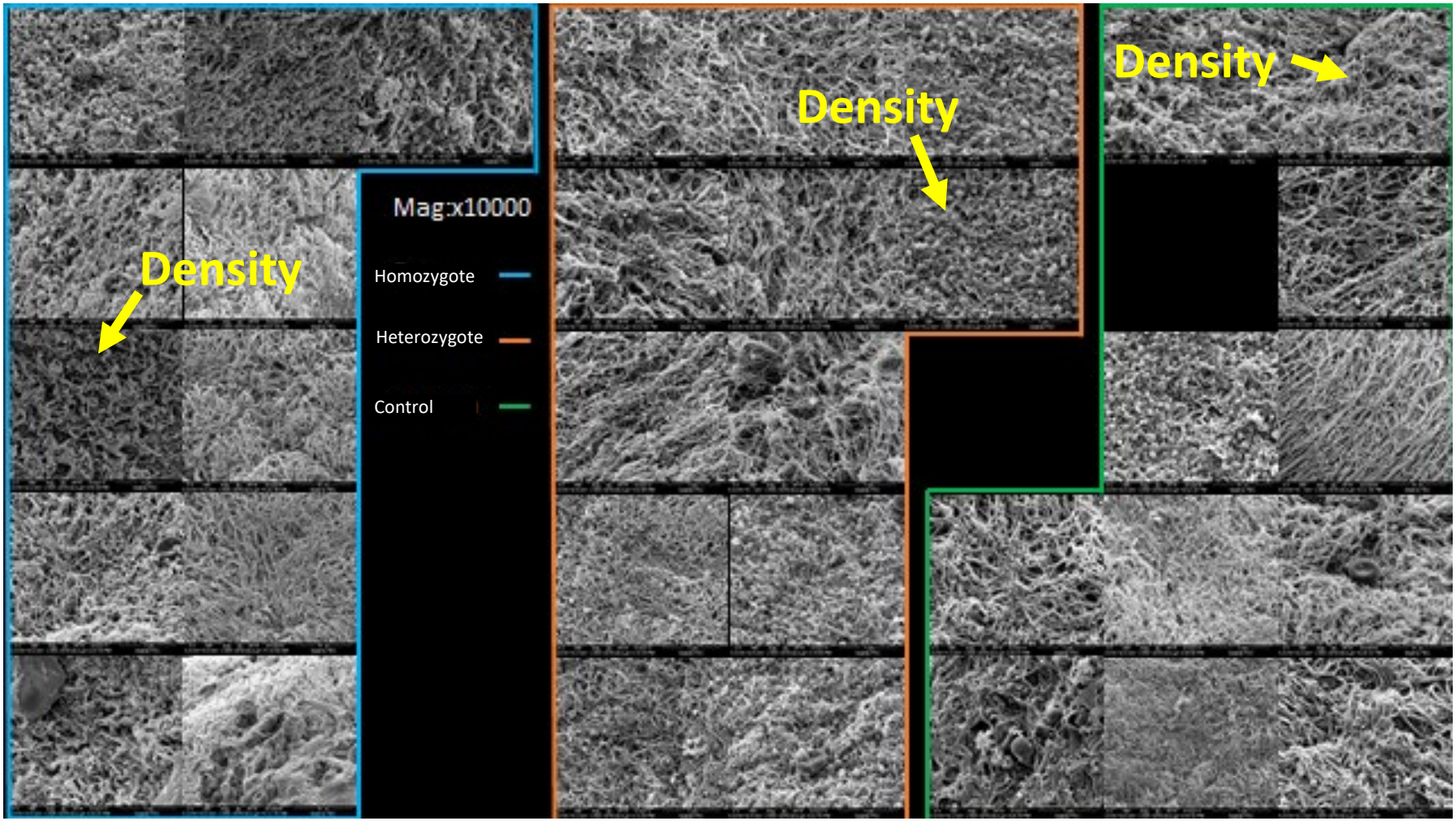
Following the modified clot retraction assay, clots were fixed, dehydrated and assessed via scanning electron microscopy (SEM). Following sixty minutes of clot retraction, samples were fixed and dehydrated before scanning electron microscopy analysis; scanning electron microscopy was kindly performed by Professor Robert Ariens. As can be seen from all of the different SEM magnifications – 5000x (figure 5.3bi), 10000x (figure 5.3bii), 250000x (figure 5.3biii), and 50000x (figure 5.3biv) – there appear to be no differences in thrombi structure. The fibrin fibre size, density, organisation and locations all appear to be similar across all genotypes, and no differences are apparent in the presence/absence of cells including platelets, red blood cells and/or white blood cells.

