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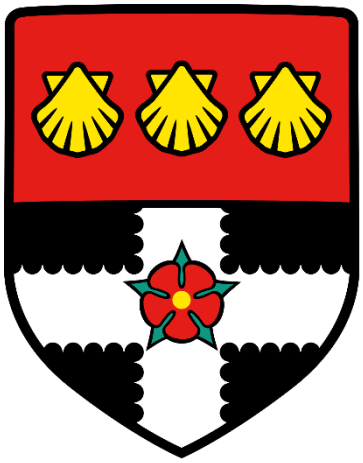

Role of the collagen molecular chaperone heat shock protein (HSP) 47 in platelet-collagen interaction

A thesis submitted for the degree of Doctor of Philosophy

By

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Declaration

I confirm that this is my own work and the use of all material from other sources has been properly and fully acknowledged.

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Abstract

Background: Heat shock protein 47 (HSP47) is an intracellular chaperone protein with an indispensable role in collagen biosynthesis in collagen-secreting cells. This chaperone has also been shown to be present on the surface of platelets. The inhibition of HSP47 in human platelets or its ablation in mouse platelets reduced their function in response to collagen and the GPVI agonist (CRP-XL), however, responses to thrombin were unaltered.

Aims: Given the importance of collagen and its interactions with platelets in triggering hemostasis and thrombosis, in this study we sought to understand the mechanisms by which HSP47 modulates platelet-collagen adhesion. We have explored the location, levels, intracellular associations of HSP47 with different platelet organelles and identified cellular events that mediate HSP47 mobilisation to platelet surface on stimulation. We examined the impact of HSP47 inhibition on the binding of platelets to various collagen receptor-specific ligands and its implication in the modulation of collagen receptors GPVI and integrin α2β1. Signalling events downstream of these receptors were also explored in presence of HSP47 inhibitor.

Results: HSP47 colocalisation with the dense tubular system was established using immunofluorescence microscopy imaging and subcellular fractionation analysis. Following platelet stimulation, HSP47 peripheral mobilisation was shown to be dependent on actin polymerisation. Inhibition of HSP47 attenuated platelet adhesion to collagen and CRP-XL, whereas, adhesion to GFOGER was unaltered. GPVI dimerisation was reduced in HSP47 inhibited platelets. Co-immunoprecipitation and microscopic studies revealed the association of HSP47 with GPVI. HSP47 has shown to support

collagen and GPVI-mediated signalling. However, outside-in signalling events in response to GFOGER stimulation were not affected by HSP47 inhibition.

Conclusions: The present study identifies a possible mechanism by which HSP47 supports platelet collagen responses and thereby contributes to haemostasis and thrombosis via GPVI dimerisation.

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List of Abbreviations

AC	Adenylyl cyclase
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
Bip	Binding immunoglobulin protein
BSA	Bovine serum albumin
Btk	Bruton's tyrosine kinase
Ca2+	Calcium [Ca2+] intracellular calcium concentration
CalDAG-GEFI	Calcium- and diacylglycerol-regulated guanine nucleotide exchange factor I
CaM	Calmodulin
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
CLEC-2	C-type lectin-like receptor 2
COX1	Cyclooxygenase 1
CRP-XL	Crosslinked collagen-related peptide
CS	Open canalicular system
Csk	C-terminal Src Kinase
C-terminal	Carboxyl-terminal
CVD	Cardiovascular disease
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DTS	Dense tubular system
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
ERp5	Endoplasmic reticulum protein 5
FAK	Focal Adhesion Kinase
FcRγ	Fc receptor γ-chain
FII	Factor II
FITC	Fluorescein isothiocyanate

FIX	Factor IX
FSC	Forward scatter
FV	Factor V
FVIII	Factor VIII
FX	Factor X
FXI	Factor XI
FXII	Factor XII
g	Gravitational force (centrifugal force)
Gads	Grb2 Related Adaptor Protein Downstream of Shc)
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Guanylyl Cyclase
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GP	Glycoprotein
GPCR	G-protein-coupled receptor
GPO	G-Glycine, P-Proline, O-Hydroxyproline
GTP	Guanosine triphosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSC	Haematopoietic stem cell
HSP	Heat shock protein
HSP47$^{-/-}$	HSP47-deficient
Ig	Immunoglobulin
IP receptor	Prostaglandin Receptor
IP3	Inositol 1,4,5-trisphosphate
IP3R	Inositol 1,4,5-trisphosphate receptor
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
JAM-A	Junctional Adhesion Molecule A
kDa	kilodaltons
KDEL	ER retention sequence, consisting of: Lys, Asp, Glu, Leu
KO	Knock-out
LAMP	Lysosomal-associated membrane protein
LAT	Linker for activation of T cells
M	Molar
MAPK	Mitogen-activated protein kinase
mg	Milligram
Mg2+	Magnesium ion

MgCl2	Magnesium chloride
Mins	Minutes
MK	Megakaryocyte
ml	Millilitre
MLC	Myosin Light Chain
mmol/L	Millimolar
mRNA	Messenger RNA
µg	Microgram
µl	Microliter
µM	Micromolar
Na2HPO4	Disodium phosphate
NaCl	Sodium chloride
NaHCO3	Sodium bicarbonate
NO	Nitric oxide
NOS	Nitric oxide synthases
ns	Not significant
N-terminal	Amino-terminal
^{o}C	Degrees Centigrade
Orai1	Calcium-Release Activated Calcium Modulator 1
PAGE	Polyacrylamide gel electrophoresis
PAR	Protease-Activated Receptor
PE	Phycoerythrin
PECAM-1	Platelet–endothelial cell adhesion molecule 1
PF4	Platelet Factor 4
pg	Picogram
PGI_2	Prostacyclin
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 3,4-bisphosphate
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKG	Protein kinase G
PLC	Phospholipase C
PM	Plasma membrane
PMSF	Phenylmethanesulfonyl fluoride
PRP	Platelet-rich plasma

PS	Phosphatidylserine
PVDF	Polyvinylidene difluoride
RabGDI	Rab GDP dissociation inhibitor
RBC	Red Blood Cell
RER	Rough endoplasmic reticulum
RhoGEFs	RhoGTPase nucleotide exchange factors
RIAM	Rap1b-interacting adaptor molecule
ROS	Reactive Oxygen Species
RT	Room Temperature
S	Serine
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium Dodecyl Sulphate – Polyacrylamide gel electrophoresis
Secs	Seconds
SEM	Standard Error of the Mean
SFK	Src family tyrosine kinase
sGC	Soluble Guanylyl Cyclase
SH	Src homology
SH2	Src homology 2
SH3	Src homology 3
SHP-1	Src homology 2 domain-containing protein tyrosine phosphatase-1
SHP-2	Src homology 2 domain-containing protein tyrosine phosphatase-2
SLP-76	Src homology 2 domain-containing leukocyte protein of 76 kDa
SMIH	Small molecule inhibitor
SNARE	Soluble NSF attachment protein receptor
SOCE	Store-operated calcium entry
SSC	Side scatter
STIM1	Stromal interaction molecule 1
STORM	Stochastic optical reconstruction microscopy
Syk	Spleen Tyrosine Kinase
TAE	Tris Acetate EDTA
TBST	Tris-buffered saline-TWEEN® 20
TNFα	Tumor Necrosis Factor alpha
TP	Thromboxane A2 receptor
TPO	Thrombopoietin
TRPC6	Transient Receptor Potential Cation Channel Subfamily C Member 6
tSNARE	Target soluble NSF attachment protein receptor
TSP-1	Thrombospondin 1

TxA_2	Thromboxane A2
TxB_2	Thromboxane B2
TxS	Thromboxane synthase
U/ml	Unit/Millilitre
v/v	Volume/Volume
VASP	Vasodilator-stimulated phosphoprotein
vSNARE	Vesicular soluble NSF attachment protein receptor
vWF	von Willebrand Factor
w/v	Weight/Volume
WPL	Whole Platelet Lysate
Y	Tyrosine

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1 Introduction

1.1 Summary of the Purpose of the Project

In the last 20 years, research has unraveled key and vital molecular players that explain the means by which platelets recognise and interact with collagen at sites of vascular damage and the consequent trigger of haemostasis. Understanding the mechanistic aspects of such interactions enable precise regulation of platelet reactivity and hence design of new therapeutic strategies to prevent prevalent thrombotic disease. Available anti-thrombotic measures of such diseases have proven effective in limiting and controlling platelet reactivity, however, the risk of developing bleeding episodes is inevitable. Such side effects have encouraged continuous search for alternative measures to control platelets which may include platelet-collagen interactions, noting that none of the currently available measures target this interaction.

A recent proteomic study led to the discovery of surface protein HSP47, a collagen-specific molecular chaperone, that contributes to the ability of platelets to respond to collagen (Kaiser et al., 2009; Sasikumar et al., 2018), nevertheless, the role and mechanism by which HSP47 influences platelet-collagen response has not been resolved.

In the present study, an in-depth look of HSP47 localisation at subcellular level was revealed for the first time in non-collagen producing human platelets. The role of HSP47 in modulating platelet-collagen response was also explored using a selective HSP47 inhibitor (SMIH). Lastly, HSP47 deficient platelets and SMIH treated platelets were employed to demonstrate the mechanistic impact of HSP47 on activatory signalling events in response to various receptor selective collagen peptides.

1.2 Platelet, history and present.

Platelets were first mentioned by Addison in 1841, who described them as "extremely minute granules in blood" (Addison, 1841). Several scientists reported the observation of such corpuscles. For instance, Osler *et al.* (1873) described platelet structure as disk-like, however, the term platelet was first used by Bizzozero, who also reported the adhesive properties of platelets when exposed to injured vessels and hence their potential role in haemostasis and thrombosis (Bizzozero, 1882; Gaetano, 2001). Studies were also conducted to understand the process of platelet formation. In 1906, megakaryocytes within bone marrow were demonstrated to generate platelets (Wright, 1906). Moreover, Wright developed a specialised polychromatic stain that enabled him to show the variation in the staining and the size of megakaryocytes and granules within platelets (Lee et al., 2002).

1.3 Platelets: an overview

Under normal physiological conditions, the bone marrow produces 10^{11} platelets per day to obtain a normal peripheral count of $1.5 – 4x10^{8}$ cells/ml range with an average life span of 8 to 10 days (Ghoshal and Bhattacharyya, 2014). Platelets are small, anucleated and discoid shaped (1-3 µm) arising from cytoplasmic fragmentation of megakaryocytes in the bone marrow (Becker and De Bruyn, 1976; Clemetson, 2012). Circulating platelets are attracted to the subendothelial surface when it is exposed upon injury, where they form a primary haemostatic plug to stop the blood loss. Several platelet stimuli are involved in this process and act by inducing changes in platelet shape, degranulation and aggregation. Platelets then serve as a procoagulant surface for the activation of a coagulation cascade resulting in the generation of thrombin. Thrombin is an enzyme that converts soluble fibrinogen into insoluble fibrin that strengthens the haemostatic plug.

Although tightly controlled, unnecessary platelet activation could result in the development of thrombosis, a prevailing condition with life-threatening consequences such as myocardial infarction and stroke (Heemskerk et al., 2002; Andrews and Berndt, 2004; Bergmeier and Hynes, 2012). Furthermore, platelets are readily activated when encountering exposed atherosclerotic, collagen-rich, plaque and form thrombus within the lesioned vessel (Barnes and Farndale, 1999). Besides cardiovascular disorders, platelets are also involved in the pathology of other clinical conditions such as cancer and diabetes (Vinik et al., 2001; Nash et al., 2002; Vazzana et al., 2012; Ghoshal and Bhattacharyya, 2014).

Despite advances in the development of successful therapeutic strategies to prevent thrombosis, this ranks among the top causes of mortality worldwide (Davì and Patrono, 2007; Ghoshal and Bhattacharyya, 2014). Given the reported contribution of platelets in pathologies of thrombosis emphasises the importance of understanding the biology of this crucial blood element. Platelet biology has been an area of great interest for researchers, whose aim is to obtain a better understanding of platelet behaviour in the control and regulation of haemostasis along with its role in the pathophysiology of thrombosis and vascular disease. This may also pave the way to explore several new therapeutic targets within platelets to treat cardiovascular disorders.

1.4 Platelet biosynthesis

Megakaryocytes are rare myeloid cells that reside in the bone marrow. These cells constitute less than 0.1% of the bone marrow nucleated cellular population where they function in the formation and release of platelets into the circulation (Ogawa, 1993; Patel et al., 2005). Megakaryocytes arise from pluripotent hematopoietic stem cells (HSCs) that further give rise to burst forming and colony forming cells (Briddell et al., 1989).

These cells then continue their development into a highly restricted lineage that leads to the formation of megakaryocyte precursors (Ogawa, 1993). The development of megakaryocytes is regulated by the cytokine thrombopoietin, which binds to the megakaryocyte-specific receptor c-MPL and regulates the process of thrombopoiesis (Italiano. and Hartwig, 2013).

Binding of thrombopoietin to the c-Mpl receptor on megakaryocyte surface induces the process of endomitosis. DNA replication is actively carried out without cell division and hence results in the transition of a megakaryocyte into a giant cell with expanded cytoplasm and polypoid nucleus (Ravid et al., 2002). Such active replication is associated with the synthesis of protein cargo to be packed into the generated platelets (Patel et al., 2005; Richardson et al., 2005; Machlus et al., 2014). The movement of microtubules to the cortex within megakaryocyte results in the formation of large pseudopodia that give rise to the proplatelet projections. The elongation of proplatelets is driven by the sliding of the microtubules over one another, which traffics cytoplasmic organelles and granules into the proplatelet tips, thereby converting the entire megakaryocyte into a proplatelet mass (Patel et al., 2005). Proplatelets extend through junctions in the sinusoid endothelial lining into the bone marrow sinuses, where further proplatelet processing and fragmentation into platelets take place prior to platelet release into circulation (Figure 1.1) (Kessal and Kardon, 1979; Tavassoli and Aoki, 1989). Each megakaryocyte produces 10 to 20 proplatelets *in vitro* and approximately 5,000 to 10,000 platelets (Ghoshal and Bhattacharyya, 2014). Following their release, platelets experiences repeated branching and splitting in a shear-dependent manner to reach discoid shape (Junt et al., 2007; Thon and Italiano, 2012b).

In addition to bone marrow, lungs have been proposed to contribute to the biogenesis of platelets (Howell and Donahue, 1937). Several studies reported megakaryocytes being present in lungs (Kaufman et al., 1965; Hansen and Pedersen, 1978; Sharma and Talbot, 1986; Levine et al., 1993). However, clear insight about lung hosting such biogenesis was obtained by Lefrançais et al. (2017) who demonstrated the release of platelets from bone marrow migrating megakaryocyte within extravascular spaces of mice lung tissue accounting for almost half of total thrombopoiesis in mice (Lefrançais et al., 2017). Moreover, the same group also showed the reverse migration of megakaryocyte progenitor cells back to the bone marrow upon introducing thrombocytopenic episode.

Upon completing their lifespan, the body exerts several approaches to clear platelet from circulation. Until recently, the classical antibody-mediated platelet clearance has been the only well characterised and established mode of platelet clearance. Platelets coated with IgGs are readily phagocytosed by Fc-receptor bearing macrophages primarily in the spleen (Crow and Lazarus, 2003). Moreover, autoantibodies against integrin αIIbβ3 (GPIIb-IIIa) or GPIbα-IX-V, von Willebrand Factor (vWF) receptor, trigger platelets clearance in immune thrombocytopenic patients (Chan et al., 2003; Stasi et al., 2008).

Other studies have also reported the role of hepatic Ashwell-Morell receptors and their contribution to platelet clearance. Removal of sialic acids from platelet surface glycoproteins unmasks various glycans and leaving ageing platelets susceptible to removal via galactose recognizing lectin (Ashwell-Morell) receptors (Rumjantseva et al., 2009; Grozovsky et al., 2015). Lastly, Mason et al. (2007) showed that disturbance in the antagonistic balance between pro-survival and pro-apoptotic agents makes platelets primed for controlled apoptosis (Mason et al., 2007).

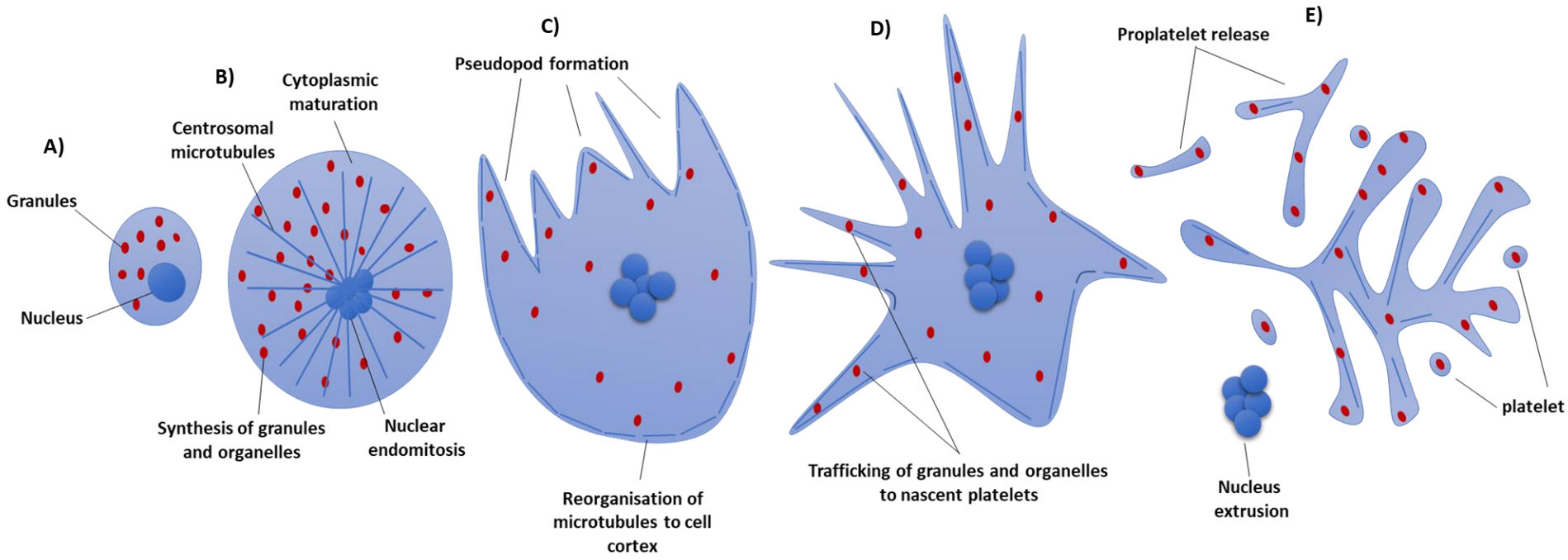


Figure 1.1: Production of platelets from megakaryocytes.

Megakaryocyte transition from immature cells **A** to the release of platelets **E.** Endomitotic replication increases the volume of the cytoplasm along with amplification in protein and organelles synthesis **B** followed by movement of the microtubules into the cortex forming pseudopods that extend to form proplatelets at the cytoplasmic tips **C**. Sliding of the microtubules drives and packs the cytoplasmic contents into the elongated proplatelets, where platelets assembly starts **D**. rapid retraction of the megakaryocyte causes the release of proplatelets. Platelets are then formed by fragmentation of the proplatelets. The extruded nucleus undergoes apoptosis **E.** Adapted from (Patel et al., 2005)

1.5 Platelet ultrastructure

Despite being tiny cytoplasmic fragments, platelets possess several ultrastructural features with complex nature. Each platelet has a mean cell volume of 8 to 10 femtoliters and with platelets being anucleated, the level of de novo protein synthesis is limited (Weyrich et al., 2009). Hence, the parental megakaryocyte provides platelets with a cargo of various proteins, organelles and minimal amounts of mRNA. Additionally, platelets have the ability to pick up plasma proteins in the vasculature, such as fibrinogen. The plasma membrane is structurally supported by a cytoskeleton made up of actin, tubulin, spectrin and filamin that maintains quiescent platelet in discoid shape (Smyth et al., 2010). Once activated, actin dynamic equilibrium is shifted from monomer and globular (G-actin) toward polymerised and filamentous actin (F-actin) allowing platelet shape changes and consequent spreading (Lefebvre et al., 1993; Bearer et al., 2002).

Additionally, a network of membranous invaginations known as the open canalicular system (OCS) allows platelet to increase surface area and serves as channels for releasing platelet contents (Escolar and White, 1991). Another important element of the platelet membrane system is a dense tubular system (DTS) that is believed to be derived from megakaryocyte smooth endoplasmic reticulum (ER) and in platelets, it stores Ca^{2+} which is readily released into the cytosol upon platelet stimulation (Ebbeling et al., 1992). The DTS also hosts the production of TxA_2, vital activatory and positive feedback mediator, from arachidonic acid with the aid of cyclooxygenase-1 (COX-1) and thromboxane synthetase enzymes (Rendu and Brohard-Bohn, 2001).

Platelet membranes express an array of receptors that contribute to the regulation of platelet function. These receptors are discussed in details in section 1.7. Within the platelet, biologically active molecules that are involved in coagulation and inflammation

are stored inside two storage granules: α and dense granules (Flaumenhaft, 2003). α granules are the most abundant granules in platelets. The number of α granules depends on platelet size and presence of any other space-occupying organelles (White, 2013), but each platelet has approximately 50 to 80 granules (Frojmovic and Milton, 1982). These granules are round to oval in shape, with an average diameter of 200 to 500 nm (White, 1968), and contain different types of proteins, including von Willebrand factor (VWF) and P-selectin that are synthesised by megakaryocytes. Other proteins, such as fibrinogen, that are taken up by platelets via endocytosis is stored in these granules. Lastly, α granules also store several transmembrane receptors, such as GPIb-V-IX, GPVI, αIIbβ3 and platelet endothelial cell adhesion molecule (PECAM-1) (Flaumenhaft, 2017). This cargo has shown to be trafficked to platelet surface upon activation (Golebiewska and Poole, 2015).

The dense granules, which are smaller and fewer in number than the α granules, are packed with high concentrations of serotonin, magnesium and calcium ions in addition to the nucleotides ATP and ADP. The release of these contents amplifies platelet activation via autocrine and paracrine actions on platelets (Thon and Italiano, 2012a; Flaumenhaft, 2013). Platelet also possesses between 1 and 3 lysosomes containing high levels of acid hydrolases and phosphatases. Such proteolytic enzymes rich contents are counteracted with glycosylated membrane proteins on the lysosome surface and hence provides protection from auto-digestion. The role of lysosomes in platelets is not well understood, however, their participation in clearing and digestion of thrombi have been suggested (Fukuda, 1991; Rendu and Brohard-Bohn, 2001).

During their lifespan, platelet mitochondria carry out oxidative phosphorylation events to generate ATP. This energy is sustained in quiescent platelets, to ensures its survival

and functionality (Zharikov and Shiva, 2013). Also, platelets store glycosomes that aids in energy generation via glycolysis **(**Figure 1.2) (Rocha et al., 2014).

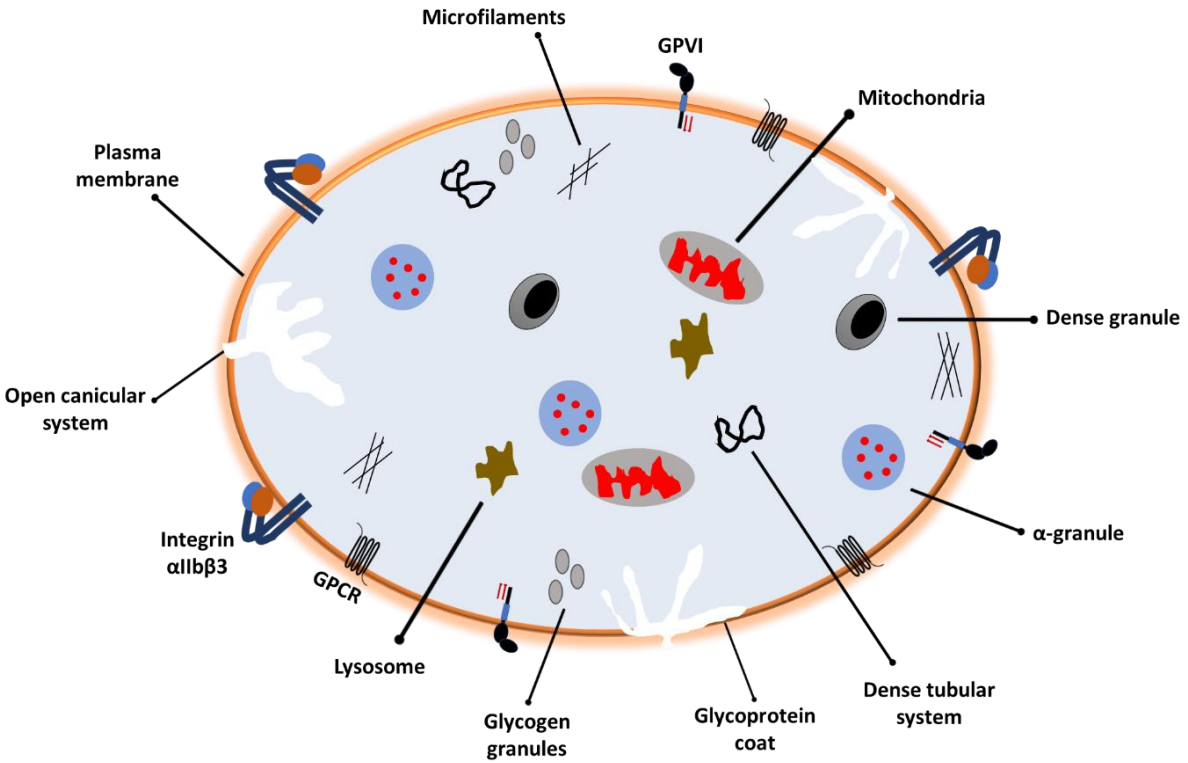


Figure 1.2: An illustrated representation of platelet ultrastructural features.

The platelet membrane is coated with glycoprotein layer and underneath this membrane there are actin cytoskeletal filaments and rings of microtubules that maintain platelet discoid shape and allow stimuli driven changes in such shape during platelet stimulation. Platelet cytosol is occupied with various organelles, for instance, alpha and dense granules, lysosomes, mitochondria, and glycogen packed granules. Also, platelets possess membranous systems, the open canalicular systems (OCS), which serves as bidirectional trafficking conduit from and into platelet, whereas, dense tubular system (DTS) acts as storage site of calcium.

1.6 Platelets and haemostasis

The small size and shape of platelets allow them to be pushed to the periphery of the vessel lumen by the flowing blood. Platelets circulate near the vascular endothelium, thereby enabling them to respond to any vascular injury and terminate blood loss in a multistep and integrated process known as haemostasis (Aarts et al., 1988; Wang et al., 2014). Injured vessels undergo muscular constriction in an attempt to narrow vessel lumen and minimise bleeding. Platelets are recruited to the exposed sub-endothelium and activation of surface receptors induces morphological changes that flatten the platelets over the injured area. This initial adhesion and thrombus formation mediate further activation of the coagulation cascade, fibrin formation and thrombus stability (White, 2013). Dynamically, haemostasis is broadly categorised into three main events: initial adhesion to thrombogenic surface, propagation and lastly, thrombus formation. Despite the adhesive nature of platelets, their activity is tightly regulated and controlled in the vasculature to avoid inappropriate thrombotic events (Figure 1.3).

1.6.1 Initiation of platelet adhesion

The subendothelial extracellular matrix contains substantial amounts of adhesive molecules, such as vWF and collagen. The initial adhesion is highly influenced by the shear stress exerted inside the vessels. The velocity of blood flow is higher in the centre of the vessel than in the periphery near the wall. However, the latter represents a position of high shear stress compared to the vessel centre (Varga-Szabo et al., 2008). Under high shear, initial tethering of the platelets is achieved by the binding of VWF to GP Ib-IX-V. This slows the flow of the platelets, allowing further adhesion through different ligand-receptor binding such as collagen-GPVI (Brass, 2010). VWF is an adhesive multimeric glycoprotein found in platelet α granules, endothelial cells Weibel-Palade bodies and in

plasma, where it circulates in an inactive form (Ruggeri, 2007). VWF binds to collagen as well as to GPIbα in the GPIb-IX-V complex (Andrews et al., 2003) and αIIbβ3 integrin (Ginsberg et al., 2005). Platelets adhere only to the surface immobilised VWF; this tightly regulates the initial platelet interaction, thereby avoiding unnecessary intravascular platelet aggregation (Ruggeri and Mendolicchio, 2007). The collagen immobilised VWF undergoes conformational changes under high shear stress, where it appears as elongated filaments rather than coiled conformation it assumes under low shear stress (Siediecki et al., 1996). This elongation exposes the binding site on the VWF A1 domain to allow GPIb-IX-V binding (Nuyttens et al., 2011). This interaction has fast on and off rate that will only keep the platelets tethered for interaction with other adhesive ligands (Varga-Szabo et al., 2008).

VWF platelet interaction causes platelet tethering (rolling), allowing the direct binding of the platelet collagen receptors GPVI and integrin α2β1 and stabilisation of platelet adhesion (Nuyttens et al., 2011). Although these two receptors are essential for platelet adhesion and activation, recent studies have revealed that integrin α2β1 serves principally as an adhesive receptor, whereas, GPVI plays a signalling role in platelet activation (Jung and Moroi, 1998; Jung and Moroi, 2000; Siljander et al., 2004; Pugh et al., 2010). These studies supported a two-site-two-step model, initially proposed by Santoro et al. based on the observation that integrin α2β1 lacks the ability to stimulate tyrosine kinase activity. The model proposed that integrin α2β1 stabilises the platelet interaction with collagen, thereby allowing another collagen-binding receptor to bind and trigger collagen-mediated platelet activation (Santoro et al., 1991).

1.6.2 Propagation (platelet activation and degranulation)

Collagen cross-linked receptors, specifically GPVI, trigger wave of activatory signals inside platelets (Gibbins et al., 1997). Consequently, an array of secondary mediators is released and synthesised, such as ADP and TxA_2, respectively. Additionally, locally generated thrombin results in the amplification of activation signals via interaction with their specific G-protein coupled receptors (GPCRs) on the platelet surface in a positive feedback pathway (Gibbins, 2004). These mediators eventually induce the transition of integrin αIIbβ3 (GPIIb-IIIa) receptor to high-affinity state in a process referred to as inside-out signalling and enable it to bind its respective ligands, such as bivalent fibrinogen. Hence, platelets are crosslinked to form aggregates and plug the injured site (Bennett, 2005; Ma et al., 2007).

1.6.3 Thrombus formation and stabilisation

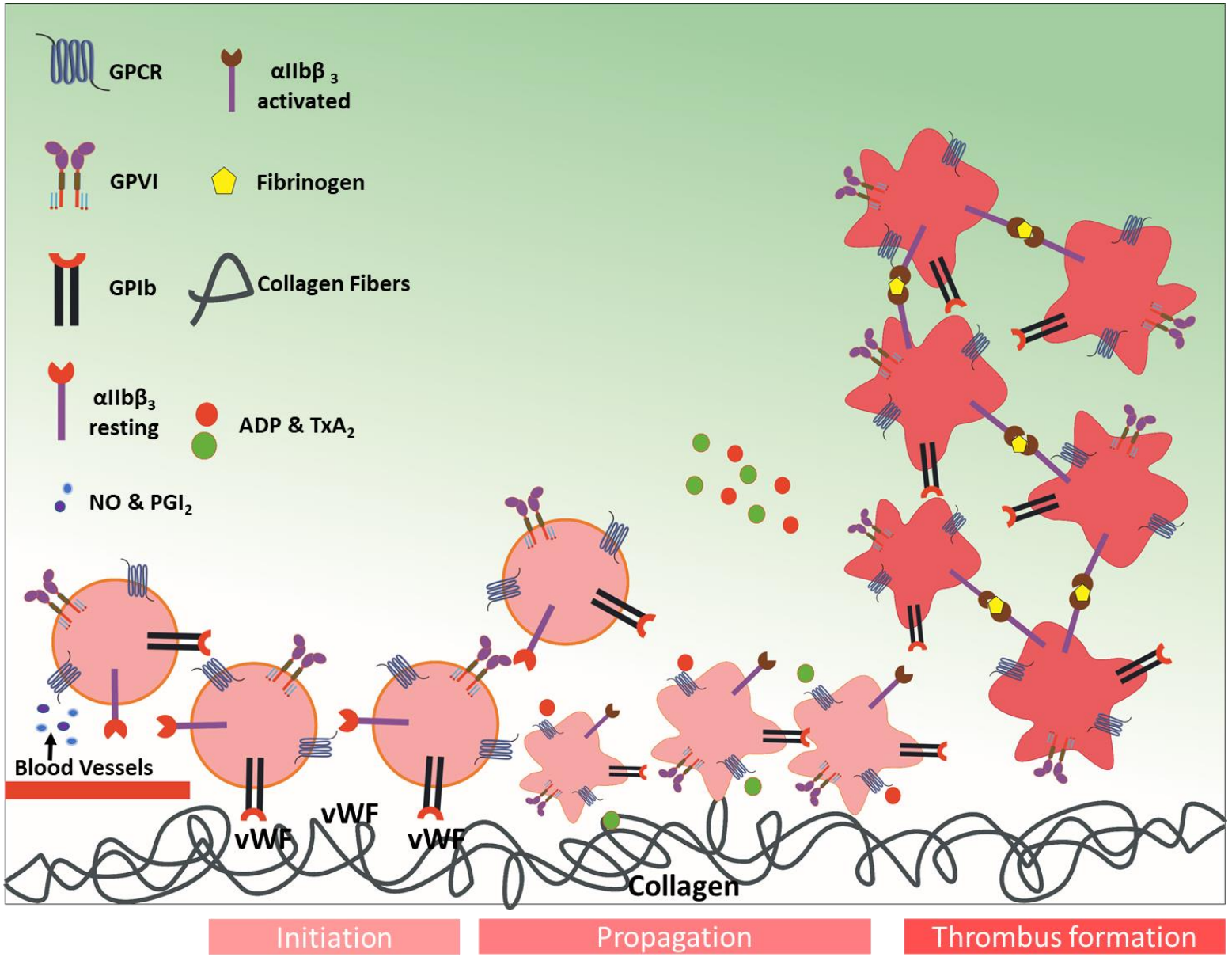
To further consolidate and reinforce the platelet plug, several physiological events take place to ensure thrombus stability. For instance, ephrin receptor tyrosine kinases have been shown to induce contact-dependent signalling among activated platelets (Prevost et al., 2005; Vaiyapuri et al., 2015). Furthermore, activated integrin αIIbβ3 evokes contractile force through reorganisation of actin cytoskeleton when bound to fibrinogen/fibrin, a process known as outside-in signalling, and thereby causes the clot to retract and stabilise (Gong et al., 2010). Beside integrin αIIbβ3 and Eph kinase mediated contact-dependent signalling, activated platelets serve as a procoagulant surface. This initiates the coagulation cascade by flipping the negatively charged phosphatidylserine (PS) to the outer membrane. Consequently, tenase and prothrombinase complexes are held at the site of injury. Such complexes catalyse the production of factor Xa and thrombin, respectively. Generated thrombin converts soluble

fibrinogen into insoluble fibrin, that is readily crosslinked by factor XIII and thereby strengthening the haemostatic plug and allowing it to withstand the shear stress of blood flowing over the injured area (Zwaal et al., 1977; Bevers et al., 1983; Brass, 2010).

As a precautious measure to control the growth of formed thrombus, a series of enzymatic fibrinolysis steps takes place to avert the accumulation of fibrin in the vessel. Uncontrolled fibrin deposition could give rise to vascular occlusive episodes with consequences such as stroke and myocardial infarction. Timely disposal of thrombus is a self-regulated process triggered by plasminogen activation via the action of tissue-type plasminogen activator (t-PA) into proteolytically active fibrin degrading plasmin (Chapin and Hajjar, 2015; Medcalf, 2015). Lastly, any products generated from fibrin degradation are removed by macrophage/monocyte system (Jennewein et al., 2011).

Figure 1.3: Diagrammatic representation of haemostasis at site of vascular injury.

Intact endothelium ensures quiescent state of circulating platelets by the release of inhibitory regulators PGI_2 and Nitric oxide (NO). VWF becomes filamentous under high shear stress, exposing the binding site for the GPIb-IX-V binding and upon vessel damage, the collagen bound filamentous VWF binds marginalised flowing platelets. Platelets tethering on GPIb-IX-V allows their rolling and hence exposure to exposed collagen. Further adhesion of platelet is then facilitated via collagen receptor GPVI which upon collagen binding triggers series of activatory signalling events causing cytoskeletal reorganisation and changes in platelet shape along with up modulation of integrin αIIbβ3 affinity and platelet degranulation. More platelets are recruited and activated by secreted TXA_2 and ADP and lastly, ligand binding conformation of integrin αIIbβ3 enables integrin binding to several ligands such as vWF and fibrinogen and hence, causing the platelets to crosslink and aggregates into plug.


GPCR
αIIbβ 3 activated
GPVI
Fibrinogen
GPIb
Collagen Fibers
αIIbβ3 resting
ADP & TxA2
NO & PGI2
Blood Vessels
vWF
vWF
vWF
Collagen
Initiation
Propagation
Thrombus formation

1.7 Receptors, agonists and signalling pathways of platelets

Platelet receptors serve as contact points between platelet and its external environment, enabling the platelet to bind and react with different adhesive proteins, other blood cells and platelet agonist molecules. Receptor-mediated interactions allow the platelets to adhere to an injured area and to activate and aggregate forming a haemostatic plug. Major agonists bind platelets primarily through two different types of receptors. The first type is tyrosine kinase linked-transmembrane receptors that include GPIb-IX-V complex, integrins and GPVI, which bind adhesion molecules such as collagen, vWF and fibrinogen and trigger tyrosine kinase-mediated signalling. The second type is G protein-coupled transmembrane receptors such as $P2Y_1$, $P2Y_{12}$, PAR1, PAR4 and TXA_2 receptors that bind soluble agonists, such as ADP, thrombin and TXA_2, and mediate platelet signalling through the initiation of G protein signalling (Watson and Harrison, 2011).

1.7.1 Tyrosine kinase linked transmembrane collagen receptors and collagen-mediated signalling

As discussed previously, platelet adhesion and aggregation on the injured vessel wall is mediated via a synergistic function of the number platelet surface receptors. This is commenced with an interaction of the platelet GPIb–V–IX receptor complex with vWF bound to exposed collagen. Such initial interaction slows down the movement of platelets and enables their stable binding with collagen receptors GPVI and integrin α2β1, leading to their stable and firm adhesion. Consequently, waves of intracellular tyrosine kinase signalling are evoked, resulting in calcium mobilisation, granule secretion, integrin αIIbβ3 activation and thrombus formation (Gibbins, 2004).

1.7.1.1 GPIb-IX-V complex

The GPIb-IX-V complex is unique to platelets and binds to vWF. It is composed of GPIbα (135 kDa) and GPIbβ (25 kDa), which are disulphide-linked and associated non-covalently with GPIX (22 kDa) and GPV (88 kDa) at a 2:2:2:1 ratio (Andrews et al., 2003; Andrews et al., 2004). Platelets express around 25,000 of these complexes per cell. Ligands, such as vWF, thrombin and factor XII, bind to the complex via extracellular N-terminus of GPIbα (Berndt *et al.*, 2001). Whereas, C-terminus of GPIbα 96 amino acids cytoplasmic tail acts as binding sites for various signalling molecules, such as actin-binding protein(filamin) and calmodulin (Ozaki *et al.*, 2005). As described earlier, the binding of GPIb-IX-V to VWF is an essential step in the initiation of haemostasis and thrombosis (Andrews et al., 2007). Besides platelets adhesion, it has been proposed that GPIb-X-IV complex is also involved in triggering tyrosine kinases dependent activatory signalling. This is based on the reported activation of Syk downstream of GP Ib. This observation was seen following the use of GPIb cross-linking antibody and in presence of vWF plus modulator botrocetin, snake venom protein that enhances the affinity of vWF for the platelet GPIbα (Asazuma et al., 1997; Yanabu et al., 1997). Falati et al. (1999) further elucidated such activation with their reported finding of GPIb association with FcR γ-chain. Moreover, the importance of this association in GPIb mediated-signalling events has been shown in the impaired aggregate formation in FcRγ-chain-deficient platelets stimulated with vWF and botrocetin (Wu et al., 2001). Other reports also showed recruitment of this complex to specialised signalling microdomain referred to as lipid raft (Shrimpton et al., 2002; Ozaki et al., 2013). GPIb-X-IV interacting filamin is associated with lipid raft fraction within platelet membrane and hence, potential interaction of this complex with other raft residing signalling molecules such as phosphoinositide 3-kinase (PI3K) and Src family kinases (SFKs) is possible. With the

later been reported to be involved in TxA_2 synthesis in response to vWF binding GPIb-X-IV (Liu et al., 2006; Li et al., 2010). Moreover, others have raised the suggestion of GPIb-IX-V constitutively binding p85 subunit of PI3K (Mangin et al., 2004; Mu et al., 2008).

1.7.1.2 GPVI receptor

GPVI receptor (64 kDa) belongs to type I transmembrane glycoprotein of the immunoglobulin (Ig) superfamily and is expressed exclusively in platelets with approximately 4000 copies per platelet (Clemetson et al., 1999; Andrews et al., 2014). It is composed of two disulphide bond linked immunoglobulin–like extracellular domains, a mucin-like stalk, a transmembrane region and a short cytoplasmic tail (51 amino acids) (Nieswandt and Watson, 2003). The transmembrane domain contains the amino acid arginine, which enables non-covalent association with the aspartic acid residue of homodimeric Fc receptor-γ chain (FcR-γ chain), which contains an ITAM in its cytoplasmic region that is of great importance in GPVI mediated signalling (Moroi and Jung, 2004). Gibbins et al. (1996) firstly identified the presence of such homodimer via spleen tyrosine kinase (Syk) association upon collagen stimulation. An immunoreceptor tyrosine-based activation motif (ITAM) is present in each FcRγ-chain. Such motif contains two YxxL sequences separated by six to eight amino acids [YxxL-$(X)_{6–8}$-YxxL) (Gibbins et al., 1997; Miura et al., 2002; Rabie et al., 2007). A proline-rich motif containing cytosolic tail of GPVI binds selectively to the SH3 domain of the active Src family tyrosine kinases, Fyn, and Lyn (Ezumi et al., 1998). In quiescent platelets, the activity of SFKs is suppressed via C-terminal Src kinase (Csk) mediated phosphorylation of C- terminal inhibitory tyrosine residues whereas, this phosphorylation is reversed upon platelet stimulation by the action of receptor-like PTP CD148 (Okada, 2012). However, a recent report by Mori et al. (2018) showed increased tail bleeding and reduced thrombus

formation in Csk deficient mice along with upregulated inhibitory ITIM-containing receptor G6b-B. This results in attenuated platelet response to vascular injury. Conversely, bleeding was normal in *CD148* deficient mice suggesting residual and sustained SFKs activity in platelets.

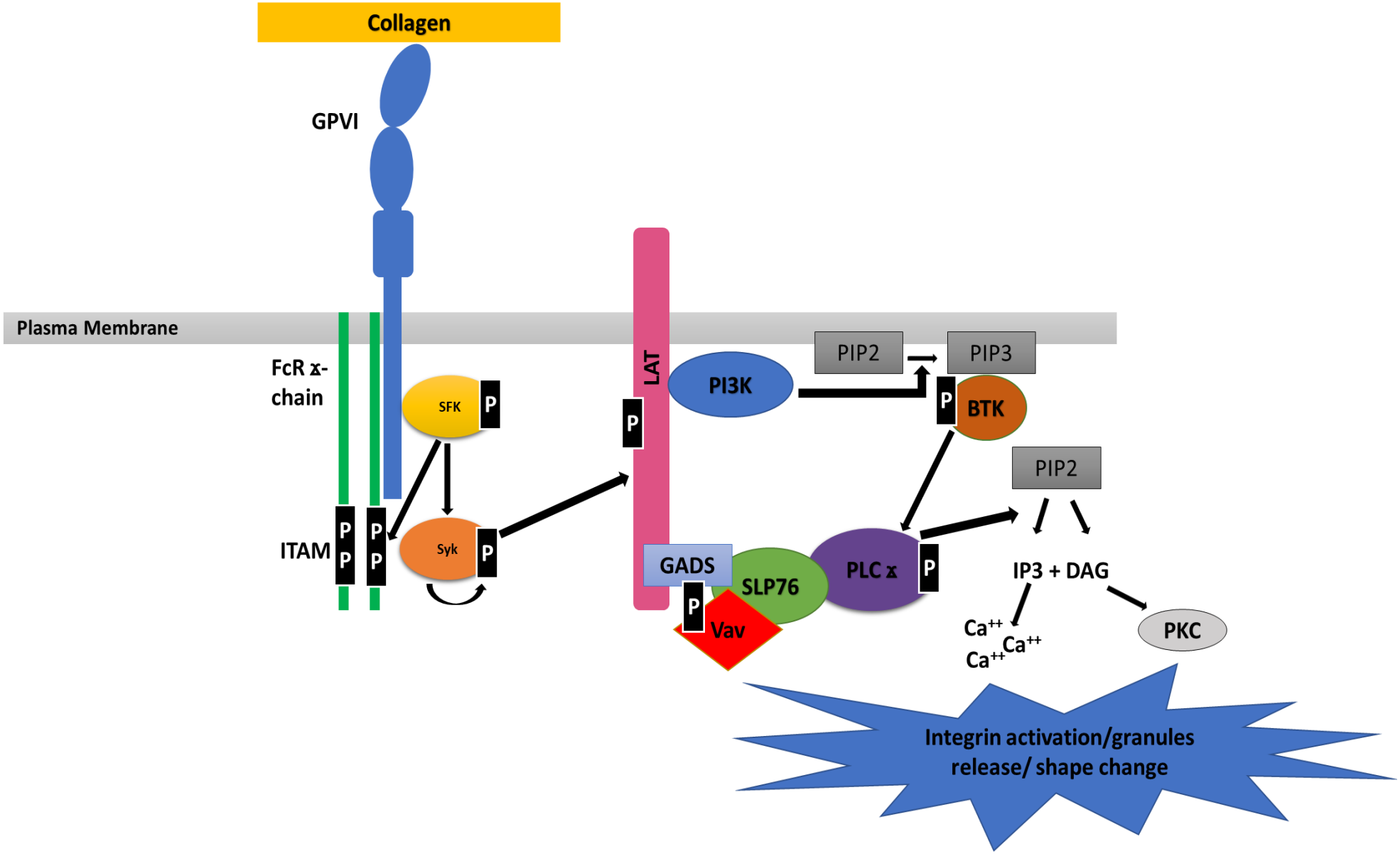
The GPVI receptor binds specifically to the Gly-Pro-Hyp sequence of collagen. Cross-linking of GPVI receptors brings the Src family tyrosine kinases, Fyn and Lyn, into contact with the Fc receptor-γ, which results in the phosphorylation of the tyrosine residue within the immunoreceptor tyrosine-based activation motif (ITAM). This phosphorylated residue serves as the docking site for tyrosine kinase Syk, which binds via its Src homology 2 (SH2) domain and becomes activated. Linker for activated T cells (LAT) is an adaptor molecule that serves as a substrate for Syk (Zhang et al., 1998a). It has multiple phosphorylation sites that recruit additional proteins, such as Src homology 2 domain-containing leukocyte phosphoprotein of 76-kDa (SLP-76), Vav family of guanine nucleotide exchange factors (GEF), Tec family kinases and grb2-related adapter protein (Gads), to form a large signalling complex. Tyrosine phosphorylated LAT interacts with and brings phosphatidylinositol-3 kinase (PI3K) and phospholipase Cγ2 (PLCγ2) into close proximity with their substrate at the plasma membrane (Gibbins et al., 1998). Upon its membrane translocation, PI3K catalyses the production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) from phosphatidylinositol-4,5-bisphosphate (PIP2) and hence allowing colocalisation of Bruton's tyrosine kinase (Btk) with its substrate PLCγ2 (Pasquet et al., 1999a; Pasquet et al., 1999b). Activated PLCγ2 induces the cleavage of phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5 trisphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 triggers the release of Ca^{++} from platelet dense tubular system (DTS). Depletion of the DTS Ca^{++} pool then induces a conformational change in STIM1, a DTS membrane protein, allowing it to bind to the

Ca^{++} entry channel, Orail, in the plasma membrane and triggering a concentration gradient-induced Ca^{++} influx across the plasma membrane. Ca^{++} activates phospholipase A2 (PLA2), which mediates the release of arachidonic acid from membrane phospholipids. The arachidonic acid is then converted to TXA2 by the enzyme cyclooxygenase 1 (COX1). At the same time, DAG activates protein kinase C, resulting in cytoskeletal changes (Brass et al., 1997; Grosse et al., 2007; Brass, 2010). Lastly, such signals result in platelet degranulation, upregulation of integrin affinity and hence the formation of platelet aggregates (Shattil et al., 1998; Jackson et al., 2003; Andre, 2012; Dunster et al., 2015) (Figure 1.4).

With such activatory capacity, GPVI plays a vital role in platelet adhesion and subsequent activation in response to collagen. Mice with GPVI or FcRγ depleted platelets failed to aggregate in response to collagen stimulation (Best et al., 2003; Kato et al., 2003a). Likewise, these platelets showed reduced adhesion and aggregation when perfused over collagen-coated surfaces. Furthermore, *in vivo* thrombus formation and collagen evoked aggregation were negatively impacted following GPVI immune-depletion (Nieswandt et al., 2001b; Munnix et al., 2005). However, reports of mild impact on tail bleeding in GPVI knock out mice have suggested compensatory effect established upon GPVI deletion or deficiency serving as protective measure from excessive bleeding in events of vascular injury (Kato et al., 2003a; Lockyer et al., 2006).

Figure 1.4: Overview of platelet activation by collagen.

Activation of GPVI leads to the phosphorylation of tyrosine residues in the FcR γ chain immunoreceptor tyrosine-based activation motif (ITAM) by Src kinases, which causes further phosphorylation of multiple proteins Linker for activated T cells (LAT), phospholipase Cγ (PLC γ) and Src homology 2 domain-containing leukocyte phosphoprotein of 76-kDa (SLP-76) by the Syk kinase and formation of downstream signalosome that acts as hub for recruitment of phosphatidylinositol 3-kinase (PI3K) and subsequent conversion of Phosphatidylinositol-4,5-bisphosphate (PIP2) into Phosphatidylinositol-3,4,5-trisphosphate (PIP3). Bruton's tyrosine kinase (Btk) interact with generated PIP3 and enable the phosphorylation and activation of PLCγ2, which then catalyses the production of Inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG). IP3 triggers mobilisation of Ca^{++}, while DAG activates protein kinase C (PKC), resulting in cytoskeletal changes, release of granules content and integrin αIIbβ3 activation causing platelets to aggregate.


Collagen
GPVI
Plasma Membrane
FcR x-chain
ITAM
P
P
P
P
SFK
P
Syk
P
LAT
P
PI3K
PIP2
PIP3
P
BTK
PIP2
GADS
P
SLP76
Vav
PLC x
P
IP3 + DAG
Ca++
Ca++
Ca++
PKC
Integrin activation/granules release/ shape change

1.7.1.3 Integrin α2β1

Integrin α2β1 (also known as GPIa/IIa) was the first receptor identified to bind collagen in platelets (Santoro, 1986) with surface expression levels of approximately 2000 to 4000 copies per platelet. Integrins share similar heterodimeric structure consisting of α and β subunits that are non-covalently bound. α2 subunits are characterised by the presence of collagen binding site, the I-domain which is situated between 2nd and 3rd repeats of the seven-bladed β propeller of the α2 subunit (Emsley et al., 2004; Saboor et al., 2013). They exist in a low affinity resting state on the cell surface and require "inside out'' signalling through other surface receptors to be converted into the ligand – high affinity – activated state (Nieswandt and Watson, 2003). Integrin α2β1 binds various collagen types such as I, III, IV, V, VI via interaction with GER (Glycine- glutamic acid- arginine) sequence (Zhang et al., 2003; Surin et al., 2008).

As mentioned earlier, engagement of collagen with GPVI triggers signalling events resulting in the activation of integrin α2β1 on platelet surface and hence enables it binding and adhesion to collagen (Nieswandt and Watson, 2003). However, the exact contribution of integrin α2β1 in platelet adhesion and consequent thrombus formation is still a matter of debate. Although delayed, integrin-β1-deficient platelets aggregate in response to collagen stimulation with normal adhesion to collagen under low and high flow condition. Hence, the authors suggested GPVI to be responsible for initial interaction with collagen and generation of signalling events triggering activation of integrin α2β1 that further stabilises platelet adhesion (Nieswandt et al., 2001a). Moreover, others reported attenuated adhesion of α2β1-null platelets to collagen in an endothelial injury to the carotid artery model (He et al., 2003). In contrast to these reports, α2-deficient platelets exhibited defected adhesion to fibrillar collagen under low shear

stress with similar defect reported with adhesion to soluble collagen (Chen et al., 2002; Holtkotter et al., 2002).

Interestingly, Inoue et al. (2003) provided evidence of the involvement of integrin α2β1 in the generation of activatory outside-in signalling events upon engagement with their selective ligand, GFOGER peptide, when presented as a monolayer. These events include key proteins similar to those implicated downstream of GPVI signalling such as Src, PLCγ2, SLP-76 and Syk. Taken together, this suggests a cooperative and complementary role of integrin α2β1 along with major collagen receptor on the platelet surface, GPVI.

1.7.1.4 C-type lectin receptor-2 (CLEC-2)

C-type lectin 2 (CLEC-2, also known as aggretin) is expressed on human platelets at high level whereas murine platelets express lower level. This 32-kDa surface receptor is composed of cytoplasmic tail with a single YxxL (hemITAM), a single transmembrane region and extracellular carboxyl-terminal C-terminal, that possesses carbohydrate-like (ligand) recognition domain, also referred to as a C-type lectin-like domain (CTLD) (Weis et al., 1998; Hooley et al., 2008). Suzuki-Inoue et al. (2006) first identified platelet expression of this receptor when studying potential receptors of rhodocytin, a snake venom toxin.