All analysis of the SEM pictures was performed visually, and further, more in-depth analysis of this data is required to elucidate any minor differences in thrombus structure that may exist.

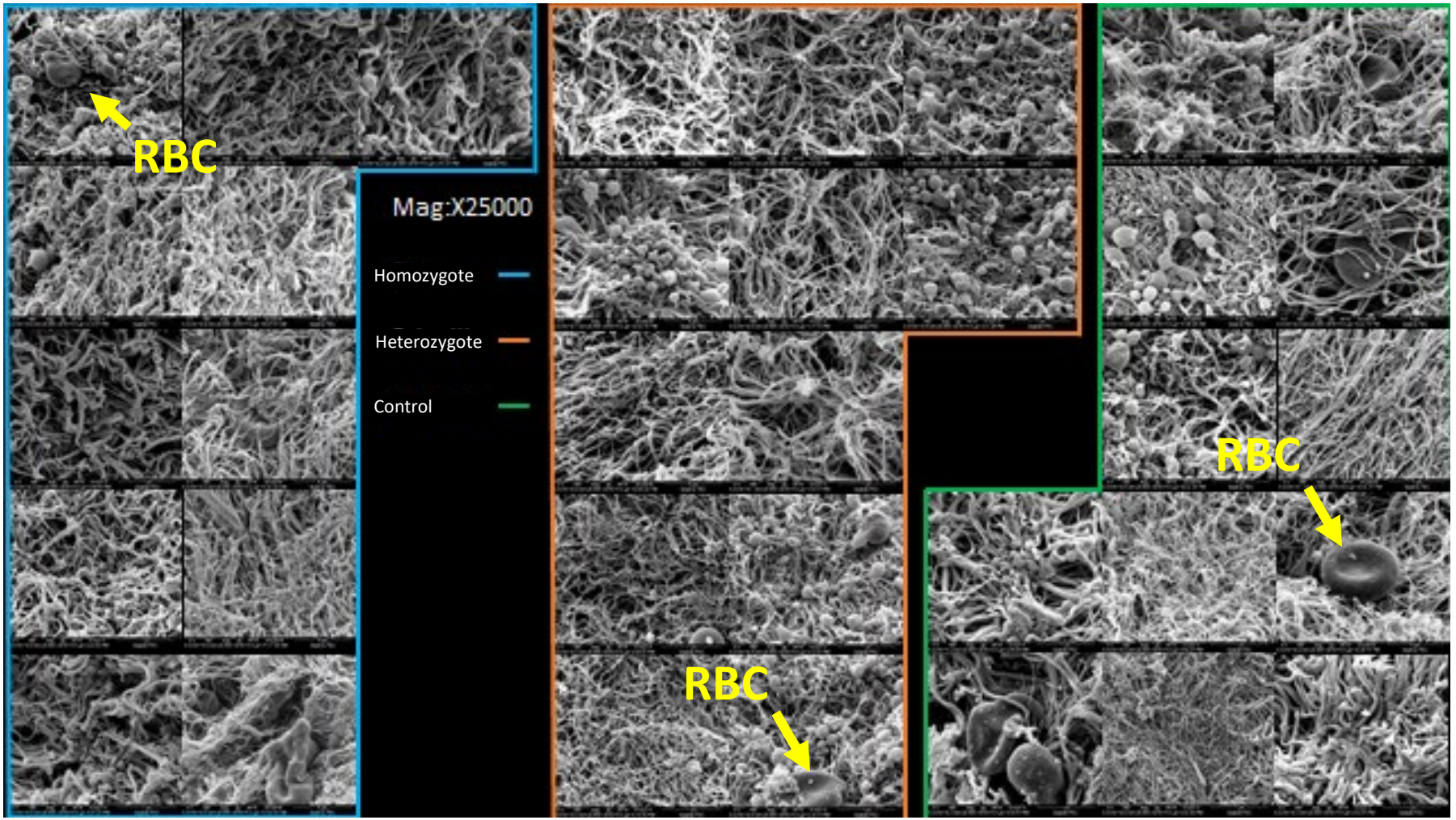
(a)