In addition to rhodocytin, tumor cells express podoplanin, a type I transmembrane sialoglycoprotein that acts as CLEC-2 endogenous ligand, which precipitates tumor-mediated platelet aggregation events (Kato et al., 2003b). It is also in normal tissues such as kidney podocytes, alveolar and lymphatic endothelial cells (Astarita et al., 2012). Following ligand binding, the YxxL motif undergoes Src kinase–dependent tyrosine phosphorylation events and hence triggering Syk activation and initiating a downstream wave of activatory signalling in a manner similar to which is seen upon GPVI

engagement (Suzuki-Inoue et al., 2006; Ozaki et al., 2009). However, unlike Syk activation in GPVI that requires dually phosphorylated YxxL, attachment and activation of Syk are facilitated via single phosphorylated YxxL (Séverin et al., 2011; Hughes et al., 2013). This is followed by tyrosine phosphorylation and activation of LAT, and assembly of the LAT signalosome, a hub for activation of further signalling adaptor and effector proteins such as, PLCγ2, SLP-76, Tec family tyrosine kinases, Vav GTP exchange factors as described in GPVI signalling (section 1.7.1.2) (Suzuki-Inoue et al., 2006; Fuller et al., 2007; Ozaki et al., 2013).

Lack of Src and Syk abolished the activation of murine platelet in response to rhodocytin (Suzuki-Inoue et al., 2006; Séverin et al., 2011; Hughes et al., 2013). *In vitro* and *in vivo* thrombus formation was substantially reduced in CLEC-2 inhibitory-antibody treated mice (May et al., 2009). Likewise, thrombus formation was attenuated in CLEC-$2^{-/-}$ mice and failed to aggregate in response to rhodocytin stimulation (Suzuki-Inoue et al., 2010). Moreover, recent evidence has shown the implication of CLEC-2 in deep vein thrombosis (DVT) (Payne et al., 2017). Using DVT model of inferior vena cava stenosis, CLEC-2 ablation provided protection against DVT. Interestingly, thrombosis was restored following the transfusion of wild type platelets into these mice. Lastly, reports showed impaired blood/lymphatic vessels separation and embryonic/neonatal lethality in CLEC-2 deficient mice (Bertozzi et al., 2010; Suzuki-Inoue et al., 2010). These findings underpin the additional role of CLEC-2 beyond haemostasis.

1.7.2 G-protein coupled receptor (GPCR) mediated signalling

To stop bleeding, a platelet monolayer propagates haemostatic plug formation via recruitment and activation of more platelets. Such recruitment is facilitated by various thrombogenic factors released by adhered and activated platelets, for instance, thrombin,

ADP and TxA_2, by which they induce their effects via binding to their respective G protein-coupled receptors (GPCRs) on platelet surface (Li et al., 2010). Platelets express several GPCRs on their surface and thrombin protease-activated receptors (PAR)-1 and PAR-4, ADP receptors P2Y1 and P2Y12 and TxA2 receptor (TP) are examples of such receptors with vital contributions to platelet activation (Offermanns, 2006). Receptors belonging to this family possess 7 transmembrane domains with an intracellular C-terminus and extracellular N-terminus. Three loops located on the N-terminus region act as a ligand binding site, while three loops on the cytoplasmic end provide binding sites for intracellular signalling proteins (Kobilka, 2007; Rosenbaum et al., 2009; Stevens et al., 2013).

As their name indicates, GPCRs are linked to an array of G-proteins through which these receptors mediate their signalling effects in an autocrine and paracrine fashion. These proteins are arranged in a heterotrimeric configuration, containing Gα, Gβ and Gγ. Lipid-based binding maintains plasma membrane association of the α and γ subunits where the former subunit is found bound to guanosine diphosphate (GDP) in quiescent/inactive state. However, GDP binding is readily replaced with guanosine triphosphate (GTP) following ligand binding and activation causing changes in the confirmation of this subunit and hence disassembly of the trimer into Gβγ and Gα (Woulfe, 2005; Tuteja, 2009).

1.7.2.1 Thrombin

Thrombin was first identified as a substance with the capability to generate fibrous blood clot and accordingly the name thrombin was driven from Greek word *thrombos*, meaning clot (Davie and Kulman, 2006). It belongs to the serine proteases family and potently causes platelet stimulation. Thrombin catalyses its chief target fibrinogen into fibrin

monomer. Local accumulation of fibrin monomers encases aggregated platelets plug and hence strengthens the formed clot (Crawley et al., 2007).

Human platelets express two forms of protease-activated receptors (PAR), thrombin receptors, PAR-1 and PAR-4. Mouse platelets lack PAR-1 and instead express PAR-3 which although incapable of mediating transmembrane signalling, however, facilitates interaction of thrombin with PAR-4. While PAR-1 displays high affinity to thrombin, higher levels of thrombin are required to activate PAR-4 (Kahn et al., 1998; Coughlin, 1999). Thrombin catalyses the cleavage of PAR N-terminus causing the exposure of a tethered ligand and triggers signalling events via coupled $G\alpha_{12/13}$ and $G\alpha_q$ with the later inducing activation of PLCβ and subsequent cleavage of PIP2 to DAG and IP3. As a result, degranulation, cytosolic calcium elevation and activation of integrin αIIbβ3 to bind fibrinogen take place. $G\alpha_{13}$, through RhoGEF induced activation of Rho kinase, is also involved in cytoskeletal reorganisation along with platelet granules release via phosphorylation of myosin light chain (Stalker et al., 2012). Moreover, G_i-coupled PAR-1 down-regulates adenylate cyclase activity and reduces the levels of cAMP levels. Such down-regulation suppresses inhibitory signalling in platelets and hence contributes to platelets activation (Figure 1.5). The importance of such receptors was shown in the reduced aggregation of PAR-4 ablated platelets in response to thrombin with prolonged bleeding time (Kahn et al., 1998; Hamilton et al., 2004; De Candia, 2012).

1.7.2.2 Thromboxane A2 (TxA2):

Thromboxane A2 (TxA2), an eicosanoid, is potent platelet agonist which is synthesised in activated platelets through cyclooxygenase-l (COX-1) and thromboxane synthase (Knezevic et al., 1993). The release of TxA2 from activated platelets induces constriction of vascular wall and aids in minimising blood loss (Halushka et al., 1987). Additionally,

platelets are recruited to the site of injury via TxA2 binding to its specific TP receptors, TPα and TPβ. Both receptors act similarly and are only different in the sequence of their C-terminus domain (Hirata et al., 1996).

Given its lipidic nature, synthesised TxA2 is capable of diffusion through the plasma membrane (Shen and Tai, 1998). TP coupled $G\alpha_q$ and $G\alpha_{12/13}$ proteins trigger activatory events following TxA2 binding, in a similar manner to what was described previously in thrombin/PAR signalling, and mediate granule release, shape changes and integrin αIIbβ3 activation (Offermanns, 2006). TxA2 contributes to a positive feedback mechanism that ensures rapid and complete platelet activation. Deficiency or ablation of TP receptors causes bleeding events and prolonged bleeding time along with failure to respond normally to TxA2 mimetic peptide analogue, U46619, respectively (Thomas et al., 1998; Mumford et al., 2010). Lastly, targeting synthesis of TxA2 by irreversible blockade of COX-1 with aspirin successfully prevents thrombotic events and is considered as gold standard anti-thrombotic agent (Awtry and Loscalzo, 2000; Davi et al., 2012).

1.7.2.3 Adenosine diphosphate (ADP)

Adenosine diphosphate (ADP) is released, at the site of an injured vessel, by the damaged endothelium, red blood cells as well as activated platelets. This vital prothrombotic mediator induces autocrine and paracrine platelet activation and thereby causing positive feedback. ADP binds human platelets via its specific purinergic receptors, P2Y1 and P2Y12. The former is expressed at low copy number, 150 copies per platelet, whereas P2Y12 levels are about 10-fold higher. While P2Y1 is coupled with $G\alpha_q$, P2Y12 is known to act through $G\alpha_i$. Besides the difference in their expression levels, ADP

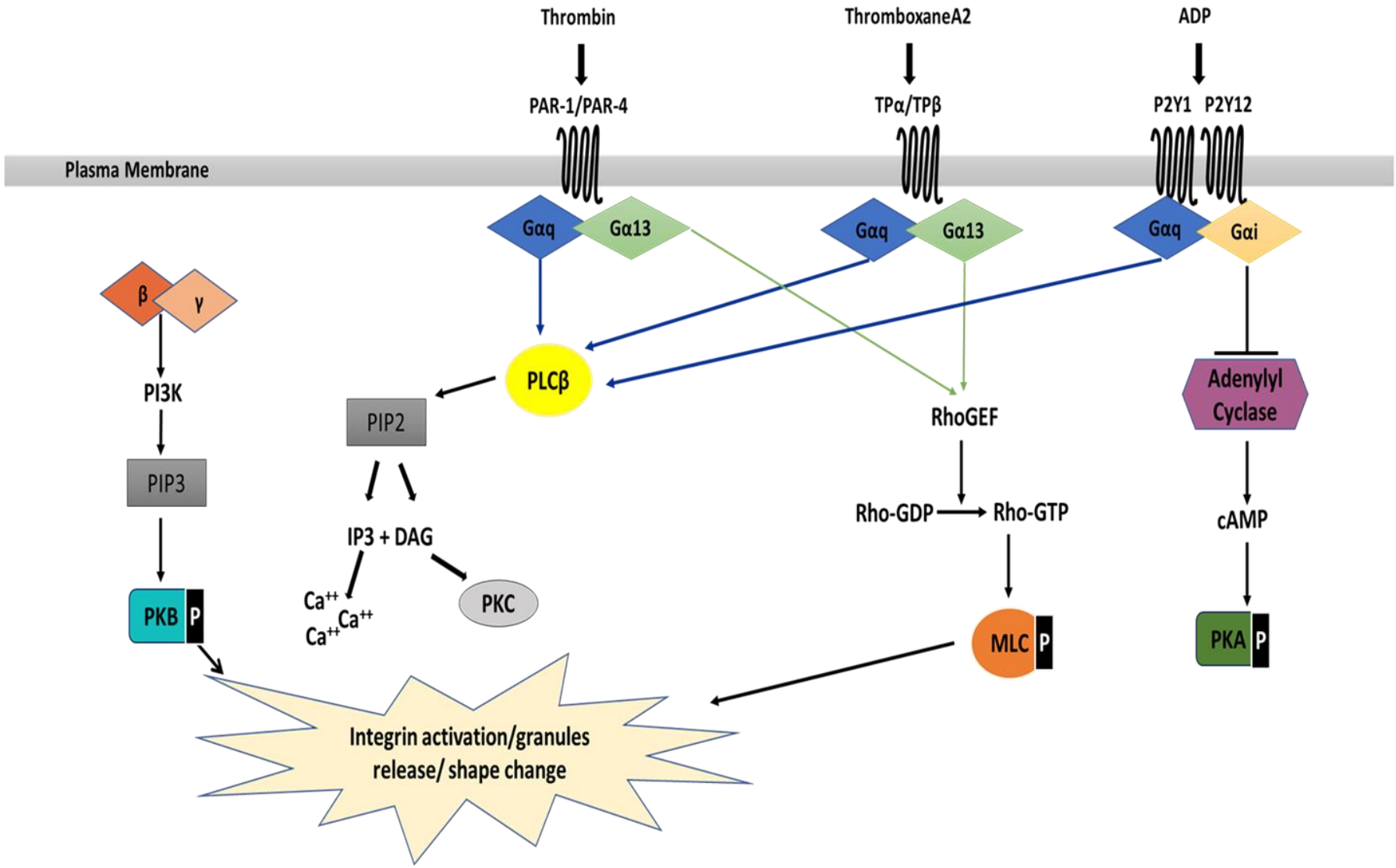
receptors are coupled with different G-protein, with P2Y1 and P2Y12 acting through $G\alpha_q$ and $G\alpha_i$ proteins, respectively (Gachet, 2006; Offermanns, 2006).

Platelet shape change, calcium mobilisation from storage sites and reversible, temporary aggregation are stimulated by P2Y1 and $G\alpha_q$ protein following ADP stimulation. This is facilitated via activation of PLCβ in a similar manner to what was previously described for thrombin (section 1.7.2.1) (Savi et al., 1998). P2Y1 ablated platelets display attenuated platelet aggregation when stimulated with ADP along with prolonged bleeding time and reduced venous thrombosis (Fabre et al., 1999; Bird et al., 2012). In contrast, P2Y12 via $G\alpha_i$ coupling negatively targets adenylyl cyclase activity and hence intracellular cAMP levels. This causes downregulation of protein kinase A (PKA) dependent signalling and subsequently, calcium release and platelet activation. Moreover, P2Y12 triggers signalling events via $G_{\beta\gamma}$ subunit causing the activation of PI3K activity and consequent production of PIP3. PI3K contributes to the regulation of the GTPase Rap1B pathway and as result, enables GTPase Rap1B mediated upregulation of integrin αIIbβ3 (Woulfe et al., 2001; Woulfe, 2005; Gachet, 2006). P2Y12 deletion or inhibition studies showed impaired ADP stimulated platelet aggregation and *in vivo* thrombus formation (Foster et al., 2001; van Gestel et al., 2003).

Lastly, although ADP receptors respond with different potency, co-activation of P2Y1 and P2Y12 is required to obtain full ADP-mediated platelet activation (Hechler et al., 1998; Gachet, 2006). Knowing the significance of such receptors makes them an attractive anti-thrombotic target. Several drugs are available in the market, such as cangrelor, ticagrelor, clopidogrel and prasugrel, and have proven effective in preventing thrombosis via irreversibly inhibiting P2Y12 receptor (Gachet, 2001; Woulfe et al., 2001).

Figure 1.5: G-protein–coupled receptor (GPCR) induced platelet activation and signalling in platelets.

In response to injury and platelet activation, degranulation events accumulate various soluble mediators acting as platelet stimuli in an autocrine and paracrine fashion through binding to their respective receptors on the platelet surface. Thrombin, thromboxane A2 (TxA2) and (ADP) interact and activate protease-activated receptors (PAR-1) and (PAR-4), TP receptor and $P2Y_1$, respectively, via coupled $G_{\alpha q}$ proteins and subsequently, signal the activation of phospholipase C (PLC) β. This is followed by PLC β mediated cleavage of phosphatidylinositol 3,4-bisphosphate (PIP2) and the formation of secondary mediators, inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG). Upon generation of such mediators, granules release and elevation of cytosolic calcium from stores enable integrin $\alpha_{IIb}\beta_3$ activation and binding to fibrinogen. PAR (-1 and -4) and TP are also coupled to $G_{\alpha 13}$ where generated signals result in the phosphorylation and activation of myosin light chain (MLC) by Rho kinase and hence changing platelet shape and further induce granules secretion. $G_{\alpha i}$ protein is found associated with $P2Y_{12}$, ADP receptor, and upon ADP binding, inhibitory effect is exerted on the adenylyl cyclase-mediated cyclic adenosine monophosphate (cAMP) synthesis and consequent suppression of protein kinase A (PKA) activity. Lastly, dimeric Gβ/γ subunits regulate activation of protein kinase B (PKB) activation via phosphoinositide 3-kinase (PI3K) activation.


Thrombin
ThromboxaneA2
ADP
PAR-1/PAR-4
TPα/TPβ
P2Y1 P2Y12
Plasma Membrane
Gαq
Gα13
Gαq
Gα13
Gαq
Gαi
β
γ
PLCβ
PI3K
PIP2
RhoGEF
Adenylyl Cyclase
PIP3
IP3 + DAG
Rho-GDP
Rho-GTP
cAMP
PKB P
Ca++
Ca++ Ca++
PKC
MLC P
PKA P
Integrin activation/granules release/ shape change

1.7.3 Calcium-mediated signalling

Intracellular Ca^{2+} plays a crucial role in regulating cellular activities and functions. Most platelet agonists share similar features of calcium mobilisation as a downstream event in their activatory pathways. In platelets, calcium-mediated signalling controls PKC activity, cytoskeletal reorganisation and integrin activation (Varga-Szabo et al., 2009). Agonist-mediated calcium elevation is mainly carried out by inositol trisphosphate receptor-induced release of calcium from intracellular storage sites in addition to the rapid influx of calcium across platelet membrane upon depletion of the calcium storage site (DTS) in a process referred as store-operated calcium entry (SOCE). In this process, the DTS membrane localised calcium sensor known as stromal interaction molecule 1 (STIM1), remains in bound state via its calcium-binding EF-hand domain, whereas calcium mobilisation in activated platelets and depletion of DTS calcium contents causes the release and translocation of STIM1 to platelet membrane where it encounters and binds calcium channel, Orai1, allowing influx of extracellular calcium (Brass and Joseph, 1985; Liou et al., 2005; Bergmeier and Stefanini, 2009; Lang et al., 2013).

Platelets display other means of elevating cytosolic levels of calcium upon platelets stimulation independent from SOCE. For instance, transient receptor potential cation channel subfamily C member 6 (TRPC6), is a non-selective cation entry channel that facilitates the calcium passage into the cytosol in the event of platelet stimulation (Hassock et al., 2002). Similarly, ADP purinoceptor, P2X1, when bound to ATP, it forms non-selective cation passage (Sage et al., 2000). Binding of calcium to the intracellular signalling molecule, Calcium- and DAG-regulated guanine nucleotide exchange factor I (CalDAG-

GEFI), induces integrin activation and platelet degranulation (Bergmeier and Stefanini, 2009). Lastly, scramblase activity is increased in elevated calcium conditions, increasing surface expression of PS and hence augmenting platelet activation and aggregate formation (Heemskerk et al., 2002).

1.7.4 Integrin αIIbβ3, activation and signalling:

The αIIbβ3 integrin, also known as glycoprotein IIb-IIIa, is found only on the platelet surface with αIIb subunit exclusively found in platelet and progenitor megakaryocyte whereas β3 subunit is expressed by several cells and tissues in high numbers (Shattil et al., 1998). The resting platelet expresses around 50,000 molecules per cell, while the levels increase up to 80,000 molecules per cell on the activated platelet surface (Brass, 2010). Integrin αIIbβ3 is also present inside α-granules and is translocated to the platelet surface upon activation when the α-granules are secreted. There, it binds different ligands containing a common Arg-Gly-Asp (RGD) motif or the KQAGDV sequence, such as fibrinogen, fibronectin and VWF, to facilitate platelet aggregation (Bennett, 1990; Humphries et al., 2006). For this to happen, the integrin undergoes conformational transformation into activated-higher affinity state as results of activatory signals generated from platelet stimulation in a process well known as inside-out signalling (Plow et al., 2000).

The integrin retains an inactive-dormant state by keeping extracellular domains in a bent position through interaction (salt bond) between α- and β-subunits membrane-proximal cytosolic regions (Luo et al., 2007b; Moser et al., 2009). However following platelet stimulation and triggering of the activation, DAG is generated along with mobilisation of calcium which is readily followed by activation of Ca^{2+} and DAG-regulated guanine

nucleotide exchange factor-I (CalDAG-GEFI). Subsequently, Rap1 is switched from its inactive GDP-bound form to the GTP-bound active form. Activated Rap1 along with talin and Rap1b-interacting adaptor molecule (RIAM) form an activation complex, enabling binding of talin to the β-subunit of the integrin. Such binding disrupts the aforementioned interaction between integrin subunits rendering the extracellular domain open for ligand binding. However, recent evidence revealed the dispensability of RIAM in the talin membrane translocation and consequent integrin activation. Platelets from RIAM ablated mice showed normal integrin activation and aggregation response when stimulated. Moreover, thrombus formation was similarly executed in control and RIAM-null mice (Stritt et al., 2015). Lastly, cytosolic protein kindlin-3 is another important player in integrin activation. Moser et al. (2008) reported the loss of integrin activation in kindlin-$3^{-/-}$ mice along with failure in developing arterial thrombosis at the sites of damaged vessel and increased bleeding tendency despite normal expression of talin.

To avoid unnecessary platelet aggregate formation, αIIbβ3 integrin activation is strictly monitored. For instance, molecular switching and activation of Rap1 are controlled by two regulatory proteins, guanine nucleotide exchange factor CalDAG-GEFI and GTPase activating proteins (GAPs). While the former ensures the exchange of GDP for GTP and triggering of Rap1 activation, the latter inhibits the activation via GTP hydrolysis (Bos et al., 2007). Stefanini et al. (2015) demonstrated the critical inhibitory role of Ras GTPase-activating protein 3 (RASA3) on Rap1 dependent platelet activation by demonstrating spontaneous platelet activation in RASA3 mutant mice along with severe thrombocytopenia and CalDAG-GEFI/Rap1-dependent platelet clearance. Based on their findings, the group suggested a possible model of RASA3 implication in Rap1 signalling. In their model, resting

platelets maintain the highly active status of RASA3 on the plasma membrane and hence, hindering Rap1 activation via CalDAG-GEFI and keeping platelet in a quiescent condition. While activated platelets result in the sensitization of P2Y12 and subsequent activation of PI3K, thereby, activated kinase inhibits the activity of RASA3 unblocking the rapid nucleotide exchange on Rap1 and integrin activation.

Individuals with defective or deficient integrin αIIbβ3 develop significant prolongation in bleeding time in a clinical syndrome known as Glanzmann thrombasthenia (Nair et al., 2002; Mutreja et al., 2017). Moreover, β_3 deficient Mice fail to form thrombi following induced vascular injury along with haemorrhagic episodes, abnormally prolonged tail bleeding and attenuated platelets aggregation (Hodivala-Dilke et al., 1999; Ni et al., 2000). With such significant impact, integrin $\alpha_{IIb}\beta_3$ is a pharmaceutical target to prevent vaso-occlusive disorders. Abciximab and eptifibatide antagonise integrin $\alpha_{IIb}\beta_3$ and thereby hinder the ability of platelets to aggregates. Although effective with more than 20% reduction in the mortality among cases undergoing percutaneous coronary intervention, these agents are prescribed with extreme caution due to the high risk of developing bleeding events (Quinn et al., 2003; Coller and Shattil, 2008; Bledzka et al., 2013; Bosch et al., 2013).

Integrin $\alpha_{IIb}\beta_3$ is capable of bidirectionally transmitting signals across platelet surface, receiving inside-out activatory signals and subsequently generating outside-in signals upon ligand binding. One of the very early detectable outside-in signalling events involves the phosphorylation and activation of various signalling and adaptor proteins (Golden et al., 1990; Huang et al., 1993) and SFKs which are constitutively associated with the cytoplasmic tail of β_3 subunit, serves as an upstream effector in the integrin signalling pathway.

In quiescent platelets, the activity of SFKs are kepts restrained via C-terminal Src kinase (Csk) induced phosphorylation on Tyr 529 residue. However, this process is rapidly reversed with the aid of protein phosphatases, for instance, protein-tyrosine phosphatase 1B (PTP-1B), upon ligand crosslinking and integrin clustering (Obergfell et al., 2002; Arias-Salgado et al., 2003). Additionally, integrin clustering facilitates *trans*-autophosphorylation of the Tyr 418 residue located in in the Src activation loop and full Src activation (Fong et al., 2016). Once activated, SFKs robustly target and phosphorylate Tyr 747 and Tyr 759 residues on the cytoplasmic tail of β_3. Such phosphorylation provides binding sites for the myosin heavy chain (myosin II) and hence, supports integrin-cytoskeletal interaction (Jenkins et al., 1998). Moreover, Tyr 747 phosphorylation negatively impacts the talin, whereas phosphorylation on Tyr 759 protects from the calcium-regulated protease (calpain) induced β_3 tail cleavage allowing platelets to spread (Xi et al., 2006; Anthis et al., 2009).

The Rho-family GTPases RhoA regulate actin dynamics and is implicated in the integrin-induced cytoskeletal changes in activated platelets. Activation of a RhoGAP by c-Src has been proposed to inhibits the activity of RhoA permitting platelet spreading over the injured area until the formation of thrombus (Arthur et al., 2000; Gong et al., 2010). On the other hand, once a thrombus is formed calpain mediates cleavage of c-Src-β_3 integrin complex relieving RhoA suppression and initiates clot retraction (Flevaris et al., 2007). Lastly, SFKs further mediate activation of several kinases and signalling proteins, downstream of the activated integrin $\alpha_{IIb}\beta_3$, including Syk, PLCγ2, focal adhesion kinase (FAK), and adhesion- and degranulation-promoting adaptor protein (ADAP) leading to the amplification of platelet activation (Obergfell et al., 2002; Harburger and Calderwood, 2009).

1.8 Negative regulation of platelet activity:

In the event of vascular damage, an immediate response is initiated. Such a process is described as being highly dynamic, integrated and multistep with a multitude of regulatory factors ensuring stabilised and limited growth of thrombi. A fine balance is constantly exerted to maintain platelets in a quiescent resting state when thrombotic activity is not needed (intact endothelium). This is facilitated by the well characterised and established endothelium-derived inhibitory regulators, nitric oxide (NO) and prostacyclin (PGI_2). Both regulators are capable of not only instantly suppressing platelet reactivity but also limiting and restricting platelet activation and thrombus growth during injury (Moncada et al., 1976; Mitchell et al., 2008; van der Stoep et al., 2014). Furthermore, other platelet borne regulators with inhibitory capacity have been identified, for instance platelet endothelial cell adhesion molecule-1 (PECAM-1), carcinoembryonic antigen cell adhesion molecule (CEACAM 1 and CEACAM 2), G6b-B and junctional adhesion molecule-A (JAM-A) (Falati et al., 2006; Newland et al., 2007; Wong et al., 2009).

1.8.1 Nitric oxide (NO)

Endothelium-derived nitric oxide synthase (eNOS) catalyses the synthesis of nitric oxide from an amino acid, L-arginine and is then diffused into circulation (Tousoulis et al., 2012). The gaseous nature of this molecule allows its diffusion across the platelet membrane. Within the cytosol, it binds soluble guanylyl cyclase (sGC), an enzyme that mediates the synthesis of cGMP from GTP and hence causes the activation of protein kinase G (PKG) (Jensen et al., 2004; Low and Bruckdorfer, 2004; Siess, 2004; Du, 2007).

This kinase is composed of catalytic, N-terminal and regulatory domains. The latter domain possesses two binding sites specific for cGMP, and when bound the enzyme undergoes conformational changes causing its activation. Differences in the N-terminal domain of PKG is responsible for the presence of different isoforms, PKGI and PKGII, with different substrate affinities and specificities (Kim et al., 2016a). Once activated, PKG targets and phosphorylates various substrates causing the inhibition of platelet activation (Bult et al., 1988; Kim et al., 2016b).

For instance, PKG attenuates platelet activation by targeting IP3 receptor-induced mobilisation of calcium from intracellular storage sites. PI3K activity is similarly impacted causing reduced levels of integrin αIIbβ3 activation (Pigazzi et al., 1999). Wang et al. (1998) reported that the function of TxA_2 receptor is downregulated via PKG-mediated phosphorylation of the receptor.

1.8.2 Prostacyclin (PGI_2)

The other principal endothelial cell-derived negative regulator is prostacyclin. This eicosanoid is produced through the sequential actions of PLA2, cyclooxygenase (COX) and prostanoid synthases on membrane lipids and is then released into circulation (Funk, 2001). PGI_2 is known to have a short half-life and is then transformed into 6-keto-$PGF_{1\alpha}$, an inactive metabolite (Majed and Khalil, 2012). However, several factors regulate the synthesis of PGI_2 including protein kinases mediated phosphorylation and modification of some nuclear transcriptional factors. For instance, thrombin mediates the activation of mitogen-activated protein kinase (MAPK). This is followed by phosphorylation and activation of PLA_2, causing elevation of membrane lipid hydrolysis and consequent COX1-mediated synthesis of PGI_2. Furthermore, thrombin stimulation increases the levels of transcription factor NF-

κB, which in turn upregulates the expression of COX1 and hence increasing the rate of PGI_2 synthesis (Wheeler-Jones, 2008). Also, the degradation of cAMP by phosphodiesterase-3 can be inhibited through NO-induced cGMP which is capable of downregulating the activity of these enzymes (Gkaliagkousi et al., 2007).

PGI_2 acts on platelets by binding specific $G\alpha_s$ coupled IP receptor (Narumiya et al., 1999). This binding rapidly activates $G\alpha_s$ protein and subsequently stimulates the synthesis of cAMP from ATP with the aid of adenylyl cyclase (AC). cAMP can then trigger the activation of protein kinase A (PKA), which in turn phosphorylates and activates several substrates in platelets. Similar to PKG, PKA negatively targets calcium mobilisation by downregulating IP3 receptors on the membrane of DTS, the intracellular calcium storage site (Miller, 2006). Rap1b, an important regulator of integrin αIIbβ3 activation, is also inhibited by PKA through direct interfering with Rap1b phosphorylation or its molecular switch, CalDAG-GEFI (Subramanian et al., 2013). Moreover, PKA sustains normal quiescent discoid shape via phosphorylation of vasodilator-stimulated phosphoprotein (VASP), an important regulator of actin reorganisation and dynamics (Jin et al., 2005). Various cardiovascular disorders have been correlated with decreased levels of PGI_2 such as stroke and myocardial infarction. Additionally, attenuated PGI_2 sensitivity has been demonstrated by patients with cardiovascular diseases (Sinzinger et al., 1981). Ablation of PGI_2 synthase results in the hyperreactivity of platelets in mice (Yokoyama et al., 2002). IP receptor knockout mice are more susceptible to developing thrombotic events when compared to WT mice (Nakae et al., 2005). Murata et al. (1997) reported increased levels of thrombus formation in $IP^{-/-}$ mice. Also, about two-thirds of these mice died from occlusive events in the carotid arteries.

1.8.3 Immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptors:

Besides endothelium antithrombotic mediators, platelets express various endogenous inhibitory receptors counteracting positive activatory signals during thrombosis and thereby limiting the progress of formed thrombus. Such receptors share common characteristic features, first, they possess immunoreceptor tyrosine-based inhibition motif (ITIM) aiding in recruiting phosphatases. Second, such recruitment enables these receptors to inhibit the activity of other activation receptors that are often linked to an immunoreceptor tyrosine-based activation motif (ITAM) or GPCRs (Daeron et al., 2008).

Immunoreceptor tyrosine-based inhibitory motif (ITIM) is located on the cytoplasmic domain of these receptors with a prototype of 6–amino acid sequencing (Ile/Val/Leu/Ser)-X-Tyr-X-X-(Leu/Val) with X denoting any amino acid (Vely and Vivier, 1997; Bolland and Ravetch, 1999). Following ligand binding and receptors clustering, Src family kinases target and phosphorylate tyrosine residues within the ITIM. The phosphorylated ITIM then acts as a docking site for the binding and recruitment of Src homology 2 (SH2) domain-containing cytoplasmic phosphatases, SHP-1 and SHP-2, allowing the enzymes to be in close proximity with their substrates (Kharitonenkov et al., 1997; Hua et al., 1998). Consequently, waves of inhibitory events target a plethora of positive regulating kinases and effector proteins such as Syk, LAT, PLCγ along with PI3K, causing further inhibition of downstream activatory events. Moreover, the aforementioned ITIM facilitated recruitment deprives activatory receptors of key modulators and effectors kinases and hence hampering the transduction and generation of their positive signals (Moraes et al., 2010). Platelet endothelial cell adhesion molecule-1 (PECAM-1) and carcinoembryonic antigen cell adhesion molecule-1 (CEACAM-1) are inhibitory receptors that have been identified in platelets as major negative

regulators (Cicmil et al., 2002; Falati et al., 2006; Alshahrani et al., 2014; Alshahrani et al., 2016). PECAM-1 is 130 kDa surface glycoprotein and belongs to the immunoglobulin gene (Ig) superfamily (Newman et al., 1990). PECAM-1 engages via homophilic ligand-binding interactions (Sun et al., 1996; Newton et al., 1997). Nonetheless, ligands other than PECAM-1 itself, have been reported to binds and activates this receptor including integrin $\alpha_v\beta_3$ and CD38 (Buckley et al., 1996; Deaglio et al., 1998). Activatory signalling events along with platelet aggregation are greatly reduced upon PECAM-1 activation prior to agonist stimulation (Jones et al., 2001; Cicmil et al., 2002). PECAM-1 deficient platelets are hyperresponsive to collagen stimulation with amplified aggregation, secretion and adhesion. increased aggregation (Patil et al., 2001). Also, deficient mice demonstrated larger thrombus formation under physiological flow (Jones et al., 2001). Likewise, an enhanced response was reported in CEACAM-$2^{-/-}$ mice with increased collagen, CRP-XL or rhodocytin mediated platelet aggregation. Thrombi formed in CEACAM-$2^{-/-}$ mice were more stable and larger than wild type (Alshahrani et al., 2014).

1.9 Antiplatelet therapies

Over the past 25 years, the number of cardiovascular diseases (CVDs) cases, such as ischemic heart disease and myocardial infarctions, has shown a great increase in Europe causing 3.9 million deaths and accounting for 45% of all deaths in Europe. The costs to manage such cases is estimated to be £186 billion a year. In 2015, Great Britain has scored CVD incidence values of 324,446 and 298,270 in men and women, respectively, causing the death of more than 79,000 affected patients and burden of approximately £11 billion management costs of which £2,5 billion was spent on medications (Wilkins E, 2017).

Of importance, arterial thrombotic events are considered a major precipitating factor in the development and pathogenesis of CVD. The contribution of platelets in such process is quite vital and critical. Disruption of this multistep process serves a pivotal tool in the ongoing battle against CVD and indeed antiplatelet agents play a crucial role in the management of such cases (Table 1.1). Aspirin is considered a "gold standard" antithrombotic agent. It has been used for over 100 years and proved effective in preventing thrombotic diseases (Weiss and Aledort, 1967; Gum et al., 2001). Aspirin causes irreversible acetylation of COX-1 enzymes, in both platelets and endothelial cells, and hence inhibiting synthesis of TxA2 (Awtry and Loscalzo, 2000).

As aggregation is the very last end-point in all activatory pathways, targeting integrin αIIbβ3 with agents, such as Abciximab, would directly inhibit platelet aggregation and consequent thrombus formation. This Fab fragment is derived from a monoclonal antibody raised against integrin αIIbβ3. However, due to the high density of this integrin on the platelet surface, high doses of abciximab are administered to reaches receptor occupancy around 80% (Popma and Satler, 1994; Giordano et al., 2016).

Other effectively targeted platelet receptors are ADP receptors. Irreversible inhibition of P2Y12 with clopidogrel reduces platelet aggregation in response to ADP stimulation by 60% (Thebault et al., 1999). Lastly, another variably effective yet indirect approach of inhibiting platelet activity is via PGI2- and NO-mediated elevation of cyclic nucleotides, cAMP and cGMP, respectively. Phosphodiesterases (PDEs) regulate the levels of these nucleotides in platelets, hence blockage of these degradative enzymes amplifies nucleotide-mediated inhibitory signals. For instance, inhibition of PDE3 with cilostazol decreases calcium mobilisation and thrombus formation (Feijge et al., 2004; Sim et al., 2004).

Table 1.1: FDA-approved antiplatelet agents

COX-1 inhibitor		
Agent	**Route**	**Reference**
Aspirin	Orally	(Weiss and Aledort, 1967)
Integrin αIIbβ3 inhibitors		
Agent	**Route**	**Reference**
Abciximab	Intravenous	(Popma and Satler, 1994)
Eptifibatide	Intravenous	(Giordano et al., 2016)
ADP receptor blockers		
Agent	**Route**	**Reference**
Clopidogrel	Orally	(Spiliopoulos et al., 2014)
Ticagrelor	Orally	(Al-Salama et al., 2017)
PDE inhibitors		
Agent	**Route**	**Reference**
Dipyridamole	Orally	(Eisert, 2012)
Cilostazol	Orally	(Asal and Wojciak, 2017)

With all the development in the field of antiplatelet pharmaceuticals, limitations in the safety and efficacy of developed agents remain a challenging issue (Angiolillo et al., 2009). Due to the working nature of such agents, the risk of developing bleeding ranging from mild to severe bleeds is unavoidable. However, most of the cases develop non-life-threatening, mild bleeding that does not require medical intervention or blood transfusion.

The success of above-mentioned Integrin αIIbβ3 inhibitor abciximab encouraged researchers to invest in designing and developing receptor blockers that would inhibit platelet-vessel adhesion, the very early step in thrombotic diseases. In mice, selective inhibition of GP Ib and VI caused the reduction in the volume of infarcts following transient occlusion of the cerebral artery. Interestingly, this inhibition was not accompanied by intracranial bleeding (Kleinschnitz et al., 2007). Efficacy of GP Ib receptor blockade was similarly explored by Gilbert et al. (2007) who reported inhibition of vWF and platelet activation in healthy donors following administration of ARC1779, vWF inhibitor. As the development of these therapeutic agents is beyond the scope of the present thesis, comprehensive and in-depth reviews dissecting this topic are available; (Jackson and Schoenwaelder, 2003; Yeung and Holinstat, 2012; Jayakumar et al., 2016; Benimana et al., 2017). Recent attention has been paid to targeting exposed collagen, during vessel injury, and its interaction with its specific receptor on the platelet surface, GPVI (Kleinschnitz et al., 2007).

Interestingly, a recent proteomics study revealed the presence of heat shock protein 47 (HSP47) on the platelet surface (Kaiser et al., 2009). This protein is known to chaperone and control biosynthesis of collagen in collagen-producing cells. The fact that collagen, the main substrate of HSP47, is the most potent and thrombogenic component in the sub-endothelium suggested a possible role of this protein in platelet-collagen interaction. Such a suggestion

was further validated and established in HSP47 inhibited and depleted platelets (Sasikumar et al., 2018).

HSP47 has demonstrated to be a promising therapeutic target in different tissues. Inhibiting excessive collagen biosynthesis in several fibrotic diseases via blocking HSP47 function is a promising mechanism for the treatment of such diseases (Masuda et al., 1994; Kakugawa et al., 2004; Hagiwara et al., 2007; Sato et al., 2008; Cottin, 2013; Ishiwatari et al., 2013). Therefore, it is essential to understand the mechanism by which HSP47 contributes to collagen-mediated platelets functions as this could facilitate the development of new anti-platelet strategies targeting platelet-collagen interaction and possibly via HSP47.

HSP47 belongs to HSPs family. Members of this family are closely involved in diverse physiological processes, and hence could potentially be used in several therapeutic applications. Pharmaceutical induction of these proteins might aid in developing resistance to harmful stimuli and serving as a novel intervention in the treatment and prevention of CVDs. However, designing and targeting these proteins is challenged with possible associated risks and benefits that should be critically taken into consideration (Jindal, 1996). The discovery, expression and role of these therapeutically promising proteins are further explained in the next section of this chapter.

1.10 Heat shock proteins (HSPs) Discovery, historical overview:

The odyssey began in 1962 in Naples where Italian scientist Ferruccio Ritossa was studying the synthesis of nucleic acid in the puffs of *Drosophila* salivary glands. He found that their larvae developed new small puffs when placed in a temperature above 36 °C that were readily regressed when the larvae were left recovering at a temperature of 25 °C (Ritossa,

1962). Since then, what instigated as a molecular curiosity, now constitutes a major area in the research of molecular and cell biology.

Tissieres et al. (1974) demonstrated an increase in the expression of a selective group of proteins when larvae were exposed to a temperature higher than that required for their normal growth. These proteins were then named heat (or stress, shock) proteins (HSPs), after the nature of stimuli that trigger their expression. However, most of these proteins are expressed at basal levels in various cells to maintain cell growth and development. Moreover, in addition to elevated temperature, other factors such as oxidative stress, viral infections and exposure to chemicals or irradiation can precipitate expression of these proteins (Pockley, 2003).

The significance of these highly conserved and ubiquitous proteins appears in their pivotal contribution in maintaining protein folding and correct functional configuration. Also, stress proteins are also involved in the repair of denatured proteins or facilitating their clearance and removal upon stress, hence the name molecular chaperones are also used to reference these proteins (Georgopoulos and Welch, 1993; Whitley et al., 1999).

1.11 HSPs nomenclature:

Stress proteins are under the umbrella of a multigene family with molecular size ranging from 8 to 150 kDa. A large number of HSP families have been discovered over the last 3 decades that are classified into HSP10, HSP27, HSP40, HSP60, HSP70, HSP90 and HSP105/110 based on their molecular weight and sequence homology. These proteins can be found in the nucleus, mitochondria, endoplasmic reticulum and cytosol (Ellis, 1999).

However, site localisation varies according to the types of these proteins (Kregel, 2002). After the nomenclature that was first used in Drosophila, HSPs are referred to according to their molecular size in sodium dodecyl sulphate (SDS) as well as precipitating inducer. For instance, HSP90 denotes heat shock inducible protein of approximately 90 kDa (Whitley et al., 1999).

1.12 HSP expression:

In addition to being induced by stress stimuli, HSPs are constitutively expressed to meet physiological requirements for cellular growth. It has been reported that constitutively expressed HSPs mainly found in the form of a multiprotein complex of the HSP and co-factor, for instance, HSP90 exists in a complex and facilitates protein folding via interaction with regulatory proteins (Buchner, 1999; Whitley et al., 1999).

Given the nature of HSPs in shifting protein configuration from a denatured and disordered form to a functionally ordered form, the demand of these proteins is often high in the events of oxidative stress or heat stimuli where proteins are consequently affected and denatured (Gething and Sambrook, 1992; Georgopoulos and Welch, 1993). Such a stressful environment upregulates HSP gene expression with the aid of heat shock transcription factors (HSFs). Four evolutionarily conserved HSPs regulating factors have been found in vertebrates (HSF1–4) with HSF-1 being a major regulator of heat shock response in mammals. Inactive HSFs are found as monomers that undergo trimerization process once activated allowing binding to promoter region of the HSP gene and subsequent gene transcription and translation. On the other hand, interaction with respective promotor site is readily reversed upon recovery from inducing stress stimuli (Wu, 1995).

1.13 HSP role in health and disease:

Much of the growing interest in the area of HSPs come from their role as molecular chaperones of protein synthesis and maturation. Biochemically, the term "molecular chaperone" is given to any protein that is able to prevent the potential interaction between complementary surfaces and disrupt any premature or improper interactions. It was first coined by Laskey et al. (1978) to describe the characteristic features of nucleoplasmin, an acidic nuclear protein, in the process of nucleosomes assembly to form isolated DNA and histones.

HSPs chaperone protein biosynthesis by binding and blocking reactive sites of the polypeptide chains. For instance, binding hydrophobic surfaces prevents the exposure of these sites to the rest of closely located reactive surfaces and the formation of inappropriate aggregates. Such binding favors correct mature folding of the synthesised proteins. This binding is reversible where the chaperone does not take any part in the final structure of the protein (Ellis and van der Vies, 1991).

The first established correlation of increased cellular HSPs expression with exposure to several amino acid analogues or antibiotic (puromycin) have raised the heat shock response hypothesis. While the former is known of producing proteins with extremely short half-lives due to the lack of correct protein folding, the latter antibiotic has been reported to induce the release of nascent polypeptide chains prematurely (Kelley and Schlesinger, 1978; Hightower, 1980). Taken together with the fact that heat or stress stimuli negatively affected protein structure and configuration, the notion was suggested that in the events of abnormally configured protein deposition, the heat shock response would be induced (Pelham, 1986).

Indeed, this was demonstrated in several lines of investigation, for instance, HSP60 and HSP70 are involved in protein translation and folding. HSPs, such as HSP90, have also been implicated in the regulation of protein kinases and some transcription factors. Members of HSP superfamily demonstrate cytoprotective behavior, where HSPs exert anti-apoptotic features. These proteins are capable of interacting with various stress signalling and apoptosis-inducing molecules, and hence, block cell death and promote cellular proliferation and survival (Lanneau et al., 2008). HSP70 ablation increases the apoptotic sensitivity of mouse embryonic cells in response to a range of stimuli (Schmitt et al., 2003). Similar effects were also reported in HSP27, HSP60 and HSP90 depleted cellular models (Compton et al., 2006; Choi et al., 2007; Kamada et al., 2007). On the other hand, overexpression of HSP27, HSP60, HSP70 or HSP90 protects the cell from apoptosis through interfering with the activation of apoptosis mediating protease, caspase when placed under stress stimuli such as DNA damage, deposition of misfolded proteins or accumulation of reactive oxygen species (Garrido et al., 1999; Mosser et al., 2000; Mosser and Morimoto, 2004; Garrido et al., 2006). Compromised cardiac functions are rapidly recovered following the exposure to preconditioning stimuli such as ischemia or hyperthermia in experimental animals (Donnelly et al., 1992; Currie et al., 1993; Hutter et al., 1994). Moreover, HSP72 overexpressing transgenic mice have shown improved cardiac contractility and recovery along with a reduction in the size of developed infarcts in a model of myocardial infarction and reperfusion (Hutter et al., 1996). Also, cellular resistance to the toxic effects caused by TNF-α following stress simulation is enhanced by overexpression of HSP27 and HSP70 (Jaattela and Wissing, 1993; Mehlen et al., 1995). HSP70 has also been suggested to confer tumorigenicity of mouse fibrosarcoma cells and enhancing their survival following

irradiation treatment (Simon et al., 1995). *In vivo* administration of HSP70 following sciatic nerve (SN) axotomy in neonatal mouse prevents axotomy-induced SN death and protects the mice from neural degeneration (Tidwell et al., 2004). The development of photoreceptor degeneration after bright light eye treatment was evaluated in mice after giving an intra-ocular injection of HSP70. The injection successfully provided protection from the aforementioned treatment and prevented associated ocular degeneration when compared to mice that received control injection (Tytell et al., 1994). In contrast to their cytoprotective roles, certain HSPs have been involved in the pathology of cardiovascular diseases. Patients with cardiomyopathy, carotid stenosis and atherosclerotic lesions have been tested positive for antibodies against the bacterial homologue of mammalian Hsp60 (Xu and Wick, 1996). This could be the result of cross-reactivity of HSPs derived immunogenic peptides and are recognized by immune cells (Schett et al., 1995).

HSP60, HSP70, HSP90 and HSP47 are similarly involved in protein folding and in preventing unfolded immature polypeptides from forming aggregates (Nagata, 1996; Saibil, 2013). However, while the former proteins are not specific for the polypeptide chain substrate, HSP47 is substrate specific (Tasab et al., 2000). The role of this collagen-specific molecular chaperone along with its physiological and pathological implication and contribution to collagen biosynthesis is introduced in the next section of this chapter. Although the aforementioned correlative reports have limitations, future *in vivo* and *in vitro* research to establish the effect of HSP superfamily deficiency or ablation could open avenues to develop novel therapeutic agents for treatment and prevention of various diseases.

1.14 Collagen:

Collagen is the most abundant mammalian structural protein. It is a major component of the extracellular matrix where it forms super-molecular structures that provide mechanical strength and elasticity in connective tissues. It has many significant roles, such as controlling cell shape and differentiation, allowing regeneration of broken bones, providing a scaffold for cells in our bodies and permitting wound healing (Ricard-Blum, 2011). About 25 different types of collagens are known (Hashimoto et al., 2002). Nine of the collagen types, I, III, IV-VI, VIII and XII-XIV, are present in the vessel wall with fibril-forming types I, III and V constituting the majority of collagens, however, network-forming collagen type IV is spatially enriched in the basement membrane of blood vessels. In the aorta and smaller arteries, collagen makes 20% and 40% of the total protein composition, respectively. Of importance is that among all collagen types within the vascular wall, collagens types, I, III and IV are the most platelet-reactive with the capacity of inducing platelet adhesion and aggregation (Surin et al., 2008).

These macromolecules vary in structure, but all the collagens share a characteristic right-handed trimeric helical structure (Kuhn, 1986). The triple helix is made up of three α polypeptide chains that can be identical or different, forming homotrimeric or heterotrimeric collagens, respectively (Gelse et al., 2003). All the chains are characterised by repeated sequences of Gly-X-Y triplets, where X and Y are usually proline and hydroxyproline, respectively. The characteristic sequence extends for approximately 1000 amino acids and is flanked by the two globular amino-terminal and carboxy-terminal peptide sequences (Kadler, 1995). The abundant glycine and proline residues in collagen allow the formation of the tight triple helix. Glycine has a small head group that accommodates the intertwining

of the collagen molecules, allowing close packing of the chains (Hendershot and Bulleid, 2000). Proline and hydroxyproline are responsible for the formation of hydrogen bonds that maintain the stability of the α chains within the helix and the helix-helix interactions in the collagen-polymer (Farndale et al., 2004).

1.14.1 Collagen-mimetic peptides: synthesis and applications:

Most of the observations reported in regards to collagen structure and features were made with the aid of short peptides that mimic the composition of naturally occurring collagen. Such peptides are easily and effectively managed compared to collagen in terms of peptide length, amino acid composition as well as physical and chemical properties of designed peptides. Collagen-mimetic peptides (CMPs), also referred to as collagen-related peptides (CRPs) and collagen-like triple helical peptides (THPs), which have allowed rapid expansion of collagen research starting from structural modelling of collagen molecules to studying the interaction between collagen binding molecules (Cejas et al., 2007). Similarly, CMPs have been a valuable tool in reaching a better understanding of collagen proteolysis, enzymes and receptor binding and even manipulation of various collagen binding receptors (Wilson et al., 2003; Lauer-Fields et al., 2007; Fields, 2010)

In the field of thrombosis and haemostasis research, various synthetic collagen-related peptides (CRP) have been designed and developed based on the sequence of GPO repeats and have shown to mimic collagen-mediated platelet activation. Moreover, the nature of these peptides and the margin of freely manipulating the number of GPO motif and peptides cross-linking, leave CRPs with greater thrombotic capacity compared to collagen (Morton et al., 1995; Asselin et al., 1997)

1.14.2 Procollagen Assembly in the Endoplasmic Reticulum (ER):

The biosynthesis of collagen is a multistep process that starts with the transcription of collagen genes within the nucleus into mRNA that is transported into the cytoplasm. There, the mRNA is translated by the ribosomes at the rough endoplasmic reticulum (RER) into procollagen. Procollagen molecules are then transported into the lumen of the endoplasmic reticulum, with the help of a signal recognition sequence that is cleaved by signal peptidases upon the entry. The procollagen molecules undergo a number of posttranslational modifications, with the help of molecular chaperones and enzymes residing within the endoplasmic reticulum (Gelse et al., 2003).

Prolyl-4-hydroxylase (P4H) is required for the hydroxylation of proline into hydroxyproline (Cohen-Solal et al., 1986). This hydroxylation is an essential step to ensure the formation of hydrogen bonds within the collagen-polymer and maintenance of its structural and thermal stability (Bateman et al., 1996). The procollagen chains begin folding from the carboxy-terminal propeptide ends (Hendershot and Bulleid, 2000), where they interact with protein disulphide isomerase (PDI) that catalyses the formation of disulphide bonds between the carboxy-terminal domains, resulting in the formation of the procollagen trimer (Wilson et al., 1998). The procollagen chains fold together within the trimer to form the triple helix flanked by the two globular carboxy and amino-terminal domains. This triple helix is unstable at physiological temperature, suggesting a role of ER residing molecular chaperones such as HSP47 in providing stability of the formed helixes (Koide et al., 2006a). These domains are thought to have a role in preventing fibril formation within the cells. The procollagen molecules are next transported from the endoplasmic reticulum through the Golgi apparatus. Before secretion of the procollagen, the two globular amino- and carboxy-

heads are cleaved off by a proteinase, aminoprotease and carboxyl protease respectively, to allow the formation of the collagen fibrils and prevents unfolding of the collagen (Hendershot and Bulleid, 2000) (Figure 1.6).

1.14.3 Heat shock protein 47 (HSP47), lifeguarding collagen biosynthesis:

Heat shock protein 47 (HSP47) is an essential pre-requisite for the formation of collagen. The necessity of HSP47 for efficient collagen production is revealed in the embryonic lethality of HSP47 knock out mice, which display abnormally oriented epithelial tissues and ruptured blood vessels (Nagai et al., 2000). This reliance has also been confirmed by other studies that reported a decreased production of type IV collagen in HSP47 $^{-/-}$ mouse embryonic stem cells, together with the secretion of highly protease sensitive collagen (Matsuoka et al., 2004). Type I collagen secretion was also affected in HSP47 $^{-/-}$ mouse embryonic cells, where the collagen formed insoluble intracellular aggregates with abnormal molecular structure (Ishida et al., 2006).

As a unique chaperone that mainly binds to collagen, various functions have been proposed for HSP47. Although the actual mechanism of the chaperone is still being debated, among the suggested functions include: Protecting the newly-synthesised pro-collagen chains from folding or aggregation until the translation is complete (Satoh et al., 1996). Stabilising newly-produced pro-collagen in the event of metabolic stress (Sauk et al., 1994), inhibiting the degradation of immature pro-collagen in the ER (Jain et al., 1994), helping the formation of three-dimensional configuration of collagen molecules by inducing the formation of a polyproline type II conformation, binding to misfolded areas within fully formed collagen and hence reduce potential aggregation (Dafforn et al., 2001), assisting the formation of

correct trimers among heterotrimeric collagens with different α-chains, with the help of other collagen chaperones, and preventing the secretion of incorrectly-folded heterotrimers (Nakai et al., 1992; Kobayashi and Uchiyama, 2010), stabilising the correctly-folded triple helix against stress stimuli, such as high temperature, and partially folded intermediates helixes that would be unstable at body temperature (Koide et al., 2006a) and lastly, retention of under-hydroxylated procollagen in the ER (Satoh et al., 1996).

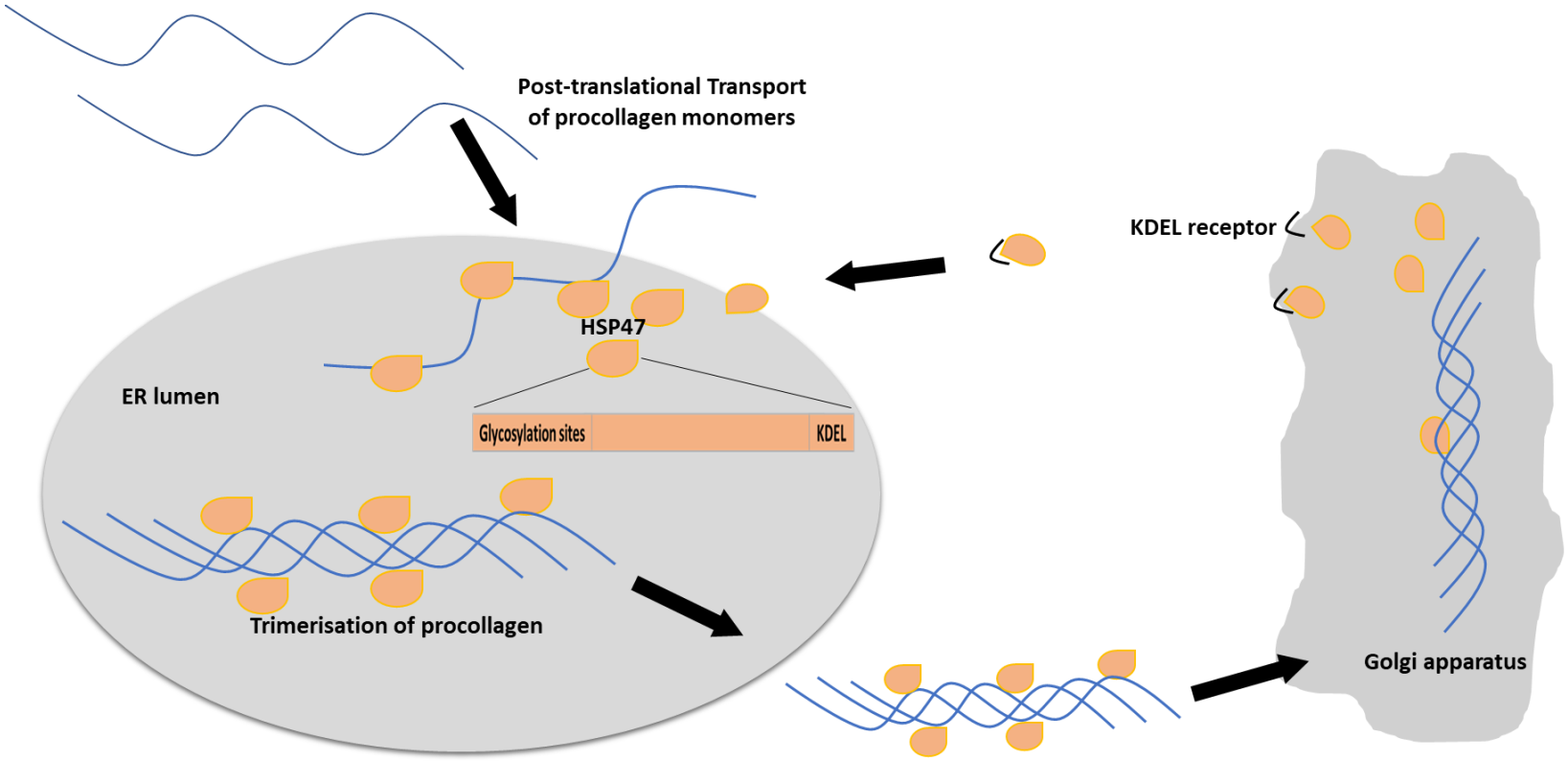


Figure 1.6: HSP47 chaperoning collagen biosynthesis.

HSP47 binds to the nascent procollagen chains in the ER, where the trimeric helical procollagen is formed. HSP47 remains bound during the transportation of the procollagen until the entry into the cis-Golgi compartment through the RDEL retention signal and the KDEL receptor in the Golgi compartment. The low pH of the cis-Golgi compartment mediates the dissociation of HSP47 which is recycled back in ER

1.14.4 Interaction with collagen:

In 2012, the crystal structure of canine HSP47 was obtained (Widmer et al., 2012). This structure displayed 97% homology with human HSP47 (Drogemuller et al., 2009), which enabled the study of the molecular aspects underlying the HSP47-collagen interaction. HSP47 binds to the Gly-X-Arg sequence of the trimeric collagen via salt bridges between Asp385 of HSP47 and Arg8 of collagen. Hydrogen bonds between Pro5/Gly6 of collagen and Arg222 of HSP47 were also reported, along with hydrophobic interactions between collagen and Tyr383 and Leu381 of HSP47, as mutations in these residues decreased the affinity of HSP47 for collagen (Widmer et al., 2012).

Interestingly, replacement of the 4-hydroxy-L-proline (Hyp) residue at position 3 in the Gly-X-Y sequence that adjoins the triplet containing the essential Arg (Gly-X-Arg) with p-benzoyl-L-phenylalanine (Bpa) results in complete loss of the HSP47 interaction, with no reported effect on the binding when the substitution occurs in any other positions. These findings emphasise the role of the Y amino acid and Arg as key residues for the recognition of HSP47. The relationship between the two residues was investigated using a collagen model peptides with different types of amino acids occupying position 3 (Yaa3-Gly-Pro-Arg-Gly).

Competitive binding assays were employed with recombinant mouse HSP47 and revealed high affinity of HSP47 binding for Pro or Thr, medium affinity with Ala, Ser and Val, and low affinity with Asn, Met and Tyr in that position. Small hydroxylated and hydrophobic amino acids were preferred over amino acids containing long or charged side chains. Different spatial arrangements of Yaa and Arg in the collagen model peptides revealed the

possible three-dimensional epitopes for HSP47 in which Yaa3 and Arg residues must be present in the same polypeptide strand (Koide et al., 2006b).

Effective interactions of HSP47 with collagen requires the presence of the Gly-X-Y sequence within the triple helical collagen (Shoulders and Raines, 2009). A study with a synthetic collagen peptide, designed based on that characteristic collagen sequence to investigate its interaction with HSP47, revealed that the Gly-X-Arg sequence favours HSP47 binding to collagen (Tasab et al., 2002). Another study that investigated the characteristic primary collagen triplet sequence and its importance in HSP47 and collagen binding reported enhanced binding of HSP47 with a Gly-Pro-Pro synthetic collagen peptide (Koide et al., 2006a).

In addition to having the Gly-X-Y sequence, collagen should exist in its helical trimeric structure for effective HSP47 binding. Several studies have confirmed the preferential binding of HSP47 to the helical trimeric collagen and HSP47 co-immunoprecipitated only with triple helical collagen peptides in a cell-based assay (Tasab et al., 2000). Similar findings were obtained in tests of the affinity of mouse and human HSP47 for collagen against trimeric and monomeric synthetic peptides, which showed increased affinity for the collagen peptide in its trimeric conformation (Koide et al., 2006a).

The requirement of the collagen tertiary structure was also demonstrated *in vivo* when a fluorophore was divided into two fragments, with the N-terminal fragment attached to HSP47 and the C-terminal fragment attached to the monomeric or trimeric collagen peptides. Binding of the two fragments resulted in the formation of a fluorescent product. The results of measurements of the product agreed with previous reports and confirmed the preferential

affinity of HSP47 for trimeric collagen (Ono et al., 2012). The preferential affinity of HSP47 for collagen can be explained based on the collagen-bound HSP47 crystal structure, which showed that the HSP47-collagen binding site is buried at 1000Å of the solvent accessible area on HSP47. With this degree of accessibility, binding is only possible if the collagen is in its trimeric conformation (Widmer et al., 2012).

The advent of many sophisticated techniques in biological research has enabled HSP47 researchers to further support or refute all the suggested functions mentioned in section 1.14.3. For example, the function of HSP47 to help stabilise the collagen chain in the event of cellular stress has been receiving more attention as a recent study on HSP47 binding motives on collagen molecules suggests the presence of high-affinity and low-affinity binding sites. These low-affinity binding sites are thought to be the sites where HSP47 molecules bind to in stress conditions, a part of the normal binding site during regular cellular events (Koide et al., 2006a; Koide et al., 2006b). Furthermore, HSP47 involvement as early as pro-collagen synthesis is now heavily debated as it was recently found to bind to single-stranded collagen peptide significantly less than the triple helical molecules (Koide et al., 2002; Koide et al., 2006a). This suggests that other collagen chaperones are involved in stabilising the single-stranded nascent pro-collagen molecules such as, BiP. The ability of HSP47 to retain under-hydroxylated collagen triple helices is also supported by further research on the importance of hydroxylation for HSP47 binding and collagen transport to the Golgi (Koide et al., 1999; Walmsley et al., 1999; Koide et al., 2006a). Despite all available literature, there are many other functions that are still being investigated, especially molecular changes induced upon HSP47 binding to collagen molecules. A detailed

explanation of this collagen chaperone along with its expression and applications in health, disease and pharmacology is discussed in the next section of this chapter.

1.15 HSP47, serine protease inhibitor (serpin) family member:

HSP47 is a slightly unusual member of the heat shock proteins as it belongs to the serine protease inhibitor (serpin) family as well. Serpins generally have a highly conserved secondary structure that is usually made up of a core of three β-sheets surrounded by nine α-helices (Huber and Carrell, 1989). As a member of the serpin superfamily, HSP47 has been shown to possess several of the archetypal serpin characteristics. Hence, it is helpful to consider the structure and behavior of the members of this family to assist in studying HSP47 functions.

1.15.1 Discovery and structure

HSP47 has been identified by several names including colligin (Kurkinen et al., 1984), CB48 (Yannariello-Brown and Madri, 1990), J6 (Wang and Gudas, 1990) and gp46 (Clarke and Sanwal, 1992) based on the cell type by which it was discovered. All other nomenclatures have rarely been used to identify this chaperone, and hence for standardisation, the name HSP47 is used throughout this study. Historically, HSP47 was first found in 1986 in heat-inducible chick embryo fibroblasts and was shown to be able to bind type I collagen (Nagata et al., 1986). HSP47 synthesis is increased upon cell incubation at 42°C, hence the name heat shock protein is employed just like the rest of HSPs family members, with an isoelectric point (pI) around 9.0. Satoh et al. (1996) reported localisation of HSP47 in the ER using immunocytochemical studies with monoclonal and polyclonal antibodies against HSP47 *in vitro*.

Of note is that HSP47 possesses an ER retention sequence, Arg-Asp-Glu-Leu (RDEL). Such sequence signifies HSP47 recycling to the ER upon dissociation in the Golgi via the action of receptors to maintain the concentration of HSP47 inside the ER (Satoh et al., 1996). HSP47 is a 400 amino acid protein with two N-linked glycosylation sites (Clarke and Sanwal, 1992), which are occupied by high mannose oligosaccharides (Hughes et al., 1987) and a C-linked Arg-Asp-Glu-Leu (RDEL) sequence that acts as an endoplasmic reticulum retention signal (Munro and Pelham, 1987). Whole HSP47 shares homology with the serpin superfamily, unlike other members of the family, HSP47 does not exhibit any protease inhibitory activity (Hirayoshi et al., 1991). The HSP47 structure is consistent with that of other serpin family members, in that it has a highly conserved structural fold of 3 β sheets in the core, surrounded by 9 α helices (Schreuder et al., 1994). The hinge, breach, gate and shutter represent the conserved structural regions characteristic of the serpin molecule (Irving et al., 2000).

1.15.2 HSP47 gene expression and its correlation with collagen expression:

HSP47 is a cell-specific protein and only expressed in collagen-synthesising cells, such as in fibroblasts (Saga et al., 1987). F9 mouse carcinoma cells and cDNA library of heat-shocked chick embryo fibroblasts were used to isolate and clone HSP47 complementary DNA (cDNA) (Wang and Gudas, 1990; Hirayoshi et al., 1991). Heat shock transcription factors (HSFs) are found in the cytosol in an inactive state. However, following exposure to stress HSFs undergo phosphorylation and form trimers which are then translocated into the nucleus where they bind specialised sequences within the promoter region within target genes known as heat shock elements (HSEs). These elements possess a minimum of three pentanucleotide modules (n GAAn) and its 80 bp apart from the transcription start (Perisic

et al., 1989; Hosokawa et al., 1993). Following HSF-HSE interaction, HSP47 transcription is initiated and generated mRNA is then translated into HSP47 in the cytosol (Figure 1.7) (Taguchi and Razzaque, 2007).

Basal amounts of HSP47 are constitutively expressed to enable constant chaperoning activities inside the cell (Gray et al., 1999). On the other hand, in non-stressed cells, the expression of HSP47 is highly correlated with the expression of collagen (Mala and Rose, 2010). In fact, HSP47 is not detected in cells that do not produce collagen, such as M1 myeloid leukaemia cells (Nagata, 1996). By contrast, HSP47 is upregulated in primary fibroblasts that synthesise collagens I and II (Hirayoshi et al., 1991). This correlational expression has also been observed in experimental liver fibrosis, where the expression of HSP47 and collagen genes increased substantially, whereas no increase occurred in normal mouse liver (Masuda et al., 1994). This co-expression was also reported in myeloblasts after the treatment with growth factor β1 (TGF β-1) (Clarke et al., 1993) and in cultured chondrocytes (Kambe et al., 1994). These reports suggest that the levels of HSP47 parallel the expression of collagen in non-stressed cells, consistent with its essential role in the biosynthesis of collagen.

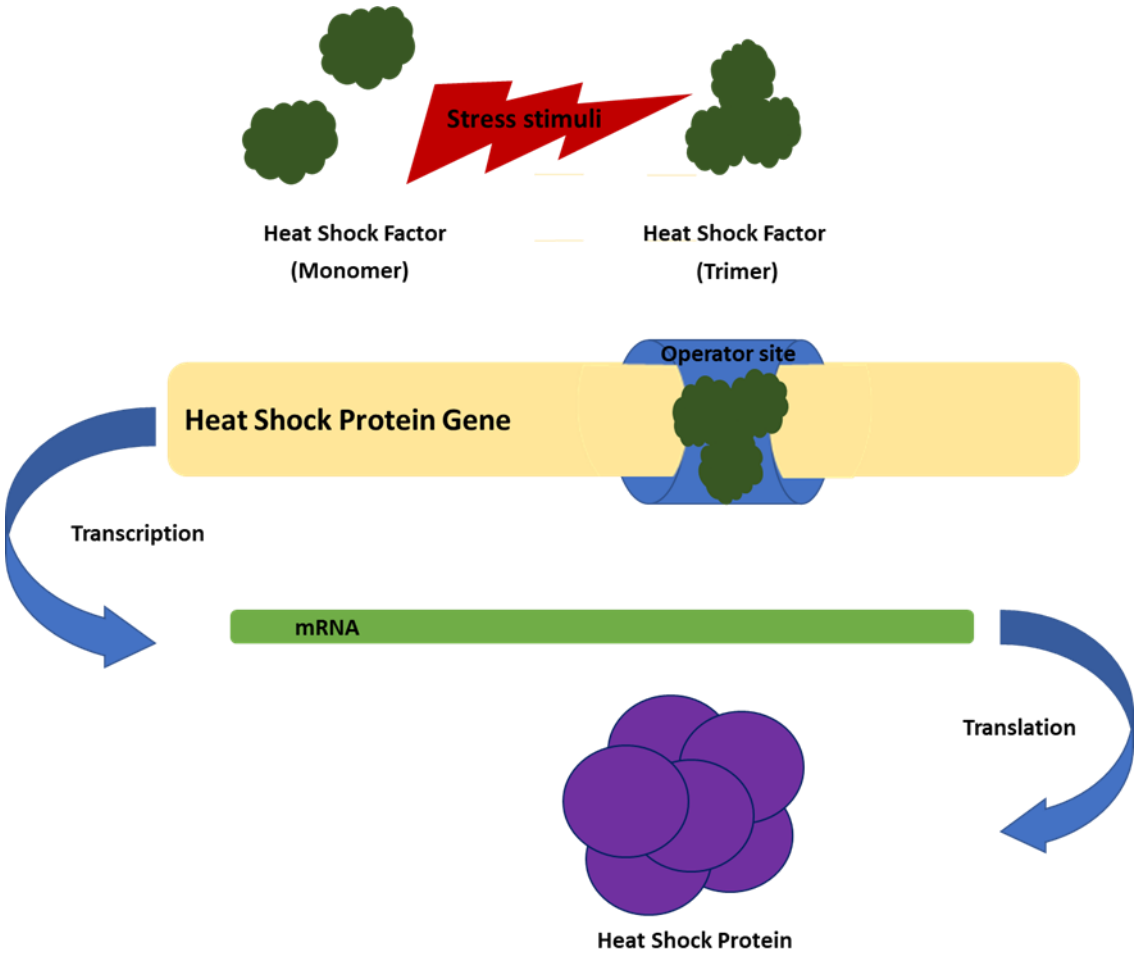


Figure 1.7: Upregulation of HSP47 expression.

Following the exposure to stress stimuli, heat shock factor (HSF) monomers move from cytosol to nucleus where they combine with other monomers to form a trimer. This trimer of HSFs is then capable of binding an operator, promotor site, on heat shock gene resulting in the initiation of transcription process and accumulation of heat shock protein messenger RNA (green). Messenger RNA moves to cytosol to be translated into heat shock protein.

1.15.3 HSP47 mode of actions, binding and releasing properties:

Collagen and HSP47 expression correlation underpin the important role of HSP47 in the processing, secretion and higher order assembly of collagen (Satoh et al., 1996; Dafforn et al., 2001; Ishida et al., 2006). HSP47-substrate binding does not require any initial phosphorylation or glycosylation, rendering it functionality as less dependent on other factors (Kurkinen et al., 1984; Jain et al., 1994). HSP47 was reported to bind gelatin, denatured type I and II collagen. Moreover, this chaperone is capable of binding both immature procollagen monomer and the triple helical collagen (Satoh et al., 1996). Although, reports suggested that HSP47 preferentially binding helical trimers over monomers (Koide et al., 2006a).

Dafforn et al. (2001) revealed that HSP47 native configuration as five-stranded β-sheet A in metastable status. Serpins tend to exhibit metastability as their native states are not considered thermodynamically stable. However, stable conformations are achieved following the cleavage of their center loop when bound to proteases (Im and Yu, 2000). To execute their chaperoning functions, chaperones such as GroEL, HSP70, GroES complex and DnaJ require ATP as a source of energy. Whereas HSP47 does not rely on such requirement to chaperone collagen biosynthesis (Nakai et al., 1992).

Binding of HSP47 to collagen *in vitro* was reported to be in a pH-sensitive manner. The binding was observed at pH 8.0 and abolished at pH 6.3 (Saga et al., 1987). The structural aspects of this pH-induced property have been studied using circular dichroism (CD) and fluorescence spectroscopy and suggested that HSP47 undergoes conversion from high to low pH via an intermediate structure. During this conversion, the β-sheet content has been

estimated to increase by up to 10-20% (Thomson and Ananthanarayanan, 2000). Such an effect of pH on HSP47 structure accounts for HSP47 binding, above pH 6.3, and release from collagen, at or below pH 6.3 (El-Taher et al., 1996). Similarly, pH dependency was also reported by Dafforn et al. (2001) when the binding of HSP47 to (PPG)10, a collagen mimic peptide, was decreased following a drop in pH from 7.0 to 6.0 and was attributed to pH-induced conformational changes.

1.15.4 HSP47 and disease:

Recent reports have shown a close correlation between increased expression of HSP47 and excessive deposition of collagens in scar tissues of several human and experimental fibrotic diseases. It is suggested that such elevation contributes processing and assembly of procollagen monomers and hence supports fibrotic lesions formation. Also, the downregulation of HSP47 expression in experimental animal models helps in reducing collagen accumulation and hinders the progression of fibrotic diseases. Given the collagen specificity of this chaperone, HSP47 makes an excellent target to selectively control collagen synthesis and production in fibrotic diseases (Nagata, 1996; Taguchi and Razzaque, 2007).

1.15.4.1 Wound healing:

HSP47 expression has been reported to increase following the induction of skin and gastrointestinal wound. For instance, increased expression of HSP47 was observed in the dermis and subcutaneous tissues of neonatal rats in response to wound induction (Wang et al., 2003). Likewise, a rat model with induced gastric ulcer reported to having fibroblasts with higher expression levels of HSP47 surrounding wounded ulcer in an attempt to prompt ulcer healing (Guo et al., 2002). Vasques et al. (2010) reported accumulation and localisation

of HSP47 in the suprabasal layer of the oral epithelium and underlying connective tissue upon induction of oral ulcer. Such localisation assists collagen accumulation during repair and remodelling phase of the induced wound.

1.15.4.2 Fibrosis:

Fibrotic diseases are characterized by excessive accumulation of collagen. Ogawa et al. (2007) reported increased HSP47 expression and consequent upregulation of collagen assembly in patients with chronic graft-versus-host disease (cGVHD). Cirrhosis is the late stage of liver fibrosis has been recorded to increase mortality in the UK, highlighting the urgent necessity for intervention and treatment, potentially via targeting HSP47 as the expression of this chaperone was reported to increase in cirrhotic liver (Masuda et al., 1994; Henderson and Iredale, 2007).

1.15.4.3 Cancer Progression

A comparative study exploring public gene expression data revealed SERPINH1 (HSP47) to be a top stomach cancer marker gene (Xu et al., 2010). Moreover, some carcinoma cases have reported elevated HSP47 expression, for instance, epithelial cells of ulcerative colitis-associated carcinoma, that accounts for the increase in collagen type I synthesis (Araki et al., 2009). Overexpression is also noted in oral squamous cell carcinoma to accompany collagen expression in the tissues surrounding the tumor, probably to prevent tumor metastasis (Lee et al., 2011). Similarly, overexpression has also been observed in pancreatic carcinoma (Maitra et al., 2002) as well as head and neck squamous cell carcinoma (Li et al., 2008). These findings amplify the growing significance and importance of HSP47 as a potential therapeutic target and biomarker for various cancerous cell types.

1.15.5 HSP47 as a therapeutic target:

The lack of effective antifibrotic therapy emphasises the need to target different molecules and therapeutic approaches (Sharbeen et al., 2015). Treatments of several fibrotic diseases by targeting HSP47 are actively being designed and developed. Among these treatments, administration of small molecule inhibitors and RNA interference (RNAi) via viral and non-viral based gene delivery system (Thomson et al., 2005). HSP47 activity can be similarly suppressed via the use of antisense oligonucleotides that are capable of hybridising unique sequence in the total pool of targeted gene in cells. Administration of antisense oligonucleotide to HSP47 suppressed peritoneal (Nishino et al., 2003) and pulmonary fibrosis (Hagiwara et al., 2007). It also reduced the deposition of collagen in the glomeruli, thereby delaying the development and progression of glomerular sclerotic processes (Sunamoto et al., 1998). Antisense HSP47 therapeutic approaches were also effective in reducing wound scarring (Ohba et al., 2003; Wang et al., 2003).

The therapeutic potential of targeting HSP47 was also tested in the treatment of liver cirrhosis. Vitamin A-coupled liposomes carrying siRNA against HSP47 used in a rat model of liver cirrhosis resulted in the reversal of liver fibrosis, as indicated by the reduction in collagen content and fibrotic areas in the rat liver (Sato et al., 2008). Similar findings were obtained in a dibutyl tin dichloride-induced pancreatic fibrosis model, where pancreatic fibrosis improved after administration of vitamin A-coupled liposomes carrying the HSP47 siRNA. The improvement was evidenced by reductions in activated pancreatic stellate cells and collagen content (Ishiwatari et al., 2013).

The role of targeting HSP47 is not limited only to the suppression of fibrosis but HSP47 therapy also improves and increases the survival of cancer patients. Deposition of collagen causes deformity of tissues, inhibiting normal function and inducing hypoxia that serves the ideal environment for the epithelial-mesenchymal transition in cancer cells, thereby increasing the tumourigenicity of the cells. Fibrosis also reduces drug delivery and increases the progression and metastasis of tumour cells (Zalatnai and Molnar, 2007; Wang et al., 2011).

The use of HSP47 inhibitors may improve fibrosis and organ malfunction in fibrotic disorders and also prevent the fibrosis-induced hypoxia and its consequences in tumours. HSP47 overexpression and its correlation with fibrosis, in conjunction with the improvement of fibrosis following the use of HSP47 inhibitors, as reported and confirmed in the literature, emphasise the importance of research into HSP47 pharmacological inhibitors. These inhibitors could be particularly useful as antifibrotic agents (Sharbeen et al., 2015).

In 2005, a high throughput assay was designed to screen for potential HSP47 inhibitor compounds. The concept of the assay was based on the ability of helical collagen trimers to forms fibrils, where turbidity, measured spectrophotometrically, was used to detect fibril formation. The addition of an HSP47 inhibiting compound increased the formation of collagen fibrils (Thomson et al., 2005). The underlying concept of the test was based on the possible role of HSP47 in collagen biosynthesis, where it prevents fibrin formation (Nagata, 1998). The screen resulted in the identification of 4 small molecule inhibitors with IC50 values ranging from 3μM to 27 μM (Table 1.2). Such values enabled the utilization of these inhibitors in demonstrating HSP47 functions in animal models (Thomson et al., 2005). In fact, compound IV refers to small molecule inhibitor (SMIH) of HSP47, was employed by

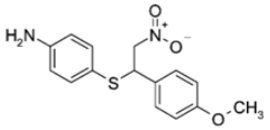
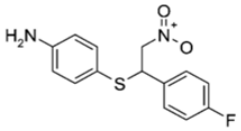
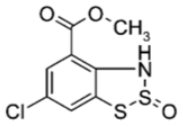
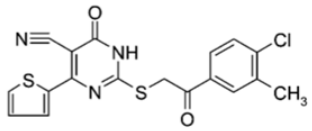
Kaiser et al. (2009) and Sasikumar et al. (2018) to establish HSP47 role in platelet functions. Likewise, the same inhibitor has also aided to study the potential role of HSP47 in platelet-collagen interaction in the present thesis. These small molecular weight compounds could provide a promising approach to target the chaperone activity of HSP47 and control the development and progression of fibrotic disorders (Taguchi and Razzaque, 2007).

Pirfenidone is an anti-inflammatory and anti-fibrotic drug that suppresses the expression of HSP47 and collagen I in lung myofibroblasts and type II pneumocytes in a mouse model of bleomycin-induced pulmonary fibrosis. In this mouse model, collagen deposition was inhibited after the administration of oral doses of pirfenidone, which resulted in reduced hydroxyproline content in homogenised lung tissues and low expression of HSP47 (Kakugawa et al., 2004). Similar findings were obtained in TGF-β-stimulated human lung fibroblasts *in vitro* (Nakayama et al., 2008). In 2011, pirfenidone was approved for the treatment of idiopathic pulmonary fibrosis (IPF) in Europe (Cottin, 2013), although its mechanism is not yet fully understood (Sharbeen et al., 2015).

Terutroban is a specific TP (TxA2 receptor) antagonist drug used to prevent hyperplasia of the aorta (Chamorro, 2009). Terutroban was used on a stroke-prone mouse model. Mice who were placed on a high sodium diet developed increased thickness of the aortic walls with increased accumulation of collagen. Immunohistochemistry of the aortic tissues revealed increased HSP47 staining. After the administration of terutroban, the HSP47 expression was reduced (Gelosa et al., 2011). This suppressive effect could be the result of indirect action on HSP47 since this drug was not designed to inhibit the effect of HSP47 (Sharbeen et al., 2015).

Although the above studies suggest an antifibrotic role for HSP47 therapy, careful consideration needs to be taken to avoid the long-term side effects of using HSP47 suppressive therapy, given the chronic nature of the fibrotic diseases. Another issue is that this type of therapy must not interfere with HSP47 cellular homeostasis, which might require the neutralisation of the toxic effect that would result from the accumulation of immature procollagen aggregates (Taguchi and Razzaque, 2007).

Table 1.2: Structure and IC_{50} of small molecules compounds identified as HSP47 inhibitors. Adapted from (Thomson et al., 2005).

Compound	Structure	IC_{50} (µM)
I		26.6
II		26.6
III		3.1
IV (Employed in the present study and referred to as SMIH)		6.3

1.16 HSP47 in platelets

In 2009, a study on platelet peripheral membrane proteins (PMPs) revealed for the first time the presence of HSP47 on the surface of activated human platelets. HSP47 was detected in PMP immunoblots from a 50KDa band, with a 14% increase in band intensity upon collagen-related peptide (CRP-XL)-mediated activation. HSP47 antibodies and flow cytometry were used to investigate the presence of HSP47 on the surface of platelets. Platelets were reactive for HSP47, with a 48% increase in reactivity in CRP activated platelets. The use of HSP47 inhibitors reduced collagen-induced platelet aggregation but had no effect on thrombin-activated aggregation (Kaiser et al., 2009).

We then sought to explore the role of this protein in platelet functions given its previously reported ability to bind collagen (Natsume et al., 1994). Consistent with above-mentioned findings, immunoblotting of biotinylated surface proteins from resting and CRP-XL activated platelets revealed HSP47 bands which suggested constitutive expression of HSP47 on the platelet surface. Immunofluorescence microscopy studies on megakaryocytes showed immunoreactivity for HSP47 on their surface, which confirmed the megakaryocytic origin of HSP47. The use of an anti-HSP47 polyclonal antibody to assess the effect of HSP47 inhibitors on collagen-induced platelet aggregation showed reduced platelet aggregation in response to collagen or CRP-XL stimulation. On the other hand, in agreement with Kaiser et al. (2009) report the use of other platelet agonists, such as thrombin, had no effect on platelet aggregation (Sasikumar et al., 2018).

Interestingly, the important role played by HSP47 in platelet-collagen interaction and subsequent platelet activation was reflected in the reduced levels of platelet adhesion to

collagen and cytoplasmic Ca^{++} levels in collagen-activated platelets following the use of HSP47 inhibitors. The *in vivo* role of HSP47 in thrombosis and haemostasis was evaluated by generating HSP47-lacking mice using the recombination strategy mediated by the expression of Cre recombinase in megakaryocytes. The intensity of thrombus formation *in vitro* was also affected by the inhibition or deletion of HSP47. The lack of HSP47 in mouse platelets reduced platelet aggregation following collagen activation and prolonged the bleeding time (Sasikumar et al., 2018), indicating a possible role of HSP47 in haemostasis.

Collectively, these observations underpin the role of platelet HSP47 in strengthening platelet responses and interactions with collagen in the formation of thrombi and haemostasis. Such ability highlights a new mechanism for this chaperone to functions in the extracellular environment and regulate cellular function.

1.17 Hypothesis and aims

Heat shock protein 47 surface exposure is unusual for non-collagen-producing cells, such as platelets. However, platelets interact and bind to collagen at the site of vessel injury, triggering a series of collagen-mediated signals and causing the activation and aggregation of the platelets. This type of interaction could be used to justify and explain the role of HSP47 on the platelet surface and its possible influence on the initial platelet-collagen adhesion and subsequent platelet activation.

This type of influence has only been observed in collagen-activated platelets, with no reported effect on platelets activated by other agonists. This selective influence could be attributed to the exclusive chaperoning activity of HSP47 towards collagen to influence receptor binding or could indicate that HSP47 serves as a collagen receptor on the platelet

surface. Another possibility is that HSP47 may influence other proteins on the platelet surface that are important for responses to collagen.

Understanding the function of HSP47 on the platelet surface and its role in collagen-platelet adhesion and subsequent platelet signalling and activation in the haemostatic response will pave the way for exploiting the possible anti-thrombotic potentials of HSP47 in a clinical setting.

The over-arching hypotheses (that are not necessarily mutually exclusive) to be explored in the present study are that HSP47 1) serves as an adhesion receptor for collagen, 2) performs a chaperone function at the platelet surface and therefore modulates the ability of the platelet-collagen receptor to respond to collagen.

These hypotheses are to be tested by answering the following questions:

1- How does HSP47 reach the platelet surface, remain there, and influence the functions of platelet collagen receptors?

2- Does the platelet surface HSP47 function as a molecular chaperone to modulate the function of integrin α2β1, GPVI in collagen binding, or to modulate the ability of collagen to bind its receptors?

3- Does HSP47 modulate GPVI and integrin α2β1 mediated signalling?

In order to address the above questions, a combination of small molecule inhibitor and cell-specific HSP47 deficient mice will be employed. Such dual approach will enable better

interpretation of the outcomes and mitigates potential off-target effects associated with the use of single inhibitors or over-reliance of on transgenic mice.

2 Materials and Methods

2.1 Materials

2.1.1 HSP47 Inhibitors

Small molecule inhibitor of HSP47 (SMIH, compound IV) from Maybridge, RH00007SC, Thermo fisher scientific (UK) was prepared in 100% DMSO as a 100 mM stock. Polyclonal rabbit anti-HSP47 (Anti-HSP47) was obtained from Life Span Biosciences (USA) as lyophilised free from sodium azide or thiomersal and prepared in ddH2O as 1mg/mL stock.

2.1.2 Antibodies

HSP47 antibodies, along with other primary and secondary antibodies, are listed in Table 2.1 with their origins, applications and the dilutions at which they were used.

2.1.3 Platelets agonists

Horm-Chemie collagen (collagen fibres from equine tendons) was from Nycomed (Munich, Germany). Collagen Type I−FITC Conjugate (from bovine skin) was from Sigma (Poole, UK). Cross-linked collagen-related peptide (CRP-XL) and GFOGER peptide were provided by Professor Richard Farndale (University of Cambridge, UK). U46619, a thromboxane A2 analog, was from Tocris Biosciences (Bristol, UK).

2.1.4 Other Reagents

Human recombinant HSP47 protein was purchased from Enzo Life Sciences (Exeter, UK), and actin polymerization inhibitor Latrunculin A from Merck Millipore (Watford, UK). The poly-l-lysine coated-12mm coverslips were obtained from VWR (Leicestershire, UK), and ProLong™ Gold Antifade Mountant was obtained from Life Technologies (Carlsbad, CA, USA). Glass microscope slides were from Thermo-Fisher Scientific (Loughborough; UK).

A µ-Slide-chambered coverslip was purchased from Ibidi (Martinsried, Germany). Paraformaldehyde (16%, methanol-free) was from Agar Scientific (Essex, UK). Alexa Fluor 488 phalloidin was from Thermo-Fisher Scientific (Loughborough; UK). Protease-free bovine serum albumin (BSA) was from First Link (Wolverhampton, UK). Phosphate-Buffered Saline (PBS) tablets were from Sigma (Poole, UK). Protein A/G Magnetic Beads for immunoprecipitation assays was from Thermo-Fisher Scientific (Loughborough; UK). Protease inhibitors (leupeptin, aprotinin, phenylmethylsulfonyl fluoride, sodium orthovanadate and pepstatin-A) were from Sigma (Poole, UK). Cangrelor and indomethacin were from Sigma (Poole, UK). MRS2179 was purchased from Abcam (Cambridge, UK). Perm Buffer III was from BD Biosciences (New Jersey, USA). 4-20% gradient Mini-PROTEAN® TGX™ precast polyacrylamide gels [15 wells (15 µl)], polyvinylidene difluoride (PVDF) membranes and dual-stained molecular weight markers were obtained from Bio-rad (Hemel Hempstead, UK). Whatman 3MM chromatography paper was from Thermo Fisher Scientific (Waltham, MA, USA). Dimethylsulphoxide (DMSO) was from Sigma (Poole, UK). A 4639-cell disruption vessel was obtained from Parr Instrument Company (Illinois, USA). All other reagents of analytical grade were obtained from Sigma-Aldrich (Poole, UK) and Thermo Fisher Scientific (Leicestershire, UK).

2.1.5 Animals

WT ($HSP47^{+/+}$) and HSP47 ablated mice ($HSP47^{-/-}$) were previously bread and characterised in our laboratory (Sasikumar et al., 2018).

Table 2.1: List of antibodies used for this study

Primary antibody	Origin	Application	Dilution	Source and catalogue number
Anti-HSP47	Mouse	Immunofluorescence microscopy (IFM) Immunoblotting Immunoprecipitation STORM microscopy	1:100 1:500 1:200 1:50	Enzolifesciences (Exeter, UK) ADI-SPA-470-F
Anti-HSP47	Rabbit	Immunoblotting	1:500	Abcam (Cambridge, UK) ab109117
Anti-GPIb	Mouse	IFM Flow cytometry	1:100 1:50	Santa Cruz technology (Heidelburg, Germany) sc-80728
Anti-GPIb	Goat	IFM	1:100	Santa Cruz technology (Heidelburg, Germany) sc-6602
Anti-integrin β3	Goat	IFM Immunoblotting	1:100 1:1000	Santa Cruz technology (Heidelburg, Germany) sc-6627
Anti-Rab GDI β	Rabbit	Immunoblotting	1:200	Santa Cruz technology (Heidelburg, Germany) sc-133939
Anti-Thrombospondin 1	Mouse	Immunoblotting	1:400	Thermo Fisher Scientific (Leicestershire, UK) MA5-13398
Anti-Calreticulin	Rabbit	IFM Immunoblotting	1:100 1:200	Merck Millipore (Watford, UK) 06-661
Anti-PDI	Rabbit	IFM Immunoblotting	1:100 1:2000	Sigma-Aldrich (Poole, UK) P7496
Anti-P selectin	Goat	IFM	1:100	Santa Cruz technology (Heidelburg, Germany) sc-6941
Anti-LAT	Mouse	Immunoblotting	1:1000	Santa Cruz technology (Heidelburg, Germany) sc-51663
Anti-GPVI	Rabbit	IFM Immunoblotting STORM microscopy Flow cytometry	1:100 1:1000 1:50 1:50	Santa Cruz technology (Heidelburg, Germany) sc-20149
Anti-GAPDH	Mouse	Immunoblotting	1:1000	Abcam (Cambridge, UK) ab8245

Anti-integrin α2β1	Mouse	Flow cytometry	1:50	Santa Cruz (Heidelburg, Germany) sc-59955
Anti-integrin αIIbβ3	Mouse	Flow cytometry	1:50	Abcam (Cambridge, UK) Ab11027
Anti-14-3-3 ζ	Mouse	Immunoblotting	1:1000	Santa Cruz (Heidelburg, Germany) sc-293415
Anti-actin	Goat	Immunoblotting	1:1000	Santa Cruz (Heidelburg, Germany) sc-1615
FITC conjugated polyclonal anti-human fibrinogen	Rabbit	Flow cytometry	1:50	Dako (Glostrup, Denmark) F0111
Anti-phospho-tyrosine 4G10	Mouse	Immunoblotting	1:1000	Merck Millipore (Watford, UK) 05-321
Anti-phospho-serine PKC substrate	Rabbit	Immunoblotting	1:1000	Cell Signalling Technology (Hitchin, UK) 2261
Phospho-Src (Y418)	Rabbit	Immunoblotting	1:1000	Thermo Fisher Scientific (Leicestershire, UK) 44-660G
Phospho-Syk (Y525/526)	Rabbit	Immunoblotting	1:1000	Abcam (Cambridge, UK) ab58575
Phospho-LAT (Y200)	Rabbit	Immunoblotting	1:1000	Abcam (Cambridge, UK) ab68139
Phospho PLCγ2 (Y1217)	Rabbit	Immunoblotting	1:1000	Cell Signalling Technology (Hitchin, UK) 3871
Phospho-Lyn (Y396)	Rabbit	Immunoblotting	1:1000	Abcam (Cambridge, UK) ab226778
GPVI dimer Fab antibody and control Fab	mouse	Flow cytometry	20 µg	Professor Stephaine (University of Cambridge, UK)
Anti-integrin β1 antibody, activated, clone HUTS-4	mouse	Immunoblotting	1:1000	Merck Millipore (Watford, UK) MAB2079Z

Secondary antibody	Origin	Application	Dilution	Source and catalogue number
Alexa Fluor® 647 anti-Goat IgG	Donkey	IFM Immunoblotting	1:500 1:1000	Life Technologies (Paisley, UK) A32849
AlexaFluor® 488 anti-mouse IgG	Donkey	IFM Immunoblotting	1:500 1:1000	Life Technologies (Paisley, UK) A21202
AlexaFluor® 488 anti-goat IgG	Donkey	IFM Immunoblotting	1:500 1:1000	Life Technologies (Paisley, UK) A11055
AlexaFluor® 568 anti-rabbit IgG	Donkey	IFM	1:500	Life Technologies (Paisley, UK) A10042
AlexaFluor® 647 anti-mouse IgG	Donkey	IFM Immunoblotting	1:500 1:1000	Life Technologies (Paisley, UK) A31571
AlexaFluor® 647 anti-rabbit IgG	Donkey	IFM Immunoblotting	1:500 1:1000	Life Technologies (Paisley, UK) A31573
Alexa Fluor® 488 AffiniPure $F(ab')_2$ Fragment Goat Anti-Mouse IgG, F(ab')2 fragment specific	Goat	Flow cytometry	50 µg	Jackson ImmunoResearch

2.2 Methods

2.2.1 Cell Preparation

2.2.1.1 Human Platelet Preparation

Whole blood (50ml) was collected from consenting, drug-free, healthy donors on the day of the experiment according to the methodology approved by the University of Reading Research Ethics Committee. Blood was drawn into 50ml syringes containing sodium citrate (3.8% (w/v)). The blood was treated with 7.5ml acid citrate dextrose (ACD; 85mM sodium citrate, 71mM citric acid and 110mM glucose) prior to centrifugation at 102g for 20 minutes to pellet the red and white blood cells. Platelet-rich plasma (PRP) was then collected carefully.

Where washed platelets were required, PRP was further treated with 10μL of prostacyclin (PGI_2, 125μg/ml, solubilised in ethanol) and centrifuged at 1,413g for 10 minutes to pellet the platelets from the plasma. The platelet-poor plasma was then removed, and the pelleted platelets re-suspended in 25ml of modified HEPES-buffered Tyrode's solution (134mM NaCl, 2.9mM KCl, 0.34mM Na2HPO4, 12mM NaHCO3, 20mM HEPES, 1mM MgCl2 and 5mM glucose, pH 7.3), 3ml of ACD and 10μL (125μg/mL) of PGI_2. Platelet count was determined using Z2™ COULTER COUNTER® (Beckman Coulter, UK) and platelets were centrifuged at 1,413g for 10 minutes. Platelet pellets were re-suspended in modified HEPES-buffered Tyrode's solution and adjusted to the desired concentration. Re-suspended platelets were allowed to rest in a 30˚C water bath for 30 minutes prior to experimentation to allow recovery from PGI_2 treatment.

2.2.1.2 Mouse Platelet Preparation

Mouse blood was obtained on the day of experimentation by cardiac puncture following termination by increasing concentration of CO_2 and cervical dislocation in accordance with Schedule 1 of the Animals (Scientific Procedures) Act 1986. Mice were sacrificed and blood was collected immediately by cardiac puncture into a syringe containing 4% citrate (at 1 (citrate): 9 (blood) ratio). PRP was isolated by centrifuging blood (diluted with 1mL modified Tyrode's-HEPES buffer) at 203 g for 8 minutes. Where washed platelets were required, PGI_2 (125 μg/mL) was added and PRP was further centrifuged at 1028g for 6 minutes. The resultant platelet pellet was resuspended in modified Tyrode's-HEPES buffer, adjusted to a concentration of 2×10^8 cells/mL and was allowed to rest for 30 minutes at 37°C.

2.2.1.3 Platelet lysate preparation

Washed human and murine platelets were isolated and prepared as described above. Platelets were then lysed in a 6X Laemmli sample treatment buffer (4% (w/v) SDS, 20% (v/v) glycerol, 0.5M Tris, 0.001% (w/v) Brilliant Blue R and 10% (v/v) 2-mercaptoethanol). Samples were heated to 95°C for 5 minutes before storing at -20°C until use.

2.2.1.4 Human platelet releasate and microvesicle preparation

Human platelets were isolated and prepared as described in section 2.2.1.1 and adjusted to a density of 8×10^8 cells/ml. Platelets were incubated at 30˚C for 30 minutes with an agonist or vehicle control. Platelets and other debris were pelleted by centrifugation at 5,000 g for five minutes and releasate was then collected, and aliquots were stored at -20 ˚C for future testing.

Where microvesicles were required, releasate was subjected to ultracentrifugation at 100,000 g for one hour at 4˚C using a TLA.100.3 rotor (Beckman Instruments, Fullerton, CA). Pelleted microvesicles were treated with 6x Laemmli sample treatment buffer and stored at -20˚C for future testing.

2.2.2 Immunofluorescence labelling and confocal microscopy

PRP was isolated and prepared as described in 2.2.1.1. PRP was fixed in 4% (v/v) paraformaldehyde-PBS (PFA-PBS) for 15 minutes and centrifuged at 950g for 11 minutes. Activated platelet preparation was treated with an agonist (5 μM U46619; in the presence of 4 μM integrilin) or vehicle control under stirring conditions at 37˚C for three minutes using an aggregometer prior to fixation and centrifugation. Platelet pellets were then re-suspended twice in 1ml of modified Tyrode's-HEPES buffer and centrifuged at 950g for 10 minutes. Final platelet pellets were then re-suspended in 500μl modified Tyrode's-HEPES buffer containing 1% (w/v) protease-free BSA.

Poly-l-lysine coated-12mm glass coverslips were placed in 6x6 well culture plates. Wet tissues were placed in the empty spaces between the wells to create humidity. Using a cut pipette tip, 90μl of platelets were dispensed onto each coverslip and left for 90 minutes at 37˚C to allow platelet adherence. Coverslips were washed three times for two minutes each time using PBS to remove non-adherent platelets, followed by blocking using 1% (w/v) BSA for 60 minutes at room temperature. Fixed platelets were permeabilised using 0.2% (v/v) Triton™ X-100 and stained with primary antibodies diluted (1:100) in 0.2% (v/v) Triton™ X-100, 2% (v/v) donkey serum and 1% (w/v) BSA and left overnight at 4˚C. Stained coverslips were washed three times for two minutes each time using PBS to remove unbound

primary antibodies, and secondary antibodies were added and left incubating in the dark for 60 minutes at room temperature. Unbound antibodies were removed by washing with PBS (3 × 2 minutes). Platelets were then fixed in 4% (v/v) PFA-PBS for five minutes and washed three times for two minutes each time using PBS, followed by mounting on glass slides using mounting media. Immunofluorescence confocal images were obtained using a Nikon A1-R confocal microscope, oil immersion confocal objectives (60x/1.35), (100x/1.4). Three filters were used to visualize different Alexa Fluor® dyes in the stained platelets: excitation 490/ emission 525 (Alexa Fluor® 488), excitation 578/ emission 603 (Alexa Fluor® 568) and excitation 650/ emission 665 (Alexa Fluor® 647). Thresholded Pearson correlation coefficient (PCC) was used to calculate the degree of colocalization between HSP47 and different markers in double immune-stained platelet after subtracting the background (Manders et al., 1993). PCC value in -1 to 1 scale, where +1 indicates perfect colocalization, 0 no colocalization and lastly -1 for anti-colocalization. All IFM images are representative of 3 independent preparations and more than 100 stained cells.

2.2.3 Stochastic Optical Reconstruction Microscopy

PRP was collected as described (section 2.2.1.1) and Tyrode's-HEPES buffer was used to dilute the PRP (1:20). The samples were stimulated with an agonist or vehicle control for 5 minutes, then unstimulated and stimulated samples were fixed with 2% (v/v) formyl saline and centrifuged at 500 g for 15 minutes. Supernatants were removed, the platelets enriched pellets were resuspended in Perm Buffer III (100 µL; BD Biosciences, Oxford, UK) and incubated for 30 minutes on ice. Platelets were then washed twice with Tyrode's-HEPES buffer for 20 minutes and centrifuged at 500 g. The supernatant was removed and the pellet was resuspended in modified Tyrode's-HEPES buffer (50 µL). Samples were then incubated

with the primary antibodies (diluted 1:50) overnight at 4 °C. This was followed by washing steps with modified Tyrode's-HEPES buffer (2 mL) and centrifugation for 20 minutes at 550 g. The samples were incubated with the secondary antibodies (diluted 1:50) in modified Tyrode's-HEPES buffer) for 30 minutes at 37 °C. Platelets were then washed with Tyrode's-HEPES buffer (2 mL) and subjected to centrifugation for 20 minutes at 550 g. Pellets were suspended in modified Tyrode's-HEPES buffer (100 µL). Lastly, platelets (100 µL) were applied to poly-L-lysine coated ibidi® slides. Platelets were allowed to adhere to slides at 4 °C overnight. Unbound platelets were removed and blinking buffer was added (Stock A: 0.90 g/mL catalase [Sigma–Aldrich], 0.182 mM Tris [2-carboxyelthyl] phosphine hydrochloride [Sigma–Aldrich], 2.27% [v/v] glycerine, 1.14 mM KCl, 0.91 mM Tris-HCl [pH 7.5], 0.045 mg/mL glucose oxidase [Sigma–Aldrich] and 5 mL diH_2O; stock B: 36 mg/mL glucose, 3.6% [v/v] glycerine and 36 mL H_2O; and stock C: 0.09 M mercaptoethylamine-HCl [Sigma–Aldrich] and 1 mL diH_2O). Platelet imaging was carried out using a 100× oil immersion lens of stochastic optical reconstruction microscopy (STORM).

2.2.4 Sucrose density gradient subcellular platelet fractionation

Prior to the day of the experiment, set of sucrose solution gradients (60%–55%, 50%–45% and 40%–35%) in PBS was prepared by layering 2 ml of each concentration of the sucrose in the designated tubes (60% at the bottom and 35% on the top). The tubes were left overnight at 4 °C to form linear sucrose gradients. Whole blood (100ml) was collected from consenting, drug-free, healthy donors on the day of the experiment. Blood was drawn into two syringes containing 5ml 3.8% (w/v) sodium citrate. The blood in each syringe was treated with 7.5 ml ACD; 85mM sodium citrate, 71mM citric acid and 110mM glucose prior

to centrifugation at 102g for 20 minutes to pellet the red and white blood cells. Platelet-rich plasma (PRP) was then removed and treated with 20µl prostacyclin (PGI_2, 125µg/ml, solubilized in ethanol). PRP was centrifuged at 1,413g for 10 minutes to pellet the platelets from plasma. The platelet-poor plasma was removed, and the pelleted platelets were re-suspended in 15 ml of tris-citrate buffer (63 mM Tris, 95 mM NaCl, 5 mM KCl, 12 mM citric acid, pH 6.5/HCl) in the presence of 10–15µl of PGI_2 and centrifuged at 1,413g for 10 minutes. Platelet pellets were re-suspended in 2.5 mL tris-citrate buffer and allowed to rest in a 30˚C water bath for 30 minutes. Platelets were then treated with 1mM EGTA (ethylene glycerol tetra-acetic acid) a calcium chelator, 10µM Indomethacin and 10U/ml Apyrase to prevent the stimulatory effects of TXA_2 and ADP respectively. Platelets were homogenized by nitrogen cavitation using a 4639cell disruption vessel (Illinois, USA) by applying pressure of 1200 psi N_2, followed by a rapid release of pressure. This homogenization was repeated three times over a period of 15 minutes. Platelet homogenate was then centrifuged at 500g for 10 minutes at 4˚C to remove partially disrupted cells and any other cellular debris. The supernatant was then gently laid on top of a linear sucrose gradient (35–60%) and subjected to ultracentrifugation at 200,000g for two hours at 10˚C. Thirteen fractions (1,000 µl each) were carefully collected from the top of the gradients. Fractions were stored at-20˚C for future testing.

2.2.5 Flow cytometry

Flow cytometric analysis was performed using a BD Accuri C6 flow cytometer (BD Biosciences, Oxford, UK) and in each analysis, values of median fluorescence intensity were calculated for 10,000 events collected from gated platelet population by limiting forward

scatter (FSC) between 1520 to 16000000 and side scatter (SSC) between 152 to 1600000. Data collected were analysed using built-in BD Accuri C6 plus software, version 1.0.264.21.

2.2.5.1 HSP47 release

To measure HSP47 within platelets, resting and activated (with 1 µg/ml CRP-XL in the presence of integrilin) human washed platelets (200 µl) at 4×10^8 cells/mL were fixed by adding an equal volume of 2% (w/v) formyl saline and permeabilised for 1 h on ice using 400 µl of BD Phosflow Perm Buffer III (BD Bioscience, Oxford, UK). Platelets were then incubated with mouse anti-HSP47 primary antibody for one hour and washed at 550g for 20 minutes. Platelets were resuspended in HEPES buffered saline and incubated with an appropriate secondary Alexa Fluor® 647-conjugated antibody (Invitrogen, Paisley UK) for an hour. All samples were analysed using flow cytometry. Appropriate isotype control was used as a negative control.

2.2.5.2 Platelet binding to FITC-collagen

Platelets were treated with HSP47 inhibitors (20 µM SMIH or 20 µg/ml inhibitory anti-HSP47) or vehicle controls (0.12% DMSO or 20 µg/ml mouse IgG) for 10 minutes at 37°C. 5 µl of vehicle or inhibitor-treated platelets were then incubated with 10 µl FITC conjugated collagen type I (10 µg/mL) (Sigma, Poole, UK) for 20 min at RT before fixation with 0.2% (v/v) paraformaldehyde. FITC-collagen bound platelets were detected using flow cytometry. Unstained platelets were used as negative control.

2.2.5.3 Analysis of GPVI dimerization

Five-fold diluted whole blood was treated with SMIH (5 and 10 µM) or vehicle control (containing DMSO 0.12% v/v) for 10 minutes at 37°C prior to treatment with modified

Tyrode's-HEPES buffer (resting) or CRP-XL (5µg/ml). 10 µl of vehicle and SMIH pre-treated platelet solution was mixed with 10 µl of GPVI-dimer specific antibody 204-11 Fab (40 µg/ml) and incubated for 10 min. 1 µl of FITC anti-mouse F(ab′)2 antibody (50 µg/ml) was added as the secondary antibody and samples were further incubated for 10 min. Each reaction mixture was then diluted with 150 µl of diluent PBS. Antibody binding was measured by flow cytometry. Platelet binding to an appropriate control, mouse Fab (Jackson ImmunoResearch), was determined as a negative control.

2.2.5.4 Analysis of cell surface receptors levels

The expression of platelet receptors in presence or absence of SMIH and HSP47 inhibitory Ab was assessed by treating PRP with 20 µM SMIH or 20 µg/ml anti-HSP47 or appropriate vehicle controls (containing 0.12% DMSO or 20 µg/ml rabbit IgG). Platelets were then incubated with antibodies directed against integrin α2β1, GPVI, integrin αIIbβ3 and GPIb for 10 minutes at dilutions shown in table 2.1. This was followed by the addition of an appropriate secondary Alexa Fluor® 647-conjugated antibody (Invitrogen, Paisley UK) for 10 minutes. Unstimulated or stimulated samples were then fixed with 0.2% (v/v) formyl saline (250 µL). The samples were analysed using flow cytometer.

2.2.5.5 Fibrinogen binding to integrin αIIbβ3

Washed platelets were treated with 20 µM SMIH, 35 µg/mL of GPVI blocker 9012 Fab or both along with appropriate vehicle controls (0.12% DMSO or 35 µg/mL control IgG Fab fragment) for 10 minutes at 37°C. Platelets were then left resting or stimulated with 0.5 µg/mL CRP-XL and incubated with Fluorescein isothiocyanate (FITC)-labelled rabbit anti-human fibrinogen antibody (Dako, Ely, UK) for 20 minutes before fixation with 0.2% (v/v)

formyl saline (250 µL). Levels of fibrinogen binding were then detected using flow cytometry. The response of EGTA (1 mM) treated platelets was measured as negative controls for the antibody responses.