Density
Mag:X5000
Homozygote
Heterozygote
Control
Density
Density

(b)


Density
Mag:x10000
Homozygote
Heterozygote
Control
Density
Density

(c)



(d)

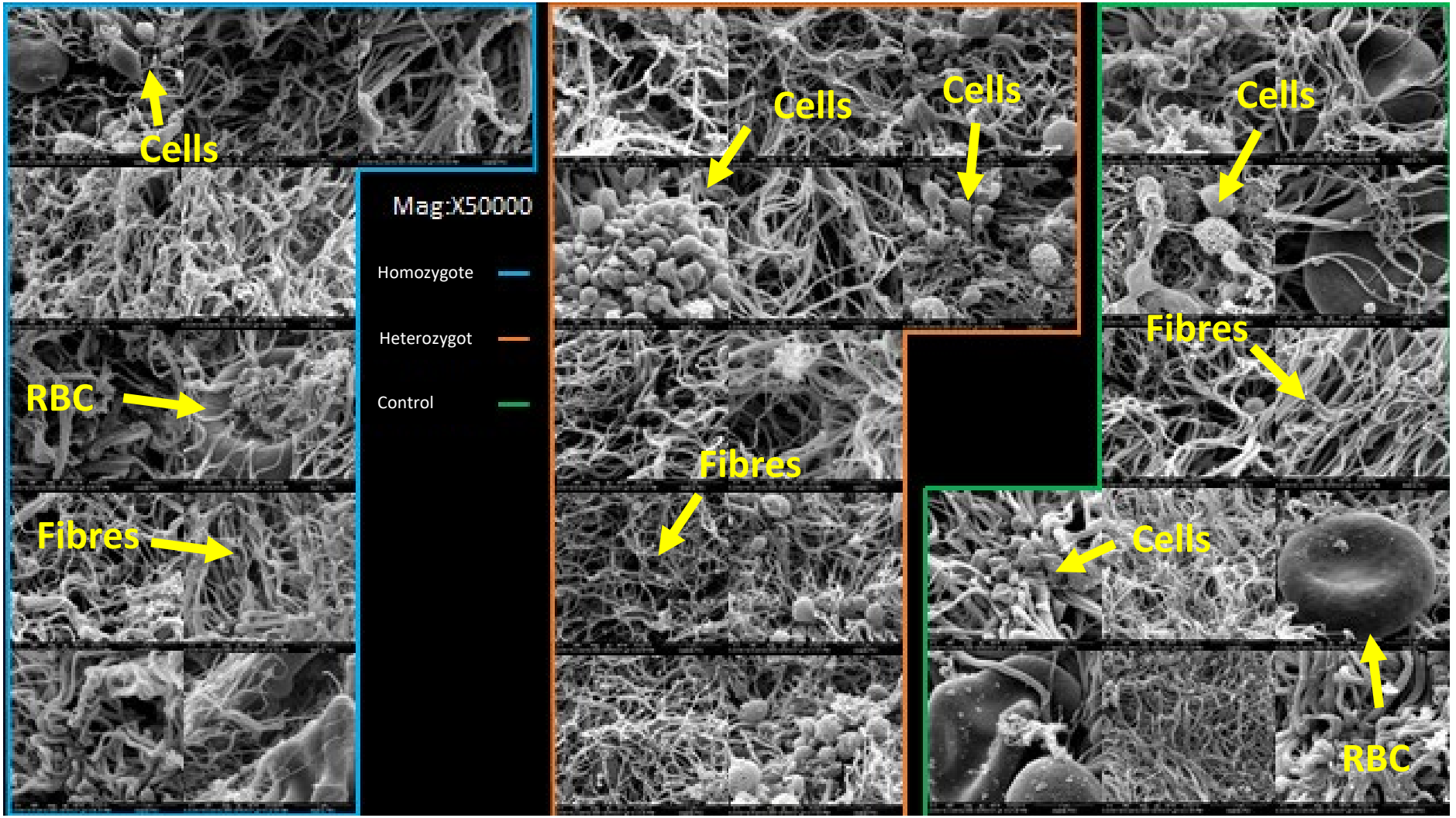


Figure 5.7 – Thrombus composition appears similar between GPVI$^{-/-}$, GPVI$^{+/-}$ and GPVI$^{+/+}$ patients. Scanning electron microscopy (SEM) images at 5000x (a), 10000x (b), 25000x (c) and 50000x (d) magnification. SEM was kindly performed by Professor Robert Ariens, and data was assessed visually (N=1 for each genotype); Density = Fibrin Fibre Density, RBC = Red Blood Cell, Cells = Platelets and White Blood Cells, Fibres = Fibrin Fibres.

5.3 Discussion

This chapter has shown for the first time that patients with a homozygous mutation in GPVI rendering it absent from the platelet surface membrane do not respond to fibrin stimulation in suspension or on a surface and is the first description of a requirement for GPVI on the surface of human platelets to support spreading on immobilised fibrinogen.

When measures specific to platelet activation and function were assessed, namely platelet aggregometry and spreading, patients with a homozygous GPVI mutation displayed a complete loss of response to the typical GPVI agonists collagen and CRP; these results are in keeping with previous models of GPVI abrogation, such as knock-out mouse models (258). Surprisingly however, family members with a heterozygous expression pattern of the GPVI mutation were almost indistinguishable from healthy controls, particularly in response to collagen (figure 5.1 and figure 5.2). This is particularly surprising, as the heterozygous GPVI patients have previously been described to have an intermediate reduction in GPVI surface expression, and heterozygous GPVI knock-out mice appear to have an intermediate reduction in response to agonists such as collagen (257, 258). These results suggest that there may be a 'critical mass' of GPVI molecules required on the surface of a platelet to support activation and signal transduction; however, it would be important in the first instance to complete full dose-response profiles for the heterozygous patients to several GPVI agonists – similarly to studies in mouse models – to determine whether a mild reactivity defect at borderline stimulatory

concentrations exists. It would remain interesting, however, to determine if there is a ‘magic number’ of GPVI molecules, as whilst these patients are the only described patients with a homozygous mutation resulting in loss of GPVI, there are several other more common conditions which might induce a reduction or complete abolition of GPVI expression at the platelet surface. Conditions such as immune thrombocytopenic purpura and systemic lupus erythematosus have been associated with a loss of platelet GPVI expression (259) and understanding the critical level of GPVI expression may aid in the future management of these, and similar, conditions.