2.2.6 Protein biochemistry analysis techniques

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting techniques were used to separate and detect proteins extracted from human and murine platelet lysates, releasates, microvesicles and subcellular fractions. For platelet signalling studies, washed human platelets (4×10^8 cells/mL) or murine platelets (2×10^8 cells/mL) were incubated with 20 µM indomethacin, 1 µM cangrelor, 100 µM MRS2179 and 1 mM EGTA reagents to block secondary signalling and diminish platelets aggregation. These platelets were then incubated with SMIH (5 or 10 µM) or vehicle control (containing DMSO, 0.12% v/v) for 10 minutes and then stimulated with agonists (such as CRP-XL or thrombin), under stirring conditions in the aggregometer (1,200 rpm at 37 °C). For GFOGER mediated signalling studies, treated platelets were stimulated with GFOGER monolayer (coated plate). Samples were lysed with 6× Laemmli reducing buffer. Prior to storage at −20 °C, the samples were heated for 5 minutes at 95 °C.

2.2.6.1 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4–20% gradient precast gels were placed in a Protean II apparatus (Bio-Rad, UK) containing tris-glycine buffer (2mM Tris, 192nM Glycine, 0.1% (w/v) SDS, pH 8.3) and samples were loaded into gels along with a molecular weight marker to ease the evaluation of protein weights in the samples. The gel was set to run at a constant voltage of 100 for 90 minutes.

2.2.6.2 Western blotting/Immunodetection

Separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by semi-dry transfer. The resolving gel containing the separated proteins was placed above methanol soaked PVDF membrane. The membrane and gels were then sandwiched between six sheets of 3mm filter paper soaked with anode buffer (300mM Tris-base, 20% (v/v) methanol; pH 10.4) placed below the membrane and six sheets of 3mm filter paper soaked with a cathode buffer (25mM Tris-base, 40mM 6-amino-n. hexanoic acid; pH 9.4) placed above the gel. The blotter was set to run at a constant voltage of 15 for 120 minutes. The PVDF membrane was then removed and blocked with 5% (w/v) bovine serum albumin (BSA) dissolved in 1% (v/v) tris-buffered saline-Tween (TBST; 20mM Tris, 137 mM NaCl, 0.1% (v/v) Tween 20, PH 7.6) for 60 minutes at room temperature. The membranes were then incubated with primary antibody diluted in 2% (w/v) BSA dissolved in TBST overnight at 4˚C (as shown in table 2.1). Unbound antibodies were removed by washing the membranes with TBST three times for five minutes each. The washed membrane was then incubated with fluorescently conjugated secondary antibodies diluted in 2% (w/v) BSA dissolved in TBST at room temperature for 60 minutes (as shown in table 2.1), followed by washing the membranes with TBST three times for five minutes each. The membranes were scanned for fluorescence detection of bound antibody using a Typhoon FLA 9500 (GE Healthcare, UK), and the obtained images were processed using Image Quant software version 8.1 (GE Healthcare, UK) that enabled the quantification of fluorescence signals.

2.2.6.3 Immunoprecipitation (IP)

Immunoprecipitation was employed to isolate and purify proteins of interest from whole platelet lysates. Washed human platelets were prepared and adjusted to the density of 8 ×

10^8 cells/ml, as described previously (section 2.2.1.1). Platelets were then stimulated with modified tyrodes HEPES buffer (resting) or U46619 (5 µM) for 3 minutes and platelets were lysed using an equal volume of ice-cold 2X NP40 buffer (300 mM NaCl, 20 mM Tris, 10 mM EDTA, 2% v/v NP40; pH=7.3) supplemented with protease inhibitors [Leupeptin (10 µg/ml), phenylmethylsulphonyl fluoride (1 mM), aprotinin (10 µg/ml), sodium orthovanadate (1mM) and pepstatin-A (25 µg/ml)]. Lysates were then precleared with 20 µl of protein A/G magnetic beads for 1 hour at 4°C. Anti-HSP47 coupled protein A/G magnetic beads were added to the precleared lysates at 4°C overnight. On the following day, beads were collected with the aid of a magnetic stand and washed twice with (1X) NP40 buffer supplemented with proteases inhibitors and once with TBST. Lastly, 100 µl of 2X Laemmli sample treatment buffer was added to the washed beads and samples were heated at 95°C for 5 minutes and stored at -20°C for future use.

2.2.6.4 Lipid raft isolation

Human platelets were isolated and prepared as described in 2.2.1.1 and adjusted to a density of 12×10^8 cells/ml. 4 ml of platelets were lysed with 2ml Triton™ X-100 ice-cold lysis buffer (25 mM MES, pH 6.5, 150 mM NaCl, 1% [w/v] Triton™ X-100) in the presence of proteases inhibitors [Leupeptin (10 µg/ml), phenylmethylsulphonyl fluoride (1 mM), aprotinin (10 µg/ml), sodium orthovanadate (1mM) and pepstatin-A (25 µg/ml)]. Lysates were mixed and kept on ice and all subsequent steps were performed at 4^0 C. An equal volume of 80 % (wt/vol) sucrose prepared in MES buffered saline was added to the lysates to yield a total concentration of 40 % (w/v) sucrose. Adjusted Lysates were transferred into the bottom of an ultracentrifuge tube. Gently, 4ml of 30% sucrose was layered on the top of the lysates, followed by 2ml 5% sucrose. The samples were subjected to ultracentrifugation

at 200,000 g at 4°C for 18 hours. In the ultra-centrifuged lysate containing tube, a whitish translucent band was visible floating in the upper part of the tube indicated successful isolation of lipid rafts. 12 equal fractions were collected from the top of the sucrose gradient and stored at -20 ^{0} C for future testing.

2.2.7 Platelet adhesion and spreading

Glass coverslips were placed in 6 well plates and coated with collagen (100 µg/ml) or CRP-XL (10 µg/ml) or GFOGER (50 µg/ml) for 1 hour. To prevent platelet-glass binding, coated coverslips were then blocked for one hour with 1% (w/v) BSA and washed 3 times with PBS. Meanwhile, washed platelets adjusted at a density of $2x10^7$ cells/ml were treated with SMIH or vehicle control (containing DMSO 0.12% v/v) for 10 minutes at 37°C. Pre-treated platelets were then seeded onto precoated coverslips and incubated at 37°C for 45 minutes. Unbound platelets were then removed from the coverslips, and adhered platelets were then washed 3 times with PBS (300 µl). Platelets were then fixed for 10 minutes with 0.2% (w/v) PFA, followed by the removal of the supernatant and 3 times washing step with PBS, prior to permeabilisation with 0.2% (v/v) Triton™ X-100 for 5 minutes. The supernatant was removed and coverslips were washed 3 times with PBS (300 µl). Platelets were stained with Alexa-Fluor 647 actin binding phalloidin for 1 hour in dark. Unbound phalloidin conjugate was removed and platelets were washed 3 times with PBS (300 µl). Lastly, fluorescence preserving Prolong Gold Antifade mounting media was used to mount the coverslips onto glass slides. Platelets were imaged using a 100× oil immersion lens on a Nikon A1-R confocal microscope (Nikon, Tokyo, Japan). Platelet adhesion data were obtained by counting the number of platelets on 5 randomly-selected images. Platelets were categorised

as adhered (not spread), spreading (filopodia forming) or spread fully (lamellipodia formed), and the relative frequency of each category was determined using ImageJ software.

2.2.8 Statistical analysis:

Statistical significance was assessed using student *t*-test for 2-grouped comparisons. Where more than two groups were studied, one-way ANOVA (with post-hoc Dunnett's multiple comparison test) was performed. All data are presented as mean ± SEM and $P \leq 0.05$ were considered to be statistically significant. Statistical analysis was conducted using Prism 6.0 software (GraphPad, San Diego, CA).

3 HSP47, a collagen-specific molecular chaperone: Study of its subcellular distribution, trafficking and mobilisation in human platelets.

3.1 Introduction:

To accomplish their specific functions, immature polypeptides must undergo a series of folding and structural modification to obtain their three-dimensional functional configurations. Cells are equipped with several enzymes and chaperones proteins that govern such vital processes. During protein synthesis, molecular chaperones bind nascent polypeptides preventing them from forming an early aggregate and ensuring correct folding and consequent native configurations (Kim et al., 2013). Various cellular stress stimuli or genetic abnormalities would challenge maintaining such configuration resulting in the formation of incorrectly folded or aggregated proteins (Fulda et al., 2010).

The endoplasmic reticulum (ER), is the main cellular protein synthesising machinery. Hence, this organelle hosts a substantial number of molecular chaperones and enzymes that enable ER to perform its role as quality checkpoint of synthesised proteins (Fu and Gao, 2014; Schwarz and Blower, 2016; Aviram and Schuldiner, 2017). This network of chaperones and signalling proteins participates in executing a number of post-translational modifications, such as cleavage of signal peptides, the formation of disulphide bonds and glycosylation, by which proteins achieve their native structure (Hebert and Molinari, 2007; Braakman and Bulleid, 2011; Araki and Nagata, 2012).

Among ER residing molecular chaperones is heat shock protein (HSP) 47. This chaperone plays a pivotal role in the biosynthesis of collagen by which chaperone interaction with procollagen monomer ensure its correct folding and hence prevention of improper aggregation (Nagata, 1996; Nagata, 1998; Ishida and Nagata, 2011; Ito and Nagata, 2017). This chaperone is characterized by various distinctive features that are not exhibited by other

members of the HSP family. For instance, other molecular chaperones, such as HSP 90, 70 and 60, have wide-ranging substrate specificity (Niwa et al., 2012), while HSP47 specifically interacts principally with procollagens. Such exclusive chaperoning is reflected by the reported expression of HSP47 in collagen-producing cells with expression levels being correlated with collagen production in these cells (Mala and Rose, 2010). However, as mentioned in chapter 1, a recent proteomics study revealed the presence of this collagen-producing cell residing chaperone among proteins isolated from the plasma membrane of platelets. Moreover, platelet aggregation in HSP47 inhibited platelets following collagen stimulation was attenuated while levels of aggregation in platelets stimulated with agonists other than collagen were not affected (Kaiser et al., 2009). Hence, this suggested a role of this protein in platelet-collagen interactions and thereby that it may contribute to thrombosis and hemostasis. Previous experiments validated the suggested notion, where HSP47 was shown to mediate collagen-stimulated platelet functions in platelets in which HSP47 was inhibited or in transgenic deficient platelets (Sasikumar et al., 2018).

An in-depth look into the localisation and mobilisation of this protein to platelet surface at subcellular level aids in understanding the mechanisms by which HSP47 controls and regulates platelet collagen binding. Accordingly, the aim of this chapter was to obtain basic information about quantitative expression levels, subcellular localization and association with various distinct subcellular compartments along with its mobilisation following platelets activation.

3.2 Presence of HSP47 in Resting and Activated Human Platelets

HSP47 existence in human and mouse platelets (Kaiser et al., 2009; Sasikumar et al., 2018) are consistent with a human and mouse mRNA transcriptome profiling study that revealed the presence of HSP47 mRNA transcripts in humans and mice (Rowley et al., 2011). However, the cellular location of HSP47 in resting and activated human platelets has not been investigated yet. The aim of this section of work was to examine the distribution of HSP47 in resting platelets and whether this distribution is changed upon platelet activation to reflect the location required to influence platelet function. Immunofluorescence staining was performed on resting and activated human platelets under permeabilised conditions, and platelets were observed using confocal microscopy.

Platelet-rich plasma (PRP) was isolated from whole human blood and fixed in 4% (v/v) paraformaldehyde for 15 minutes. For activated platelets, 5 μM U46619 was added to the PRP in presence of integrilin prior to fixation. Such agonist would induce platelet activation with minimal changes in platelet shape and hence allows better visualisation of HSP47 distribution. Fixed resting and activated platelets were stained with permeabilisation in 0.2% (v/v) Triton™ X-100 using goat anti-GPIb antibody to demarcate the boundary of the platelets and the HSP47 mouse antibody, HSP47 antibodies used in the present study have been previously tested on HSP47 ablated platelets ($HSP47^{-/-}$), and shown to bind specifically to HSP47 (Sasikumar et al., 2018). GPIb and HSP47 antibodies were detected using Alexa Fluor® 488 anti-goat and Alexa Fluor® 647 anti-mouse IgG, respectively. As negative controls, platelets were incubated with goat and mouse IgG and then detected using Alexa Fluor® 488 anti-goat and Alexa Fluor® 647 anti-mouse IgG and confirmed the absence of non-specific binding (Figure 3.1).

Permeabilised platelets displayed diffused staining of HSP47 throughout the intracellular space (Figure 3.1, a). However, upon activation, HSP47 was mobilised towards the plasma membrane showing concentrated ring-like staining with proportionally lower levels of immunofluorescence compared to the resting platelet (Figure 3.1, b). Although such findings do not discriminate whether observed mobilised HSP47 is on platelet surface or re-localised to the area below the plasma membrane, this is consistent with HSP47 movement to the cell surface upon platelet activation reported previously (Kaiser et al., 2009; Sasikumar et al., 2018).

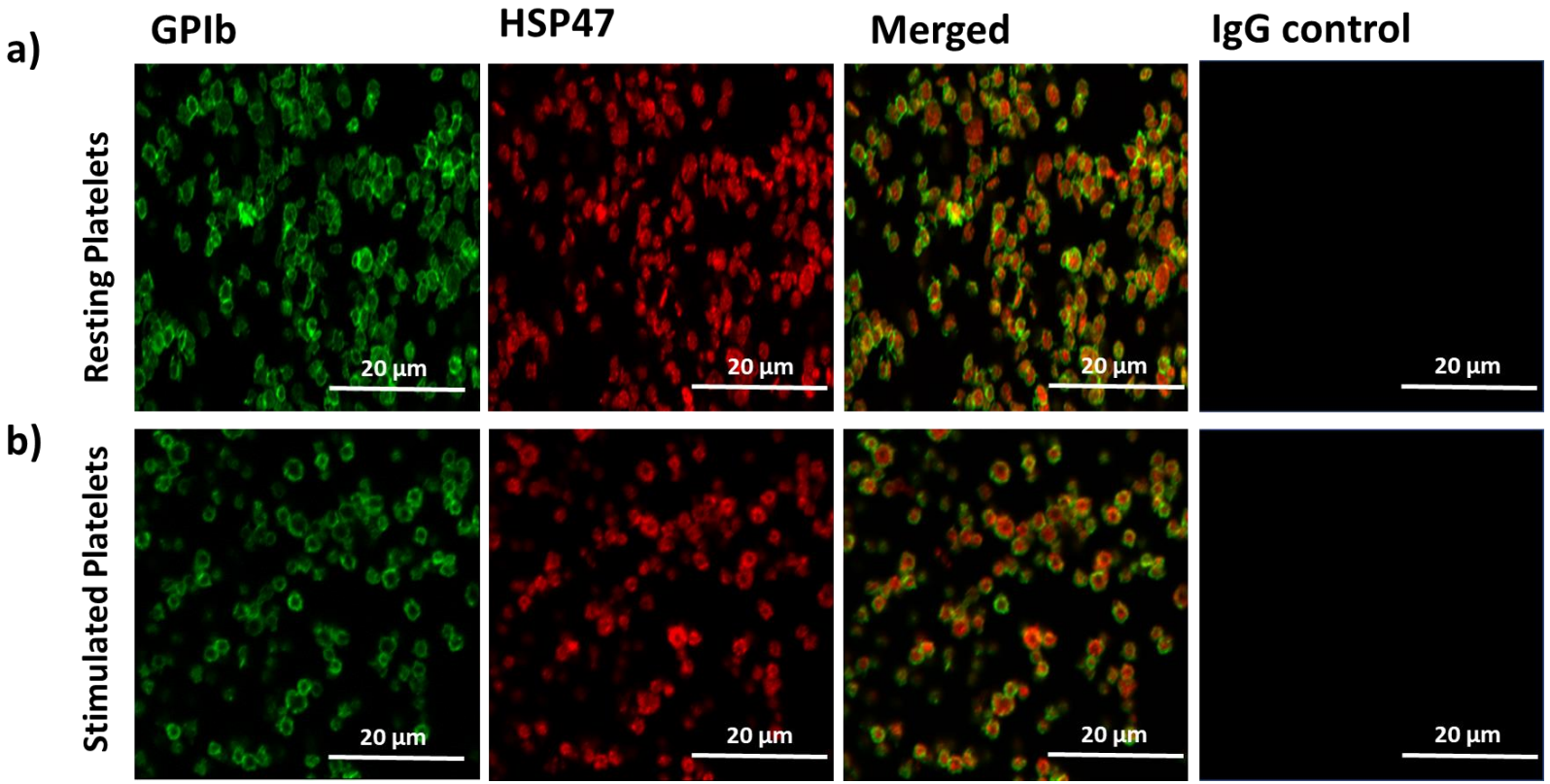


Figure 3.1: Immunofluorescence staining of permeabilised resting and activated platelets revealed the distribution of HSP47 inside the platelet.

(a) Resting and **(b)** stimulated platelets (5µM U46619) were fixed and permeabilised using 4% (v/v) formaldehyde and 0.2% Triton™ X-100, respectively. This was followed with multiple washing steps and washed platelets were subjected to blocking for one hour and then incubated with 1 µg/mL anti-HSP47 and anti-GPIb. Binding was detected using Alexa Fluor® 488 (GPIb; green) and Alexa Fluor® 647 (HSP47; red) conjugated secondary antibodies. The images were obtained using a 100× oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of >3 separate experiments using platelets from different donors.

3.3 Quantification of HSP47 in Human Platelets

Quantification of HSP47 levels in human platelets could contribute to the initial study of HSP47 interaction with potential partners or surface anchors and determine whether it is likely to be present at the sort of levels that might be possible to have functional effects on platelet. Quantitative western blotting was employed to establish the level of HSP47 present in human platelet lysates.

Washed platelets (4×10^8/ml) from seven different donors were lysed with an equal volume of 2X NP40 buffer in presence of proteases inhibitors. A set of serially diluted recombinant HSP47 protein standards was prepared and treated with Laemmli sample treatment buffer along with platelet lysates and were separated by SDS-PAGE for western blotting. Immunoblots were scanned and quantified using ImageQuant TL software (Figure 3.2a and b). The fluorescence intensity from each standard was used to plot a standard curve that was used to estimate the concentration of HSP47 in platelet lysates (Figure 3.2c). For each lysate sample, the mass generated from the plot was converted to molar concentration and the number of copies were calculated by multiplying the number of moles by Avogadro's number. The level of HSP47 among donors was not highly variable with a mean of 7040 ± 1015 copies per platelets.

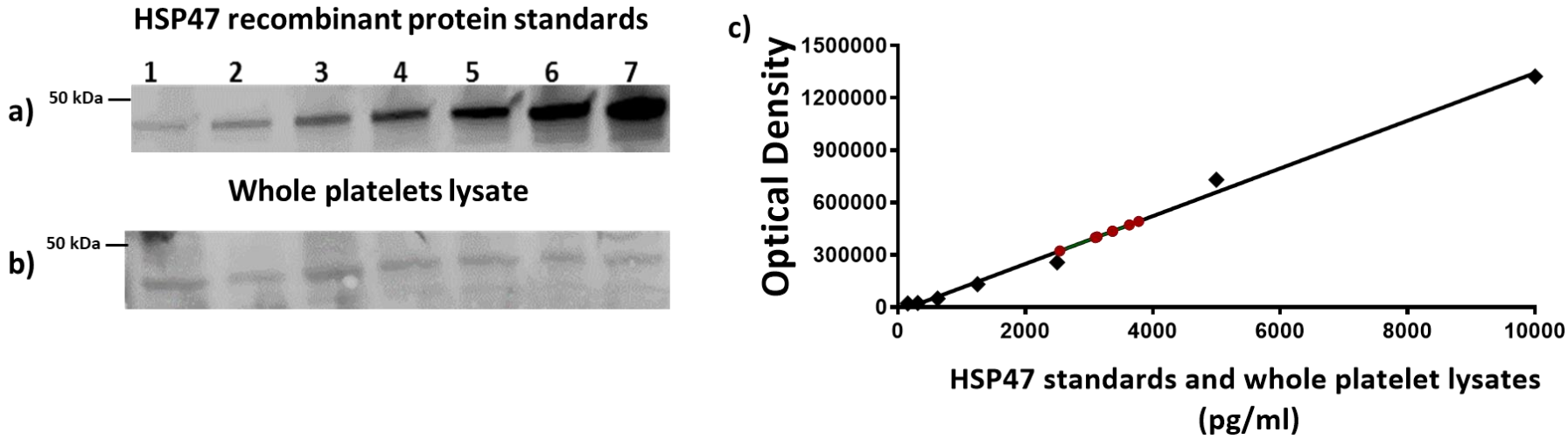


Figure 3.2: HSP4 is expressed as 7000 copies per platelet.

Washed platelets from seven different donors were lysed in a lysis buffer, supplemented with a proteases inhibitor cocktail. Alongside the platelet lysates, a set of seven serially diluted standards of HSP47 recombinant proteins (156.25, 312.5, 625, 1250, 2500, 5000, 10000 pg/ml) were prepared. **(a)** standards and **(b)** samples were treated with Laemmli sample treatment buffer and separated for western blotting. The immunoblots were probed for HSP47 and detected using Alexa Fluor® 647-conjugated species-specific secondary antibodies. Immunoblots were scanned for fluorescence using a Typhoon FLA 9500 (GE Healthcare, UK), and the obtained images were processed using ImageQuant TL software (GE Healthcare, UK) that enabled the quantification of the fluorescent signals. **(c)** Fluorescent signal levels of the standards (black diamonds) were used to generate the standard curve on which the sample fluorescent levels (red circles) were plotted and quantified.

3.4 Co-localisation of HSP47 with Distinct Platelet Subcellular Markers

Following confirmation of the presence of HSP47 in resting and activated platelets, and in an effort to reach a better understanding of how HSP47 reaches the platelet surface where it is anticipated to influence platelet collagen adhesion, we looked more closely into the possible co-localisation of HSP47 with different subcellular granules and organelles.

In mature platelets, the dense tubular system is derived from megakaryocyte endoplasmic reticulum (White, 1972), an organelle responsible of hosting HSP47 chaperoning activities in collagen-producing cells (Nagata, 1996), so it was pertinent to look into the possible localisation of HSP47 in such organelles by observing the immunofluorescent staining of HSP47 along with calreticulin and protein disulphide isomerase (PDI), proteins reported to be residents of dense tubular system (van Nispen tot Pannerden et al., 2009). Sasikumar et al. (2018) confirmed megakaryocytic origin of HSP47, and hence the possibility that like the rest of platelet proteins, HSP47 might be synthesized in megakaryocyte and packed inside secretory granules such as alpha granules was considered. Therefore, alpha granule marker P-selectin double stained with HSP47 to look for possible colocalization. Moreover, detection of the surface receptor GPIb was included to track the surface recruitment of HSP47 to platelet surface.

Platelet-rich plasma was isolated and fixed in 4% (v/v) paraformaldehyde for 15 minutes. 5 μM U46619 was used to stimulate platelets in presence of integrilin prior to fixation. Fixed resting and activated platelets were stained with permeabilisation of 0.2% (v/v) Triton™ X-100 using goat anti-GPIb antibody, goat anti-β3 integrin antibody, rabbit anti-calreticulin antibody, rabbit anti-PDI antibody, rabbit anti-P-selectin antibody and mouse anti-HSP47

antibody. Primary antibodies were detected using Alexa Fluor® 488 anti-goat, Alexa Fluor® 568 anti-rabbit, and Alexa Fluor® 647 anti-mouse IgGs. Platelets were visualised using confocal microscopy and thresholded Pearson correlation coefficient (PCC) was calculated. The PCC is well-established measure of colocalisation between different fluorophores in the stained sample. This colocalisation measure has a range of +1, positive colocalisation, to -1, negative colocalisation, whereas a value of 0 denotes lack of colocalisation between fluorophores (Adler and Parmryd, 2010).

Reduced colocalization of HSP47 and surface receptor GPIb was noted in resting platelets (PCC = 0.56 ± 0.08; Figure 3.3 a). On the other hand, HSP47 did highly colocalise with surface receptor GPIb upon activation (PCC = 0.91 ± 0.05; Figure 3.3 b). Such observation is consistent with our previous immunofluorescence studies demonstrated diffused distributed staining of HSP47 in resting platelets and its mobilisation to the membrane upon platelet activation.

There was no colocalisation detected between HSP47 and alpha granules, which is represented by the double immunostaining of HSP47 and P-selectin that showed no overlap between the two proteins (PCC = 0.43 ± 0.01; Figure 3.4 a) in resting condition. On the other hand, the colocalization coefficient value increased following platelet activation due to the mobilization of both proteins to the platelet periphery (PCC = 0.84 ± 0.11; Figure 3.4 b).

Moreover, HSP47 showed a great degree of co-localisation with calreticulin (*PCC* = 0.94 ± 0.018; Figure 3.5 a) and PDI (*PCC* = 0.97 ± 0.015; Figure 3.6 a) in resting platelets. This co-localisation was also seen in activated platelets in which calreticulin (Figure 3.5 b) and PDI

(Figure 3.6 b) was redistributed and mobilised to the platelet membrane in a similar pattern exhibited by HSP47, suggesting its association with the dense tubular system.

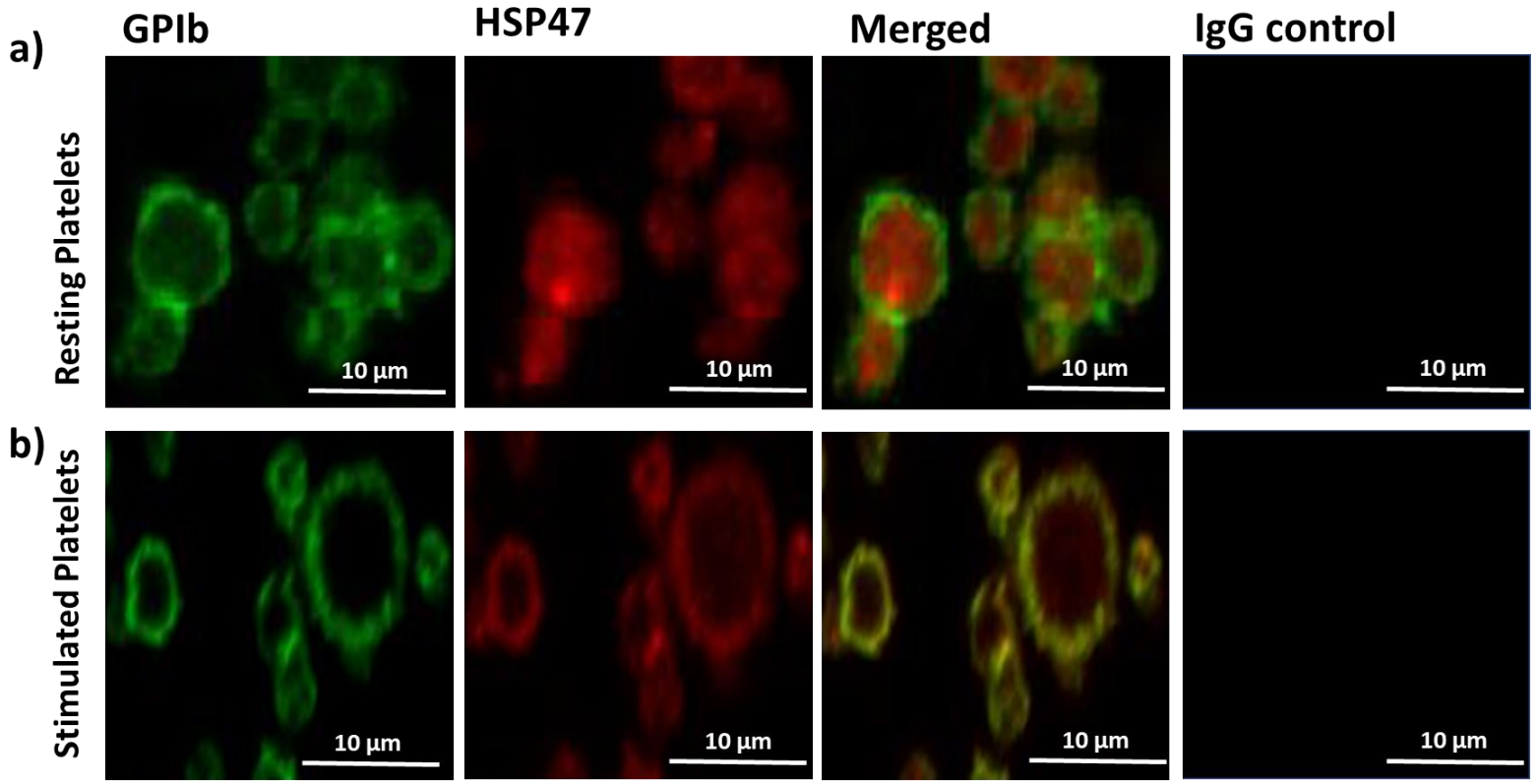


Figure 3.3: HSP47 highly colocalises with surface receptor GPIb in activated platelets, but not in the resting condition.

Platelets were stimulated with **(a)** modified Tyrode's-HEPES (resting) and **(b)** 5µM U46619 (stimulated) followed by fixation in 4% (v/v) formaldehyde. 0.2% Triton™ X-100 was used to permeabilise fixed platelets and samples were washed and blocked prior to incubation with 1 µg/mL anti-HSP47 and anti-GPIb. Antibody binding was detected using Alexa Fluor® 488 (GPIb; green) and Alexa Fluor® 647 (HSP47; red) conjugated secondary antibodies and images were obtained using a 100× oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of >3 separate experiments using platelets from different donors.

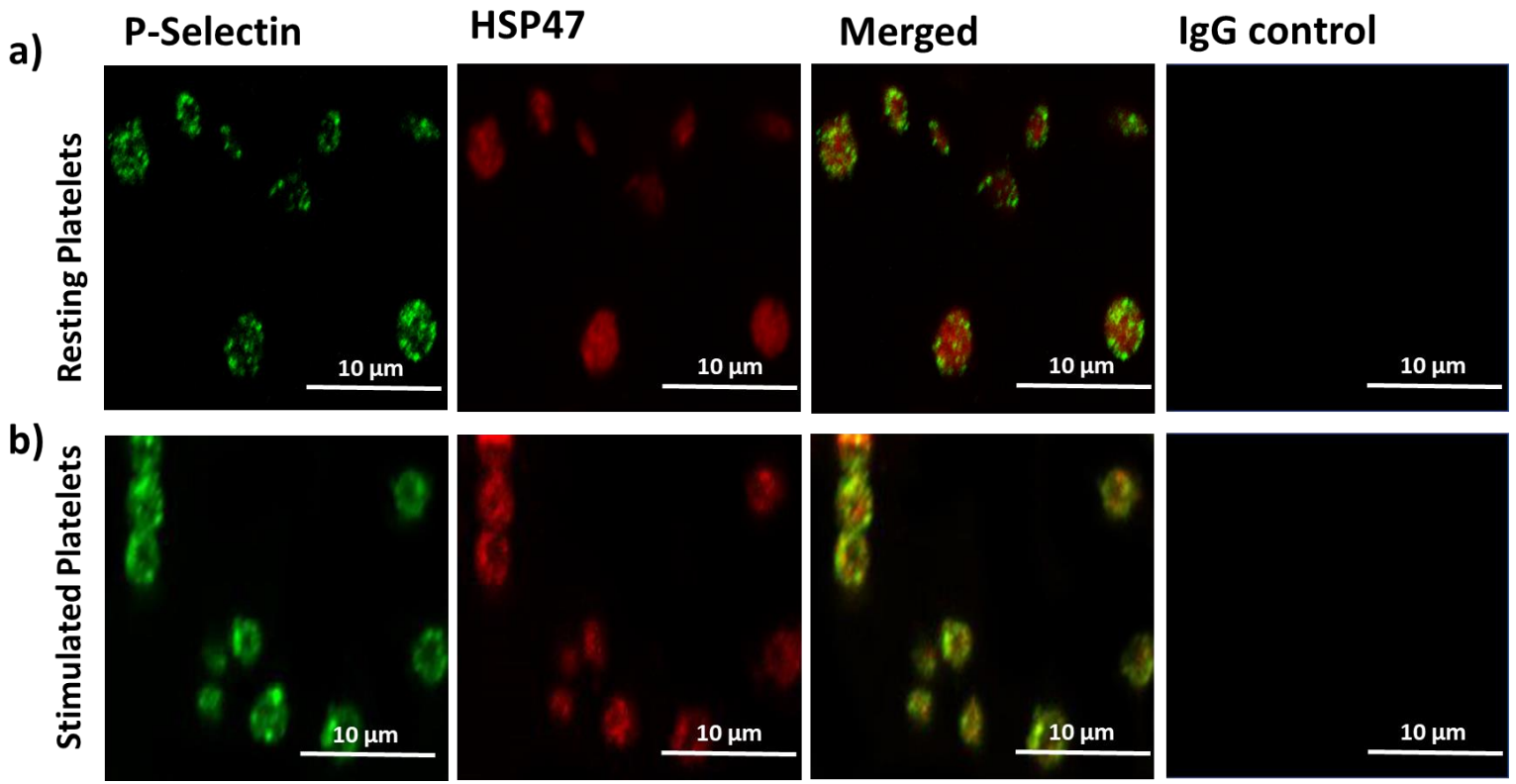


Figure 3.4: HSP47 is not associated with alpha granules in resting platelets.

The IMF images of permeabilised **(a)** resting and **(b)** stimulated platelet (5µM U46619) incubated with 1 µg/mL anti-HSP47 and anti-P-selectin. P-selectin and HSP47 Bound platelets were then detected using Alexa Fluor® 568 (green) and Alexa Fluor® 647 (red) conjugated secondary antibodies, respectively. The images were obtained using a 100× oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of >3 separate experiments using platelets from different donors.

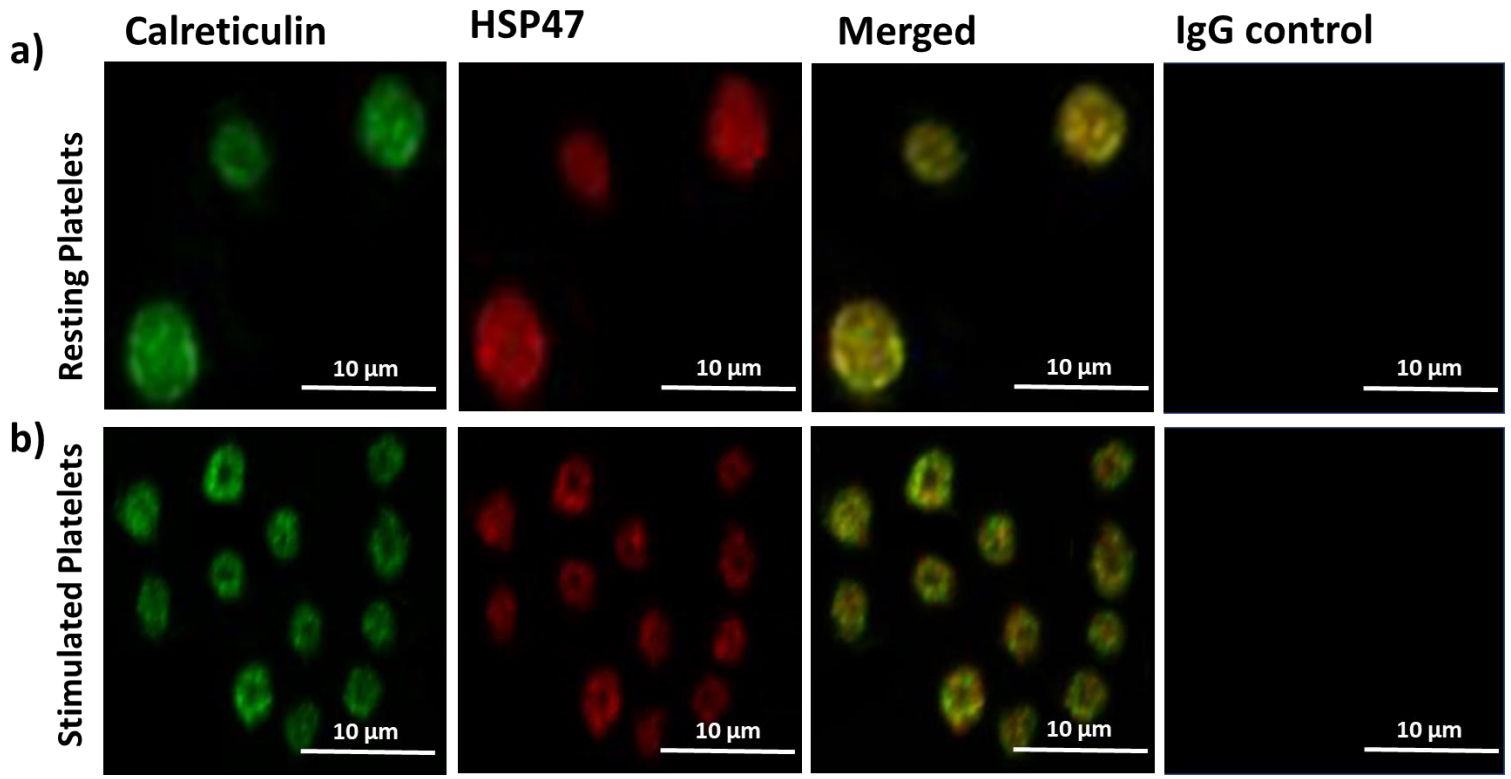


Figure 3.5: HSP47 is associated with dense tubular system marker, calreticulin in resting and activated platelets.

(a) Resting and **(b)** (5µM U46619) stimulated platelets were fixed in 4% (v/v) formaldehyde and permeabilised in 0.2% Triton™ X-100. Samples were then blocked and washed prior to addition of 1 µg/mL anti-HSP47 and anti- Calreticulin. Unbound antibodies were washed away and antibodies bound platelets were detected using Alexa Fluor® 488 (Calreticulin; green) and Alexa Fluor® 647 (HSP47; red) conjugated secondary antibodies and images were obtained using a 100× oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of >3 separate experiments using platelets from different donors.

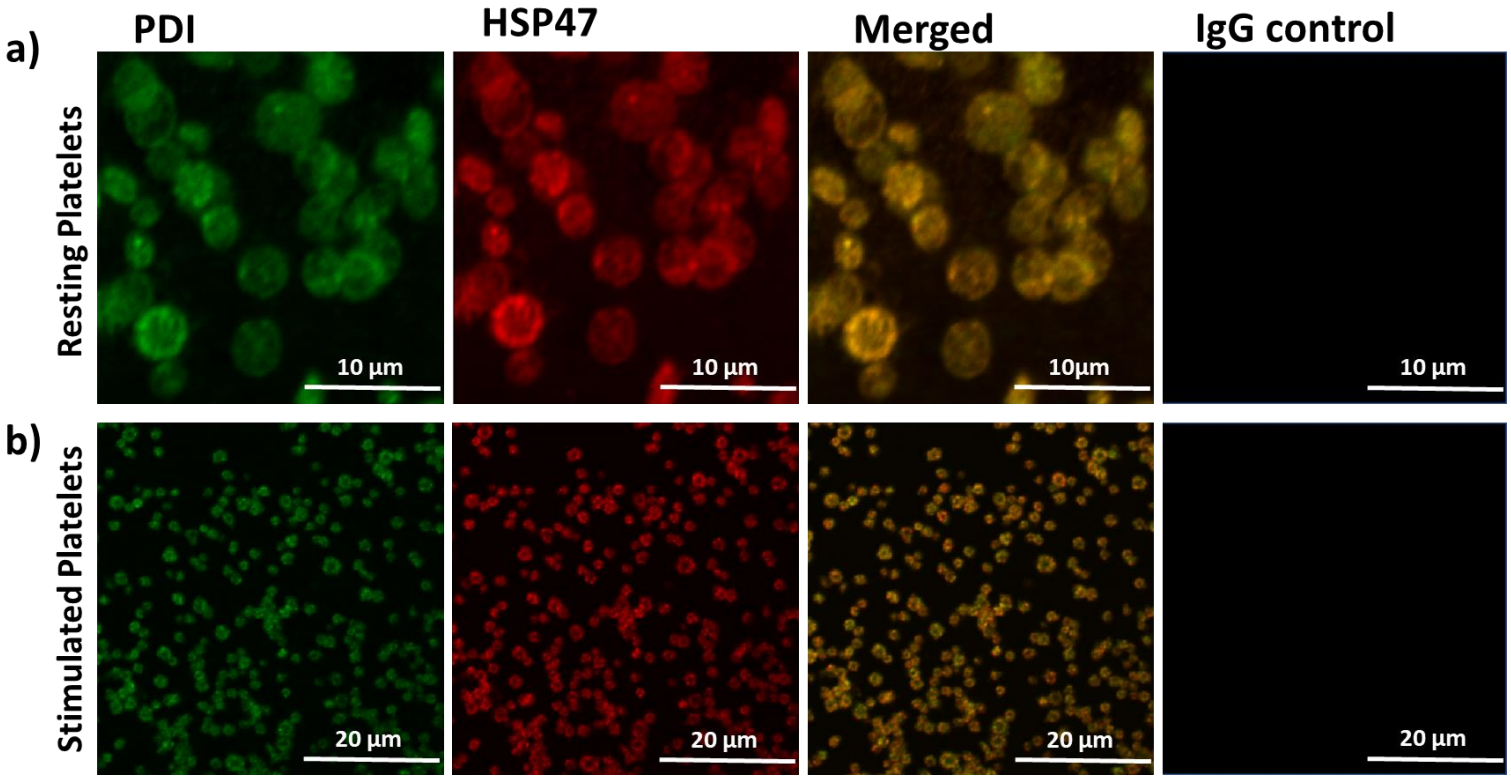


Figure 3.6: HSP47 and PDI, dense tubular system marker, colocalises in resting and stimulated platelets.

Platelets were stimulated with **(a)** modified Tyrode's-HEPES or **(b)** 5µM U46619 and followed with fixation in 4% (v/v) formaldehyde and permeabilisation with 0.2% Triton™ X-100. Blocking agent was added to the samples and followed with the addition of 1 µg/mL anti-HSP47 and anti- PDI. Samples were then washed to remove unbound antibodies. Alexa Fluor® 488 (PDI; green) and Alexa Fluor® 647 (HSP47; red) conjugated secondary antibodies were then added to detect antibody binding. Images were obtained using a 100× oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of >3 separate experiments using platelets from different donors.

3.5 Distribution of HSP47 in Platelet Subcellular Fractions

Immunofluorescence studies established the localisation of HSP47 in permeabilised resting and activated platelets and its co-localisation with the dense tubular system markers, calreticulin and PDI. To further delineate such an association and investigate the subcellular localisation of platelet HSP47 more closely, examination of HSP47 distribution in platelet homogenates was carried out using a linear sucrose density gradient centrifugation to separate the membrane and cytosolic proteins from the platelet granules and organelles.

Washed human platelets were homogenised by nitrogen cavitation using a cell disruption vessel. Homogenates were laid over a linear sucrose density gradient and subjected to ultracentrifugation at 200,000 g for two hours at 10˚C. Fractions collected from the top to the bottom of the gradient, representing the low- and high-density fractions, were separated by SDS-PAGE, western blotting and immunoblotted for HSP47 along with marker proteins that represent distinct subcellular compartments. β3 integrin represents surface proteins and was present abundantly in the lighter fraction 1 and 2 as well as the heavier fractions 8-13 which is due to the distribution of β3 integrin containing α-granules in the heavier fractions. This finding is consistent with the reported β3 integrin distribution in platelets fractions (Jonnalagadda et al., 2014). Calreticulin and PDI are known to be present in the dense tubular system (Crescente et al., 2016) and were concentrated in the light fractions (1,2 and 3). Similarly, cytosolic marker Rab GDP dissociation inhibitor (RabGDI) was confined to the light fractions 1 and 2, and lastly, alpha granules marker thrombospondin-1 (TSP-1) distributed heavily in the higher density fractions while their unexpected presence in the

lower fractions is due to the possibility of granule rupture during nitrogen cavitation as previously reported (Crescente et al., 2016).

Consistent with our immunofluorescence colocalisation of HSP47 with DTS residing proteins, HSP47 was highly concentrated in the low-density fractions (1-7) with distribution pattern similar to that of calreticulin and PDI. Moreover, no notable detection of HSP47 in the higher density fractions in which platelet granule proteins are expected to reside suggesting that HSP47 occupies a low-density compartment inside the platelet (Figure 3.7).

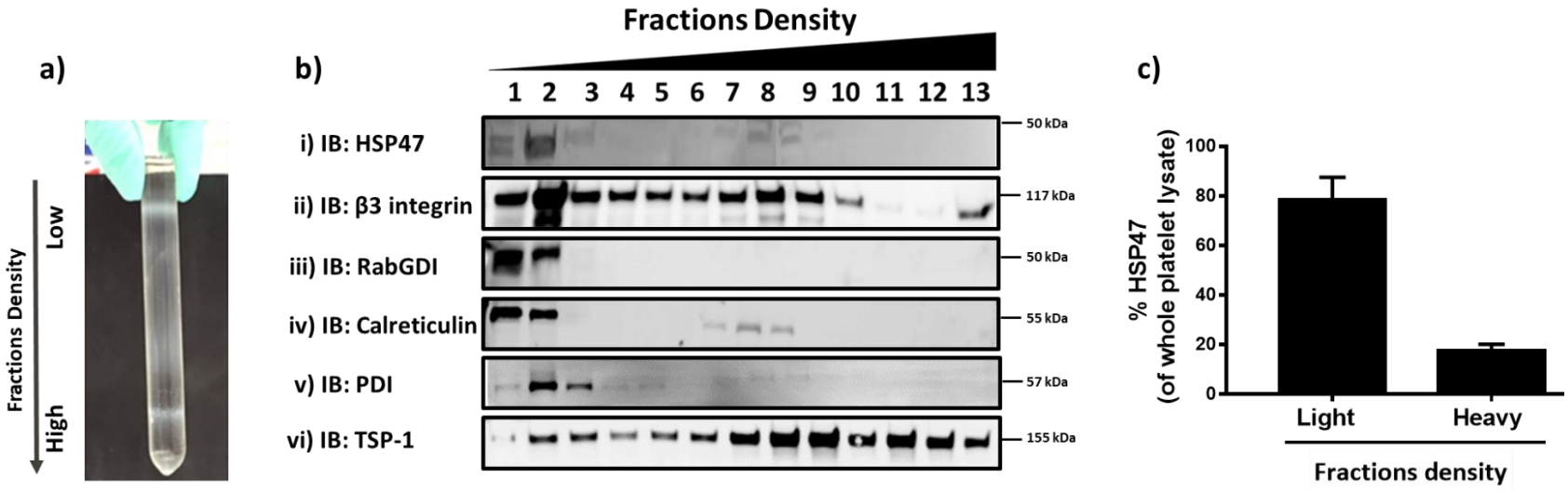


Figure 3.7: Distribution of HSP47 in the lighter platelet subcellular fractions

Platelet homogenate was laid on a sucrose density gradient and subjected to ultracentrifugation. **(a)** Representative picture of the sucrose gradient after centrifugation is shown. 13 fractions were then collected from the top of the gradient, with top 1 to 6 fractions representing light density platelet fractions whereas, the bottom 7 are platelets fractions with heavier density. **(b)** Fractions were separated by SDS-PAGE and immunoblotted for HSP47, β_3 integrin, RabGDIb, calreticulin, PDI and TSP-1. Immunoblots were scanned using a Typhoon FLA 9500 (GE Healthcare, UK). Results are representative of three individual experiments and data represent **(c)** HSP47 distribution in platelet fractions is expressed as percent of total HSP47 in whole platelet lysate.

3.6 HSP47 association with platelet lipid rafts:

The plasma membrane is distinguished by a specialised (20–50 nm) microdomain known as lipid rafts or glycolipid-enriched microdomains (GEMs). It is characterized by a high content of cholesterol and sphingolipid. Acyl chains of the sphingolipid are tightly associated along with hydrophobic cholesterol (Simons and Ikonen, 1997; Rietveld and Simons, 1998). These micro-domains are vitally involved in platelet function by providing a platform for several agonist receptors and signalling molecules (Hoylaerts, 2003). More importantly, several studies reported the implication of lipid rafts in platelet activation and signalling events mediated via GPVI and FcγRIIa (Watson et al., 2001; Locke et al., 2002; Wonerow et al., 2002; Bodin et al., 2003). Because of the established and reported role of HSP47 in platelet function and reduced platelet aggregation following the inhibition of HSP47 upon collagen stimulation (Kaiser et al., 2009; Sasikumar et al., 2018), work was extended to investigate the possibility that HSP47 is localised in raft fractions.

Lipid rafts were isolated based on low density and detergent resistance at cold temperatures and ability to float in sucrose gradients. 1 % Triton™ X-100 was used to lyse the platelets as this concentration of Triton has been reported to be suitable for successful lipid raft isolation (Rabani et al., 2016). The lysate was placed on a sucrose gradient and subjected to ultracentrifugation at 200,000 g for 18 hours at 4°C. The raft fraction was isolated as whitish opalescent band in the lighter part of the gradient and isolated fractions were separated by SDS-PAGE, western blotting and immunoblotted for HSP47 along with raft marker protein LAT (Zhang et al., 1998b) and non-raft marker beta 3 integrin (Raslan and Naseem, 2015) as shown in (Figure 3.8).

Raft fractions were distinguished from the rest of isolated fractions by the notable presence of LAT, a raft marker that is known to reside in such a region, along with the absence of β3 integrin in LAT marked raft fractions and its distribution in non-raft fractions (Pollitt et al., 2010). There was a notable presence of HSP47 in the raft fractions with a higher level of distribution in non-raft fractions. Such association suggests potential interaction of HSP47 with collagen receptors and or its signalosome proteins in the platelet rafts.

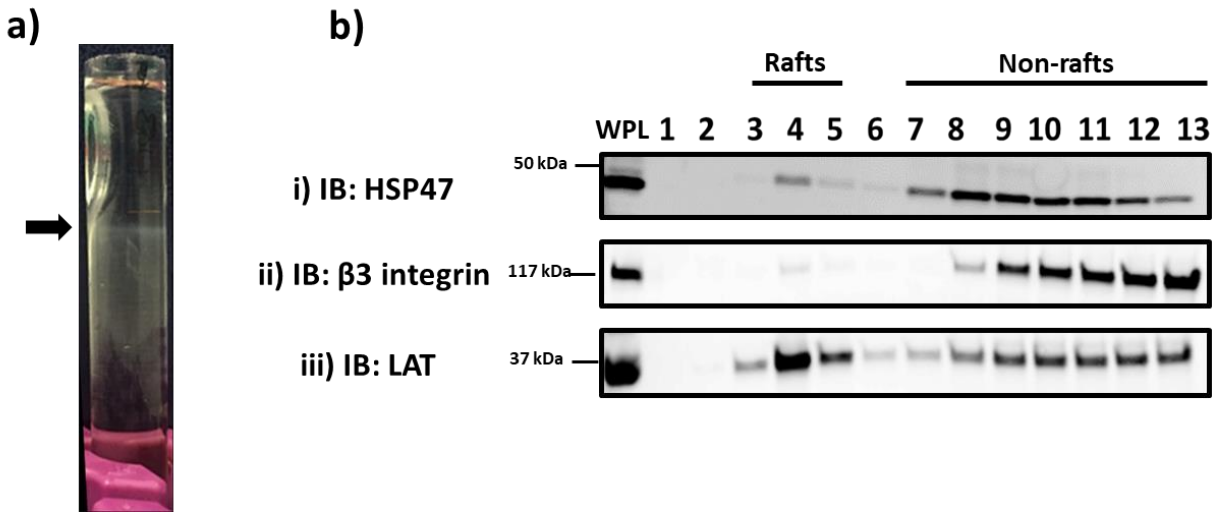


Figure 3.8: HSP47 is associated with lipid rafts in platelet.

Washed platelets (12×10^8) were lysed in the presence of 1% Triton™ X-100 and separated into lipid raft and non-raft fractions by sucrose gradient ultracentrifugation. **(a)** A representative picture of the gradient obtained after centrifugation is shown. **(b)** The localisation of **(i)** HSP47 was determined by immunoblotting. The lipid raft fractions were identified using **(iii)** an anti-LAT blot, and non-raft fractions were identified using **(ii)** an anti– β3 integrin blot. A Typhoon™ FLA 9500 fluor-imager was used to image the immunoblots (GE Healthcare, UK). The results are representative of 3 individual experiments.

3.7 Mobilisation of HSP47 upon Platelet Activation

HSP47 is mobilised to the platelet membrane upon activation. A number of studies reported the potential roles of actin polymerisation in platelet protein mobilisation and release (Lefebvre et al., 1993; Flaumenhaft et al., 2005; Crescente et al., 2016). According to our localisation studies, HSP47 occupies subcellular structure DTS and not traditional granules. So how does HSP47 mobilize to platelet surface? And does it require cytoskeletal changes induced by actin polymerisation to be mobilized like alpha granules cargo? To answer these questions, we explored the mobilisation of HSP47 to platelet surface in the presence or absence of inhibitors of actin polymerisation. Latrunculin A was used as an actin polymerisation inhibitor at a concentration of 200 µM, which was confirmed to be sufficient in reducing alpha granule marker P-selectin expression on platelet surface (Crescente et al., 2016). Platelets were stimulated with 5 µM thromboxane A2 analogue (U46619) in the presence or absence of latrunculin-A and stained for HSP47. Platelets were also co-stained for P-selectin, whose mobilisation and surface exposure are known to be dependent on actin polymerisation (Crescente et al., 2016), to serve as a control for the effectiveness of latrunculin-A in reducing the release of alpha granules proteins. P-selectin and HSP47 stained platelets were detected using Alexa Fluor® 568 and Alexa Fluor® 647, respectively, and visualised using confocal microscopy.

In the absence of latrunculin-A, P-selectin (Figure 3.9, aii) and HSP47 (Figure 3.9, cii) showed normal mobilisation to the platelet membrane upon activation. However, latrunculin-A reduced the mobilisation of both proteins following platelet activation and platelet displayed cellular distribution of P-selectin (Figure 3.9, aiii) and HSP47 (Figure 3.9,

ciii) similar to that seen in resting condition (Figure 3.9, ai and ci). This result indicates that actin polymerisation is required for HSP47 mobilisation.