As discussed, there is a complete loss of aggregatory response to both the typical GPVI agonists and fibrin in the homozygous GPVI patients (figure 5.1). Interestingly, when platelets were assessed via spreading analysis, there was a substantial reduction in platelet spreading on fibrin, however platelet adherence – as measured by the number of platelets per field of view – was not as strongly affected (figure 5.5). Whilst the differences in platelet spreading have been observed previously (140), albeit in mouse models, differences in adherence have previously only been measured via methods such as platelet flow over fibrin coated surfaces; this analysis displays significant defects in platelet adherence to fibrin both in GPVI knock-out mice and human GPVI-depleted platelets (141). One obvious difference between these two protocols is that one is a static assay whilst one has the presence of platelet flow, making it likely more physiological, however a comparison of the two methods may provide valuable information on potential differences in the importance of the GPVI-fibrin adherence interaction in high pressure, high flow

vessels, such as arteries, and low pressure, low flow vessels, such as veins. Whilst no such discrepancy is observed in platelet spread on agonists such as collagen (figure 5.2), this disparity in the response to fibrin in this study may possibly be explained by the presence of the traditional platelet fibrin receptor, integrin αIIbβ3. Indeed, there is a large drop in the number of adherent platelets observed for all genotypes and agonists when platelets pre-treated with the integrin αIIbβ3 inhibitor integrilin are compared with untreated platelets (figures 5.2-5.5). Taken together, this information suggests that both GPVI and αIIbβ3 are important for the response of platelets to fibrin, but that the receptors may play different roles and be important at different times; indeed the spreading analysis seems to suggest that GPVI appears to be important for the both the activation and spreading of platelets in response to fibrin, whereas αIIbβ3 appears to be important for tethering, adherence, also the spreading of platelets. It is also interesting that the patients from family 1 appear to be more severely affected in the spreading experiments, particularly when platelets are pre-incubated with inhibitors of platelet function. For example, the GPVI$^{+/-}$ patient from family 1 often displays impairments in line with the GPVI$^{-/-}$ patients, following pre-treatment with inhibitors. It would be interesting to assess this family for other underlying mutations which may affect platelet function, as well as comparing the surface expression levels of GPVI between the two families.