We previously confirmed the association of HSP47 with dense tubular system markers, calreticulin and PDI, in co-immunofluorescence microscopic and platelet subcellular fractionation studies. Additionally, PDI mobilisation was reported to be dependent on actin polymerisation (Crescente et al., 2016). We decided to look more closely into the co-localisation of HSP47 and PDI in a double stained platelet under the effect of actin polymerisation inhibition and their mobilisation upon activation. In the absence of latrunculin-A, translocation and mobilisation of PDI (Figure 3.9, bii) and HSP47 (Figure 3.9, cii) was achieved following platelet activation. On the other hand, the latrunculin-A effect on HSP47 translocation (Figure 3.9, ciii) was similar to that seen on PDI (Figure 3.9, biii) which is shown in the reduced mobilisation of both proteins exhibiting a high level of co-localisation and retaining their resting distribution (Figure 3.9, bi and ci).

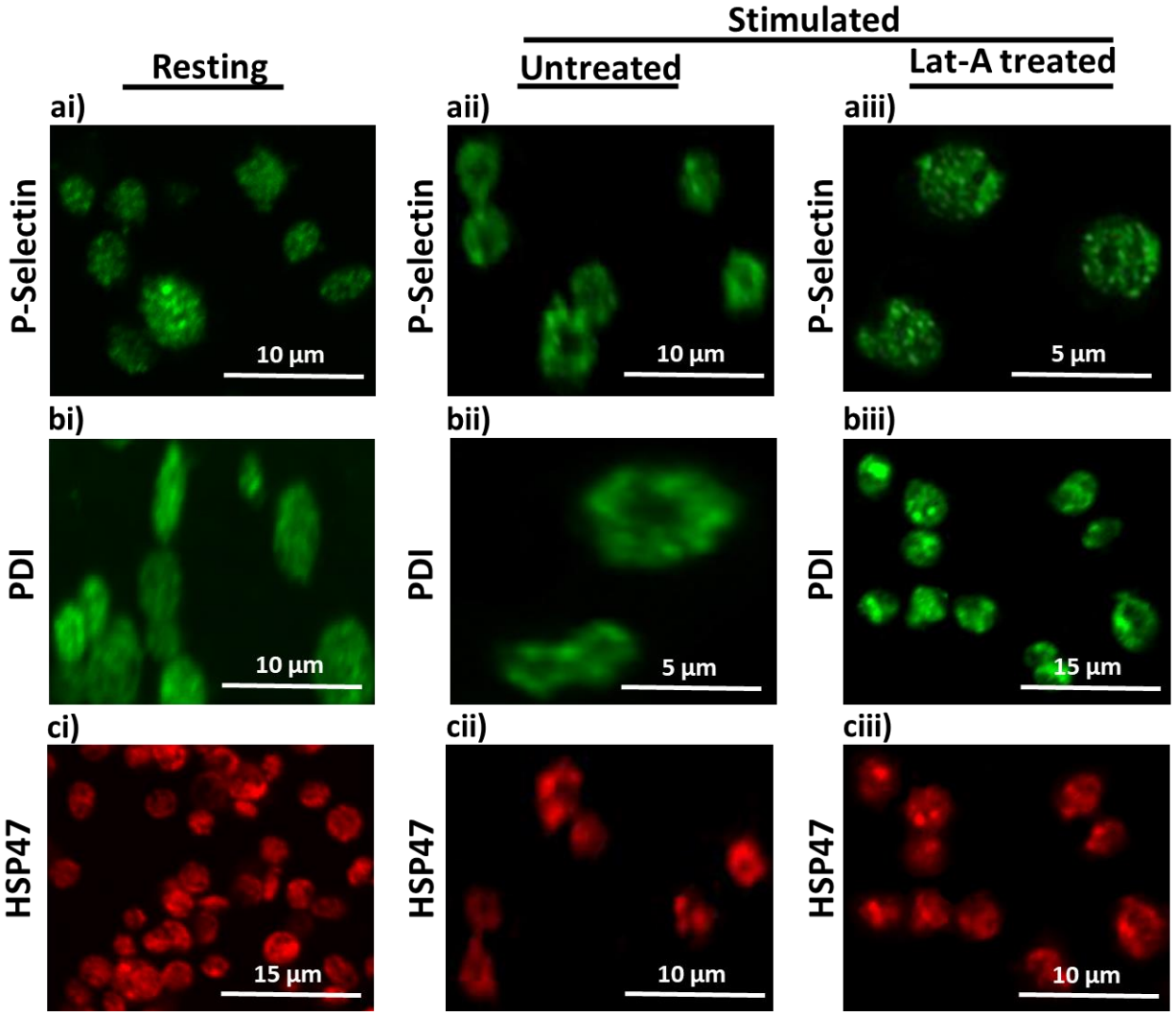


Figure 3.9: HSP47 mobilisation upon platelet activation requires actin polymerisation.

Platelets were stimulated with modified Tyrode's-HEPES **(ai, bi and ci)** or 5 µM U46619 in the absence **(aii, bii and cii)** or presence **(aiii, biii and ciii)** of 200 µM latrunculin-A (incubated with platelets for 10 minutes) were fixed in 4% (v/v) formaldehyde and permeabilised using 0.2% Triton™ X-100. Samples were then blocked and incubated with 1 µg/mL **(a)** anti-P-selectin, **(b)** anti- PDI or **(c)** anti-HSP47. Multiple washing cycles were performed on the samples to ensure the removal of unbound antibodies. Alexa Fluor® 568 (P-selectin or PDI; green) and Alexa Fluor® 647 (HSP47; red) were then added to detect Antibody bound platelets. The images were obtained using a 100× oil immersion lens and a Nikon A1-R confocal microscope. Data are representative of >3 separate experiments using platelets from different donors.

3.8 Human Platelets Release HSP47

Platelet activation by different agonists results in the mobilisation and release of paracrine and autocrine mediators such as ADP, TXA2, epinephrine and the generation of thrombin (Li et al., 2010). Such mediators trigger signalling pathways initiated by G protein-coupled receptors (GPCRs) (Offermanns, 2006). Moreover, platelet adhesion to collagen induces tyrosine kinase-mediated signalling through GPVI receptors (Gibbins, 2004). Triggering these signalling pathways results in platelet shape changes, secretion of platelet granules, inside-out activation of integrin αIIbβ3, and subsequently causes platelets to aggregate (Offermanns, 2006). HSP47 distribution and mobilisation to the platelet membrane upon activation was observed in our immunofluorescence studies. We decided to examine the fate of the mobilised HSP47 following platelet activation. Flow cytometry was employed to measure the median fluorescence intensity of HSP47 in permeabilised platelets in resting and activated conditions in order to determine whether HSP47 levels would remain the same as resting or decrease following platelet activation indicating HSP47 release. Resting and stimulated (1 µg/ml CRP-XL) washed platelets (4×10^8/mL) were fixed and treated with a permeabilisation buffer. Primary antibodies were added to detect HSP47 inside the platelets. HSP47 antibody binding was detected using species-specific Alexa Fluor® 647-conjugated antibody and analysed by flow cytometry where reduction of the median fluorescence intensity of HSP47 upon activation was an indication that it may be released from the cell.

Flow cytometry analysis of permeabilised activated platelets showed that HSP47 median fluorescence intensity level was reduced by 56.62% ± 10.69 when compared to the

resting levels (Figure 3.10a). Such reduction is consistent with reduced HSP47 immunofluorescence staining previously observed in our confocal imaging of activated platelets (Figure 3.1, b).

Flow cytometry finding indirectly indicated the potential release of HSP47 upon platelet activation by comparing the reduction in median fluorescence intensity of resting and activated platelets. To explore this further, experiments were performed to determine whether HSP47 could be detected in platelets releasate using western blotting. Human-washed platelets were treated with different concentrations of CRP-XL (0 - 2µg). The stimulation was performed under non-aggregating conditions, in presence of 1mM EGTA, 10µM Indomethacin and 10U/ml Apyrase, to avoid any reduction in the release process due to possible trapping of releasate proteins on the platelet surface or within an aggregate. The platelet releasate was collected by centrifugation and separated by SDS-PAGE and the potential presence of HSP47 was explored by immunoblot analysis. As a positive indicator of platelet release, TSP-1 was also detected by immunoblot analysis. The absence of GAPDH in the releasate confirmed its purity and exclude the possibility of HSP47 release due to platelet rupturing during preparation. In agreement with flow cytometry studies, immunoblotting of the platelet releasate confirmed the release of HSP47 from platelets following activation (Figure 3.10b and c).

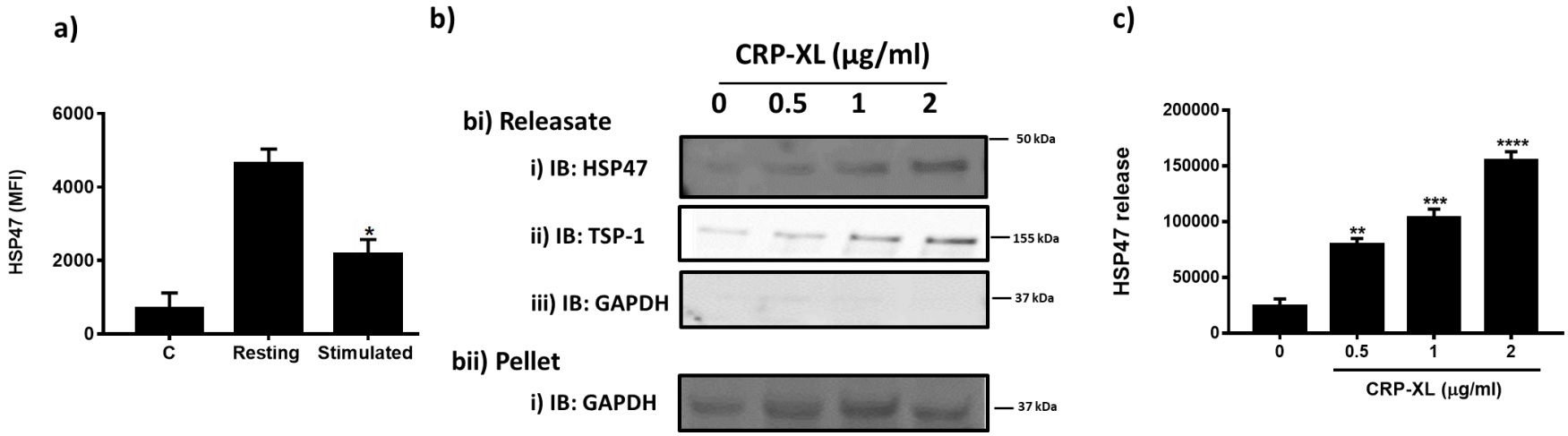


Figure 3.10: HSP47 is released in a dose-dependent manner.

Following fixation and permeabilisation, resting or (1 µg/ml CRP-XL) stimulated washed platelets were incubated with HSP47 antibodies and detected using Alexa Fluor® 647-conjugated antibodies. Appropriate isotype control was included as negative control. Samples were then analysed by flow cytometry. **(a)** levels of HSP47 binding in isotype control, resting and stimulated samples are shown. **(b)** Washed platelets (8×10^8/ml) were stimulated with the indicated concentrations of CRP-XL under stirring conditions for 3 minutes, and 0.1% sodium azide was added to stop the reaction. **(bi)** Releasate was collected by centrifugation, separated on SDS-PAGE and immunoblotted to detect **(i)** HSP47, **(ii)** TSP-1, and **(iii)** GAPDH. Similarly, **(bii)** pellet was separated and immunoblotted with **i)** GAPDH to exclude platelet rupture during preparation. The presence of TSP-1 was a confirmation of the platelet release, while GAPDH served as a negative control. **(c)** Data represent quantified HSP47 release in resting and stimulated samples. Immunoblots were scanned using a Typhoon FLA 9500 (GE Healthcare, UK). Data represent mean ± SEM (n=5), $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ were calculated using one-way ANOVA.

3.9 Platelets Release HSP47 on Microvesicles

Platelet release is not solely confined to soluble molecules. Furthermore, plasma membrane-derived microvesicles are shed into circulation. The potential role and contribution of microvesicles in cardiovascular disorders and coagulation is supported extensively in the literature (Sims et al., 1989; Gemmell et al., 1993; Castaman et al., 1997; Nieuwland et al., 1997; Nieuwland et al., 2000; Nomura et al., 2000). Microvesicle membranes may contain molecules that were originally present on the platelet surface, have been mobilised or translocated and shed upon activation and lastly, proteins derived from the inside of platelet (VanWijk et al., 2003).

Given our establishment of HSP47 membrane mobilisation and release, we wanted to determine if HSP47 being released upon the shedding of microvesicles following platelet activation. Washed human platelets (8×10^8/ml) were stimulated with 3 µg/ml CRP-XL for 30 minutes at 37°C and then removed by centrifugation. Microvesicles were pelleted from platelets free supernatant by ultracentrifugation. The releasate pellet and supernatant were separated by SDS-PAGE and immunoblotted for HSP47. To ensure the success of microvesicle isolation, β3 integrin was used as microvesicle marker since this is present on the platelet surface. HSP47 was detected in microvesicle fraction (Figure 3.11); however, it was highly enriched in the supernatant fraction, indicating that the majority of the HSP47 population is released in a soluble form.

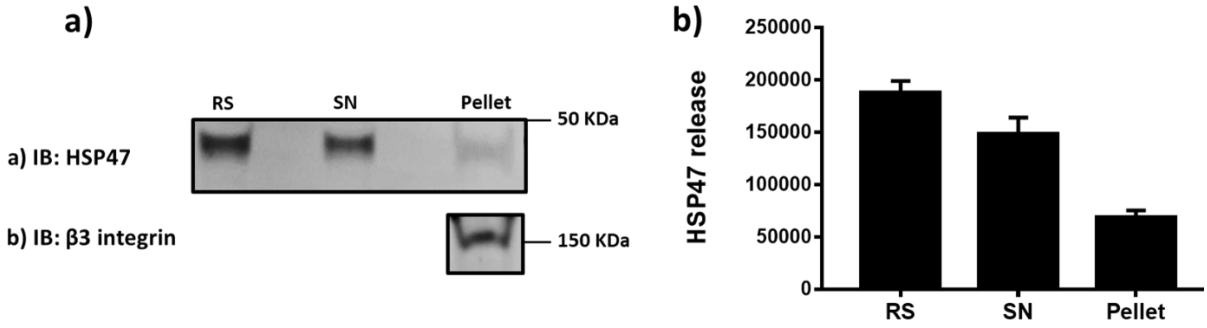


Figure 3.11: HSP47 is released on platelet-derived microvesicles.

Washed platelets (8×10^8/ml) were stimulated with 3 µg/ml CRP-XL for 30 minutes at 37°C. Platelets were pelleted by centrifugation, and the releasate was further subjected to ultracentrifugation to isolate the microvesicle pellet. **(a)** The microvesicle pellet was re-suspended in the Laemmli sample treatment buffer along with the supernatant fraction (SN) and pre-fractioned releasate (RS). Lysates were then separated on SDS-PAGE and immunoblotted for HSP47 and β3 integrin which was used as a control for platelet-derived microvesicles. **(b)** Bars represent quantified HSP47 release in releasate, supernatant and MVs containing pellet. Data represent mean ± SEM (n=5). Immunoblots were scanned using a Typhoon FLA 9500 (GE Healthcare, UK).

3.10 Small molecule inhibitor selectively inhibits HSP47 activity in platelets

Low molecular weight compound IV was identified as HSP47 inhibitor that showed an inhibitory capacity with low IC50 (6.3μM) (Thomson et al., 2005). This HSP47 inhibitor [referred to as IOH and SMIH in published papers (Kaiser et al., 2009; Sasikumar et al., 2018)] has been reported to inhibit the function of HSP47 in platelets. Hence, SMIH was chosen to investigate the role of this chaperone in collagen-platelet interactions in the present study. To rule out any potential off-targets, the selectivity of SMIH toward HSP47 was tested. The specificity of SMIH was examined by comparing the CRP-XL-stimulated fibrinogen binding to integrin $\alpha_{IIb}\beta_3$ in control wild type ($HSP47^{+/+}$) and $HSP47^{-/-}$mouse platelets in the presence of SMIH. Also, selectivity was also tested by examining the effects of multiple HSP47 inhibitors on platelet collagen binding using SMIH and anti-HSP47, an inhibitory antibody that was previously proven selective (Sasikumar et al., 2018).

Mouse PRP (obtained from both $HSP47^{-/-}$ and $HSP47^{+/+}$) was incubated with FITC-conjugated anti-human fibrinogen antibody, then with SMIH (20 μM) or vehicle containing control (0.12% DMSO) for 10 minutes. 0.5 μg/mL of CRP-XL was used to stimulate the PRP for 20 minutes at room temperature with occasional mixing. Samples were then fixed in 0.2% (v/v) formyl saline and analysed by flow cytometry (10,000 platelet-gated events).

SMIH inhibited CRP-XL-mediated fibrinogen binding in $HSP47^{+/+}$ mouse platelets by 46% when compared with vehicle control treated platelets (Figure 3.12). Similarly, levels of fibrinogen binding in $HSP47^{-/-}$ platelets were inhibited by 38% compared to wild type (Figure 3.12). On the other hand, SMIH did not induce any further inhibition in CRP-XL-stimulated $HSP47^{-/-}$ mouse platelets (Figure 3.12). With multiple inhibitors approach,

platelets were treated with 20 μM SMIH, 20 μg inhibitory anti-HSP47, combined inhibitors and vehicle controls (0.12% DMSO or 20 μg rabbit IgG) for 10 minutes at 37°C. Vehicle or inhibitor pre-treated platelets were then incubated with (10 μg/mL) FITC conjugated collagen type I for 20 min at RT before fixation with 0.2% (v/v) paraformaldehyde and FITC-collagen bound platelets were detected using flow cytometry. Number of FITC-collagen bound platelets was attenuated in SMIH by 24% when compared to vehicle control treated sample with no greater effect noted following the addition of inhibitory antibody (Figure 3.13).

Additional examination of HSP47 inhibitor treatment of platelets was carried out by comparing the surface levels of GPIb (Figure 3.14 ai, bi), integrin $\alpha_{IIb}\beta_3$ (Figure 3.14 aii, bii), integrin $\alpha_2\beta_1$ (Figure 3.14 aiii, biii) and GPVI (Figure 3.14 aiv, biv), evaluated in the presence and absence of SMIH or inhibitory antibody to assess if aforementioned treatment would influence the expression levels of these receptors in platelets. Similar surface levels of these receptors were seen in SMIH (Figure 3.14 a) and inhibitory antibody (Figure 3.14 b) treated platelets when compared to the vehicle containing samples (DMSO or IgG) in resting and activated (CRP-XL, 0.5 μg/mL) conditions. Taken together, these data confirmed the specificity of SMIH actions along with the interpretation of SMIH treatment attributed to HSP47 activity.

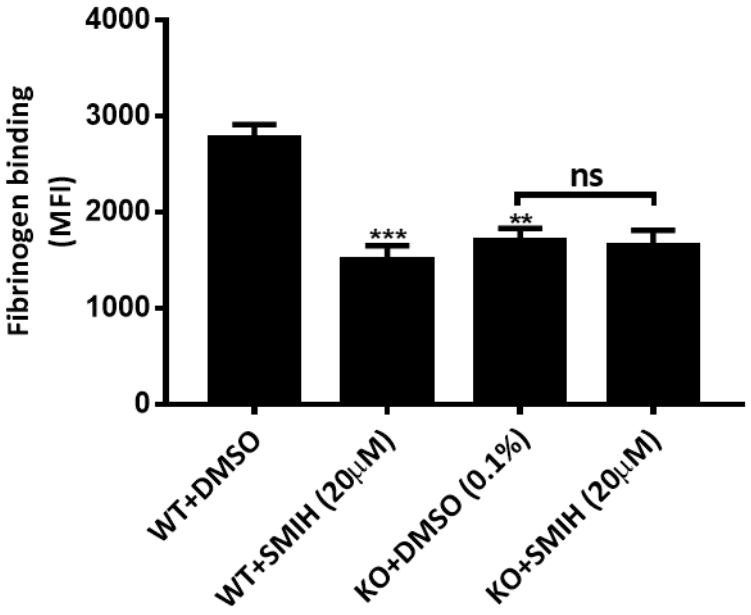


Figure 3.12: HSP47 activity is selectively inhibited by SMIH.

PRP from HSP47$^{+/+}$ or HSP47$^{-/-}$ knockout mice was incubated with FITC-labelled rabbit anti-fibrinogen antibody prior to treatment with vehicle DMSO control (0.12%) or SMIH (20 μM) for 10 minutes. Then, the samples were stimulated with CRP-XL (0.5 μg/mL) for 20 minutes. The cells were fixed in 0.2% formyl saline and the samples were examined on a flow cytometer. Results are represented as the mean± SEM (n = 3). **P ≤ 0.01, ***P ≤ 0.001 were calculated using one-way ANOVA. ns: not significant.

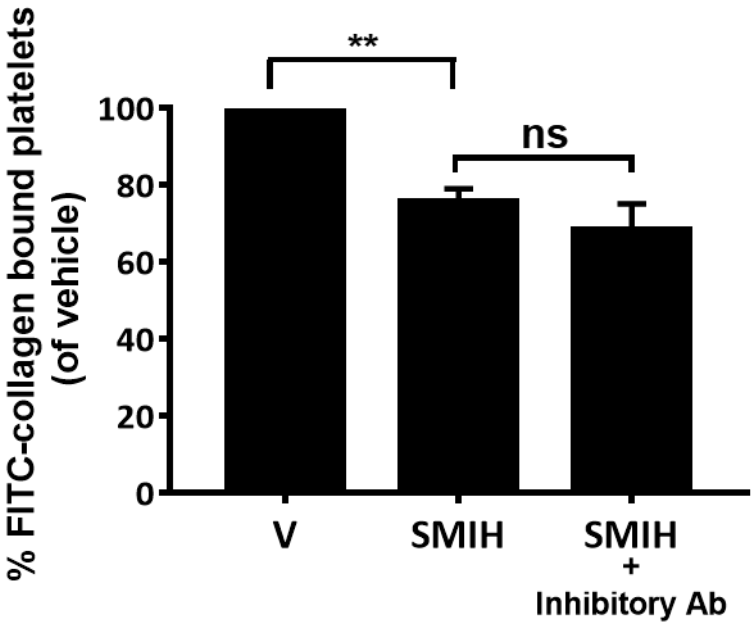


Figure 3.13: Addition of anti-HSP47 antibody failed to impact HSP47 activity in SMIH treated platelets.

Washed platelets (4×10^8) in the presence and absence of SMIH (20 μM) or the combination of all inhibitors [inhibitory antibody (20 μg/ml) and SMIH (20 μM)] were incubated with FITC-collagen (10 μg/ml) at room temperature and after 20 minutes 0.2% PFA was used to fix the samples and 10,000 platelets gated events were analysed for FITC-collagen binding using flow cytometer. Results are represented as mean ± SEM ($n \geq 3$). **P ≤ 0.01 was calculated using One-way ANOVA. ns: not significant.

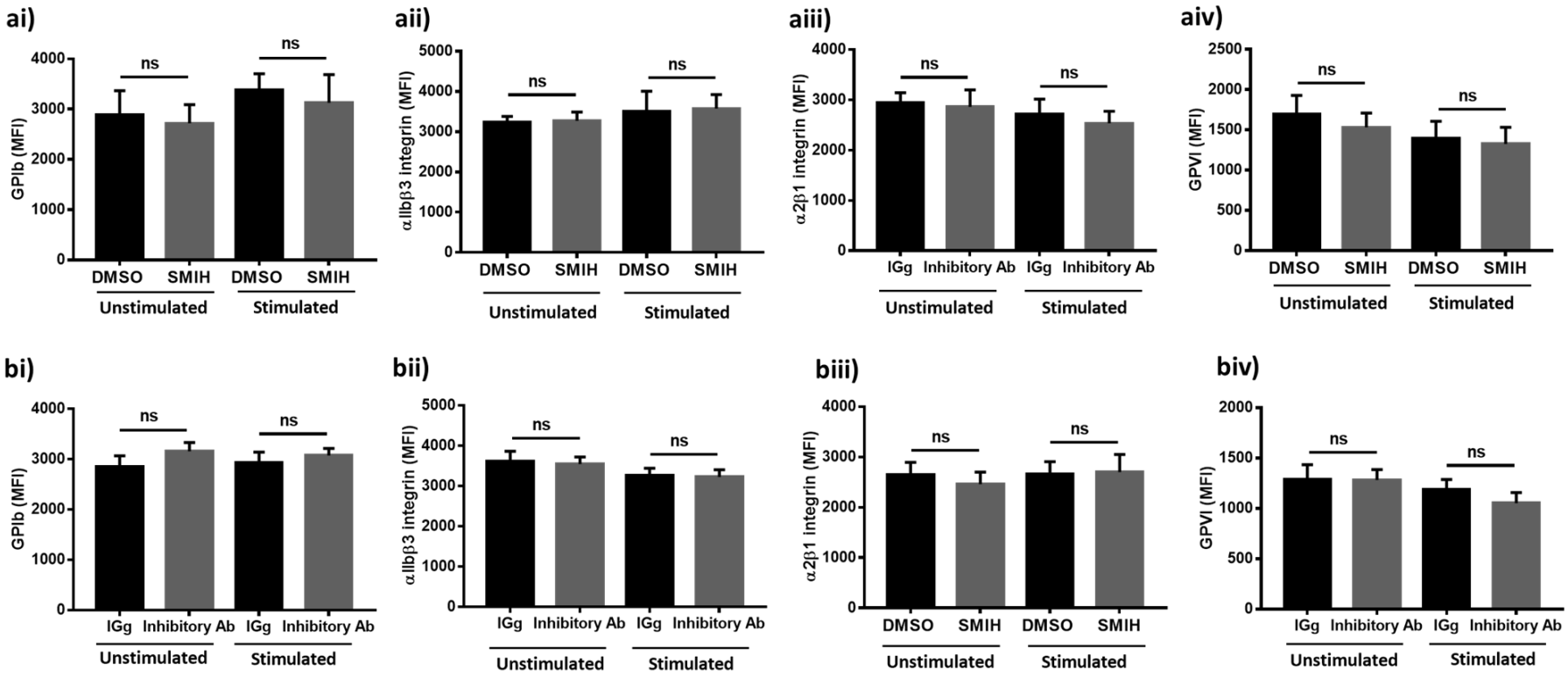


Figure 3.14: Levels of platelet receptors are unaffected by HSP47 inhibition.

PRP was treated with SMIH (20 µM) or HSP47 inhibitory antibody (20 µg/ml) for 10 minutes or vehicle controls (DMSO [0.12%] or IgG [20 µg/ml]) and the surface levels of platelet receptors on resting and (CRP-XL, 0.5 µg/mL) activated platelets were analysed by flow cytometry. Displayed data are MFI of **(ai, bi)** GPIb, **(aii, bii)** integrin $\alpha_{IIb}\beta_3$, **(aiii, biii)** integrin $\alpha_2\beta_1$ and **(aiv, biv)** GPVI receptor antibody binding levels obtained by SMIH, inhibitory antibody and vehicle-treated samples. Data were analysed with Student's *t*-test and displayed as the mean ± SEM (n = 3), ns: not significant.

3.11 Discussion:

Platelets are different from any other mammalian cells in that they are not originated through cell fission of individual precursors. Instead, platelets start developing in the bone marrow where progenitor megakaryocytic cells undergo repeated endomitosis transforming into a giant (>20 μm diameter) multi-nucleated cells. During their maturation, megakaryocytes increase protein production capacity along with the development of an intracellular specialised membranes system. Synthesized secretory proteins such as von Willebrand factor (vWF) is transported from the Golgi apparatus to multivesicular bodies, before packaging in secretory granules along with other endocytosed proteins, for instance, fibrinogen. Platelet generation starts with the development of proplatelet extensions in the giant megakaryocytes pushing into sinusoidal vessels in which thousands of platelets are released into the vasculature (White, 1972; Patel et al., 2005; Junt et al., 2007).

Less than 1% of circulating platelets contains noticeable remnants of Golgi apparatus or ER (White, 2013). Because of the lack of traditional ER system along with ER retrieval mechanism, it is perhaps less surprising that normally ER residing proteins escape to platelets surfaces. Protein disulphide isomerase (PDI), endoplasmic reticulum protein 5 (Erp5) and other thiol isomerases have been reported to be expressed in platelets with increased surface exposure following platelet activation in spite of their possession of KDEL sequence, ER retention motifs (Essex et al., 1995; Jordan et al., 2005; Holbrook et al., 2010). Similarly, such a motif is also present in the C-terminal of HSP47 along with increased surface mobilisation in stimulated platelets (Kaiser et al., 2009).

3.11.1 Presence of HSP47 in Resting and Activated Human Platelets

HSP47 is a collagen molecular chaperone. Its collagen-binding properties were first discovered in 1986 in chick fibroblasts (Nagata et al., 1986), and its vital role in collagen biosynthesis was revealed in the embryonic lethality of HSP47 knockout mice (Nagai et al., 2000). The expression of HSP47 is highly related to that of collagen in collagen-producing cells (Hirayoshi et al., 1991). Moreover, HSP47 was not found in non-collagen-producing cells, such as M1 myeloid leukaemia cells (Nagata, 1996). The presence of HSP47 was reported in platelets, non-collagen-producing cells (Kaiser et al., 2009), although HaemAtlas database indicates the expression of type IV collagen mRNA by megakaryocytes that may contribute to collagen secretion into the bone marrow (collagen niche) (Watkins et al., 2009). Inhibition of HSP47 or the employment of HSP47-deficient platelets affected several functional aspects of platelets in response to collagen or CRP-XL, using a different range of *in vitro* and *in vivo* assays, but not other agonists (Kaiser et al., 2009; Sasikumar et al., 2018). Taken together, these studies established the important role of HSP47 in collagen-induced platelet functions. In the present study, we aim to understand the mechanisms and contribution of HSP47 to platelet adhesion and activation.

We started our investigation by looking into the distribution of HSP47 in platelets at the microscopic level, which would provide a powerful clue to understanding the localisation and mobilisation route of HSP47 to platelet surface where expected HSP47 chaperoning or receptor action is performed. Using immunofluorescence studies, we confirmed the presence of HSP47 in resting and activated platelets following permeabilisation. In a resting platelet, HSP47 was distributed throughout the cytoplasm and, in a more condensed distribution,

underneath the plasma membrane. However, a ring-like staining pattern was exhibited in activated platelets, where the protein was shown to be mobilised toward the platelet periphery. This variation in the pattern of immunostaining was reflected on the co-localisation studies of HSP47 with surface receptor GPIb, in which activated platelets exhibited a higher level of co-localisation compared to the resting state (Figure 3.1).

3.11.2 Quantification of HSP47 in human platelets:

We showed that HSP47 is present at approximately 7,000 copies per platelet. The estimate is higher than that reported in the most recent platelet proteomics study, which estimated the level of HSP47 at 3,800 copies per platelet (Burkhart et al., 2012). This variation could be attributed to the fact that proteomics quantification is estimated and based on spectral counting-based quantification, which may be affected by several analytical and post-analytical factors, such as in tryptic digestion and data interpretation and analysis. Additionally, HSP47 possesses two glycosylation sites at the N-terminus, and such a modification could hamper the access of trypsin and therefore limit the peptide yield. Looking at the levels of many important cell surface proteins, such as integrin α2β1 and GPVI, at 2,000 to 4,000 and 3,700, respectively (Best et al., 2003; Varga-Szabo et al., 2008), it is possible that these proteins may serve as binding partners for HSP47.

3.11.3 HSP47 colocalization

Collagen-producing cells store HSP47 in an endoplasmic reticulum, where it is transported along with its collagen substrate to the Golgi apparatus and recycled back through the ER retention sequence (Nagata, 1996). In contrast, anucleated platelets contain a specialised membranous dense tubular system that is considered a remnant of ER (White, 1972). A co-

localisation analysis revealed the association of HSP47 with calreticulin and PDI (Figure 3.5 and Figure 3.6), proteins known to reside in the dense tubular system compartment (van Nispen tot Pannerden et al., 2009), which supports its counterpart ER localisation in a nucleated cell (Nagata, 1996). HSP47 showed no association with alpha granules, which was established by a lack of overlap between HSP47 and P-selectin localisation (Figure 3.4), therefore supporting its distinct association with DTS. Further platelet fractionation using sucrose gradient assays confirmed that HSP47 and markers of DTS were present in the same fractions, in which the protein was highly concentrated in lower density fractions in a pattern similar to that of PDI and calreticulin.

Plasma membranes include highly specialised lipid-enriched micro-domains that have been widely studied with increasing interest as it represents a platform for signalling pathways such as collagen-mediated pathway in platelets (Bodin et al., 2003). Reorganization of cholesterol domains within the platelet membrane following collagen stimulation observed by Boesze-Battaglia et al. (1996), indirectly proposed implication of lipid rafts in GPVI-mediated signalling. Such proposal was further confirmed where platelet lipid rafts demonstrated a vital contribution to GPVI and FcRγ-mediated activatory signalling pathways (Bodin et al., 2003; Lee et al., 2006; Quinter et al., 2007). Furthermore, accumulation of lipid rafts in filopodial projection of spreading platelets was shown to be highly correlated with the recruitment of tyrosine kinase c-Src and the tetraspanin CD63 to these regions (Heijnen et al., 2003). It has been reported that ligation of CLEC-2 induces its translocation to lipid rafts and such movement is required for subsequent activatory phosphorylation and signalling events (Pollitt et al., 2010). Similar involvement of lipid rafts in platelet spreading was suggested due to delayed clot retraction in sphingomyelin ablated

platelets harvested from sphingomyelin synthase knockout mouse (Kasahara et al., 2013). Chemical disruption of platelet lipid rafts has been shown to result in attenuated platelet activation in response to ADP stimulation (Quinton et al., 2005). Interestingly, the literature reports members of HSP family displaying lipid raft localisation and/or recruitment. HSP60 was reported to become recruited to lipid rafts within the plasma membrane of tumor cell and cardiac myocyte following acute damage (Gupta and Knowlton, 2002; Lin et al., 2007; Campanella et al., 2012). Proteomic studies profiling lipid rafts unraveled the presence of various signalling proteins and factors involved in trafficking and mobilisation of cellular cargo and HSP90 and 70 were among these proteins (Foster et al., 2003; Li et al., 2004). Furthermore, generation of HSP60 containing exosomes from cardiac myocyte was hindered upon disruption of lipid rafts suggesting its association to this microdomain (Lin et al., 2007). More importantly, for lipid raft localisation, proteins must be linked to 16-carbon saturated palmitic acid in a post-translational modification process known as palmitoylation (D'Avanzo, 2016). Large numbers of signalling regulating enzymes and adaptors proteins such as CD8β, CD4 and members of Src-family kinases, Lck and Fyn, are palmitoylated (Resh, 2006). In fact, LAT protein used in the present study to confirm the identity of the raft fractions is reported to undergo such modification (Zhang et al., 1998b). In a palmitoylation sites prediction analysis, HSP47 showed two potential palmitoylation sites at position 11(LLLLSAFCLLEAALA) and 156 (SSKQHYNCEHSKINF) (Ren et al., 2008), supporting the possibility that this chaperone associate with lipid raft microdomains, an association that was confirmed in the present study. Association of HSP47 with such specialized signalling microdomain points toward possible interaction of HSP47 with rafts

residing collagen signalosome proteins and receptors to finally support platelet activation in response to collagen.

3.11.4 Mobilisation and release

Our immunofluorescent confocal images showed the mobilisation of HSP47 to the platelet periphery upon activation. Further observation in the nature of such mobilisation revealed that the protein is trafficked in an actin polymerisation-dependent manner, similar to that of alpha-granule release (Figure 3.9). Moreover, the fluorescent intensity of the mobilised HSP47 in activated platelets was lower compared to resting conditions, which led us to propose that HSP47 is released from the platelets, a notion that was confirmed and supported by the flow cytometry and western blotting analysis (Figure 3.10). Generally, besides their intracellular distribution, heat shock proteins show an extracellular localisation. Several mechanisms may precipitate the release of HSPs. For instance, detection of HSPs in serum was associated with inflammatory and infectious disorders in humans (Barreto et al., 2003; Saito et al., 2005). HSP70 has been found to be actively released from tumor cells and this release was further induced in response to external stress stimuli such as inflammation in an *in vitro* analytical setting (Barreto et al., 2003). Our immunoblotting and flow cytometry analysis revealed the release of HSP47 from activated platelets, suggesting the existence of another internal pool of HSP47 in the platelets and most likely in the open canalicular system. It is also possible that a minor population of HSP47 is weakly associated with the platelet surface and hence is cleaved off upon stimulation. Lastly, the presented selectivity and specificity of SMIH toward HSP47 along with the lack of impact on various platelets receptors validate the significance of such treatment to HSP47 inhibition in treated platelets.

Elucidation and a close examination of HSP47 at the microscopic level and understanding of its route and fate in activated platelets form the first step in understanding the mechanism and nature of HSP47 novel role in haemostasis and thrombosis. The established surface expression and its association with collagen signalling protein enriched lipid raft along with evoked surface mobilisation from the subcellular store and release following platelet activation mark the beginning of several functional possibilities and potentials of HSP47 in platelet-collagen interactions. In the next chapter, possible interaction of HSP47 with binding partners in human platelets and the role of this chaperone in modulating platelet-collagen interaction is thoroughly studied and investigated.

4 HSP47 interacts with collagen receptor GPVI supporting its dimerization and hence contributing to platelet-collagen interaction.

4.1 Introduction

Platelets circulate in close proximity with the vessel wall, however, the integrity of the vasculature system prevents unnecessary platelet adhesive events (Stalker et al., 2013). A breach to the vascular integrity exposes subendothelial matrix allowing platelet to encounter various extracellular matrix components such as collagen. This highly thrombogenic macromolecule binds platelets via direct or indirect interactions (Farndale et al., 2004). There are about 25 isoforms of collagen in human (Hashimoto et al., 2002), for instance, collagen type I, III, IV ,V, VI, VIII, XII, XIII and XIV, and collagen types I and III are part of the vascular wall (Barnes and Farndale, 1999).

Bounameaux (1959) showed for the first time the ability of platelets to couple on collagen fibers. Since then, wider aspects of platelets-collagen binding have been discovered along with various subsequent events taking place upon collagen binding including cytoskeletal reorganization, granules release, structural modulation of integrin αIIbβ3 allowing it to bind to fibrinogen and eventually the formation of a platelet aggregate. The involvement of GPVI in collagen-mediated platelet activation was first revealed in a Japanese patient suffering from thrombocytopenia and autoimmunity who showed defective platelet response to collagen (Sugiyama et al., 1987; Moroi et al., 1989; Ishibashi et al., 1995; Ichinohe et al., 1997).

In the arterial circulation where shear stress is high, various set of platelet receptors collectively participate to enable platelet adhesion on exposed collagen under such stress. GPIb, part of the Glycoprotein Ib-IX-V complex, plays a crucial role in the tethering and capture of platelets flowing in arterial circulation by binding its collagen immobilised ligand,

von Willebrand factor. Such tethering allows subsequent rolling of platelets on the exposed collagen and hence binding of GPVI (Watson et al., 2000). Transgenic mice with platelets expressing modified GPIbα showed defective recruitment, adhesion and consequent thrombus formation on exposed collagen under arterial flow condition (Bergmeier et al., 2006).

GPVI triggers the shift of integrins to a high-affinity state thus establishing the stable adhesion via the adhesive receptor, integrin α2β1 (Siediecki et al., 1996; Watson et al., 2000). Integrin α2β1 contribution was evidently demonstrated in the collagen interaction with platelets lacking the integrin (Nieuwenhuis et al., 1985; Nieuwenhuis et al., 1986) or upon the use of blocking antibody (Coller et al., 1989), suggesting its role in hemostasis and collagen adhesion under static and flow conditions (Santoro, 1986; Saelman et al., 1994).

Sasikumar et al. (2018) established a role for HSP47 in collagen-mediated platelet functions *in vitro* and *in vivo*. Nonetheless, little is known regarding the mechanism by which this chaperone supports platelet collagen binding and subsequent platelet activation. In this chapter, investigations were carried out to study the proposed modulation of collagen receptors by HSP47 to bind collagen. Additionally, potential HSP47 mediated-chaperoning on collagen was examined.

4.2 Inhibition of HSP47 attenuates platelets adhesion on collagen and CRP-XL, not GFOGER

Differential interpretation of interaction and adhesion studies through individual platelet receptors is challenged with the fact that one collagen fiber is capable of binding and potentially crosslinking multiple receptors simultaneously on the platelet surface. However,

the breakthrough in the synthesis and development of synthetic collagen peptides with distinctive selectivity toward individual collagen receptors has enabled the roles and signalling stimulated by different collagen receptors to be isolated and studied. Collagen-related peptide CRP-XL, a potent platelet agonist binds specifically to GPVI, whereas six-amino acid residue GFOGER peptide binds to integrin α2β1 and supports platelet adhesion (Morton et al., 1995; Asselin et al., 1997; Kehrel et al., 1998; Knight et al., 1998).

Previous work has reported inhibited thrombus formation on collagen-coated surface under flowing condition in presence of HSP47 inhibitor (Sasikumar et al., 2018). Since GPIb plays a vital role in supporting platelet collagen adhesion in arterial shear rate, the aforementioned experiments were repeated on vWF coated surfaces, and platelet adhesion was not altered by HSP47 inhibition. Together, these findings indicate that HSP47 supports platelet-collagen adhesion independently of GPIb, via other collagen receptors.

To tease this apart, the effect of HSP47 inhibition on the binding of individual collagen receptors, GPVI and integrin α2β1 to their respective immobilized specific ligands CRP-XL and GFOGER peptides, respectively, was examined. Also, assessing the adhesion in a static condition allowed the evaluation of platelet spreading on distinct ligands.

Glass coverslips were coated with collagen (100 μg/ml), CRP-XL (10 μg/ml) or GFOGER (50 μg/ml) for 1 hour followed by incubation with BSA for 30 minutes to prevent platelet from binding to the glass. Washed platelets (2×10^7 cells/ml) were incubated with SMIH (5 and 10 μM) or vehicle-control (containing DMSO 0.12% v/v) for 10 minutes at 37°C. Platelets were then seeded on the coated coverslips at 37°C. After 45 minutes coverslips were washed with PBS and fixed with 0.2% (w/v) paraformaldehyde and then washed with PBS.

Prior to platelets staining, 0.2% (v/v) Triton™ X-100 was used to permeabilise adhered platelets and then samples were washed with PBS. Alexa-Fluor® 647-conjugated phalloidin, that binds to actin filaments, was added and Prolong Gold Antifade mounting media was used to mount coverslips onto glass slides. The 100× oil immersion lens of the Nikon A1R confocal microscope (Nikon, Tokyo, Japan) was used to image samples (excitation at 650 nm, emission at 665 nm) and five randomly selected images were taken of each sample in a single focal plane. Platelet spreading in these images was categorised as adhered (not spread), filopodia projection formation (about to spread) and lamellipodia forming platelets (completely spread) and lastly, relative frequencies of each category were calculated.

As shown in Figure 4.1 a, vehicle-treated platelets adhered to immobilized collagen with filopodial projections and lamellipodial formation fully exhibited by platelets. Such concentration of immobilised collagen was reported to mediate platelet adhesion and subsequent spreading (Bye et al., 2015). Treatment with SMIH resulted in a significant reduction of platelet adhesion to collagen-coated coverslips. In comparison to vehicle-treated control, platelet adhesion was inhibited by 77% and 90% following treatment with 5 and 10 μM of SMIH, respectively (Figure 4.1 b). Platelet spreading on collagen was similarly affected with the reduction observed in all three categories of spread platelet in SMIH treated samples (Figure 4.1 c). Collagen binds to GPVI and integrin α2β1 on platelet surface which hampers interpretation and prediction of HSP47 effect on individual collagen receptors. Therefore, the impact of SMIH on platelet adhesions to immobilised CRP-XL and GFOGER peptides, GPVI and integrin α2β1 selective agonists, respectively, was assessed.

Images of platelet adhesion and spreading on CRP-XL following the treatment with vehicle control (containing DMSO 0.12% v/v) or SMIH are shown in Figure 4.2 a. Immobilised,

CRP-XL (10 μg/ml) has been reported to support platelet adhesion and spreading (Morton et al., 1995; Verkleij et al., 1998; Poulter et al., 2017) and this was demonstrated in vehicle-treated platelets on CRP-XL coated coverslips. Incubation with SMIH (5 and 10 μM) inhibited platelet adhesion to CRP-XL-coated coverslips by approximately 40% and 51%, respectively, when compared to adhesion levels in vehicle treated sample (Figure 4.2 b). Additionally, the percentage of lamellipodia forming platelets was reduced by 43% and 89% in 5 and 10 μM treated platelets, respectively, (Figure 4.2 c). Inhibition of platelet binding to immobilised CRP-XL indicates HSP47 supporting platelet collagen binding via GPVI.

To explore the impact of HSP47 on platelet-integrin α2β1 interaction, vehicle control (containing 0.12% DMSO) and SMIH treated platelets were seeded on coverslips precoated with GFOGER. At a concentration of 50μg/ml and as immobilised monolayer form, GFOGER peptide is capable of supporting platelet adhesion and spreading (Inoue et al., 2003; Pugh et al., 2010; Jarvis et al., 2012). This was indeed successfully shown in vehicle-treated platelets which adhered to GFOGER coated coverslips (Figure 4.3 a). In comparison to vehicle treated sample, pre-treatment of platelets with SMIH (5 and 10 μM) did not alter platelet adhesion and spreading to GFOGER (Figure 4.3 b and c).

The lack of impact of inhibition of HSP47 on platelet-GFOGER binding and spreading along with its influence on collagen and CRP-XL interaction with platelets under static condition suggest the participation of HSP47 in platelet-collagen adhesion and subsequent downstream activation and thrombus formation in a GPVI dependent manner. These results, therefore, raised the possibility that HSP47 may modulate collagen receptors to bind collagen or/and whether this protein exerts its chaperoning activities on collagen and render it more appealing to be bound by its receptors on the platelet surface. Henceforward, experiments

were performed to explore the regulation of platelet collagen receptors, GPVI and integrin α2β1 by HSP47.

Figure 4.1: HSP47 inhibition reduces platelet adhesion and spreading on collagen.

Human washed platelets (2×10^7 cells/ml) were treated with SMIH (5 and 10 μM) or vehicle-control containing DMSO (0.12% v/v) for 10 min at 37°C. Treated platelets were added onto collagen (100 μg/ml)-coated coverslips for 45 mins at 37°C. Samples were fixed and permeabilised with 0.2% (w/v) paraformaldehyde and 0.2% (v/v) Triton™ X-100, respectively. To stain the platelets, Alexa-Fluor® 647 conjugated phalloidin was added for 1 hour in the dark and coverslips were washed and mounted onto slides using Prolong Gold Antifade mounting media. **(a)** Nikon A1-R confocal microscope with a 100× oil immersion lens was employed to visualise samples and 5 randomly selected images were captured in each sample. **(b)** The number of platelets adhered in each sample are shown. **(c)** Platelets spreading was analysed and categorised into: adhered but not spread; filopodia, projection-forming platelets and lastly lamellipodial, fully-spread platelets) and percentage of each category was computed relative to the total number of adhered platelets. Data represent mean ± SEM (n=3), **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 were calculated by one-way ANOVA.

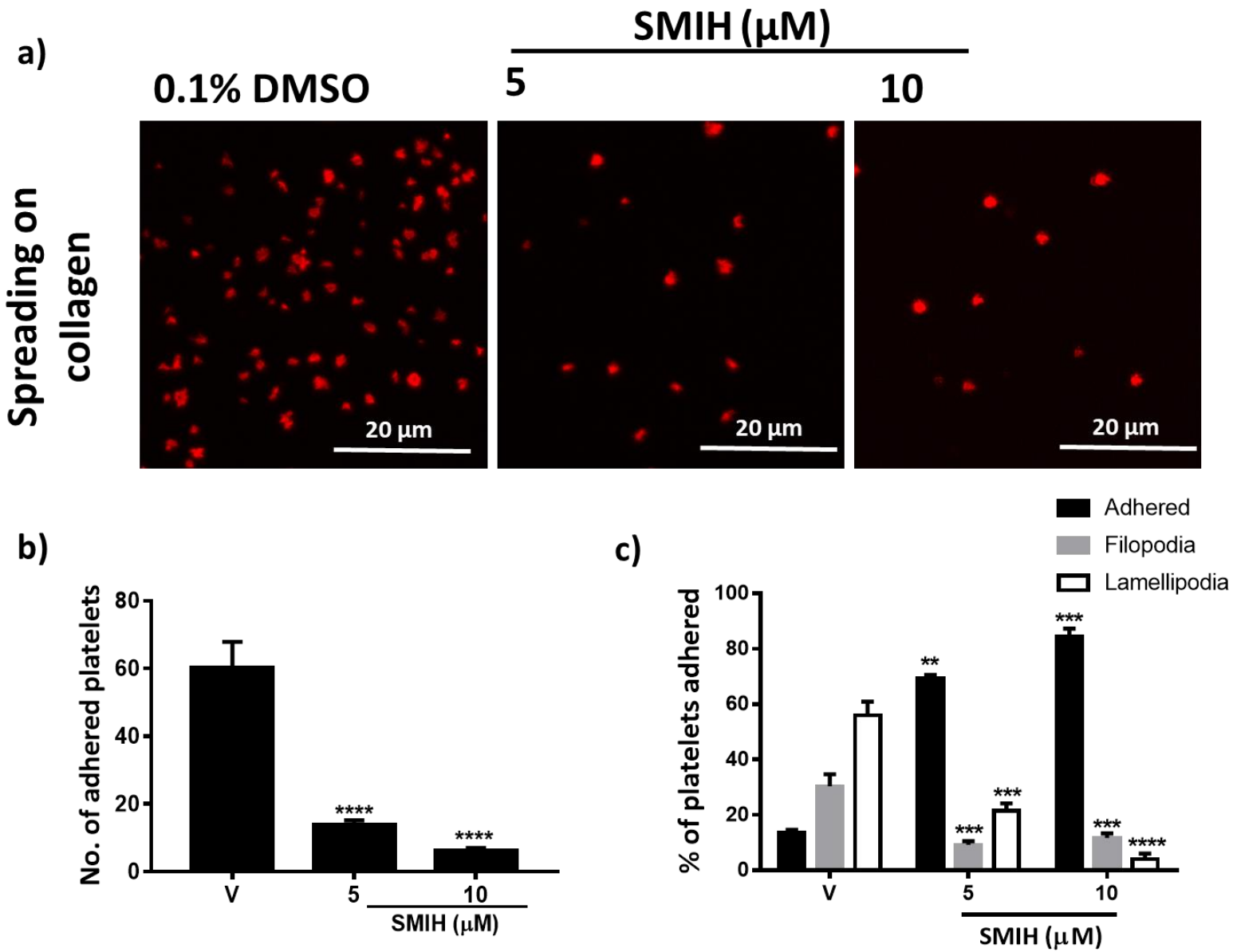

a)
SMIH (µM)
0.1% DMSO
5
10
Spreading on collagen
20 µm
20 µm
20 µm
b)
No. of adhered platelets
80
60
40
20
0
****
****
V
5
10
SMIH (µM)
c)
Adhered
Filopodia
Lamellipodia
% of platelets adhered
100
80
60
40
20
0
**
***
***
***
***
****
V
5
10
SMIH (µM)

Figure 4.2: Platelet adhesion and spreading on GPVI selective ligand, CRP-XL, is impeded by HSP47 inhibition.

SMIH (5 and 10 μM) or vehicle-control (containing, DMSO 0.12% v/v) was incubated with human washed platelets (2×10^7 cells/ml) for 10 min at 37°C. Glass coverslips were pre-coated with CRP-XL (10 μg/ml) and platelets treated with SMIH and vehicle control were added onto coated coverslips for 45 minutes at 37°C. Unbound platelets were removed and adhered platelets were fixed with 0.2% (w/v) paraformaldehyde followed by permeabilisation step with 0.2% (v/v) Triton™ X-100 and staining for one hour with Alexa-Fluor® conjugated 647 phalloidin. Coverslips were mounted on glass slides using Prolong Gold Antifade mounting media. **(a)** The visualisation of adhered platelets was performed using a Nikon A1-R confocal microscope with a 100× oil immersion lens and Five random images were captured of each sample. **(b)** An average number of adhered platelets in-vehicle control and SMIH treated samples are shown. **(c)** platelets spreading were assessed in each sample and adhered, filopodia and lamellipodia, referring to not spread, in the process of spreading and completely spread platelets, respectively, were computed as percentage relative frequency of the total number of adhered platelets. Data represent mean ± SEM (n=3), *P ≤ 0.05, **P ≤ 0.01, and ****P ≤ 0.0001 were calculated by one-way ANOVA.

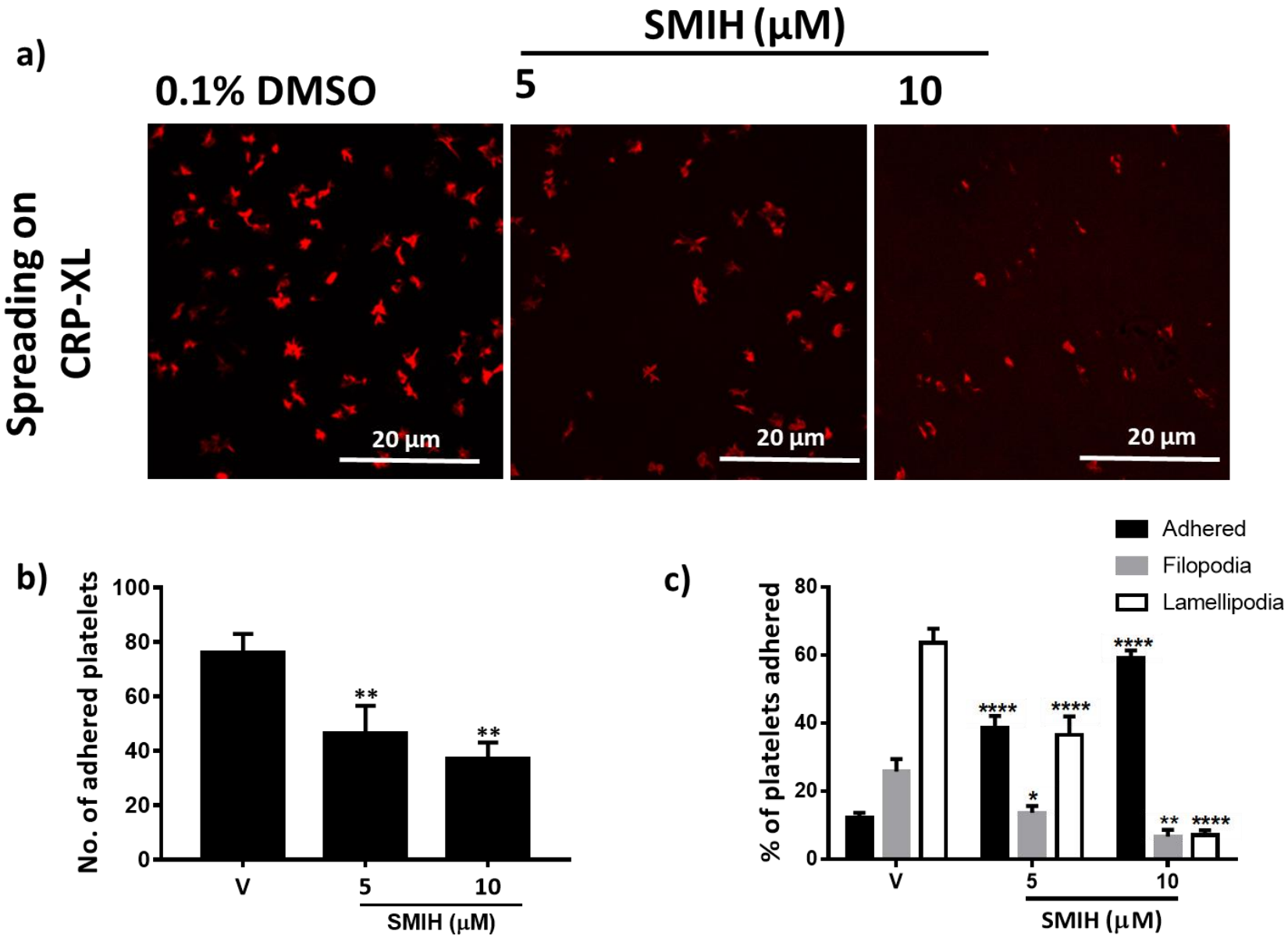
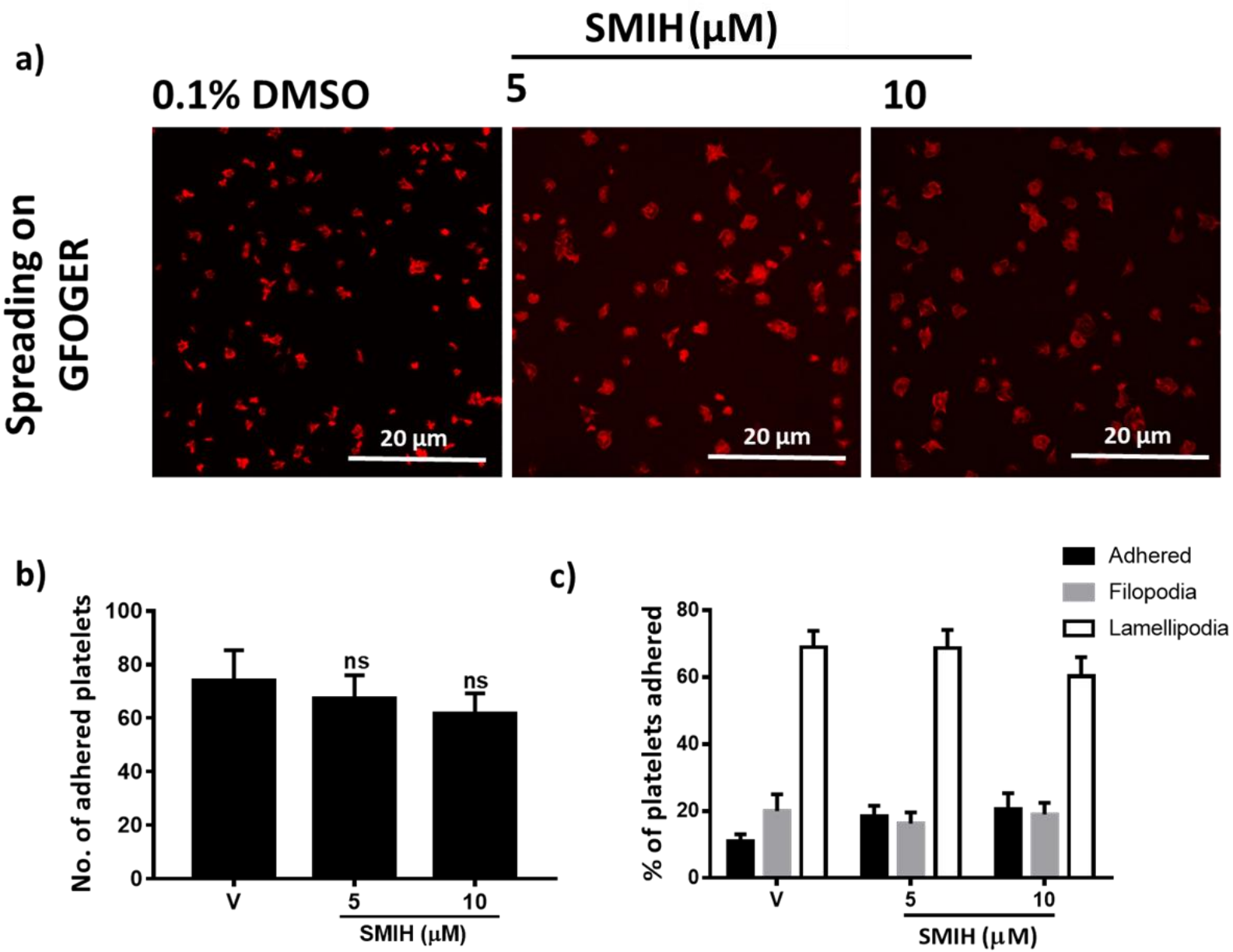

a)
SMIH (µM)
0.1% DMSO
5
10
Spreading on CRP-XL
20 µm
20 µm
20 µm
b)
No. of adhered platelets
100
80
60
40
20
0
**
**
V
5
10
SMIH (µM)
c)
Adhered
Filopodia
Lamellipodia
% of platelets adhered
80
60
40
20
0
****
*
****
****
**
****
V
5
10
SMIH (µM)

Figure 4.3: Targeting HSP47 with SMIH does not affect platelet adhesion and spreading to integrin α2β1 selective ligand, GFOGER.

SMIH (5 and 10 μM) or DMSO containing vehicle-control (0.12% v/v) pre-treated human washed platelets (2×10^7 cells/ml) were added onto GFOGER peptide (50 μg/ml) precoated coverslips at 37°C. After 45 minutes samples were fixed with 0.2% (w/v) paraformaldehyde and permeabilised with 0.2% (v/v) Triton™ X-100 and followed with staining using Alexa-Fluor 647® conjugated actin binding phalloidin for 1 hour in dark. Using Prolong Gold Antifade mounting media, coverslips were mounted onto slides prior to confocal microscopy visualisation with a 100× oil immersion lens. **(a)** Five images were captured at random locations. **(b)** Bar chart represents the average number of platelet adhesion in each sample. **(c)** Relative frequency of adhered (not spread), filopodia (about to spread) and lamellipodia (spread) forming platelets spreading were calculated to the total number of adhered platelets. Data represent mean ± SEM (n=3), one-way ANOVA. ns: not significant.


a)
SMIH(μM)
0.1% DMSO
5
10
Spreading on GFOGER
20 μm
20 μm
20 μm
b)
No. of adhered platelets
100
80
60
40
20
0
ns
ns
V
5
10
SMIH (μM)
c)
Adhered
Filopodia
Lamellipodia
% of platelets adhered
80
60
40
20
0
V
5
10
SMIH (μM)

4.3 HSP47 modulates and interacts with GPVI receptor

GPVI plays a fundamental role as a platelet activatory receptor in response to collagen stimulation (Andrews et al., 2014; Stegner et al., 2014) and has been implicated in ischaemic stroke, arterial thrombosis in addition to its role in supporting haemostasis during inflammation (Gros et al., 2015; Induruwa et al., 2016). GPVI is non-covalently linked with Fc receptor γ-chain (FcRγ) in platelet membrane (Gibbins et al., 1996). The small extracellular domain of FcRγ is not sufficient to support collagen binding. Platelet-collagen binding is facilitated via two extracellular Ig-like domains of GPVI. Moreover, FcRγ mediates downstream signalling events through phosphorylation of its immunoreceptor tyrosine-based activation motif (ITAM) present on its cytoplasmic domain (Watson et al., 2001; Berlanga et al., 2002; Bori-Sanz et al., 2003).

Dimeric GPVI recognizes and binds collagen with high affinity, with some of GPVI constitutively existing as a dimer on quiescent platelets. Also, reports showed an increase in dimer levels following platelet stimulation (Jung et al., 2012; Loyau et al., 2012). Section 4.2 showed the negative impact of SMIH on platelets adhesion on CRP-XL coated surface. Therefore, it was plausible that HSP47 acts and influences GPVI dimerization in addition to the potential interaction of HSP47 with this collagen-mediated activatory receptor. These investigations are elaborated in this section.

4.3.1 HSP47 supports GPVI dimerization on platelet surface

GPVI dimer is formed following the fusion of the extracellular domain of GPVI with Fc domain of IgG on near GPVI via disulfide bond. A number of lines of evidence suggest that

GPVI functions as a dimer and such configuration elevates receptor affinity for collagen (Jandrot-Perrus et al., 2000; Horii et al., 2006). When tested for the binding with several collagenous substrates, dimeric GPVI binds Gly-Pro-Hyp (GPO) triplet sequences in collagens whereas a substantial reduction in the binding and affinity for collagen substrates associated with monomeric GPVI suggesting that the dimeric configuration as the binding form for collagen. Having a pre-existing population of GPVI in dimeric configuration may be important for the initial interaction of platelets with collagen at sites of injured vessels. Moreover, prompt dimer mediated binding triggers downstream activatory signalling events which result in increased numbers of dimers on platelet surface (Jung et al., 2012). Such elevation serves as a feedback mechanism potentiating and further strengthening platelet-collagen interaction and activation. Using non-inhibitory, recombinant dimer-specific 204-11 Fab (a kind gift from Dr.Stephanie Jung, University of Cambridge), GPVI dimer formation was assessed in resting and stimulated platelets following treatment with SMIH.

5-fold diluted whole blood was pre-treated with EDTA and incubated with SMIH (5 and 10µM) or vehicle (contains 0.12%v/v DMSO) prior to the stimulation with CRP-XL (5µg/ml). EDTA treatment prevents the cleavage of GPVI upon activation and hence enables its detection following platelet stimulation (Gardiner et al., 2012). Dimer-specific 204-11 Fab (40 µg/ml) was added to platelets and incubated for 10 min at room temperature followed by addition of FITC anti-mouse IgG F(ab′)2 antibody (50 µg/ml, final). Antibody binding levels were measured by flow cytometry (Accuri C6 flow cytometer, BD Biosciences). Non-specific binding was assessed using an isotype-matched IgG (Jackson ImmunoResearch).

In agreement with previous reports (Jung et al., 2012; Poulter et al., 2017), the dimerisation of GPVI in resting platelets was confirmed along with an increase in the level of dimerization

upon platelet activation, with 204-11Fab binding to platelets stimulated by CRP more than to unstimulated platelets (Figure 4.4 aii, bii). Treatment of resting platelets with SMIH (5 and 10µM) significantly reduced the basal level of dimerisation by 50% and 74%, respectively (Figure 4.4 ai, aii). Likewise, activated platelets pretreated with SMIH (5µM) showed 34% decrease in dimerization when compared to vehicle-treated platelets. Also, treatment with a higher concentration of SMIH (10µM) further inhibited level of dimerisation by 66% (Figure 4.4 bi, bii). This observation unravels pivotal and chief controlling aspect of HSP47 on the functional collagen binding GPVI structure and hence explains attenuated collagen and CRP-XL adhesion events, downstream of dimeric GPVI, in HSP47 inhibited platelets (section 4.2).

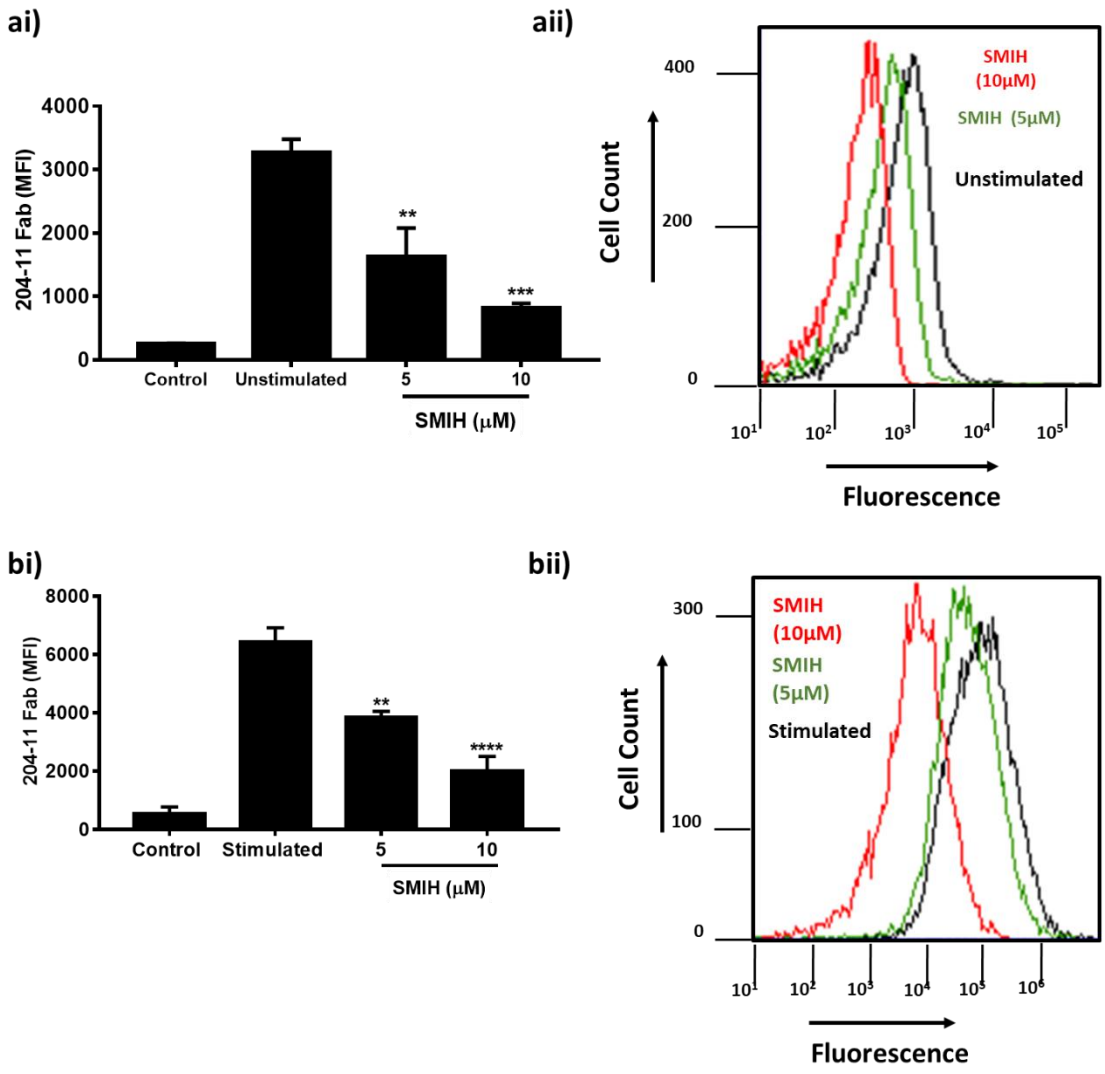


Figure 4.4: HSP47 supports GPVI dimerization in resting and stimulated platelet.

Whole blood was treated with SMIH (5 and 10μM) or vehicle for 10 minutes and treated with Tyrode's HEPES buffer unstimulated or stimulated with CRP-XL (5μg/ml) in presence of EDTA (2mM). Dimer specific m Fab 2014-11 or control human Fab was added and FITC-labelled anti-m Fab was added to detect antibody binding using flow cytometry. Binding of FITC-labeled 204-11 Fab to the vehicle and SMIH (5 and 10μM) pre-treated **(ai)** unstimulating and **(bi)** stimulated platelets are shown. FITC-labeled control mouse Fab was used as a control. Representative histograms of dimerisation in **(aii)** unstimulating and **(bii)** stimulated platelets in presence of vehicle or SMIH (5 and 10μM). Data represent mean ± SEM (n≥3), **P ≤ 0.01, ***P ≤ 0.001 and ****P ≤ 0.0001 were calculated by one-way ANOVA.

4.3.2 HSP47 associates with GPVI

GPVI localisation to lipid rafts has been established in the literature (Wonerow et al., 2002). Constitutive association of GPVI with membrane rafts has been reported by Ezumi et al. (2002), however, others have reported such association only when platelets are stimulated (Locke et al., 2002). Interestingly, a similar association with this signalosome enriched microdomain was exhibited by HSP47 as shown in section 3.6. Given such common rafts association together with the established impact of HSP47 on the basal levels of GPVI dimerisation, work was carried out investigate potential interactions between HSP47 and GPVI by which HSP47 may impact the functional structure of GPVI.

Co-immunoprecipitation (Co-IP) studies were performed, where an anti-HSP47 mouse monoclonal antibody was used to isolate HSP47 from resting and U46619 (5µM) activated human platelets. In this experiment, we avoided using collagenous agonists to stimulate platelets as both HSP47 and GPVI bind to these ligands which may leads to a false positive interpretation of the co-immunoprecipitation results. This was followed by a western blot analysis using an anti-GPVI rabbit polyclonal antibody to determine whether HSP47 and GPVI interact with each other. Control procedures were performed using an isotype-matched irrelevant antibody (Figure 4.5).

As shown in Figure 4.5 a, an equal amount of HSP47 present in both resting and stimulated platelet lysates. GPVI was found to co-immunoprecipitate with HSP47 from both resting and activated platelets (Figure 4.5 a). We also noted that stimulated platelets showed lower levels of HSP47-GPVI association when compared to the levels observed in resting condition (Figure 4.5 c), which may reflect dissociation and /or reduced levels of association

following platelet stimulation. In contrast, when reversing immunoprecipitation and detection antibodies (not shown), HSP47 was undetectable in GPVI immunoprecipitates under both resting and stimulated conditions.

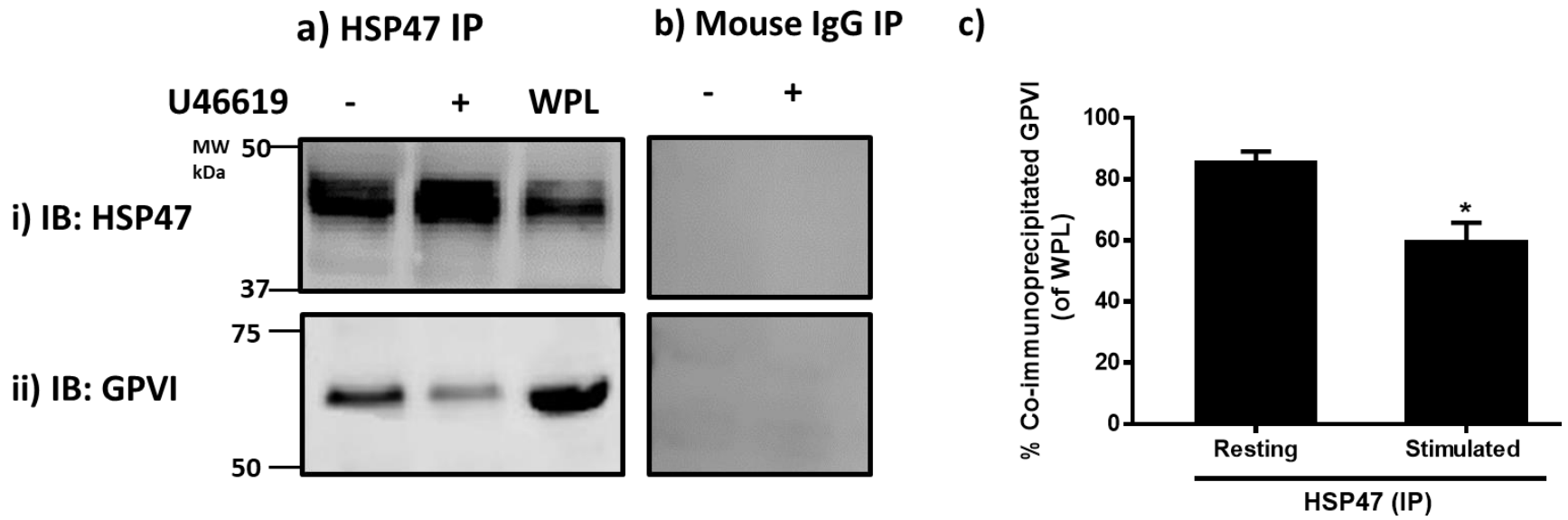


Figure 4.5: HSP47 interacts with GPVI in resting and stimulated platelets.

Interaction of HSP47 with GPVI was investigated in human platelets using a Co-IP assay. Human washed platelets ($8x10^8$) were stimulated with HEPES tyrodes buffer or U46619 (5µM) and lysed in 2 Nonidet P-40 lysis buffer. Protein A/G magnetic beads pre-coupled to mouse anti-HSP47 were incubated with precleared lysates for 3 hours before washing and resuspension in SDS-PAGE sample buffer. Proteins were resolved on SDS-PAGE, transferred to nitrocellulose, and probed for **(a)** immunoprecipitated HSP47 and **(b)** co-immunoprecipitating GPVI. **(c)** Quantified data represents the percentage of GPVI in resting and stimulated HSP47 IP samples (the amount of GPVI protein available for co-ip in WPL represents 100% GPVI). Isotype-matched immunoprecipitations for mouse antibody confirmed the absence of nonspecific binding to the IgG. Data represent mean ± SEM (n=3), *P ≤ 0.05 was calculated by *t*-test. WPL, whole platelet lysate; IP, immunoprecipitation; IB, immunoblot.

4.3.3 Visualization of HSP47-GPVI interaction

4.3.3.1 HSP47-GPVI colocalisation in human platelet

Co-immunoprecipitation studies showed HSP47 interaction with GPVI (Figure 4.5). Co-immunofluorescent labelling followed by visualisation using confocal microscopy was employed to gain a closer look into the subcellular association of these proteins in human platelets. Whole blood was centrifuged and PRP was allowed to rest at 37°C for 30 minutes. The thromboxane A2 analogue, U46619 (5µM), was used to stimulate platelets in presence of integrilin, an integrin αIIbβ3 antagonist, to prevent aggregation of stimulated platelets. U46619 was chosen to stimulate platelets without drastic cytoskeletal shape changes that could avert from obtaining a clear visualisation of colocalising signals in stimulated platelets. Stimulated and resting platelets were then fixed and permeabilised using 4% (v/v) formaldehyde and 0.2% (v/v) Triton™ X-100, respectively. Platelets were then probed with mouse anti-HSP47 antibody and rabbit anti-GPVI antibody. Whereas HSP47 and GPVI primary antibodies were omitted in negative control platelets. Lastly, Alexa Fluor®647- and Alexa Fluor®488-conjugated secondary antibodies were added to detect HSP47 and GPVI, binding as shown in red and green, respectively (Figure 4.6). In resting platelet HSP47 was found to distribute in the cytosol along with close localisation to platelet membrane where surface receptor GPVI was predominantly distributed (Figure 4.6 a). On the other hand, in stimulated platelets HSP47 mobilised toward platelet membrane and colocalised with GPVI (Figure 4.6 b).

To assess the level of HSP47 and GPVI colocalisation, Pearson correlation coefficient (PCC) was employed. HSP47 and GPVI signals scored average PCC values of 0.62±12 and 0.80±07

in resting and stimulated platelets, respectively, reflecting their colocalisation. Unlike the reduction in HSP47-GPVI association seen in co-ip studies (Figure 4.5), stimulated platelet scored high PCC value which may be attributed to the surface mobilisation of HSP47 upon stimulation.

Having established HSP47-GPVI interaction at a microscopic level, work was expanded to examine the colocalisation on collagen fibers coated surface. In alignment with the previous observation of HSP47 interaction and colocalisation with GPVI (Figure 4.5 and Figure 4.6), HSP47 and GPVI seem to show a certain extent of colocalisation in collagen clustered platelets (Figure 4.7). Additionally, demonstration of HSP47 and GPVI colocalisation was also depicted in the profile line intensities of the fluorescent signals. This parameter, line profiles intensities of pixels generated from fluorophore probed targets, measures its distance in the image and hence compares the degree of colocalisation of the probed targets (Kreft et al., 2010). A general trend of overlapping was observed between HSP7 and GPVI fluorescent signals as shown in Figure 4.7.

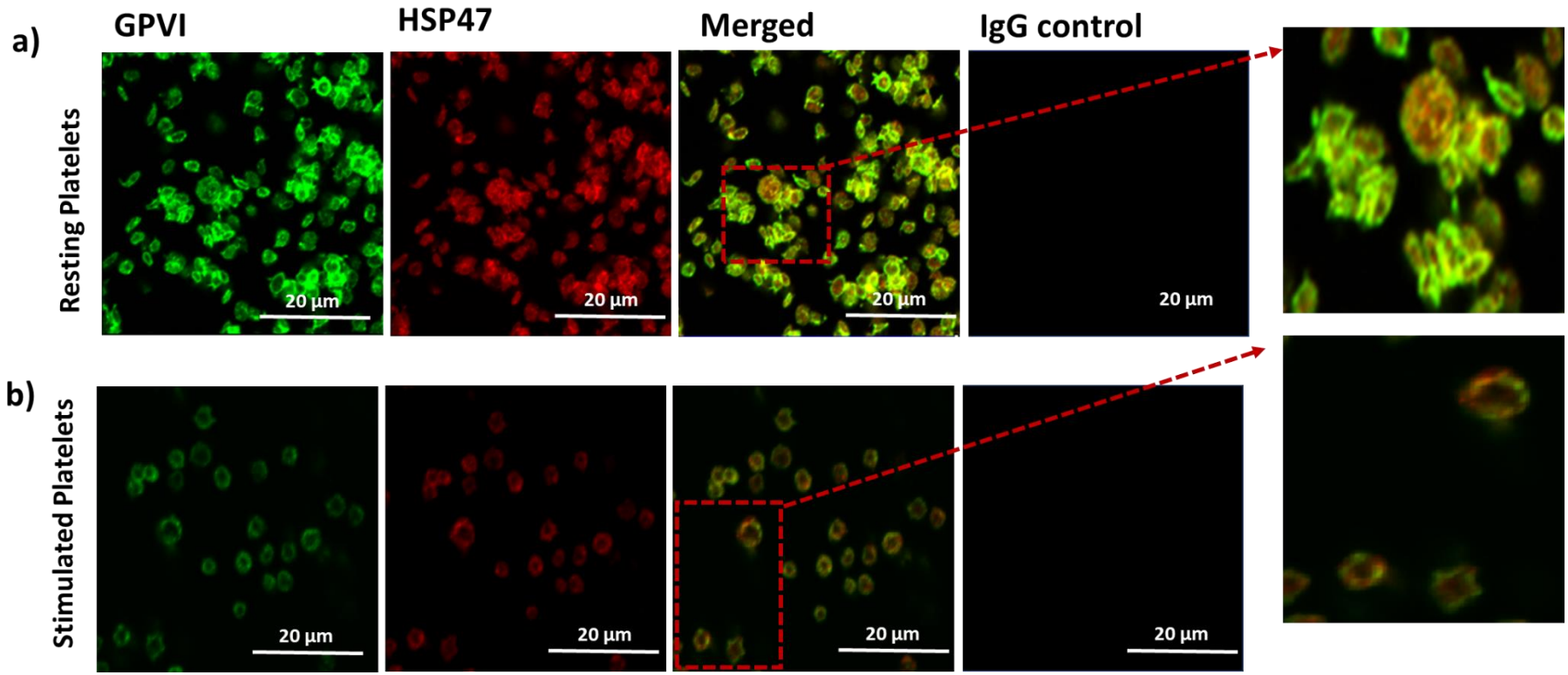
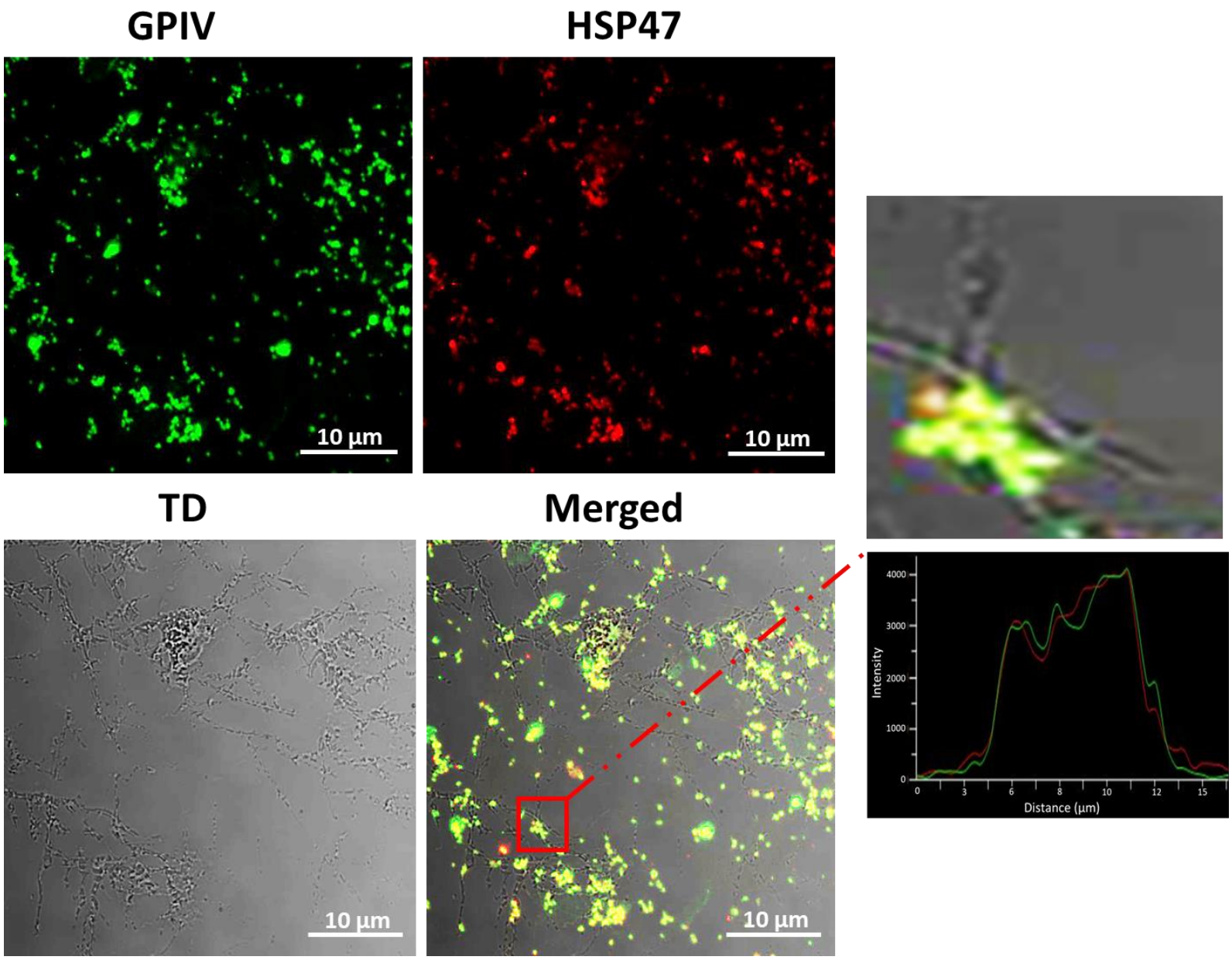


Figure 4.6: HSP47 and GPVI are colocalised within human platelets.

Immunofluorescence studies of HSP47 and GPVI were examined using confocal microscopy. Resting and (5µM U46619) stimulated human platelets were fixed with 4% (w/v) paraformaldehyde and permeabilised using 0.1% (v/v) Triton™ X-100. HSP47 (in red), GPVI (in green) were stained using anti-HSP47 and anti-GPVI antibodies and detected using Alexa-647 and Alexa-488 conjugated secondary antibodies, respectively. Platelets were visualised using a Nikon A1-R confocal microscope (100× oil immersion lens). Representative images of the distribution of HSP47 and GPVI in **(a)** resting and **(b)** stimulated platelets. Data are representative of >3 separate experiments.

Figure 4.7: HSP47 and GPVI colocalises within platelets spread on collagen fibers.

Co-fluorescent studies of HSP47 and GPVI colocalisation was visualised on collagen fiber spread platelets using confocal imaging. Human washed platelets (2×10^7) were added onto glass coverslips precoated with collagen (100 µg/ml). The supernatant was removed adhered platelets were fixed with 4% (w/v) paraformaldehyde and permeabilised using 0.1% (v/v) Triton™ X-100. HSP47 (in red), GPVI (in green) were stained using anti-HSP47, anti-GPVI antibodies respectively and probed proteins were then detected using corresponding secondary antibodies conjugated to Alexa-647, Alexa-568 were used to visualize HSP47 and GPVI, respectively. Collagen adhered platelets were visualised using a Nikon A1-R confocal microscope (100× oil immersion lens). Representative images showing the distribution of HSP47 and GPVI in collagen spread platelets along with transmitted light (TD) image showing collagen fibers coating. The profiles of the fluorescent intensities represent HSP47 (red) and GPVI (green) in the red boxed collagen spread platelet. Data are representative of >3 separate experiments.


GPIV
HSP47
10 µm
10 µm
TD
Merged
10 µm
10 µm
4000
3000
2000
1000
0
Intensity
0
3
6
8
10
12
15
Distance (µm)

4.3.3.2 Surface Interaction of HSP47-GPVI Studied using Super-Resolution Microscopy (STORM)

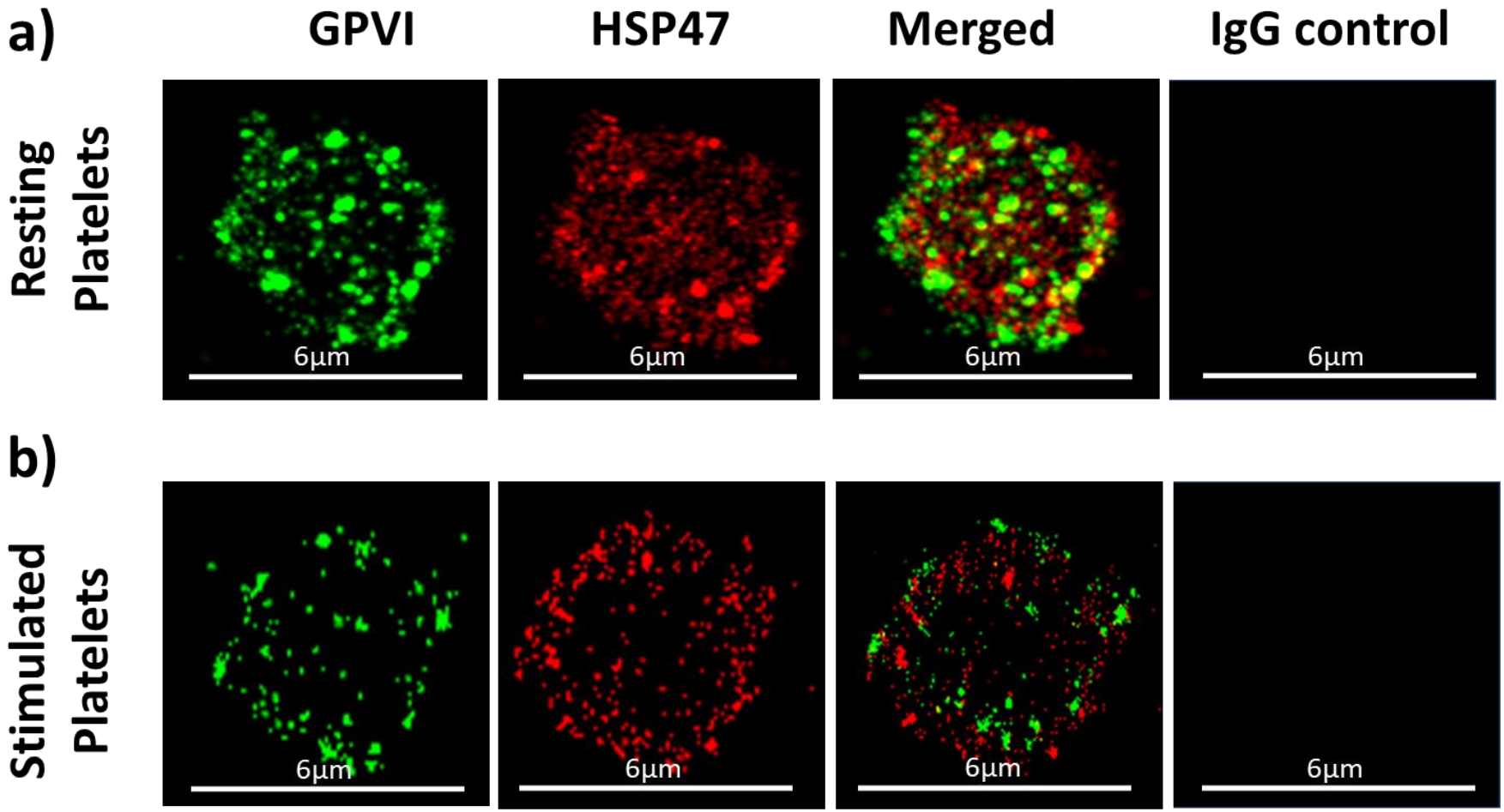
Confocal microscopy imaging of HSP47 and GPVI co-fluorescent studies suggested their colocalisation. However, since the resolution of confocal microscopy is limited to around ~250 nm (White et al., 1987; Westmoreland et al., 2016), delineation in small-sized platelets, 2–5 μm in diameter, (Ghoshal and Bhattacharyya, 2014) could be challenging. Search was broadened for other optical systems that would overcome such pitfall. Stochastic optical reconstruction microscopy (STORM) delivers imaging with superb resolution enabling subcellular non-structural visualisation within 20 to 25 nm resolution range (Olivier et al., 2013) making it ideal tool to address such colocalisation within platelet. Therefore, this section employed STORM to visualise HSP47 and GPVI colocalisation in permeabilised resting and activated human platelets.

Following the differential centrifugation and collection of PRP from the whole blood, PRP was allowed to rest at 37 °C for 30 minutes. PRP was then diluted in HEPES buffer and resting and (5μM) U46619 stimulated platelets, in the presence of integrilin, were fixed in 2% [v/v] formyl saline followed by centrifugation at 500 *g* for 15 minutes. The pellet was resuspended in permeabilisation buffer (BD Phosflow Perm Buffer III) and resuspended platelet suspension was washed twice in HEPES buffer. Mouse monoclonal anti-HSP47 and rabbit anti-GPVI antibodies were added and primary antibodies were omitted as negative controls. Alexa Fluor® 647 (red) and Alexa Fluor® 568 (green) conjugated secondary antibodies were used to stain HSP47- and GPVI-labelled platelets. Platelets were visualised using a Nikon N-STORM using a 100× oil immersion lens and NIS-Elements software was employed for data acquisition.

STORM images showed dual localisation of HSP47 and GPVI in resting platelets where HSP47 and GPVI molecules observed distributed on platelet surface with populations of both proteins scattered cross the cytosol (Figure 4.8 a). We found overlapping colocalising signals toward platelet surface. However, HSP47 and GPVI showed different distribution upon platelet activation with clustered arrangement appeared concentrated at platelet periphery (Figure 4.8 b), which may account for lower levels of association observed in section 4.3.2.

Figure 4.8 STORM imaging of HSP47-GPVI colocalisation in resting and stimulated human platelets.

HSP47 and GPVI colocalisation as observed in STORM optical system. **(a)** Resting and **(b)** (5µM) U46619 stimulated platelets were fixed in 2% (v/v) formyl saline and followed with permeabilisation with BD Phosflow™ Perm Buffer III. Platelets were then incubated with HSP47 and GPVI antibodies and Alexa Fluor® 647 (red) and Alexa Fluor® 555 (green) conjugated secondary antibodies were then added to stain HSP47- and GPVI-labelled platelets, respectively. Nikon N-STORM, NIS-Elements and a 100× oil immersion lens were used for visualisation and image acquisition. Data are representative of >3 separate experiments.


a)
GPVI
HSP47
Merged
IgG control
Resting Platelets
6µm
6µm
6µm
6µm
b)
Stimulated Platelets
6µm
6µm
6µm
6µm

4.3.4 The inhibitory effect of SMIH on collagen-induced platelet activation is dependent on GPVI.

In the present study, several approaches were employed to explore the physical interaction of HSP47 with GPVI. However, this conclusion doesn't exclude the ability of HSP47 to interact with other platelet receptors or proteins. GPVI blockers provide an excellent approach to address such a hypothesis and to examine HSP47-GPVI dependency. Murine monoclonal antibody-derived Fab (9O12) has been well established and characterized to specifically block human GPVI (Lecut et al., 2003). It is high-affinity and inhibitory effect not only completely blocks collagen-induced platelet aggregation but also prevents thrombus formation under both static and flow conditions (Mangin et al., 2012).

The aim of this section was to determine whether diminished platelet activation occurring during HSP47 inhibition in response to collagen stimulation (Sasikumar et al., 2018), was due to the reduction in activation of GPVI or that HSP47 is also involved in down-regulating other aspects of platelets function. Therefore, the levels of fibrinogen binding to integrin αIIbβ3 was evaluated upon treatment with SMIH in presence of inhibitory 9012 Fab.

Human PRP was allowed to rest for 30 minutes at 37°C, followed by the addition of FITC-labelled rabbit anti-human fibrinogen antibody diluted in modified Tyrodes HEPES buffer. The samples were incubated with vehicle (DMSO 0.12% v/v and Fab control), 9012 Fab (35 µg/mL) or 9012 Fab (35 µg/mL) combined with SMIH (20 µM) for 10 minutes. In an aim to reach absolute confidence in excluding other potential actions of HSP47 on various platelet functions, a higher concentration of SMIH was used. Vehicle and inhibitor-treated platelets were then stimulated with CRP-XL (0.5 µg/mL) and after 20 minutes, samples were fixed with

0.2% (v/v) formyl saline. Median fluorescence intensity was measured for 10,000 platelet-gated events using a flow cytometer.

The addition of 9012 Fab blocker to PRP reduced the fibrinogen binding by ~90% in platelets stimulated with CRP-XL (0.5µg/ml). However, no further reduction was seen upon the addition of SMIH (20µM) (Figure 4.9). Taken together, these findings suggest that HSP47 supports collagen-mediated platelet functions in a manner that is dependent on GPVI activation.

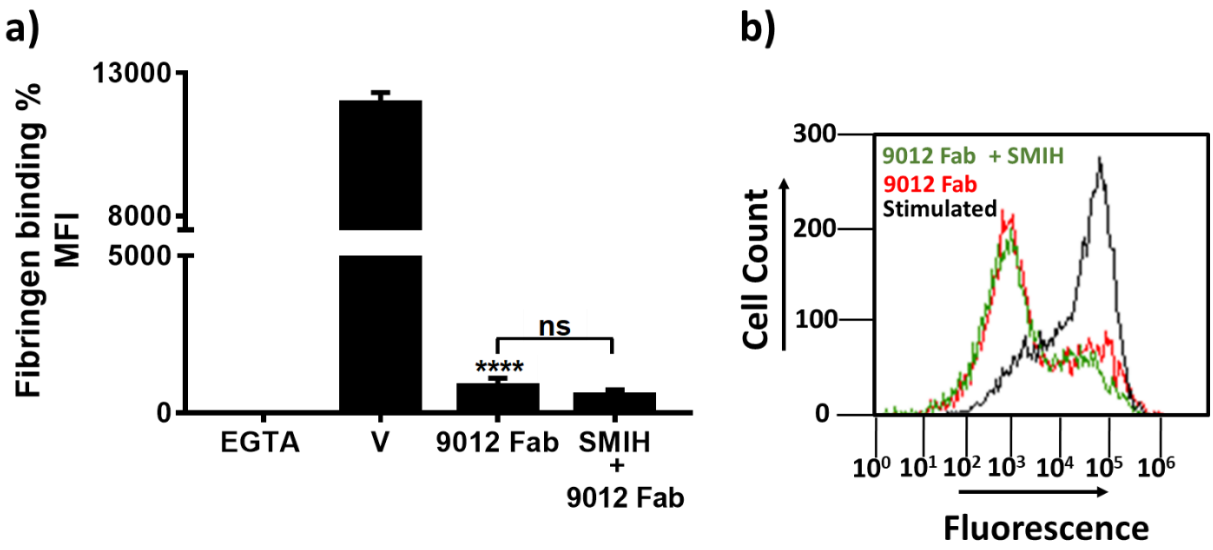


Figure 4.9: Effects of SMIH on fibrinogen binding to integrin αIIbβ3 are mediated through the inhibition of GPVI activation.

Human PRP was incubated with vehicle (containing, DMSO 0.12% v/v with Fab control) or Fab 9012 (35 µg/mL) or combination of Fab 9012 (35 µg/mL) with SMIH (10 µM) for 10 minutes. Platelets were stimulated with CRP-XL (0.5 µg/mL) for 20 minutes and analysed by flow cytometry. Integrin $\alpha_{IIb}\beta_3$ activation was measured by the binding of anti-fibrinogen antibody to the platelets. **(a)** Bar chart represents the median fluorescence intensity of fibrinogen binding in the presence of vehicle or Fab 9012 or combination of Fab 9012 with SMIH. Resting platelets response to FITC-conjugated anti-human fibrinogen antibody treatment served as negative control and also shown (EGTA). **(b)** Histograms of fibrinogen binding for CRP-XL stimulated platelets in the presence of vehicle (black) and CRP-XL -stimulated platelets in the presence of Fab 9012 (red) or combination of Fab 9012 with SMIH (green). Data are shown as mean ± SEM (n = 3) was calculated by one-way ANOVA ****$P \leq 0.0001$.

4.4 HSP47 supports platelet adhesion independently of integrin α2β1

SMIH treatment did not alter platelet binding to integrin α2β1 selective ligand, GFOGER (section 4.2). Moreover, HSP47 inhibition had no effect on GPVI blocked platelets when tested for fibrinogen binding to integrin αIIbβ3 (section 4.3.4). To further confirm a lack of direct effect of HSP47 on integrin α2β1, work was extended to examine integrin β1 activated conformation upon platelet activation in presence of SMIH.

In this study, washed platelets (4×10^8 cell/mL) were treated with vehicle containing DMSO (0.12% v/v) or SMIH (5 and 10 μM) for 10 minutes. Treated platelets were then added onto GFOGER-coated wells (50 μg/ml) at 37°C. Samples were lysed in Laemmli sample buffer after 30 minutes and adhered cells were scraped off using a glass rod. Platelets were also stimulated with CRP-XL (1μg/ml) and collagen (3μg/ml) for 90 seconds under stirring condition and platelets were then lysed with Laemmli sample buffer. The activated conformation of integrin β1 was measured by Western blotting using monoclonal conformation antibody specific for the active conformation of human β1 integrins (Luque et al., 1996).

Adhesion of platelets to GFOGER induced a marked increase in the level of active conformation of human β1 integrins at 30 min in comparison to resting sample (Figure 4.10 a). SMIH treatment had no effect on integrin activation as shown in the similar activation levels when compared with vehicle-treated sample (Figure 4.10 a). In comparison to the vehicle sample, pre-treatment of platelets with SMIH (5 and 10 μM) for 10 minutes inhibited collagen (3μg/ml) and CRP-XL (1 μg/ml) (Figure 4.10 b and c, respectively) mediated integrin β1 activated conformation by 41%, 73% and 60%, 71%, respectively. Based on these

observations, we can conclude that integrin activated confirmation is indirectly supported by HSP47 via GPVI.

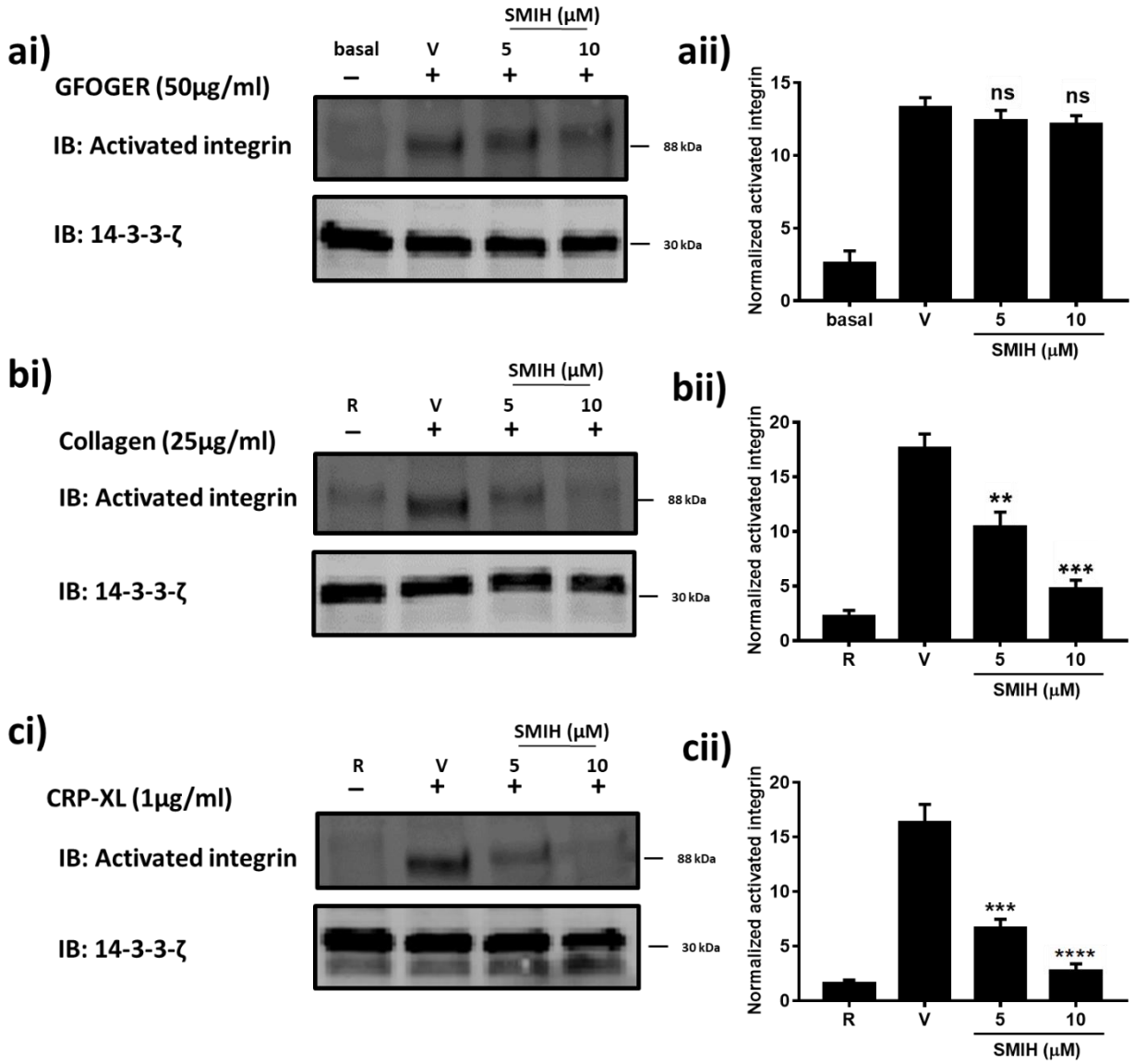


Figure 4.10: HSP47 modulates integrin β1 activated conformation indirectly via GPVI.

Washed platelets ($4x10^8$ cells/ml), pre-treated with SMIH (5 and 10 μM) or vehicle-control (containing, DMSO 0.12% v/v) were exposed to 1%BSA or GFOGER-coated wells (50 μg/ml) of a tissue culture plate and allowed to adhere at 37°C, or stimulated in suspension with CRP-XL (1 μg/mL), or collagen (3 μg/mL) for 90 seconds under stirring condition. Laemmli sample buffer was used to lyse the samples, which were separated by SDS-PAGE and then transferred to PVDF membranes. Integrin β1 activated confirmation-specific antibody was used to determine receptor activation levels. Representative immunoblots are shown for the integrin activated confirmation levels in **(a)** GFOGER, **(b)** CRP-XL and **(c)** collagen in the vehicle and SMIH pre-treated platelets. 14-3-3-ζ was detected by immunoblotting as the loading control and quantifications of the activation levels are presented as the mean relative to 14-3-3-ζ. The results represent the mean ± SEM ($n \geq 3$). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ were calculated by one-way ANOVA. ns: not significant.