Perhaps one of the more surprising results from previous research into GPVI using deficient mouse models is the minor physiological bleeding phenotypes observed; similar mild bleeding diatheses have been observed in the compound heterozygous and homozygous GPVI patients (141, 257, 258). When considering the textbook

descriptions of platelet activation and thrombus formation, and the integral roles that collagen (initial platelet tethering and activation at sites of vascular damage) and fibrin (fibrin mesh formation and clot retraction) have been recorded as playing (260), it is surprising that a loss of GPVI does not induce a catastrophic loss of haemostatic capability. However, when a modified clot retraction assay was performed in both healthy controls, $GPVI^{+/-}$ patients and $GPVI^{-/-}$ patients (figures 5.6-5.7), there appeared to be no differences in the rate of thrombus formation (figure 5.6) nor in the observable macroscopic and microscopic thrombus components and composition (figure 5.7); for example, the fibrin fibres and presence of red and white blood cells appeared comparable between all genotypes. However, the analysis of this data has been performed visually, and as such is not particularly robust; more in-depth analysis of these experiments is required to elucidate the full profiles of $GPVI^{+/-}$ and $GPVI^{-/-}$ samples compared to controls. Further analysis of the dynamics of thrombus formation and retraction in these patients would also be interesting when considering the results of previous studies, which have shown a loss of GPVI mediates an often significant defect in thrombus stability (140, 141).

This data does suggest, however, that whilst GPVI may be important in platelet activation and thrombus formation, there are other redundant systems in place which can contribute to both initial platelet tethering and activation – systems such as the VWF-GP1ba signalling axis (261) – alongside the mediation of platelet-fibrin interactions – receptors such as αIIbβ3 (262). Coupled with the importance of fibrin in the coagulation cascade (65) – a complementary mechanism of haemostasis – alongside the release of agonists such as thrombin and ADP from activated platelets

and expression of potent pro-coagulant proteins such as tissue factor on damaged endothelial cells, it appears that the importance of GPVI in maintaining haemostasis may be overcome by other complementary systems *in vivo*.

Another interesting and unexpected result from these experiments was the defect in platelet adherence and spreading on fibrinogen in homozygous GPVI deficient patients (figure 4). This hypothesis has been partially explored previously, for example in the studies by Mammdova-Bach et al and Alshehri et al (140, 141), and it appears to have been proven correct by a recent publication using work taken from this chapter, alongside complementary techniques (238). This study showed that – further to the spreading experiments performed in this chapter – mice expressing human GPVI undergo full spreading on a fibrinogen coated surface; mouse platelets do not typically undergo full spreading on fibrinogen coated surfaces. Other work in this study included surface plasmon resonance experiments (clearly showing that fibrinogen binds to monomeric but not dimeric GPVI), Ca^{2+} mobilisation assays (showing clear calcium increases following fibrinogen stimulation of human GPVI expressing murine platelets), and flow adhesion experiments (describing an impairment in platelet aggregate growth on a fibrinogen coated surface) using human GPVI expressing murine platelets pre-treated with a GPVI blocking antibody fragment.

The data from this chapter alongside that performed in the above study clearly describe the presence of a significant interaction between fibrinogen and GPVI. Interestingly, both fibrinogen and fibrin have been shown to bind to monomeric GPVI

rather than dimeric GPVI with almost equal binding affinities (237, 238), raising a number of interesting questions and opening several novel avenues for research. For example, what is the physiological rationale behind GPVI binding to collagen, fibrinogen and fibrin, which are all exposed or expressed at distinct times during thrombus formation? Furthermore, what is the impact of the preferential binding of fibrinogen and fibrin to monomeric GPVI, when GPVI dimerisation and clustering are integral steps for collagen induced GPVI-mediated platelet activation? It will be interesting to further characterise these interactions, and to determine any physiological or pathophysiological roles which they may play.

Overall, the data in this chapter demonstrates that GPVI is a signalling receptor not just for collagen and CRP but also for fibrin, and that a complete loss of surface GPVI expression abolishes the responsiveness of platelets to all three agonists. It has also contributed significantly to the finding that GPVI appears to interact with fibrinogen, and whilst this interaction does not appear to catalyse any robust platelet activity or signal transduction in suspension, it appears to induce activation and platelet spreading when interacting with fibrinogen adhered to a surface (figure 4). This disparity in stimulatory capability is likely due to the simulated cross-linked nature of fibrinogen when incubated on a surface, compared to the presence of monomeric fibres in suspension. Whilst the lack of response to collagen and fibrin in $GPVI^{-/-}$ platelets is apparent in a barrage of platelet specific activation assays, there appeared to be no differences in the kinetics of thrombus formation or in thrombus composition in a modified clot retraction assay, in keeping with the literature which suggests that a deficiency in GPVI does not appear to induce any

thrombotic complications or severe bleeding diatheses (257, 258). The results from the modified clot retraction assay should be interpreted cautiously however, as only a visual assessment was performed and more in-depth analysis is required to generate definitive data from these experiments.

It will be interesting to assess in further detail the full physiological effects of a loss of GPVI on haemostatic capability, particularly any differences that may be present between collagen, fibrin and fibrinogen mediated GPVI activation. It would also be of potential interest to compare heterozygous and homozygous patients to determine the critical number of GPVI molecules required at the platelet surface to sustain signalling and platelet activation, as described above. Overall, the data in this thesis chapter has identified and confirmed fibrin and fibrinogen as novel agonists for GPVI and raises numerous possibilities for further research into the role of the novel GPVI-fibrin and GPVI-fibrinogen interactions in haemostasis, and the potential dual role for the receptor in both initial thrombus formation as well as thrombus propagation and retraction.

Chapter 6

General Conclusions

6.1 Key Results Summary

The main aims of this thesis were to:

- Investigate any potential adapter function of Syk, and explore tyrosine phosphorylation events downstream of GPVI and CLEC-2 using a novel Syk kinase-dead transgenic mouse model (Syk K396R),
- Explore the known hyporeactivity of neonatal platelets, particularly in response to collagen, and determine if this impaired responsiveness extends to CLEC-2 induced platelet activation
- Investigate the possibility that fibrin may be a novel ligand for GPVI

Throughout this thesis, I have performed experiments against the above aims, and the results are summarised briefly below. Firstly, using a novel kinase-dead mouse model, I have shown that for platelet activation following both GPVI and CLEC-2 stimulation, a functional Syk kinase-domain is integral. Whilst some phosphorylation is maintained on several key regulatory tyrosine residues throughout Syk (176-181, 183) in the absence of a functional kinase domain. this maintenance of potential adapter function is does not appear to be correlated with downstream signal transduction or functional responses (figure 3.6).

Secondly, using mice at different embryonic and postnatal developmental stages, I have shown that platelets are hyporeactive to (hem)ITAM stimulation via GPVI and CLEC-2, but not GPCR stimulation throughout gestation and early postnatal life. Furthermore, I have shown that the hyporeactivity following GPVI and CLEC-2

stimulation is only partially correlated with reductions in the surface expression of the two receptors. I have also shown that a model of *de novo* platelet production following depletion partially reconstitutes the platelet phenotype observed in developing mice

Lastly, using platelets from two un-related families in Chile who have presented with the same GPVI mutation – an adenine insertion (c.711_712insA) resulting in a premature stop codon - I have confirmed that fibrin is an agonist for GPVI, and have presented evidence that fibrinogen is also an agonist for GPVI. Lastly, despite the above results, $GPVI^{-/-}$ patients appeared to display normal clot retraction kinetics and thrombus compositions, although more experiments and in-depth analysis is required to confirm these results.