4.5 HSP47 treatment of collagen does not enhance platelet adhesion on collagen

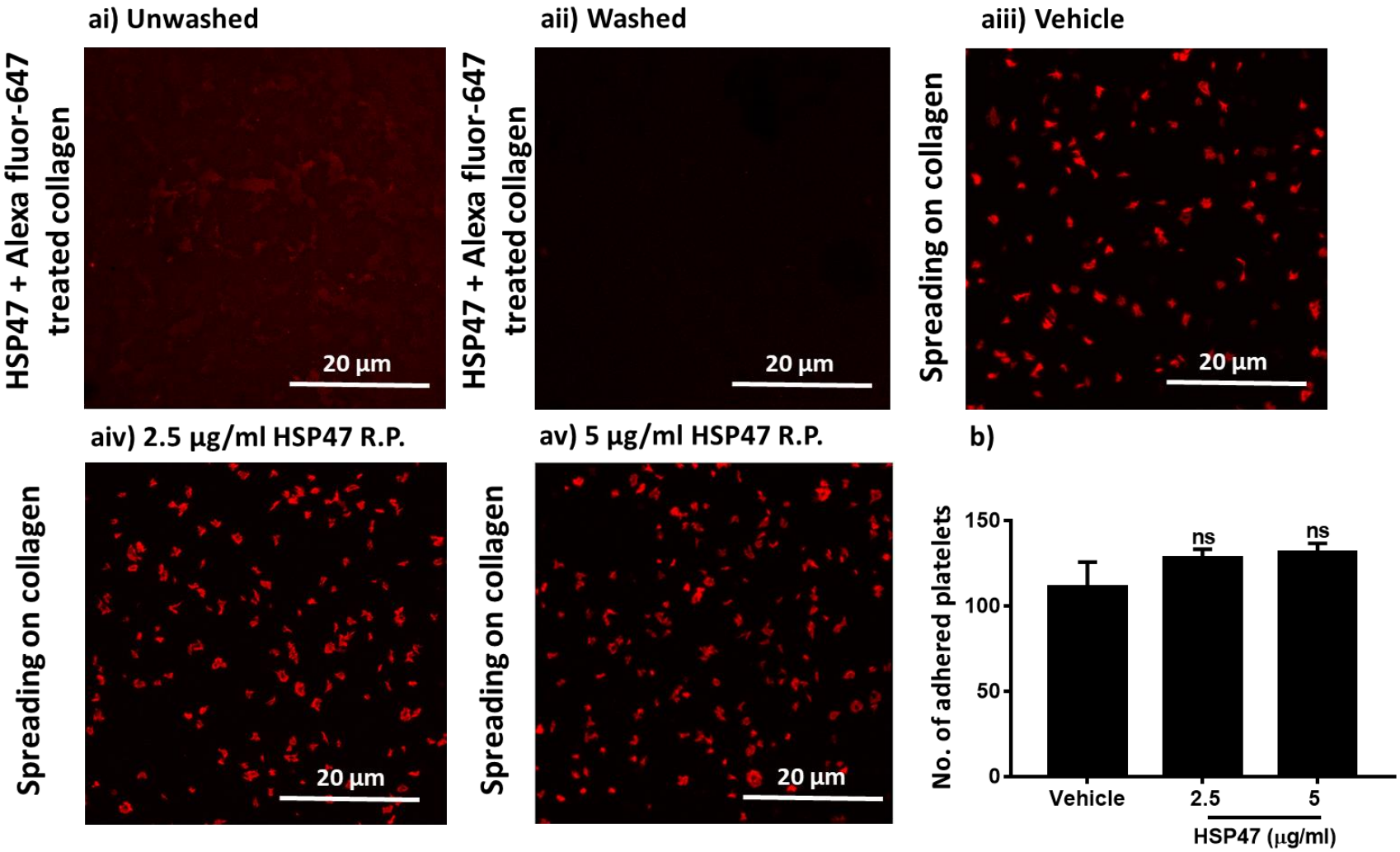
Our data suggest that HSP47 may support GPVI dimerization, subsequent integrin α2β1 activation and hence contributes to firmer and stabilised platelet collagen binding. Knowing the chaperone function of HSP47 in collagen-producing cells (Lamande and Bateman, 1999), work was carried out to examine whether HSP47 would modulate collagen and hence influence its binding by platelet receptors. In this section potential enhancement in platelet static adhesion on HSP47 recombinant protein pre-treated collagen was evaluated.

Glass coverslips were pre-coated with collagen (100 μg/ml) for 1 hour and incubated with BSA for 30 minutes to prevent platelet-glass binding. Coverslips were then treated with HSP47 recombinant proteins (2.5 and 5μg/ml) and equivalent level of BSA was added to untreated collagen-coated coverslips. After 30 minutes coverslips were subjected to prolonged washing steps to remove the protein. Unwashed and washed collagen-coated coverslips were stained for HSP47 and used as positive and negative control of HSP47 treatment. Washed platelets (2×10^7 cells/ml) were added onto the treated and untreated coated coverslips at 37ºC. After 45 minutes, the supernatant was removed, and coverslips were washed with PBS. Samples were fixed with 0.2% (w/v) paraformaldehyde, washed with PBS, and adhered platelets were permeabilised with 0.2% (v/v) Triton™ X-100 followed by washing with PBS. The platelet cytoskeleton was probed with Alexa-Fluor 647 phalloidin and Prolong Gold Antifade mounting media was used to mount coverslips onto slides using. Visualisation was performed using a Nikon A1-R confocal microscope (100× oil immersion lens). Five randomly selected images were captured of each sample.

The presence of HSP47 staining in unwashed control confirmed its binding and hence proposed collagen treatment (Figure 4.11 ai). On the other hand, complete removal of the protein was achieved in washed coverslip as indicated by the lack of staining for HSP47 (Figure 4.11 aii). Exposure of collagen to two levels of HSP47 recombinant proteins (2.5 and 5µg/ml) did not enhance platelet binding to collagen when compared to the levels of platelet binding to untreated collagen (Figure 4.11 aiii, aiv and av). Together, it seems that HSP47 is less likely to contribute to collagen-platelet interaction via modulating collagen, a phenomenon that is observed during collagen biosynthesis in collagen-producing cells (Nagata, 1996; Lamande and Bateman, 1999; Tasab et al., 2000).

Figure 4.11: Platelet adhesion is not enhanced by pre-treatment of collagen with HSP47.

Pre-coated collagen (100 μg/ml) coverslips were treated with BSA or HSP47 recombinant protein (2.5 and 5 μg/ml) followed by multiple washing steps. Human washed platelets (2×10^7 cells/ml) were then added onto collagen-coated coverslips at 37ºC and after 45 minutes supernatant was removed and adhered platelets were fixed and permeabilised with 0.2% (w/v) paraformaldehyde and 0.2% (v/v) Triton™ X-100, respectively. Alexa-Fluor 647 phalloidin was used to stain platelets actin cytoskeleton for 1 hour and coverslips were mounted onto glass slides using Prolong Gold Antifade mounting media. As controls for HSP47 treatment and washing procedure, unwashed and washed treated collagen were stained for HSP47 and detected using Fluor®647. Nikon A1-R confocal microscope with a 100× oil immersion lens was used to aid in the visualisation of samples and five randomly selected images were captured of each sample. Representative image of platelet adhesion on **(aiii)** untreated and **(aiv, av)** treated collagen is shown. **(b)** An average number of platelets adhered in each sample are shown. Images of **(ai)** unwashed and **(aii)** washed HSP47 treated collagen-coated coverslips controls are also shown. Data represent mean±SEM (n=3), one-way ANOVA. ns: not significant. Abbreviation: R.P.: recombinant protein


ai) Unwashed
HSP47 + Alexa fluor-647 treated collagen
20 µm
aii) Washed
HSP47 + Alexa fluor-647 treated collagen
20 µm
aiii) Vehicle
Spreading on collagen
20 µm
aiv) 2.5 µg/ml HSP47 R.P.
Spreading on collagen
20 µm
av) 5 µg/ml HSP47 R.P.
Spreading on collagen
20 µm
b)
No. of adhered platelets
150
100
50
0
ns
ns
Vehicle
2.5
5
HSP47 (µg/ml)

4.6 Discussion

Over the last decades, research has focused on unravelling the identities of the collagen interaction receptors along with their individual contribution in this integrated process of haemostasis. However, the diversity of these receptors and multiplicity of platelet responses to collagen, along with other proteins discovered in platelets have questioned the presence of other not yet revealed platelets proteins that might have a role in this highly integrated process of collagen recognition (Nieswandt and Watson, 2003).

Collagen molecular chaperone HSP47 is among these newly unraveled platelets proteins that have been proposed to be implicated in this pivotal collagen interaction. Such a proposal was based on the reported effects of HSP47 inhibition or depletion on collagen-mediated platelets functions. However, studies were limited to the establishment of HSP47 role in several collagen-induced platelet functional assays *in vivo* and *in vitro* and hence underlying mechanism of actions has not been established (Kaiser et al., 2009; Sasikumar et al., 2018). Out of the available and limited literature of HSP47 in platelets, the scope of the present study was to investigate the mechanism and nature of actions behind the established role of this chaperone in platelet regulation.

4.6.1 HSP47 participates in the binding of GPVI to CRP-XL and hence contributes to platelet-collagen interaction.

Previous studies established the role of HSP47 in mediating various aspects of platelets activation in response to collagen. Additionally, in the light of reported evidence of HSP47 supporting platelet adhesion to collagen (Sasikumar et al., 2018), a proposal was raised that

such protein is important in regulating collagen-mediated platelet functions via early events when circulating platelets encounter collagen exposed at the site of damaged vessels.

In the present study platelet adhesion to collagen and CRP-XL coated surfaces was reduced in presence of SMIH. However, platelet treatment with SMIH did not affect the ability of platelet to adhere to GFOGER (section 4.2). Moreover, it has been reported that platelet adhesion on vWF coated surface under arterial flow conditions was not affected in the presence of HSP47 inhibitors and hence it seems that HSP47 is not involved in the initial tethering of platelets via GPIb (Sasikumar et al., 2018). Taken together, these findings suggest HSP47 contribution to an adhesive event in platelet upon the encounter with collagen via GPVI. Lastly, such action may explain reported platelet functions affected by the inhibition or ablation of HSP47 (Kaiser et al., 2009; Sasikumar et al., 2018).

4.6.2 HSP47 supports GPVI dimerization and interacts with the receptor in platelet

Basal levels of GPVI dimer in resting platelets was significantly reduced upon inhibition of HSP47. A similar reduction in the dimerization was also seen in activated platelets. These observations were made with the help of m-Fab 204-11 that specifically recognizes dimeric GPVI. Also, this inhibition is less likely due to m-Fab 204-11 binding to collagen binding site of dimeric GPVI as this Fab did not inhibit dimer binding to immobilized triple helical (GPO)10-containing peptide (Jung et al., 2012).

GPVI co-immunoprecipitated with HSP47 and this interaction was further confirmed in confocal and STORM images. Also, the reduction of HSP47-GPVI association following platelets activation may reflect a mechanistic aspect of HSP47 function. Hence, in the light of these observations, we proposed that such interaction in resting platelets would facilitate the maintenance of basal GPVI dimerization by HSP47 and participate in priming platelet

for rapid collagen response. In fact, substrate-chaperone dissociation is exhibited by HSP47 in collagen-secreting cells during collagen synthesis (Thomson and Ananthanarayanan, 2000), therefore, it is possible that platelet HSP47 exerts similar phenomenon. Lastly, we were unable to co-immunoprecipitate HSP47 with GPVI using GPVI antibodies for immunoprecipitation. A possible reason is that epitope of the immunoprecipitating GPVI is at the interaction site of the two proteins, hence hindering the pulldown of HSP47 along with GPVI.

The mechanisms underlying HSP47 sustaining dimeric configuration of GPVI in quiescent and stimulated platelets remain to be clarified. In the literature, targeting platelet cytoskeleton and actin dynamics using various inhibitors of actin polymerisation, such as latrunculin A, reduced levels of GPVI dimer in resting and activated platelets (Poulter et al., 2017) suggesting a vital contribution of platelet cytoskeleton in the formation of GPVI dimer. Interestingly, literature reports the implication of HSP47 in the cytoskeletal reorganization of the actin filaments. The increased expression level of actin filaments was reported in phenotypically-altered, HSP47-producing renal cells in chronic diabetic rats (Liu et al., 2001). Another study revealed that collagen-binding HSP47 is associated with alteration of actin filaments in interstitial and epithelial cells (Razzaque et al., 1998). Additionally, cytoskeletal reorganization of the actin filaments has been reported to be affected by the activity of HSP47 in Caco-2 cells (Han et al., 2002). More importantly, the actin antagonist latrunculin A, reduced HSP47 surface mobilisation upon platelets stimulation (section 3.6). Together, these findings propose a possible mechanism of HSP47 supporting GPVI dimeric state via reorganization of platelet cytoskeleton. However, such a proposal requires further validation.

A pivotal role for HSP47 in maintaining GPVI dimerization could explain the observed effects of HSP47 inhibition on platelet adhesion (section 4.2) and on other reported aspects of collagen-mediated platelet function (Kaiser et al., 2009; Sasikumar et al., 2018) as the use of GPVI dimer inhibitory Fab has been reported to reduces GPVI dimer interaction with collagen fibers, collagen-mediated platelet aggregation (Jung et al., 2009) and platelet adhesion to collagen-coated surfaces (Jung et al., 2012) and hence highlighting the physiological importance of regulating GPVI dimeric configuration on platelet surface.

4.6.3 HSP47 contribution to collagen-mediated platelet activation depends on GPVI.

Integrin α2β1 activated configuration was down-regulated by inhibition of HSP47 when stimulated with collagen, CRP-XL, but not with GFOGER. This suggests that HSP47 affects integrin activation indirectly through GPVI. Moreover, to confirm dependency on GPVI, blocking antibody Fab fragment 9O12, directed against human GPVI, was used. This antibody is reported to markedly decreased various collagen and CRP-XL mediated platelets responses (Lecut et al., 2003; Muzard et al., 2009; Mangin et al., 2012). In addition to its inhibitory properties, this blocker displays high level of specificity and selectivity toward GPVI (Lecut et al., 2003), rendering it an excellent choice to address the aforementioned dependency. Given the reported attenuation of platelet aggregation in response to collagenous agonists only (Sasikumar et al., 2018), HSP47 dependency on GPVI was assessed by inspecting the activation levels of integrin αIIbβ3 on stimulated platelets indirectly using a fibrinogen binding assay (Ma et al., 2007). SMIH failed to induce a further inhibitory effect on the levels of fibrinogen binding to integrin αIIbβ3 on activated platelets pre-treated with Fab 9O12 blocker. These findings emphasise that the underlying role by which HSP47 regulates platelet functions is dependent on GPVI modulation. Furthermore,

reported reduced convulxin induced platelet aggregation in presence of SMIH (Kaiser et al., 2009) and the fact that convulxin and collagen have different structures (Francischetti et al., 1997), suggested an alternative mode of action by HSP47 in the regulation of platelet activation other than the well-known and reported collagen binding chaperoning activities of this protein (Tasab et al., 2000). These findings together with the lack of enhancement noted in platelet adhesion to HSP47 recombinant protein pre-treated collagen confirmed the aforementioned mode of action.

In summary, despite the existence of several effective antithrombotic agents, thrombotic disorders are yet foremost burning concerns in western countries. Further understanding of platelets initial response to injury and the involvement of platelet receptors in this multifactorial process attracts notable research interests in a pursuit to achieve wider comprehension of the primary haemostatic events and underlying molecular mechanisms preceding thrombus formation. This chapter focused on understanding the nature of HSP47 contributions in platelet-collagen interaction. We conclude that HSP47 modulates platelet collagen adhesion and activation in a manner that is dependent on GPVI and independent of other platelet receptors. Moreover, HSP47 was shown to be essential to maintain a basal level of GPVI dimerization. Lastly, within the employed experimental approach, HSP47 has not demonstrated any modulation on collagen substrate which may result in detectable modulation of platelet-collagen interactions.

5 HSP47 regulates GPVI-mediated signalling in platelets.

5.1 Introduction

Platelets maintain minimal contact with each other while circulating in the vasculature but contact occurs rapidly brought encountering damaged vessels to enable platelets aggregation and consequent thrombus formation (Humbert et al., 1996). However, unnecessary platelet contacts and activation result in the generation of thrombotic events and hence compromising blood flow in the circulation. Such blockage is often seen following rupture of atherosclerotic plaque lesions and exposure of subendothelial collagen leading to ischemic episodes and development of various thrombotic disorders such as stroke and heart attack (Adiguzel et al., 2009). GPVI has been established as a major collagen receptor inducing vital activatory signalling events (Gibbins et al., 1997; Nieswandt and Watson, 2003) along with its involvement in precipitating several thrombotic disorders (Induruwa et al., 2016).

Signalling pathways proximal to GPVI receptor commences with a multistep cascade of tyrosine phosphorylation events (Gibbins et al., 1996; Andrews et al., 2014; Stegner et al., 2014). Collagenous substrates induce clustering of GPVI receptors and consequently, activation of Src family kinases (SFKs) which then catalyse the phosphorylation of the tandem tyrosine residues in the immunoreceptor tyrosine-based activation motif (ITAM) containing FcRγ-chain (Gibbins et al., 1997). Spleen tyrosine kinase (Syk) is then recruited and auto-phosphorylated allowing subsequent phosphorylation of linker for activation of T cells (LAT) (Pasquet et al., 1999b). This results in the assembly of LAT-signalosome which serves as docking site for the recruitment of phospholipase Cγ2 (PLCγ2), phosphatidylinositol-3 kinase (PI3K) and phosphorylation of phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-3,4,5-trisphosphate (PIP3) (Gibbins et al.,

1998; Stegner and Nieswandt, 2011; Andrews et al., 2014; Stegner et al., 2014) enabling the colocalisation of Bruton's tyrosine kinase (Btk) and its PLCγ2 substrate at plasma membrane (Quek et al., 1998).

The phosphorylation and activation of PLCγ2 results in cleavage of PIP2 and generation of secondary messengers, inositol (1,4,5)-trisphosphate (IP3) and diacylglycerol (DAG), which promotes calcium mobilisation and activation of protein kinase C (PKC), respectively. Further downstream events take place in activated platelets including degranulation, upregulation of integrin αIIbβ3 affinity and lastly, platelet aggregation (Nieswandt and Watson, 2003; Li et al., 2010; Stalker et al., 2012; Dunster et al., 2015). Platelet aggregation is augmented through the actions of molecules that are released during activation thereby ensuring a rapid and full response. ADP and TxA2 are among these molecules that are released from stimulated platelets and bind to $P2Y_1$, $P2Y_{12}$ (ADP) and TP (TxA_2) receptors (Jantzen et al., 1999; Li et al., 2003).

Sasikumar et al. (2018) reported inhibition or depletion of HSP47 resulted in the attenuation of various platelets activities, such as aggregation, degranulation, outside-in signalling, Ca^{2+} mobilisation, haemostasis and thrombus formation (*in vitro* and *in vivo*). Such aspects of platelet functioning are vital for platelet activation and thrombus formation and are directly influenced by different signalling pathways in platelets. Moreover, studies carried out in the previous chapter revealed that interaction of HSP47 with the collagen receptor GPVI and its role in supporting receptor dimerization. Together these accumulated findings led us to scrutinise further implication of HSP47 in the regulation of signalling events downstream of platelet collagen receptors.

5.2 SMIH down-regulates platelet signalling mediated by collagen

Collagen is the most abundant protein in the ECM of blood vessels. Its insoluble and triple helical configuration provides excellent scaffolding of the subendothelial matrix within vascular walls (Smethurst et al., 2007). Collagen-induced platelet activation commences with the interaction of the platelet GPIb–V–IX receptor complex with vWF bound to exposed collagen at the site of injury. This allows a stable interaction of platelets with collagen via collagen receptors GPVI and integrin $\alpha_2\beta_1$, which leads to firmer adhesion. The clustering of GPVI leads to the stimulation of intracellular tyrosine kinase signalling that leads to Ca^{2+} mobilisation, granule secretion, integrin $\alpha_2\beta_1$ activation and upregulation of integrin $\alpha_{IIb}\beta_3$ and thereby formation of platelet aggregates (Gibbins et al., 1997; Gibbins, 2004).

In the light of our previously reported attenuated collagen-induced platelet functions findings (Sasikumar et al., 2018), it may be possible that the inhibitory effects of SMIH may result from the modulation of signalling events downstream of GPVI. Therefore, the impact of SMIH on the collagen signal transduction pathway was examined.

Human washed platelets (4×10^8 cell/mL) were treated with EGTA (1 mM), indomethacin (20 µM), cangrelor (1 µM) and MRS2179 (100 µM). Indomethacin and MRS2179 eliminate signalling through $P2Y_1$, $P2Y_{12}$ and TP receptors resulting in the removal of complicating effects of secondary mediators and a greater focus on GPVI. Such treatment was applied in all presently approached signalling studies as it allows interpreting the effect of the activatory signals generated from the primary stimuli (agonist) used to stimulate platelets. Additionally, maintaining non-aggregating conditions prevents the mobilisation and

translocation of signalling proteins to the insoluble domain of platelets cytoskeleton and hence facilitate the detection and immunoblotting with site-specific phospho- antibodies (Hubbard et al., 2003). Platelets were then incubated with vehicle (containing DMSO 0.12%) or SMIH (5 or 10 µM) for 10 minutes before activation with collagen (25 µg/m) for 90 seconds. Collagen concentration and stimulation time of platelets were based on reported dose-response and time course studies of collagen-mediated tyrosine phosphorylation in washed platelets (Asselin et al., 1997). Finally, the tyrosine phosphorylation of collagen signalling proteins was observed by immunoblotting.

Collagen-induced tyrosine phosphorylation in vehicle controls (containing DMSO 0.12%). Pre-incubation with SMIH treated samples was associated with significantly lower total tyrosine phosphorylation levels compared to vehicle controls. Approximately 61% lower phosphorylation was observed after treatment with 5 µM SMIH, with further reduction by 84% in samples treated with 10 µM SMIH (Figure 5.1).

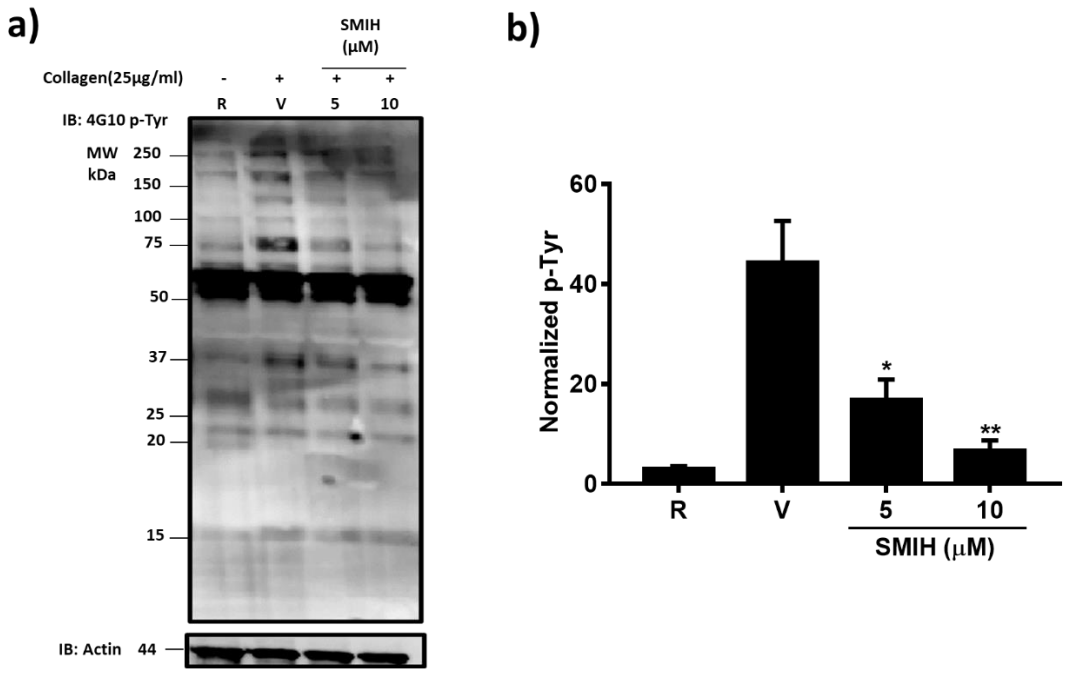


Figure 5.1: SMIH inhibits tyrosine phosphorylation in collagen-stimulated platelets.

Platelets (4×10^8 cells/mL) were pre-treated with vehicle (containing 0.12% DMSO) or SMIH (5 and 10 μM) for 10 minutes, followed by stimulation with collagen (25μg/mL) for 90 seconds in the presence of indomethacin (20 μM), EGTA (1 mM), cangrelor (1 μM) and MRS2179 (100 μM). Samples were lysed using Laemmli sample buffer and whole cell lysates were separated by SDS-PAGE and transferred to PVDF membranes. To detect tyrosine phosphorylation, membranes were probed with anti-phosphotyrosine antibody (4G10). As a control for protein loading, blots were re-probed for actin **(a)** Representative immunoblot is shown. **(b)** Bar chart represents mean normalised tyrosine phosphorylation values relative to actin (the middle band was excluded from the quantification). Results are shown as the mean ± SEM ($n \geq 3$). $*P \leq 0.05$, $**P \leq 0.01$ were calculated by one-way ANOVA. Abbreviations: R: resting; V: vehicle.

Following the observed reduction in total collagen-mediated tyrosine phosphorylation in response to SMIH treatment, experiments were expanded to track the impact of SMIH treatment on earlier signalling events, upon GPVI clustering and activation, including Src family kinases, Syk, LAT and PLCγ2.

In agreement with characterised and reported proteins involved in collagen-mediated signalling pathway (Gibbins et al., 1996; Asselin et al., 1997; Bye et al., 2017), vehicle-treated samples displayed elevated phosphorylation of Src (Y418), Lyn (Y396), Syk (Y525/526), LAT (Y200) and PLCγ2 (Y1217) (Figure 5.2 a, b, c, d, and e). Conversely, tyrosine phosphorylation of Src at pY418, which is its auto-phosphorylation site (Bye et al., 2017) was reduced by 62% and 75% in 5 μM and 10 μM SMIH pre-treated platelets, respectively (Figure 5.2 ai and ii). Pre-treatment with 5 μM and 10 μM SMIH inhibited phosphorylation levels of Lyn (Y396) by 56% and 90%, respectively (Figure 5.2 bi and ii). Likewise, the autophosphorylation sites on Syk at Y525/526 site (Sada et al., 2001) was impacted by SMIH treatment. When compared to vehicle control treated samples, pre-treatment with 5 and 10 μM SMIH for 10 minutes inhibited collagen-stimulated tyrosine phosphorylation of Syk by 50% and 62%, respectively (Figure 5.2 ci and cii).

Activated Syk catalyses the phosphorylation of transmembrane protein LAT which in turn, initiates the assembly of signalosome complex, a docking site for further activation of downstream signalling proteins. Phosphorylation of LAT at Y200, the equivalent of Y171 site and phosphorylated by SFKs and Syk (Paz et al., 2001; Jiang and Cheng, 2007), was detected in vehicle control sample in response to collagen stimulation (Figure 5.2 di and dii) which is consistent with previous report (Pasquet et al., 1999b). On the contrary, LAT

phosphorylation was inhibited by 52% and 74% in, 5 and 10 μM, SMIH pre-treated samples, respectively (Figure 5.2 di and dii).

PLCγ2 is one of the signalling proteins that is recruited to the site of LAT signalosome complex. Consistent with the reduction in the preceding signalling events, a similar reduction was also observed in the phosphorylation of PLCγ2 at Y1217, a site for phosphorylation by Bruton's tyrosine kinase (Btk) (Watanabe et al., 2001), with 60% and 67% inhibition in 5 and 10 μM SMIH incubated samples, respectively (Figure 5.2 ei and eii).

Inhibition of CRP-XL-mediated Ca^{2+} mobilisation was also seen upon HSP47 inhibition (Sasikumar et al., 2018). Ca^{2+} mobilisation is triggered by IP3, a product generated from the cleavage of PIP2 by PLCγ2, whereas DAG mediates PKC family activation (Yacoub et al., 2006; Varga-Szabo et al., 2009; Bye et al., 2016). These events take place following PLCγ2 activation. With observed effects of HSP47 on PLCγ2, the phosphorylation of PKC substrates was also evaluated.

An antibody raised against phosphorylated PKC substrate recognition sequence was used to assess the effect of SMIH on PKC activity. As anticipated, collagen stimulation caused an elevation of PKC substrate phosphorylation in vehicle-treated platelets. PKC substrate phosphorylation was reduced by approximately 59% and 85% in the presence of 5 and 10 μM SMIH, respectively (Figure 5.3).

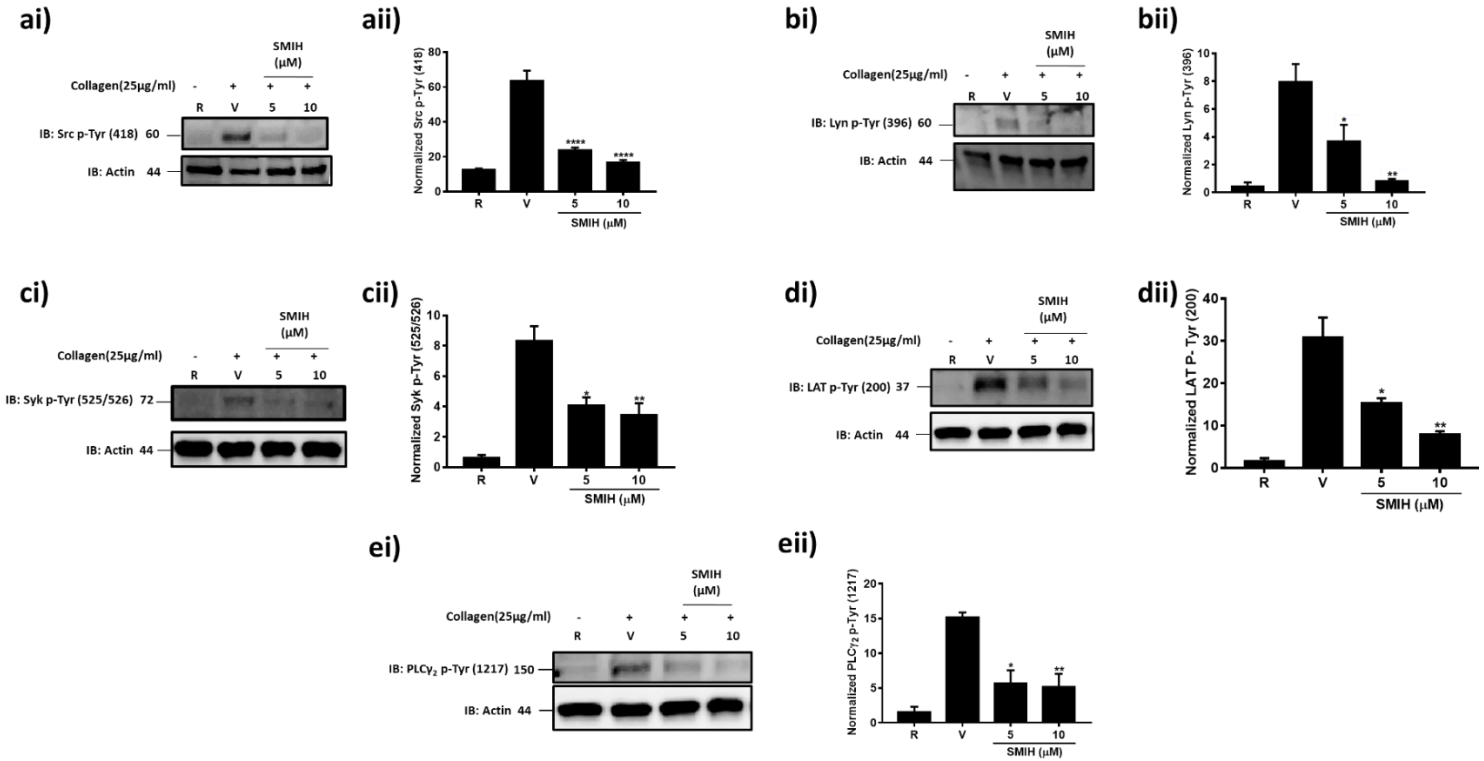


Figure 5.2: HSP47 supports collagen-stimulated Src family kinases, Syk, LAT and PLCγ2 tyrosine phosphorylation.

In the presence of indomethacin (20 μM), EGTA (1 mM), cangrelor (1 μM) and MRS2179 (100 μM), washed platelets (4×10^8 cells/mL) pre-treated with vehicle (containing 0.12% DMSO) or SMIH (5 and 10 μM) were activated with collagen (25 μg/mL) and after 90 seconds, Laemmli sample buffer was added to lyse the samples. Whole cell lysates were then resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with site-specific phospho-antibodies for Src (Y418), Lyn (Y396), Syk (Y525/526), LAT (Y200) and PLCγ2 (Y1217) to determine phosphorylation levels. Representative immunoblots for the phosphorylation levels of **(ai)** Src, **(bi)** Lyn, **(ci)** Syk, **(di)** LAT and **(ei)** PLCγ2 are shown following treatment with vehicle or SMIH. As a loading control, membranes were re-probed with antibodies against actin. Bar charts are mean phosphorylation values of **(aii)** Src, **(bii)** Lyn**, (cii)** Syk, **(dii)** LAT and **(eii)** PLCγ2 in samples treated with SMIH relative to actin. Results representing the mean ± SEM (n ≥ 3) and *$P \leq 0.05$, **$P \leq 0.01$ and ****$P \leq 0.0001$ were calculated by one-way ANOVA. Abbreviations: R: resting; V: vehicle.

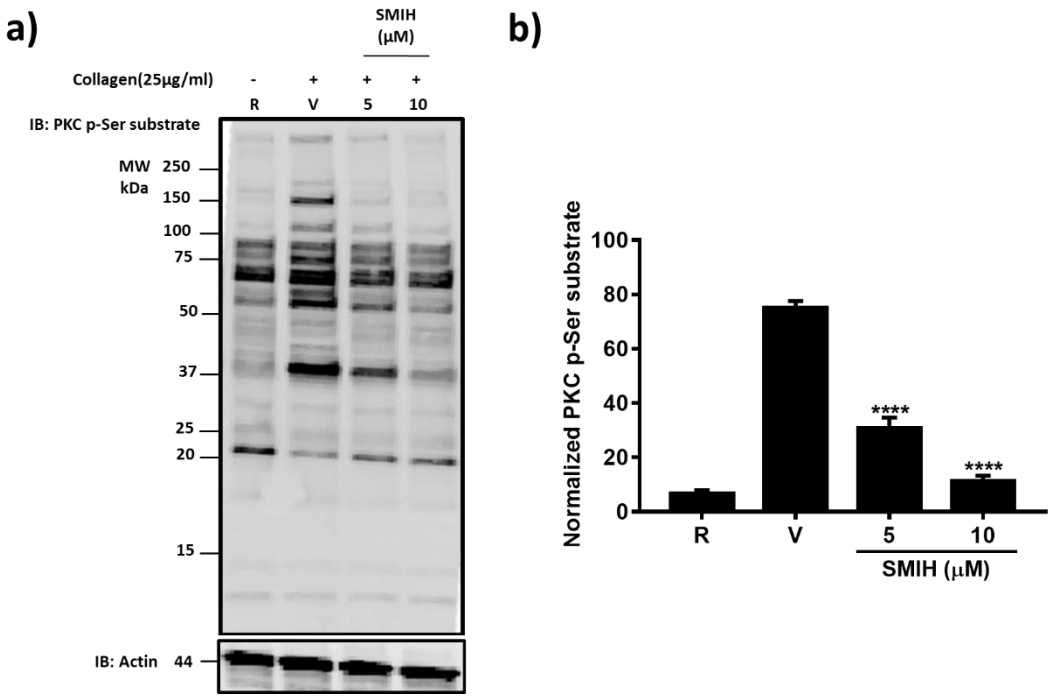


Figure 5.3: SMIH attenuates collagen-stimulated PKC substrate recognition sequence phosphorylation.

Washed platelets (4×10^8 cells/mL) were treated with vehicle (containing 0.12% DMSO) or SMIH (5 and 10 μM) for 10 minutes, then activated with collagen (25 μg/mL) in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM) for 90 seconds before lysing samples with Laemmli sample buffer. SDS-PAGE was used to separate the lysates, followed by blotting to PVDF membrane. Blots were then incubated with an antibody directed against phosphorylated PKC substrate recognition sequence was used to detect its phosphorylation level. **(a)** A representative immunoblot is shown for the phosphorylation levels of PKC substrate in vehicle and SMIH pre-treated samples. **(b)** Bar chart represents mean PKC substrate phosphorylation levels normalised to actin which served as a control for protein loading. The results represent the mean ± SEM ($n \geq 3$). ****$P \leq 0.0001$ was calculated by one-way ANOVA. Abbreviations: R: resting; V: vehicle.

5.3 HSP47 Supports GPVI-mediated Signalling

The influence of HSP47 to modulate collagen-mediated signalling was observed following treatment with HSP47 inhibitor, SMIH (section 5.2). Platelets, however, bind collagen directly via both GPVI and integrin α2β1 (Nieswandt and Watson, 2003; Emsley et al., 2004) and hence, multiple steps and stages are involved to synergistically achieve satisfactory interaction between circulating platelets and exposed collagen.

There has been considerable growth in the development of various collagen peptides with selective affinity to individual collagen receptors. The group of Prof. Richard Farndale (University of Cambridge), has been instrumental in the identification of these peptides where they successfully generated large libraries of triple helical oriented peptides covering the whole sequences of collagen types I and II. With the help of this library, GPO-containing peptide termed collagen-related peptide (CRP), was synthesised and when cross-linked to form CRP-XL, it serves as highly potent GPVI- selective agonist (Knight et al., 1998).

Failure of integrin α2β1 inhibitory antibodies to inhibit CRP-XL evoked tyrosine phosphorylation further ascertain that such phosphorylation is independent of the inhibited integrin α2β1 (Asselin et al., 1997; Gibbins et al., 1997). Knowing the ability of HSP47 to support GPVI dimerization, implies the possibility that effect seen in collagen-mediated signalling (section 5.2) was via GPVI receptor. Therefore, we sought to explore the impact of HSP47 on signalling upon stimulation of human and mice platelets with GPVI-selective agonist CRP-XL.

The impact of HSP47 on GPVI signalling was examined by first incubating human washed platelets (4×10^8 cell/mL) with indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100

µM) and EGTA (1 mM) prior to platelet stimulation. This was followed with vehicle (containing DMSO 0.12%) or SMIH (5 and 10 µM) treatment for 10 minutes before stimulation with CRP-XL (1 µg/mL) for 90 seconds and tyrosine phosphorylation of GPVI signalling proteins was observed by immunoblotting. The concentration of agonist was chosen based on a previous study reporting that CRP-XL-generated tyrosine phosphorylation was observed in immunoblotting studies when platelets were stimulated with 1 µg/mL under non-aggregating condition (Unsworth et al., 2017). Additionally, CRP-XL has been reported to maximally induce tyrosine phosphorylation at a range of 1-3 µg/mL following 90 seconds of stimulation (Asselin et al., 1997).

CRP-XL-stimulated tyrosine phosphorylation of several proteins detected within vehicle control-treated samples. Pre-treatment with SMIH (5 and 10µM) was found to inhibit early events downstream of GPVI mediated signalling with reduced tyrosine phosphorylation of key proteins by 32% and 42%, respectively when compared to vehicle (Figure 5.4). These data are consistent with the effects of SMIH on collagen-stimulated platelets (section 5.2).

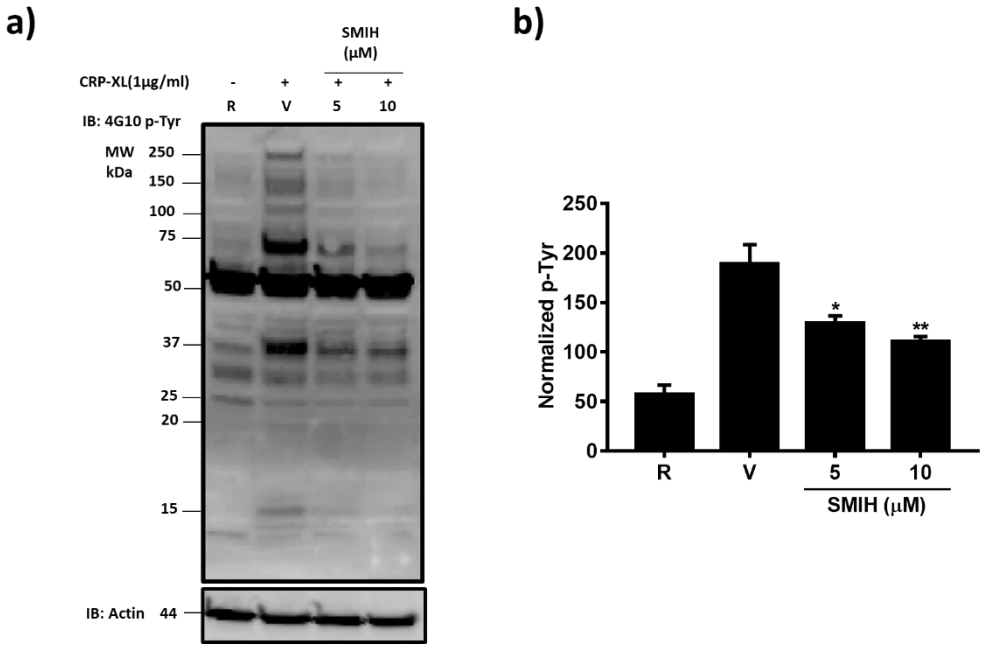


Figure 5.4: SMIH inhibits tyrosine phosphorylation in CRP-XL-activated platelets.

Platelets (4×10^8 cells/mL) were pre-treated with vehicle (containing DMOS 0.12%) or SMIH (5 and 10 μM) for 10 minutes and stimulated with CRP-XL (1 μg/mL) in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM). After 90 seconds, samples were lysed with Laemmli sample buffer and lysate samples were then resolved by SDS-PAGE followed by western blotting onto PVDF membranes. Blots were probed with a phospho-tyrosine antibody (4G10). **(a)** Representative immunoblot of resting, vehicle control and SMIH treated samples is presented. **(b)** Bar charts are mean normalised tyrosine phosphorylation values relative to actin. Actin was detected by immunoblotting as a protein loading control. The results are shown as the mean ± SEM ($n \geq 3$). $*P \leq 0.05$ and $**P \leq 0.01$ were calculated by one-way ANOVA. Abbreviations: R: resting; V: vehicle.

CRP-XL at a concentration of 1 µg/mL resulted in the induction of phosphorylation of Src family kinases (Src and Lyn) in vehicle-treated samples. Pre-treatment with 5 and 10 µM SMIH inhibited the level of phosphorylation of Src by 90% and 97%, respectively (Figure 5.5 a). Similarly, Lyn (Y396) levels decreased by approximately 94% following SMIH treatment (Figure 5.5 b). Src family kinases mediated phosphorylation of the immunoreceptor tyrosine-based activation motif (ITAM) in the FcR γ-chain was observed in vehicle treated sample (Figure 5.5 c). This is in alignment with reported phosphorylation of this motif in CRP-XL-stimulated samples (Gibbins et al., 1997; Quek et al., 2000). However, levels were diminished substantially by 71% and 76% following treatment with 5 and 10 µM of SMIH, respectively (Figure 5.5 c).

Similarly, pre-treatment with 5 and 10 µM of SMIH resulted in the reduction of phosphorylation levels of Syk, at its autophosphorylation site Y525/526 (Sada et al., 2001) by 43% and 54%, respectively (Figure 5.6 a) in response to CRP-XL stimulation. The phosphorylated form of Syk proceeds to phosphorylate the transmembrane protein LAT (Gibbins et al., 1998). Vehicle-treated samples displayed tyrosine phosphorylation of LAT at pY200 and SMIH inhibited such phosphorylation by 78% and 89% at 5 and 10 µM concentrations of SMIH (Figure 5.6 b).

PLCγ is recruited and phosphorylated via LAT signalosome. Such recruitment and phosphorylation mark crucial onset of later events in GPVI signalling manifested by its role in the generation of second messengers, IP3 and DAG, that directly regulate crucial platelet activation events such as calcium mobilisation and degranulation (Watson et al., 2005). Such phosphorylation is demonstrated in vehicle treated sample in which Y1217 residue of PLCγ, which is a Bruton's tyrosine kinase (Btk) phosphorylation site (Watanabe et al., 2001), was

highly phosphorylated following CRP-XL stimulation. Phosphorylation of PLCγ was downregulated by SMIH in a dose-dependent manner. Likewise, PLCγ phosphorylation in 5 and 10 µM SMIH pre-treated samples was inhibited by 52% and 64%, respectively (Figure 5.6 c). Lastly, HSP47 showed similar influence on later events in collagen signalling pathway in CRP-XL evoked sample as demonstrated in the reduction of PKC substrate phosphorylation levels following SMIH treatment (5 and 10 µM) by 49% and 64% when compared to vehicle treated control samples (Figure 5.7).

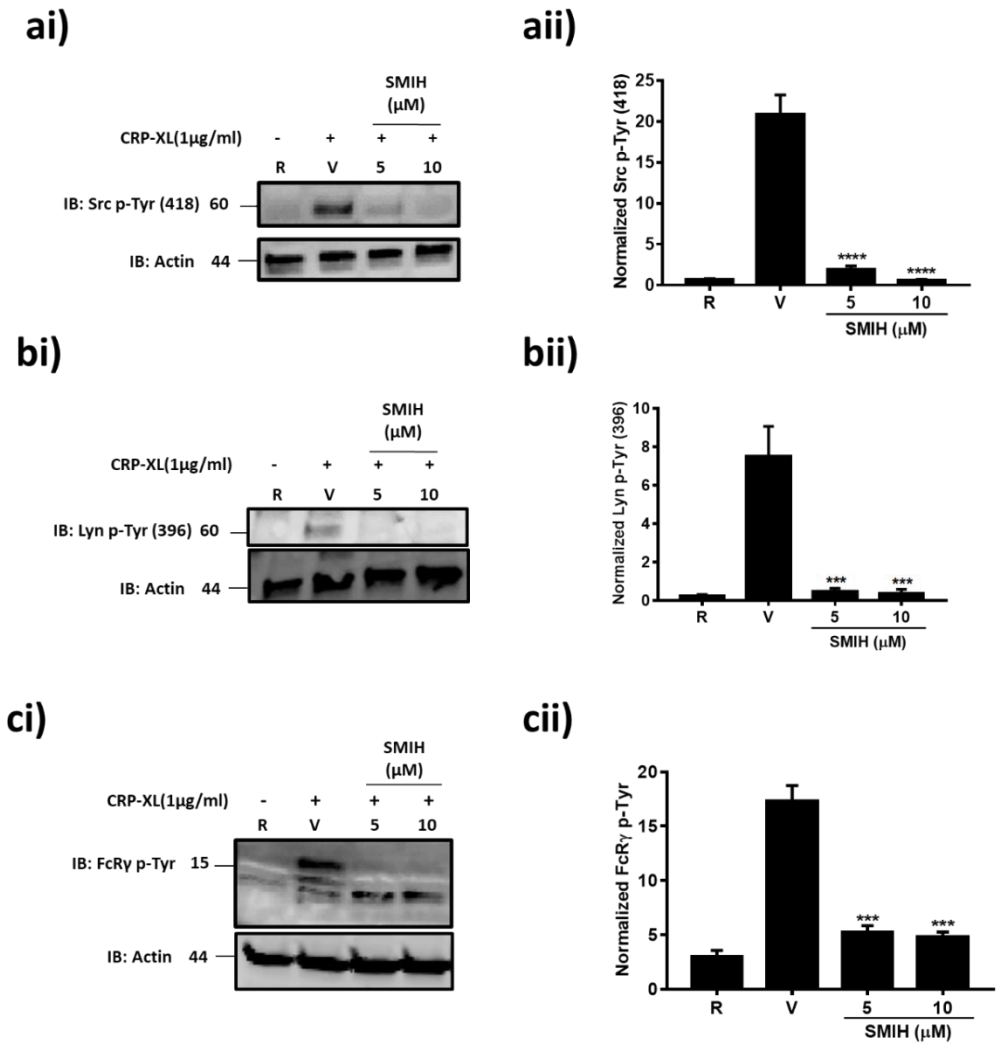


Figure 5.5: Phosphorylation of FcR-γ and its upstream effector Src family kinases is inhibited by SMIH in CRP-XL stimulated platelet.

Washed platelets (4×10^8 cells/mL) were treated with vehicle (containing DMSO 0.12%) or SMIH (5 and 10 μM) for 10 minutes and activated with CRP-XL (1μg/mL). Stimulation was carried out for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA acid (1 mM) before the addition of Laemmli sample buffer. Whole cell lysates were then separated by SDS-PAGE and transferred to PVDF membranes. Tyrosine phosphorylated FcR γ-chain, Src and Lyn were detected by anti-phosphotyrosine antibody (4G10), a site-specific phospho-antibodies for phosphorylated Src (Y418) and Lyn (Y396), respectively. Representative immunoblots are shown for the phosphorylation levels of **(ai)** Src, **(bi)** Lyn and **(ci)** FcR-γ-chain and following treatment with vehicle and SMIH. Phosphorylation levels of **(aii)** Src, **(bi)** Lyn and **(cii)** FcR-γ-chain are presented as the mean relative to total protein and actin, respectively, that was also used as a control for protein loading. The results are mean ± SEM ($n \geq 3$). ***$P \leq 0.001$ and ****$P \leq 0.0001$ were calculated by one-way ANOVA. Abbreviations: R: resting; V: vehicle.

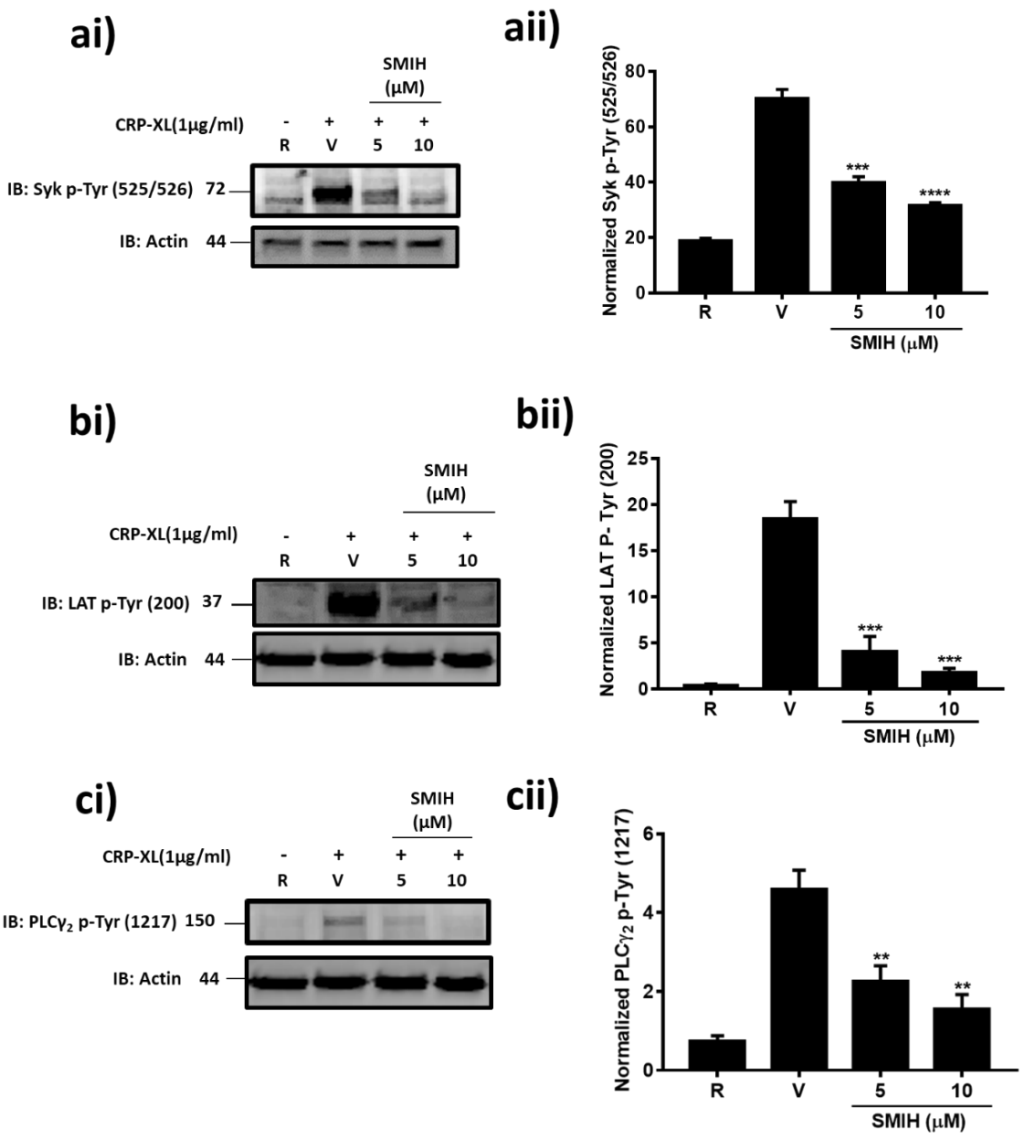


Figure 5.6: CRP-XL-stimulated Syk, LAT and PLCγ2 tyrosine phosphorylation was inhibited by SMIH.

Vehicle (containing DMSO 0.12%) or SMIH (5 and 10 μM) treated washed platelets (4×10^8 cells/mL) were stimulated with CRP-XL (1μg/mL) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA acid (1 mM) before lysing the samples with Laemmli sample buffer. Lysates were separated on SDS-PAGE and transferred to PVDF membranes. Site-specific phospho-antibodies for Syk (Y525/526), LAT (Y200) and PLCγ2 (Y1217) were used to determine their phosphorylation levels. Immunoblots for the phosphorylation levels of **(ai)** Syk, **(bi)** LAT and **(ci)** PLCγ2 following treatment of platelets with vehicle or SMIH are shown. Phosphorylation levels of **(aii)** Syk, **(bii)** LAT and **(cii)** PLCγ2 are presented as the mean normalised to levels of actin detected. Results represent the mean ± SEM ($n \geq 3$). $^{**}P \leq 0.01$, $^{***}P \leq 0.001$ and $^{****}P \leq 0.0001$ were calculated by one-way ANOVA. Abbreviations: R: resting; V: vehicle.

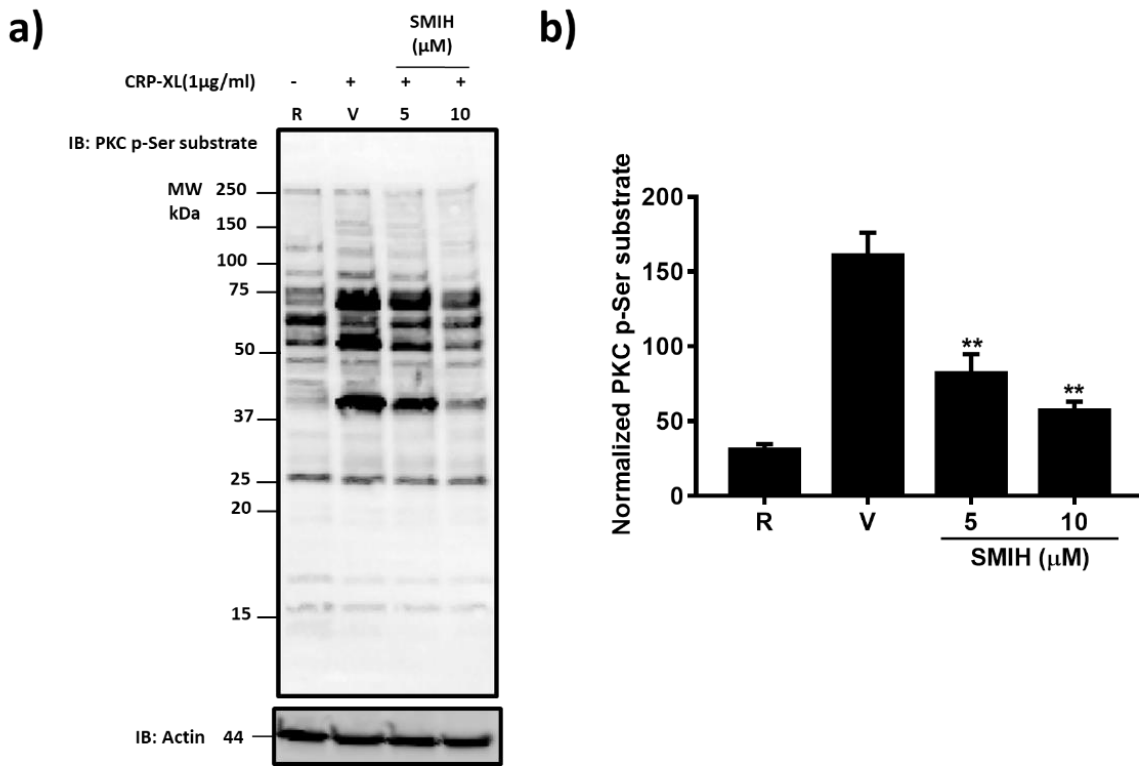


Figure 5.7: PKC substrate recognition sequence phosphorylation is negatively impacted by SMIH in response to CRP-XL stimulation.

Washed platelets (4×10^8 cells/mL) were treated with vehicle (containing DMSO 0.12%) or SMIH (5 and 10 μM) for 10 minutes before stimulation with CRP-XL (1 μg/mL) for 90 seconds in the presence of indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA acid (1 mM). Laemmli sample buffer was used to lyse the samples, followed by samples separation using SDS-PAGE and transfer to PVDF membranes. Membranes were then probed with phosphorylated PKC substrate recognition sequence antibody to determine its level of phosphorylation. **(a)** Representative immunoblots are shown of PKC substrate phosphorylation levels. **(b)** Bar chart represents mean PKC substrate phosphorylation levels normalised to actin protein detected by immunoblotting as a loading control. The results represent the mean ± SEM ($n \geq 3$). $^{**}P \leq 0.01$ was calculated by one-way ANOVA. Abbreviations: R: resting; V: vehicle.

The availability of the HSP47 deficient platelets offered the potential to further explore the role of HSP47 in GPVI-mediated signalling. WT mice platelets exhibited robust tyrosine phosphorylation in response to CRP-XL stimulation at 1µg/ml similar to that reported in the literature (Jarvis et al., 2004). On the contrary, HSP47 deficient platelets displayed a reduction in the phosphorylation levels of several signalling proteins in response to CRP-XL stimulation (Figure 5.8). This was consistent with the reduction observed in SMIH-treated platelets targeting an early signalling phase (Figure 5.5), as shown by the reduction of phosphorylated Src and Lyn by 74% and 55%, respectively, in comparison with wild type (Figure 5.9 a and b).

Accordingly, other signalling proteins downstream of Src family kinase (Lyn) were also studied. Autophosphorylation of Syk along with phosphorylation levels of LAT and PLCγ were significantly decreased in CRP-stimulated platelets by 37%, 50% and 54%, respectively, when compared to WT (Figure 5.9c, d and e). Lastly, in comparison with WT, the activity of PKC was significantly inhibited by 33% in HSP47 deficient platelets, which further validates the role of HSP4 in early signalling events in response to GPVI stimulation (Figure 5.10).

Taken together, these findings unravel the mechanisms behind attenuated collagen-mediated platelet functions in HSP4 inhibited and ablated platelets, where HSP47 is implicated in an early binding of platelets to collagen which facilitate subsequent signalling events. Nevertheless, HSP47 contribution is not restricted to early signalling events as the activity of later signalling proteins downstream of PIP2 hydrolysis was negatively impacted as observed in the attenuated activity of PKC. More importantly, such an early contribution

provides insight into the consequences of the previously observed role of HSP47 in GPVI dimerization (section 4.3).

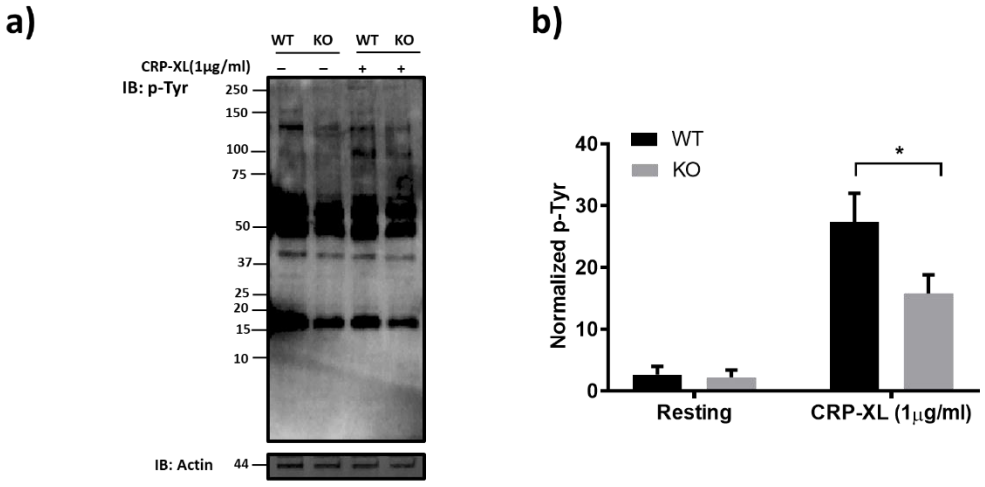
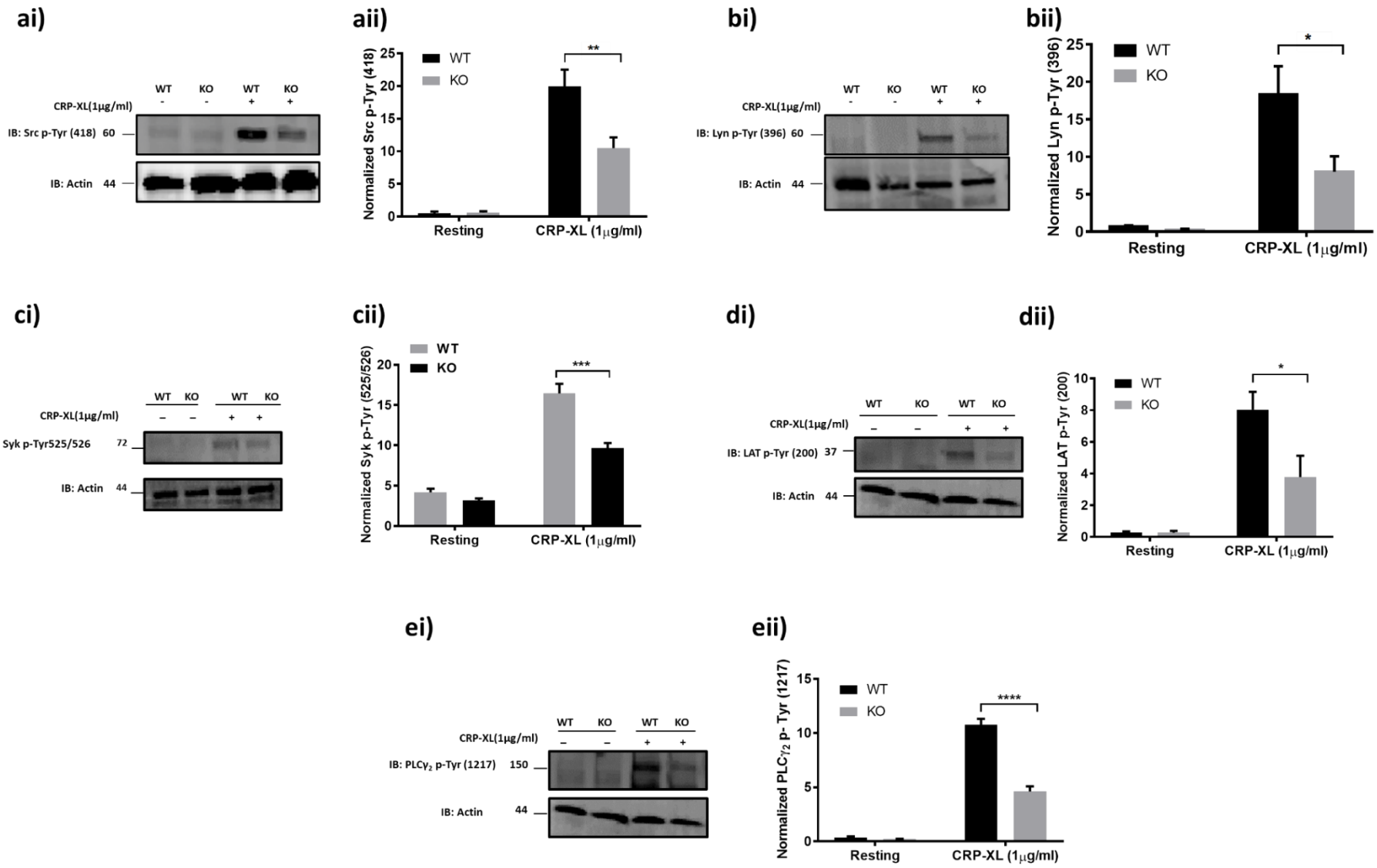


Figure 5.8: CRP-XL evoked tyrosine phosphorylation is reduced in HSP47 ablated platelet.

Platelets (2×10^8 cells/mL) from $HSP47^{+/+}$ and $HSP47^{-/-}$ mouse were activated with CRP-XL (1 µg/mL) for 90 seconds in the presence of indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA (1 mM). Laemmli sample buffer was used to lyse the samples and whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and probed with an anti-phosphotyrosine antibody (4G10) to detect tyrosine phosphorylation. **(a)** A representative immunoblot is presented. **(b)** Bar charts are mean normalised tyrosine phosphorylation values relative to levels of actin detected (the middle band was excluded from the quantification). Results are mean ± SEM ($n \geq 3$). $*P \leq 0.05$ was calculated by Student's *t*-test.

Figure 5.9: Src, Lyn, Syk, LAT and PLCγ2 tyrosine phosphorylation are negatively affected by HSP47 deletion.

$HSP47^{+/+}$ and $HSP47^{-/-}$ mouse platelets (2×10^8 cells/mL) were activated with CRP-XL (1 µg/mL) in the presence of indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA acid (1 mM). after 90 seconds, Laemmli sample buffer was used to lyse the samples, which were next separated by SDS-PAGE and transferred to PVDF membranes. Site-specific phospho-antibodies for Src (Y418), Lyn (Y396), Syk (Y525/526), LAT (Y200) and PLCγ2 (Y1217) were used to determine their phosphorylation levels. Representative immunoblots for the phosphorylation levels of **(ai)** Src, **(bi)** Lyn, **(ci)** Syk, **(di)** LAT and **(ei)** PLCγ2 are shown. Phosphorylation levels of **(aii)** Src, **(bi)** Lyn, **(cii)** Syk, **(dii)** LAT and **(eii)** PLCγ2 are presented as the mean relative to levels of actin detected. Results represent the mean ± SEM ($n \geq 3$). *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$ and ****$P \leq 0.0001$ were calculated by Student's *t*-test.


ai)
WT KO WT KO
CRP-XL(1µg/ml) - - + +
IB: Src p-Tyr (418) 60
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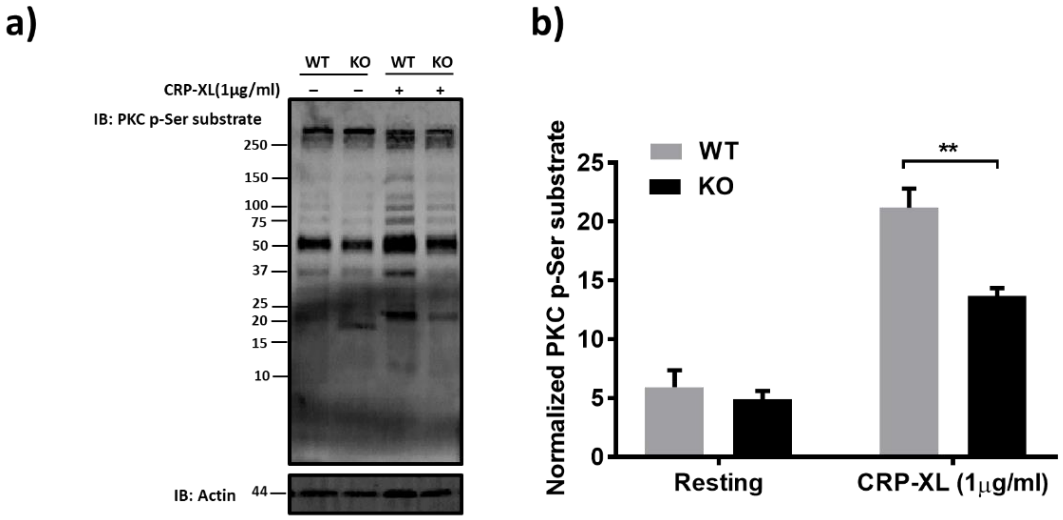

Figure 5.10: HSP47 deficient platelets show reduced levels of PKC substrate recognition sequence phosphorylation

Platelets (2×10^8 cells/mL) from HSP47$^{+/+}$ and HSP47$^{-/-}$ mouse were activated with CRP-XL (1 µg/mL) for 90 seconds in the presence of indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA acid (1 mM). Samples were then lysed with Laemmli sample buffer and whole cell lysates were then separated by SDS-PAGE and transferred to PVDF membranes and antibody against the phosphorylated PKC substrate recognition sequence was used to determine its level of phosphorylation in the blot. **(a)** Representative blots are shown for the phosphorylation levels of PKC substrate in HSP47$^{+/+}$ and HSP47$^{-/-}$ platelets. **(b)** Bar chart represents mean PKC substrate phosphorylation levels relative to levels of actin detected. Results represent the mean ± SEM ($n \geq 3$). $^{**}P \leq 0.01$ was calculated by Student's *t*-test.

5.4 The Inhibitory Effects of SMIH on Platelets Activation are not dependent on integrin α2β1

HSP47 was shown to support the binding of GPVI receptor to its selective ligand (CRP-XL) whereas, platelet binding to integrin α2β1 selective ligand (GFOGER) was unaffected by HSP47 inhibition condition (section 4.2). Moreover, down-regulated CRP-XL-mediated signalling following HSP47 inhibition and deletion seen in human and mouse platelets all support the conclusion that HSP47 contributes to platelet-collagen interaction and subsequent activation and is dependent on GPVI. To confirm the aforementioned GPVI dependence in the actions of HSP47, signalling investigations were further extended using GFOGER, an integrin α2β1 selective agonist.

Integrin α2β1 preferentially binds, glycine- glutamic acid- arginine- (GER) containing sequences in collagen and has low-affinity toward GPO enriched sequences (Knight et al., 1998; Zhang et al., 2003). GFOGER, peptide presented on backbone of repeat GPP sequences, was identified as a ligand that recognises and binds selectively to integrin α2β1 by Morton and colleagues (Morton et al., 1995). The identification of these receptor selective peptides has allowed further investigation and a better understanding of the individual and synergistic effects of collagen receptors (Pugh et al., 2010). Besides its primary adhesive role, GFOGER engaged integrin α2β1 and stimulated tyrosine kinase-dependent outside-in signalling events. Such an activatory role was reported to be involved in the formation of filopodia and lamellipodia in the process of platelet spreading (Inoue et al., 2003).

The impact of HSP47 on integrin $\alpha_2\beta_1$ signalling was examined by first incubating washed platelets ($2x10^7$cell/mL) with indomethacin (20 μM), cangrelor (1 μM), MRS2179 (100 μM) and EGTA (1 mM), then followed by vehicle (containing DMSO

0.12%) or SMIH (5 and 10 μM) treatment for 10 minutes. Treated platelets were then exposed to GFOGER-coated wells (50 μg/ml) and allowed to adhere at 37°C for 30 minutes. The experimental set up applied here was based on the report that GFOGER stimulates tyrosine phosphorylation when presented as a monolayer at a concentration of 50 μg/ml for 30-minute incubation time (Inoue et al., 2003). After 30 minutes, samples were lysed in Laemmli sample buffer and adhered cells were scraped off. Lysates were separated using SDS-PAGE and lastly, tyrosine phosphorylation was detected by Western blotting using the anti-phosphotyrosine mAb 4G10.

GFOGER stimulated a marked increase in the level of tyrosine phosphorylation at 30 minutes in vehicle samples. Conversely, in comparison to the vehicle treated sample, pre-treatment of platelets with SMIH (5 or 10 μM) for 10 minutes did not alter GFOGER (50μ/ml) initiated total tyrosine phosphorylation levels (Figure 5.11). The lack of effect of HSP47 inhibition on integrin α2β1 mediated outside-in signalling using GFOGER is consistent with the un-affected platelet binding to GFOGER following SMIH treatment (Section 4.2).

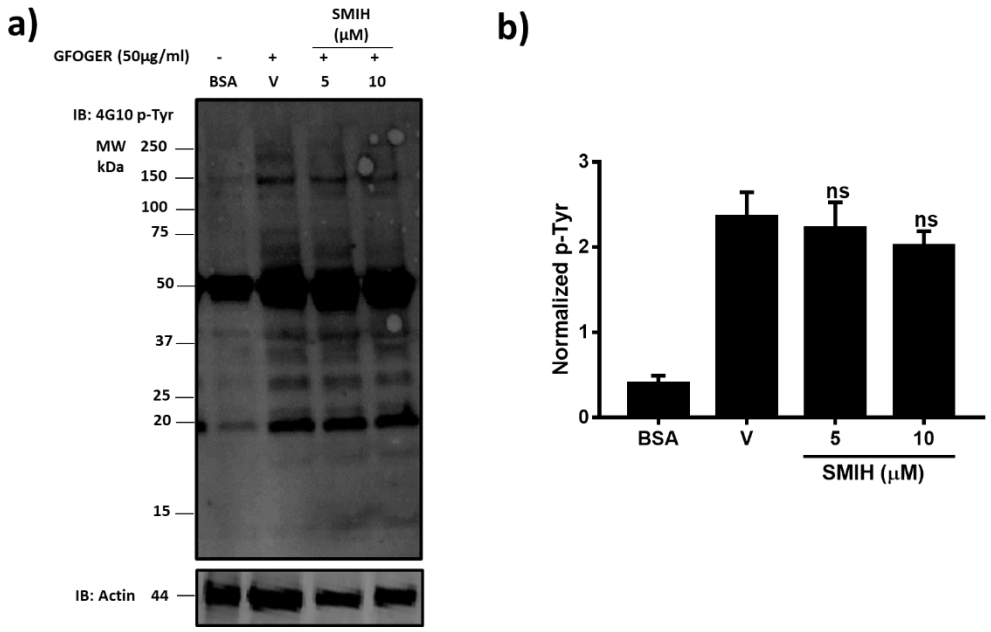

Figure 5.11: Integrin α2β1 mediated outside-in signalling is not altered by HSP47 inhibition.

Washed platelets (2×10^7 cells/ml), pre-treated with SMIH (5 or 10 μM) or vehicle-control (containing DMSO 0.12%) were seeded onto 1% BSA or GFOGER-coated wells (50 μg/ml) and allowed to adhere at 37°C. Samples were lysed in Laemmli sample buffer after 30 minutes, separated by SDS PAGE, transferred to PVDF membranes and immunoblotted with an anti-phosphotyrosine antibody (4G10) to detect tyrosine phosphorylation. **(a)** A representative immunoblot is presented. **(b)** Bar charts are mean normalised tyrosine phosphorylation values relative to the levels of actin detected (the middle band was excluded from the quantification). Results are displayed as the mean ± SEM ($n \geq 3$), one-way ANOVA. ns: not significant. Abbreviations: V: vehicle.

5.5 HSP47 does not modulate G-protein coupled receptor (GPCRs) signalling

It has been reported that HSP47 inhibition did not exert any impact on thrombin-mediated platelet functions (Sasikumar et al., 2018). To confirm a lack of a role for HSP47 in the regulation of signalling by other receptors, platelet signalling evoked by thrombin in presence of SMIH was studied.

PAR-1 and PAR-4 thrombin receptors are coupled with $G_{\alpha q}$ proteins (Brass, 2003). Upon activation of these receptors, several signalling events are triggered to phosphorylate and activate PLCβ. When activated, PLCβ catalyses phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolysis into inositol trisphosphate (IP3) and diacylglycerol (DAG). These secondary mediators increase the level of intracellular Ca^{2+}, activate PKC, induce granules release and upregulate integrin $\alpha_{IIb}\beta_3$ (Offermanns, 2006; Stalker et al., 2012).

The influence of HSP47 on GPCR signalling was examined by first incubating washed platelets (4×10^8 cell/mL) with indomethacin (20 µM), cangrelor (1 µM), MRS2179 (100 µM) and EGTA (1 mM). Platelets were then treated with vehicle (containing DMSO 0.12%) or SMIH (5 or 10 µM) for 10 minutes before stimulation with thrombin (0.1 U/mL) for 90 seconds. Thrombin at (0.1 U/mL) was used to stimulate platelets to enable further detection of downstream tyrosine phosphorylation by western blotting (Fuste et al., 2002).

High phosphorylation levels were observed in vehicle treated sample (containing DMSO 0.12%). Interestingly, no notable differences in total tyrosine phosphorylation levels were observed between the platelets treated with SMIH (5 and 10 µM) and those treated with vehicle for 10 minutes after stimulation with thrombin (Figure 5.12).

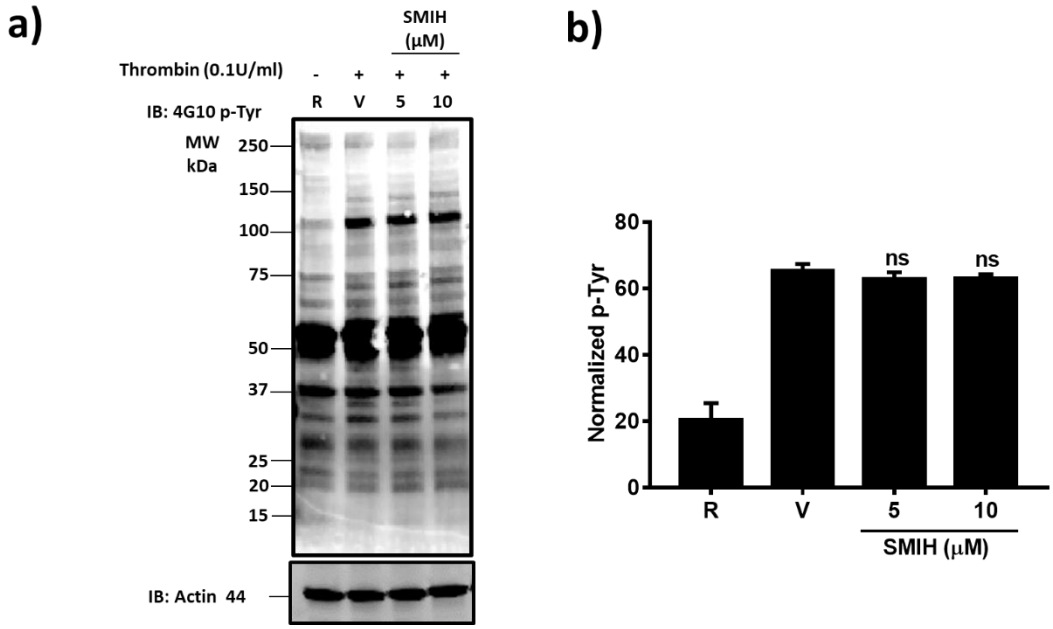

Figure 5.12: Thrombin-mediated platelet tyrosine phosphorylation is unaffected by HSP47 inhibition.

Washed platelets (4×10^8 cells/mL) were treated with vehicle (containing DMSO 0.12%) or SMIH (5 or 10 μM) for 10 minutes, followed by 90 seconds simulation with thrombin (0.1 U/mL) in the presence of EGTA (1 mM), cangrelor (1 μM), MRS2179 (100 μM) and indomethacin (20 μM). Laemmli sample buffer was used to lyse the samples and whole cell lysates were separated by SDS-PAGE, transferred to PVDF membranes and blots were probed with an anti-phosphotyrosine antibody (4G10) to detect tyrosine phosphorylation. **(a)** A representative immunoblot is shown. **(b)** Bar charts are mean normalised tyrosine phosphorylation values relative to the levels of actin detected (the middle band was excluded from the quantification). Results are displayed as the mean ± SEM ($n \geq 3$), one-way ANOVA. ns: not significant. Abbreviations: R: resting; V: vehicle.

5.6 Discussion:

The ability of platelets to form aggregates upon encountering collagen at the site of vascular damage signifies its primary and chief role in the integrated process of hemostasis. The capability of platelets to recognise stimuli and respond, in terms of its adhesive, activatory and thrombus forming roles, results from a complex and interconnected network of tightly controlled signalling pathways. Such a network of activatory and counteracting inhibitory signals maintains haemostatic balance (Gibbins, 2004).

The previous chapter established the influence exerted by HSP47 on GPVI dimerisation along with its role in supporting platelet adhesion to collagen and GPVI selective ligand, CRP-XL. Additionally, in the light of findings observed in GPVI blockade studies, HSP47 was proposed to support platelet activation in a GPVI dependent manner. In this chapter, the mechanisms by which HSP47 modulates inside-out and outside-in activatory signalling events were assessed.

5.6.1 HSP47 supports platelets activation via modulation of collagen-mediated signalling:

Given the reported potent inhibitory effects of HSP47 inhibition on a variety of platelet functions, thrombosis and haemostasis in response to collagen (Sasikumar et al., 2018), the role of HSP47 to regulate underlying platelet signalling pathways were studied. SMIH was found to inhibit tyrosine phosphorylation of the FcRγ-chain and therefore, HSP47 appears to have a stimulatory role in CRP-XL-stimulated signalling pathway.

We have also shown that SMIH is able to inhibit the phosphorylation of Src in a concentration-dependent manner. Consequently, SMIH is able to inhibit collagen-

stimulated tyrosine phosphorylation of the FcRγ-chain. Additional molecules further down the collagen and CRP-XL activation pathway were examined for the effect of SMIH on their phosphorylation and hence activation. SMIH was shown to inhibit the tyrosine phosphorylation of Syk, which is likely to be in part a consequence of the inhibition of Src family kinases (Src and Lyn), and thereby the signalling downstream. Tyrosine phosphorylation and activation of Syk is thought to lead to the phosphorylation of tyrosine residues in the transmembrane protein LAT and PLCγ2 (Gibbins et al., 1998; Pasquet et al., 1999b) which were also inhibited by SMIH. Such role of HSP47 in the generation of activatory signals downstream of GPVI as demonstrated in the diminished phosphorylation level of Src family kinases was confirmed following HSP47 inhibition.

The generation of mice with HSP47 ablated platelets has allowed a new approach to assess the HSP47 role on the platelet functions (Sasikumar et al., 2018). Moreover, the use of knockout mice lacking proteins of interest has been intensively employed to study the significance and importance of deleted proteins in platelets and in other cell lines. HSP47 deficient platelets displayed a similar pattern of attenuated GPVI-mediated phosphorylation events. This was demonstrated in the reduced phosphorylation levels of Src when compared to $HSP47^{+/+}$. Likewise, subsequent Src downstream signalling proteins, Syk, LAT and PLCγ were inhibited. Undoubtedly, combined analysis using HSP47 inhibitor and HSP47 ablated platelets provides further validated evidence of the importance of HSP47 in the earlier stages of the GPVI signalling.

5.6.2 HSP47 contribution to collagen signalling is not dependent on integrin α2β1:

The vital role of SFKs resides in their ability to participate in activatory signalling events downstream of various receptors, such as vWF/GPIb-IX-V receptor complex, GPVI, and

integrin α2β1, and hence contributing to platelet activation (Senis et al., 2014). However, it has been reported that initial interaction mediated through binding of immobilised vWF to its receptor GPIb on platelet surface was unaffected in HSP47 inhibited conditions (Sasikumar et al., 2018). Such finding supports the involvement of HSP47 in platelet-collagen adhesion following initial tethering via vWF and excludes any possible effects of HSP47 on the activity and phosphorylation of SFK downstream of GPIb-IX-V engagement. Similarly, HSP47 had no detectable impact on integrin α2β1 outside-in signalling in GFOGER adhered platelets. This maintained platelet signalling could be in part of an earlier initial interaction of platelets to GFOGER ligand not altered by HSP47 inhibition (sections 4.2 and 5.3).

Targeting HSP47 with a set of increasing concentrations of several selective inhibitors, SMIH and inhibitory HSP47 antibody, had no effect on platelet aggregation induced by 0.1U/ml thrombin (Sasikumar et al., 2018). This is consistent with the inability of SMIH to reduce thrombin-stimulated signalling events. Additionally, previous experiment with lower levels of thrombin (0.3, 0.1, 0.01, 0.03 and 0.01U/ml) induced similar aggregation responses in vehicle and HSP47 inhibitor-treated samples (Sasikumar et al., 2018). Therefore, it is less likely that lack of effect of HSP47 observed in thrombin-stimulated samples was due to the high level of thrombin (0.1U/ml) overcoming inhibitory capacity of SMIH.

Together, these data demonstrate that HSP47 inhibition interferes with signalling proteins responsible for prompting the onset of collagen-mediated signals following the engagement of GPVI and therefore contributing to subsequent platelet activation and thrombus formation.

6 General discussion

6.1 Introduction

Thrombus formation and cessation of bleeding after injury to the vasculature require efficient platelet function. Platelets are equipped with sets of integrin and non-integrin glycoprotein specialised surface receptors that respond to insoluble stimulating components of the extracellular matrix such as collagen and laminin, and other several soluble stimuli, such as ADP and epinephrine. Engaged receptors result in the activation of cascades of effector enzymes and adaptor proteins to finally ensure optimal responsive platelets and thrombus formation at the site of injured vessels (Ghoshal and Bhattacharyya, 2014).

Different intracellular signalling events are triggered downstream of tyrosine kinase-linked or G-protein- coupled receptors, although different, both pathways overlap at certain signalling events in the integrated process of haemostasis (Watson and Gibbins, 1998; Brass, 1999). Collagen protein induces tyrosine kinase signalling pathway upon crosslinking GPVI-FcR γ-chain receptor complex (Poole et al., 1997), whereas ADP and protease thrombin are examples of platelet agonists that bind and activate G-protein-coupled receptors in platelets (Brass, 1999).

In platelets, intracellular signalling events are strictly regulated reflecting the significance of such process. Excessive up-regulation in signalling events could precipitate an acute coronary thrombotic episode, whereas opposite events of down-regulated signals risk the development of bleeding complications (Bye et al., 2016). Although challenging, current therapies successfully interfere with the cellular and intracellular signalling pathways and thereby hindering the extent of platelet activation and consequent clot formation. While available agents have been proved effective, there are always bleeding risks that need to

be considered. Hence there is an urging need to develop new effective therapeutic agents with fewer side effects (Yeung et al., 2018).

The exclusive expression of GPVI on platelets and its mother progenitor cell (megakaryocyte) in the bone marrow, makes this receptor a promising therapeutic target to downregulate platelet reactivity without adverting collateral off-target effects on other cells and tissues. In animal studies, the use of GPVI inhibitory antibodies has effectively reduced experimentally induced thrombosis *in vivo* without any reported bleeding in rats (Li et al., 2007). Collagen, GPVI ligand, is a component of atherosclerotic plaque and subendothelial matrix, hence, interfering with GPVI-collagen binding would serve as a potential therapeutic strategy to prevent unnecessary platelet activation.

In addition to the well-characterized platelet receptors participating in collagen adhesion and activation, a number of additional proteins in platelets have been found to play a significant role in such process (Nieswandt and Watson, 2003). For instance, levels of protein tyrosine phosphorylation in GPVI/FcRγ-chain-deficient platelets were slightly induced in response to collagen stimulation (Savage et al., 1998). Others reported that mice platelets with low levels of GPVI/FcRγ-chain respond to collagen stimulation in presence of integrin $\alpha_2\beta_1$ blocking antibodies (Poole et al., 1997). These findings have raised a question whether all components and players for platelet-collagen interaction have been identified.

The discovery of HSP47 expression in platelets and its role in supporting collagen-mediated platelets functions warrant the investigation of this protein as a potential and novel antithrombotic target. HSP47 was first discovered in a proteomic study that aimed to identify platelet proteins recruited to membrane following GPVI stimulation (Kaiser et al., 2009). It was then shown that targeting HSP47 with an inhibitor resulted in

attenuated platelet aggregation in response to collagen, while aggregation of thrombin evoked platelets remained unaffected (Kaiser et al., 2009; Sasikumar et al., 2018). In the present study, the hypothesis was raised that HSP47 contributes to platelet-collagen interaction and activation and hence, regulation of thrombosis and haemostasis.

6.2 Heat shock protein 47, subcellular localisation and distribution in human platelets.

Endoplasmic reticulum (ER) residing HSP47 is a collagen molecular chaperone and indispensable prerequisite for collagen biosynthesis. It maintains correct folding and configuration of synthesised collagen via its transient interaction with procollagen monomers in the ER until collagen reaches the cis-Golgi or ER–Golgi intermediate compartment (ERGIC) where low pH causes the dissociation of HSP47 from collagen. HSP47 is readily retrieved back to ER via its ER retention sequence (RDEL) (Kambe et al., 1994; Masuda et al., 1994; Nagata, 1996). Such interaction maintains the correct folding of collagen and prevents unfolding events or the formation of early procollagen aggregates. The vital chaperoning role of HSP47 probably explains the high correlation of its expression with the levels of collagen production in various cells and tissues (Kambe et al., 1994; Masuda et al., 1994; Nagata, 1996).

Kaiser et al. (2009) and Rowley et al. (2011) confirmed the presence of HSP47 protein and transcripts in human platelets, respectively. Moreover, HSP47 has been quantified in a quantitative proteomics study where single platelets were estimated to contain 3800 molecules of HSP47 (Burkhart et al., 2012). However, platelets are non-collagen producing cells which made localisation and significance of HSP47 in platelets unclear. This unusual localisation raised the attention of the potential of this collagen chaperone in regulating platelet collagen functions.

In a previous study we established the megakaryocytic origin of this chaperone in platelets along with its impact on attenuating collagen-mediated platelet functions *in vitro*. Additionally, platelet-specific HSP47-deficient mice were generated and these platelets displayed similar diminished platelet functions in response to collagen (Sasikumar et al., 2018). To further explore the mechanism underlying negative impact of HSP47 inhibition or deletion on collagen evoked platelet functions, in the present study we examined the subcellular localization of HSP47 in platelets. Also, we examined the cellular events responsible for their movement to the platelet surface on activation where they are anticipated to participate in a platelet-collagen encounter. This would provide clues as to the mechanisms through which the roles of HSP47 are ultimately controlled.

Immunofluorescence and flow cytometry studies confirmed the surface expression of HSP47 on platelets (Sasikumar et al., 2018). As discussed above, HSP47 contains the carboxy-terminal amino acid residue sequence RDEL, which is an endoplasmic reticulum (ER) retention sequence. This sequence maintains retrieval and intracellular localisation of this chaperone into the ER (Ito and Nagata, 2017). The release of this protein to the platelet surface is therefore surprising. However, studies have reported the expression of a number of ER retention motifs containing proteins in platelets, for instance, PDI, endoplasmic reticulum protein (ERp) 5, 57, 72, 44 and 29. These proteins were also recruited and mobilised to platelet surface in response to agonist stimulation and have shown to support platelets function *in vivo* and *in vitro* (Essex et al., 1995; Jordan et al., 2005; Holbrook et al., 2010).

Also, the surface expression of HSP47 has been reported in the literature. Murine parietal endoderm cells express HSP47 on their surface where collagen binding capacity of this

chaperone was first discovered (Hogan et al., 1984; Kurkinen et al., 1984). Likewise, surface expression was also reported on epidermoid carcinoma cell lines and HCS-2/8 chondrosarcoma cells (Hebert et al., 1999; Hattori et al., 2003; Hattori et al., 2005). Being constitutively present on platelet surface implies the potential influence of HSP47 on collagen responses. For this to happen, HSP47 is expected to be present at levels quite enough to exert such effects. Hence, we sought to quantify HSP47 in human platelets. HSP47 was quantified and 7000 copies are estimated to be present per platelet.

In addition to surface localisation, HSP47 was noted to occupy subcellular space in confocal imaging of resting platelets. To further explore such localisation, the colocalization of HSP47 with distinct subcellular structures inside platelets was investigated using immunofluorescence studies. HSP47 displayed a high level of co-localisation with the dense tubular system (DTS) residing proteins PDI and calreticulin in resting platelets with similar surface mobilisation upon platelet stimulation.

Given the limitation in the resolution of confocal microscopy employed to address aforementioned colocalization in such small-sized platelets, this was further explored through subcellular fractionation analysis of platelet lysates. This approach has proven effective in studying similar colocalisation of several proteins in human platelets (Mairhofer et al., 2002; Crescente et al., 2016). In agreement with immunofluorescence studies, immunoblotting for HSP47 in the isolated platelet fractions revealed similar colocalisation with DTS markers. Moreover, HSP47 is well known to reside in the endoplasmic reticulum (ER) in nucleated cells (Kambe et al., 1994). As shown in microscopic studies concerned with platelet structure, platelets possess a DTS, a closed-channel network of the residual endoplasmic reticulum (White, 1972). Together this

validates HSP47 colocalisation with DTS observed in immunofluorescence and fractionation studies.

HSP47 has been reported to be expressed on stimulated platelets (Kaiser et al., 2009). Also, our immunofluorescence studies showed increased surface mobilisation of HSP47 upon activation. Given the DTS association of this chaperone, HSP47 may utilise mobilisation route toward the surface other than classical route exhibited by alpha granules cargo. Actin polymerisation was required for HSP47 to be mobilised to platelet periphery as shown in the reduction of HSP47 mobilisation in presence of latrunculin A (actin polymerisation blocker) (Spector et al., 1999; Crescente et al., 2016). Interestingly, the reliance on actin dynamics for peripheral mobilisation was reported to be required for thiol isomerase (PDI) (Crescente et al., 2016) which showed colocalisation with HSP47 in DTS in human platelets.

6.3 HPS47 is associated with lipid rafts and its release is induced upon platelet stimulation.

Lipid rafts serve as the assembly and sorting platform for signal transduction complexes, increase cell interactions and enhance intercellular crosstalk downstream of platelet receptors such as GPVI (Locke et al., 2002). Given the negative impact reported in various collagen-stimulated platelet functions in HSP47 deficient mice and upon the use of several HSP47 inhibitors (Kaiser et al., 2009; Sasikumar et al., 2018), the potential for HSP74 to interact with collagen receptor signalosome enriched platelet rafts was then assumed. Platelet lipid rafts were isolated based on its low density and insolubility in Triton™ X-100 detergent (Rabani et al., 2016). HSP47 was found to be present in raft containing fractions. Such an association would facilitate HSP47 participation in signalling events downstream of raft occupying collagen receptors. Besides membrane

and rafts association, our immunoblotting and flow cytometry analysis showed that HSP47 is released upon platelet stimulation with minor pool being released on microvesicles. Similarly, members of the HSP family has been reported to perform various roles and chaperoning functions extracellularly in response to several stimuli (Schmitt et al., 2007).

6.4 HSP47 supports platelet adhesion to collagen by modulating GPVI dimerization.

As mentioned earlier, previous studies established a potential role for HSP47 in collagen-induced platelet activation, but further elucidation of the mechanisms by which such chaperone modulates platelet functions required clarification (Kaiser et al., 2009; Sasikumar et al., 2018). Different forms of collagen with various preparations, receptor-specific synthetic collagens peptides such as GFOGER and CRP-XL along with receptors targeting inhibitory antibodies enabled better understanding and elucidation of the events underlying platelet stimulation by collagen at a molecular level. Therefore, adhesion to CRP-XL and GFOGER, GPVI and integrin α2β1 selective ligands, respectively, coated surfaces were assessed following inhibition with HSP47 using SMIH. Pre-treatment with SMIH reduced platelet adhesion and spreading to CRP-XL but not GFOGER. Such findings imply a direct or indirect effect of HSP47 on GPVI function.

To explore the mechanisms underlying attenuated platelet adhesion to CRP-XL, work was carried out to investigate potential modulation of GPVI dimerisation by HSP47. Such dimerisation has shown to increase the collagen binding competency of GPVI receptor (Jung et al., 2012). GPVI dimeric form was reduced in resting and stimulated platelets upon HSP47 inhibition. Direct physical interaction of HSP47 with GPVI which may explain the modulation of this receptor by HSP47 was then considered. This was

indeed shown in the positive interaction of HSP47 and GPVI observed in co-immunoprecipitation and microscopic studies. Since this dimeric form of GPVI is required for both platelet adhesion to collagen and subsequent activation (Jung et al., 2009; Jung et al., 2012), the present study suggests that previously established role of HSP47 in collagen-mediated functions may be due to its capability to support GPVI dimerization.

Although levels of adhesion and spreading to GFOGER in SMIH and vehicle control platelets were similar, an indirect impact on integrin α2β1 by HSP47 via GPVI is still possible. Following platelet stimulation, GPVI induced inside-out signalling events provoke structural changes in the extracellular domain of integrin α2β1. This results in integrin activation as represented by increased affinity for ligands. Such process controls and regulates the activation of integrins in platelets (Chen et al., 1994). Collagen and CRP-XL-stimulated platelets displayed a reduction in integrin β1 activated configuration levels in presence of SMIH. However, levels were unaffected by HSP47 inhibition when platelets were stimulated with GFOGER, integrin α2β1 selective ligand. Together, it seems unlikely that HSP47 has a direct effect on integrin α2β1 functional activation. Instead, integrin activation is indirectly impacted by HSP47 via GPVI.

6.5 HSP47 contribution to platelet activation is GPVI-dependent

As mentioned above, evidence from platelets adhesion to several collagen receptors selective ligands suggest a role of HSP47 in supporting platelets collagen responses mainly through GPVI. The availability of selective GPVI blocking agents (Muzard et al., 2009), allowed further investigation into the nature of HSP47 role in platelets activation. Platelets treated with GPVI blocking agent (Fab-9012) displayed a reduction in the levels

of integrin αIIbβ3 activation in response to agonist stimulation. However, the addition of SMIH failed to induce further reduction which implies HSP47 functioning via GPVI.

We also considered the chaperoning nature of HSP47 toward collagen in collagen-producing cells (Tasab et al., 2000; Matsuoka et al., 2004) and the possibility of having a similar scenario in platelet-collagen interaction. Therefore, work was extended to examine potential modulation of collagen by HSP47 which may results in enhanced platelets adhesion on collagen. Platelets adhesion was performed on HSP47 recombinant protein treated collagen and levels of adhesion was assessed against non-treated collagen-coated surfaces. We found that collagen pretreatment with recombinant HSP47 protein did not enhance platelet adhesion and therefore, collagen modulation by HSP47 is less likely to happen. These observations along with our reported finding of unaffected adhesion of SMIH treated platelets to vWF (GPIb ligand) (Sasikumar et al., 2018) indicate GPVI dependent action of HSP47 in platelets.

6.6 HSP47 inhibition attenuates collagen-mediated signalling events

Reversible protein phosphorylation is among highly important and well investigated posttranslational modifications. Indeed, 30 to 50% of intracellular proteins are estimated to undergo such modification at certain time points (Kalume et al., 2003). Almost all physiological events such as cellular growth, proliferation, differentiation and apoptosis are under the control of this regulatory and mechanistic process (Blume-Jensen and Hunter, 2001).

Platelets respond to extracellular stimuli by regulating protein function via regulation of phosphorylation process of a multitude of phosphoproteins mediated by family of protein kinases (Kunapuli et al., 2017). Protein kinases are phosphotransferases that catalyse the

transfer of γ-phosphate of ATP to tyrosine, serine, or threonine amino acids residues (Steinberg, 2004). Consequently, a cascade of intracellular activatory signals is triggered and an elevated level of second messengers, diacylglycerol (DAG) and Ca^{2+} are generated resulting in the activation of protein kinases and activation of platelets (Varga-Szabo et al., 2009; Li et al., 2010).

In recent years protein kinases have attracted growing interest as therapeutic drug targets where various inhibitors have been successfully designed and developed to modulate the activities of these enzymes (Cohen, 2009; Bynagari-Settipalli et al., 2010). Knowing the onset and site of actions by which phosphorylation controls proteins activity aid in understanding the mechanistic aspect of proteins action and possibly the discovery of new therapeutic targets.

Clustering of GPVI receptor via collagen or CRP-XL induces trans-auto-phosphorylation of the Src family kinases (SFKs) such as Lyn and Fyn (Senis et al., 2014). This is followed by SFK-dependent phosphorylation of FcRγ-chain-associated immunoreceptor tyrosine-based activation motif (ITAM). Consequently, tyrosine kinase Syk is recruited and auto-phosphorylated (Berlanga et al., 2002; Ellison et al., 2010). Inhibition of HSP47 attenuated platelet phosphorylation levels by hindering these early phases in collagen-mediated signalling as observed in the reduced phosphorylation of SFK Lyn. Interestingly, a similar effect on GPVI mediated signalling was observed in HSP47 deficient mouse platelets. Since HSP47 deficient mice have been characterized to possess normal platelet count and normal levels of platelet receptors including collagen receptors (Sasikumar et al., 2018), this further suggests that HSP47 functions in supporting GPVI-mediated signalling.

We previously established a lack of effect of HSP47 blockade on platelet aggregation in response to thrombin, GPCR agonist (Sasikumar et al., 2018). To confirm the lack of HPS47 effects on thrombin-mediated platelets activation, levels of thrombin-mediated phosphorylation events (Coughlin, 2000; Sidhu et al., 2014) were assessed following SMIH treatment. Consistent with the aforementioned finding, inhibition of HSP47 activity in human platelets did not alter thrombin generated phosphorylation events. Also, as mentioned earlier, HSP47 inhibition did not affect platelets adhesion on integrin α2β1 selective ligand peptide GFOGER. Hence, we then sought after exploring the impact of SMIH on the generation of integrin α2β1-mediated phosphorylation events in platelets (Inoue et al., 2003). No difference was recorded in outside-in signalling events generated upon the ligation of integrin α2β1 with its selective agonist peptide, GFOGER. Collectively, these findings are consistent with a role of HSP47 in the modulation of platelet function through GPVI.

6.7 Conclusion and future work

With the current lack of efficacy in the anti-platelet therapies, there is continuous need to look for alternative strategies to control platelet reactivity and this may include targeting platelet response to collagen. Evidence provided in the present study identifies the collagen molecular chaperone HSP47 as a new important player in platelet-collagen interaction and provide novel insight into the mechanistic aspects underlying such role. Given the newly identified GPVI dependent actions of HSP47 and its important role in regulating thrombosis and haemostasis, HSP47 may serve as a potential target for the development of anti-thrombotic agents. The present study established the role of HSP47 in platelets. However, the implication of HSP47 in megakaryocytes was beyond the scope

of the present research and hence several and wider aspects need to be addressed to better understand the originating role of this chaperone in megakaryocyte.

In the bone marrow, megakaryocytes interact with a various component in the extracellular matrix. Such interaction is vital to maintaining the maturation of these progenitor cells and consequent platelet production (Balduini et al., 2008; Malara et al., 2014). HSP47 has been implicated in fibrosis in a range of tissues such as liver (Kakugawa et al., 2004), while the HSP47 associated protein-lysine 6-oxidase (LOX) has been reported to contribute to the expansion of megakaryocyte and bone marrow fibrosis (Eliades et al., 2011). Moreover, analysis of HaemAtlas transcriptomic data (Watkins et al., 2009) reveals the expression of collagen in megakaryocyte. Hence, it would be relevant to investigate whether HSP47 is required for normal development of megakaryocytes and its involvement in fibrosis that is associated with myeloproliferative disorders. Impact of HSP47 ablation on the development of megakaryocyte can be addressed by assessing the ploidy and maturation of megakaryocytes in HSP47 ablated mouse bone marrow. This would reflect the early role of this chaperone in thrombopoiesis.

Bone marrow stroma has been reported to contribute to the process of pro-platelet formation (Avecilla et al., 2004). Extracellular components such as collagen and fibrinogen have been implicated in such a process. This has been shown in the reduction of proplatelet formation *in vitro* upon the addition of GPIbα, integrin αIIb and integrin αIIbβ3 antagonists to cultured megakaryocyte (Takahashi et al., 1999; Larson and Watson, 2006). Such reduction suggests that proplatelet formation may be controlled by matrix-receptor signalling in addition to the attachment surface provided by receptor-matrix interaction. While platelets count is unaffected by HSP47 ablation in KO mice, if the function of megakaryocytes is impacted by HSP47 ablation, this would be reflected

on the rate of platelets production. Such an impact has been reported in PECAM-1 deficient mice that showed reduced megakaryocytes migration toward sinusoids (Dhanjal et al., 2007). This could be addressed by examining the migration of megakaryocytes on the collagen surface in the presence or absence of HSP47. Also, this can be approached by inducing thrombocytopenic episode in HSP47 deficient mouse and then recovery of platelets counts would be monitored.

6.8 Concluding remarks

The work presented in this study revealed the surface expression of HSP47 along with other subcellular pool associating with DTS organelle. As other platelet surface expressed proteins, the expression of this chaperone was induced upon platelets stimulation, hence its implication in the thrombotic event is possible. Moreover, HSP47 was shown to be associated with lipid raft in platelet membrane indicating a potential influence on collagen receptors and downstream activatory signalling events. This was demonstrated later with HSP47 inhibition affecting platelet adhesion to collagen, and CRP-XL, but not GFOGER. Moreover, the study provided evidence for the underlying mechanism by which HSP47 contributes to platelet-collagen interaction and consequent activation via GPVI dimerization. This is suggested to be facilitated via physical interaction with GPVI receptor (Figure 6.1).

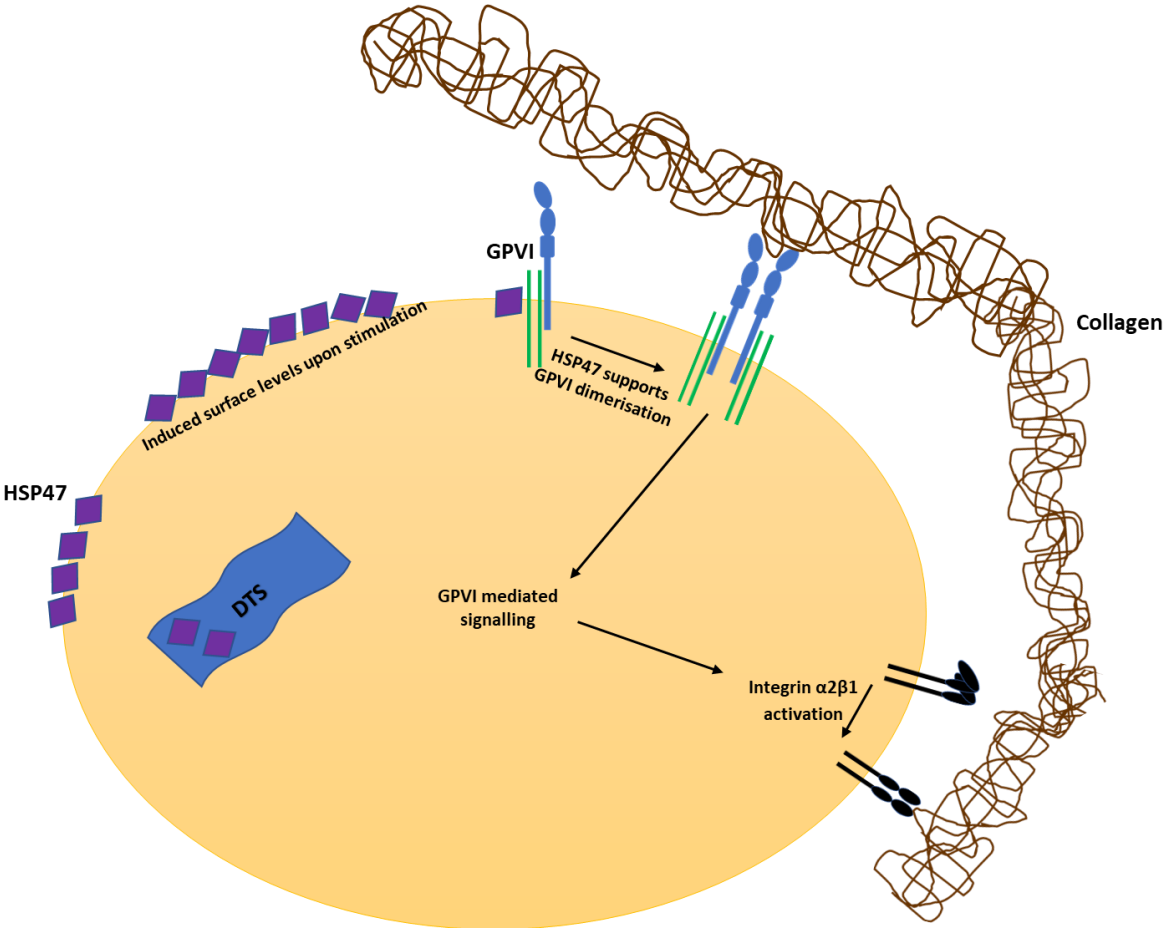

Figure 6.1: Schematic representation of HSP47 role in collagen-mediated platelet function.

Surface levels of HSP47 is increased in response to stimulation. Moreover, this chaperone occupies DTS compartment in platelets. HSP47 facilitates collagen receptor GPVI to bind to its substrate collagen causing platelet activation and upregulation of integrin α2β1 affinity toward collagen. Present evidence suggests role of HSP47 in GPVI dimerisation as possible mechanism by which this protein supports platelet function. This leads to the trigger of tyrosine phosphorylation of various signalling molecules downstream of collagen receptors resulting in platelet activation, aggregation and thrombus formation.

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