6.2 Conclusions and Discussion

The data in chapter 3 of this thesis has shown that for platelet activation following both GPVI and CLEC-2 stimulation, a functional Syk kinase-domain is integral. It appears that, despite the differences in reliance on Syk for proximal signalling events downstream of GPVI and CLEC-2 – with CLEC-2 being reliant on Syk for initial hemITAM phosphorylation (151) – both pathways are equally reliant on the kinase function of Syk for downstream signalling and platelet functional responses. This data furthers the body of information regarding the regulation of Syk following receptor engagement, which includes cell line studies describing the importance of a functional Syk kinase-domain in supporting signalling (263), alongside in vitro and in vivo studies assessing the novel Syk regulatory mechanisms (136, 236).

However, whilst the experiments performed within chapter 3 clearly demonstrate that a loss of Syk kinase function abolishes platelet functional responses following GPVI and CLEC-2 stimulation, the current work only assesses specific tyrosine residue phosphorylation as a surrogate marker of adapter capability. It would be interesting to perform experiments, such as co-immunoprecipitation experiments, to determine any differences in the association of Syk with partner proteins involved in signal transduction, such as LAT and PLCγ2, alongside other regulators of Syk activity such as c-Cbl and TULA-2. Assessment of these known interactions in Syk kinase-dead and wild type mice may help to determine which interactions are dependent upon auto-phosphorylation of Syk, and also aid in developing our knowledge of the organisation of the signalling pathways downstream of GPVI and CLEC-2. Overall, it is hoped that this data may help to guide further research and possible novel therapeutic design.

As stated, it has been shown that CLEC-2 is integral for development; as described mice deficient in CLEC-2 or associated signalling proteins display significant lymphatic development defects, and even present with defects in cerebrovascular development (22, 23). Alongside this observation, it has previously been shown that neonatal platelets display a significant hyporeactivity in response to many typical platelet stimuli, notably in response to the GPVI agonist collagen (43, 242). These two observations formed the basis of the hypothesis that, as GPVI signalling is apparently impaired in neonates, it is possible that CLEC-2 signalling may also be impaired; this impairment would be counterintuitive when considering the important of CLEC-2 in development. During the development of this study, it was shown by

Baker-Groberg et al that human neonates have significantly reduced responses to both CRP and rhodocytin at 24h post-birth (253), seemingly confirming this counterintuitive hypothesis, however, only single dose agonists were used, and no time-course of reactivity was assessed. Our study is the first to utilise mice at different gestational and post-natal time points to determine a time-course of platelet hyporeactivity. We also found that there was a significantly reduced response to GPVI and CLEC-2 stimulation immediately following birth, however we also showed that for CLEC-2 stimulation with rhodocytin, this hyporeactivity could be overcome with high doses of agonists; this was not the case for GPVI stimulation with CRP. It was also found that CLEC-2 reactivity appeared to recover quicker than GPVI reactivity – suggesting the possibility of differential regulation of signalling downstream of the two receptors – and although we found reductions in the surface expression of GPVI and CLEC-2, these only partially matched the patterns and severity of hyporeactivity. Whilst we could not assess the expression and reactivity of key downstream signalling proteins using the mouse model – due mainly to the low blood volumes available – we did manage to assess these proteins in both term and pre-term neonatal humans, in collaboration with the group of Dr Jose Rivera-Pozo in Spain (255).

From the combined studies, we found that human neonates displayed similar impairments in receptor expression and platelet reactivity to the mice, and also that pre-term neonates have a worse phenotype than their term counterparts. We also managed to assess the expression and reactivity of Syk, LAT and PLCγ2 in these samples, and discovered that both expression and phosphorylation of all of these

proteins was impaired in neonatal platelets, again with a worse phenotype in pre-term vs term samples (255). Taken together, these two sets of experiments suggest that platelet (hem)ITAM hyporeactivity is likely mediated by both impaired receptor expression and impaired expression and function of key signalling molecules, and that the reactivity of these pathways is strongly developmentally regulated. However, whilst we appear to have described the mechanisms controlling the observed hyporeactivity of neonatal platelets to hemITAM agonists, the physiological rationale for this phenomenon remains to be elucidated. This is particularly important to determine when we consider the importance of CLEC-2 in development alongside its described impaired reactivity. One possible explanation – lent credence by the observation that infusion of adult, normo-reactive platelets into embryonic mice results in almost immediate diffuse thrombosis (254) – is that the vascular environment is significantly different in rapidly developing vessels, compared with established vessels as seen in adults. It would be interesting to assess the exposure levels of proteins such as collagen and fibrin, which are commonly found in the extracellular matrix, in developing embryonic vessels, as this might help to determine whether the observed hyporeactivity may be a protective mechanism. It is also interesting to consider the reactivity responses to CLEC-2 in more detail, as the impaired response appears to be overcome at higher agonist concentrations and appears to approach adult reactivity levels quicker than the reactivity of GPVI. These results, considered in the context of the current literature, raise the hypothesis that platelet reactivity may be dampened throughout development to prevent diffuse spontaneous thrombosis (254), but that a currently unknown mechanism is present to allow high concentrations of CLEC-2 agonists to

overcome this reactivity impairment. However, more work is needed to fully elucidate the rationale for reduced platelet hemITAM reactivity in development, and explain why this impairment is not correlated with increased incidence rates of CLEC-2 mediated developmental dysfunctions.

Finally, building on the work previously describing fibrin as an agonist for GPVI (140, 141), I have shown that patients with a homozygous mutation in GPVI do not respond to fibrin either in suspension or on a surface. Whilst it was hypothesised that these patients would display a lack of response to fibrin, the surprise finding that GPVI is important for platelet spreading on fibrinogen coated surfaces was extremely novel; data from this chapter was formed an integral part in a recently published study definitively proving that GPVI can bind fibrinogen (238). We also found that, despite impaired fibrin-GPVI and fibrinogen-GPVI interactions in GPVI$^{-/-}$ platelets, thrombus formation kinetics and thrombus composition do not appear to differ significantly in the absence of GPVI. Whilst it should be noted that only basic optical analysis was performed for these experiments, and more in-depth analysis is required, these results suggest that GPVI may not be integral for thrombus formation – in keeping with current literature.

The finding that both fibrin and fibrinogen can interact with GPVI, alongside the traditional interaction with collagen, raises interesting questions regarding the timings of these interactions. It is important to determine when each of these interactions dominates – i.e. collagen-GPVI interactions are likely to occur during initial tethering and activation of platelets at sites of vascular damage whereas fibrin-

GPVI interactions are likely to occur during thrombus growth and retraction – to determine their importance and impact on thrombosis and haemostasis. It will be particularly interesting to explore the importance of the fibrinogen-GPVI interaction, as it appears to only initiate signal transduction via GPVI when immobilised on a surface and not in suspension.

It was previously believed that fibrin and fibrinogen interacted with platelets solely via the integrin αIIbβ3, typically seen an adhesion receptor. This is interesting, as it raises the question of the role of GPVI; is the receptor most important as a signalling receptor, or can it function as an adhesion receptor? It is also interesting to consider subsequent work performed in this area, which has shown that fibrin and fibrinogen bind with high affinity to the monomeric form of GPVI (237, 238). This data is in direct opposition to the mechanism of binding and activation of GPVI following collagen stimulation. Collagen has a high affinity for the dimeric form of GPVI, and upon binding and activating GPVI induced significant receptor clustering (129, 130). These stark differences in binding affinities and GPVI signal regulation again suggests distinct roles for GPVI-collagen and GPVI-fibrin(ogen) interactions. Further experiments using platelets from the homozygous patients – such as biochemical analysis following platelet spreading and super-resolution microscopy to assess receptor kinetics and organisation following differential stimulation of GPVI – may help to elucidate some of the key differences between these GPVI interactions, and provide some hints as to their physiological relevance.

6.3 Final Remarks

Overall, these results highlight the fact that despite their apparent involvement in significantly different biological processes, the regulation of GPVI, CLEC-2 and their common signalling pathway appear to be intimately linked. It has also shown that platelet reactivity and composition vary drastically throughout development, which is interesting considering their integral role in lymphatic and cerebrovasular development. Lastly, it has helped to identify new ligands and regulatory mechanisms for GPVI and CLEC-2.

The information uncovered throughout this thesis reinforces the important role that platelets play in a diverse range of physiological processes, and also highlights the fact that many of the processes controlling and regulating these interactions remain incompletely understood. It is hoped that the data generated over the course of this body of work may form the basis of further studies to fully elucidate the mechanisms controlling the integral contributions of these specs of ‘cellular dust’ in thrombosis, haemostasis, and beyond.

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