Factors that regulate thrombin generation in the blood: studies in healthy subjects and patients with thrombotic diseases

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Abstract

Cell-derived microparticles (MPs) have been demonstrated to play a major role in haemostasis and thrombosis, inflammation, vascular reactivity and angiogenesis. They are released from virtually all body cell types during activation or apoptosis. This project aimed to characterise the procoagulant properties of MPs derived from platelets and other vascular cells and evaluate the mechanisms whereby platelets generate procoagulant MPs.

A lack of standardisation of pre-analytical variables has hindered MP analysis. Systematic analysis revealed that platelet contamination should be efficiently removed from MP preparations using appropriate centrifugation protocols and that repeated freezing and thawing has no significant impact on the procoagulant activity of MPs providing the plasma is free of platelets and cell fragments, but does cause a slight decay of clotting factor activity.

Microparticles derived from platelets, endothelial cells and macrophages had significant procoagulant phospholipid (PPL) activity with macrophage-derived>platelet-derived >endothelial cell-derived microparticles. However, the tissue factor (TF) activity was different for each cell type with strong activity for macrophage-derived MPs, moderate activity for endothelial cell-derived MPs, but platelet-derived MPs did not express any TF activity.

It was demonstrated that platelet immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors; GPVI, FcγRIIA and CLEC-2, but not the G protein-coupled receptors (GPCRs), are the primary receptors eliciting platelet procoagulant response and MP formation. However, GPCRs play a crucial role in the feedback mechanism of platelet procoagulant response. Additionally, activation via CLEC-2 was demonstrated to induce platelet procoagulant responses and MP formation in a similar manner to GPVI.

Heparin-induced thrombocytopenia (HIT) is caused by the interaction between HIT-immune complexes with platelet FcγRIIA leading to platelet activation, MP generation and thrombocytopenia. MPs generated by HIT-immune complexes in suspected HIT patients was demonstrated to exhibit similar procoagulant activity to those generated through the other two platelet ITAM-containing receptors. Using their procoagulant activity measured in thrombin generation assay, it provides a rapid functional diagnostic assay for HIT with good correlation and association with other clinical and laboratory investigations.

Mohammed A. Alsahli

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Dedication

In the memory of my parents and sisters

During the second year of my PhD and on the 29th March 2012, in their way back home after they spent a nice time travelling with our extended family in the med-term holiday, a tragic car accident happened to my family where my father, Ali, my mother, Hussa, and my sister, Shorooq were killed in that accident.

Words cannot even begin to express our sorrow to lose our beloved parents and lovely little sister. Their death left us all devastated.

They indeed devoted themselves to us as their kids, to their trusted family and friends. For sure they are being dreadfully missed by everyone who had the good fortune to enjoy their friendship.

I also should not fail to remember my sister Monerah who passed away during my first few months of my PhD after long term fighting with disease.

May Almighty God rest their souls in peace, Amen

I dedicate this thesis to them.

Statement by the candidate

This thesis is the result of work carried out mainly during the period of registration. It is substantially the original work of the candidate. Where it is not, this is clearly stated in the text.

..……..

Mohammed Alsahli
November 2014

Publications arising from this thesis

Published abstracts have been presented at national and international conferences

1- Alsahli MA, Watson C, Popat BM, Horner K, Hopkins B, Davidson S, Kitchen S, van Veen JJ, Pavord S, Swallow G, Goodall AH. PROCOAGULANT ACTIVITY OF PLATELET-DERIVED MICROPARTICLES: A NOVEL METHOD TO DIAGNOSE HEPARIN-INDUCED THROMBOCYTOPENIA. British Society for Haemostasis and Thrombosis and Belgian Society on Thrombosis and Haemostasis Joint Meeting; Nottingham, UK. 10-11th Oct 2013. (Oral presentation).

2- Alsahli MA, Hamali HA and Goodall AH. CHARACTERISATION OF THE PROCOAGULANT PROPERTIES OF MICROPARTICLES DERIVED FROM DIFFERENT CELL TYPES. XXIV Congress of the International Society on Thrombosis and Haemostasis; Amsterdam, the Netherlands. June 29-July 4 2013. Abstract PA 3.04-2 (Poster presentation).

3- Alsahli MA, Appleby JA, Pollitt AY, Turnbull RE, Hughes CE, Hamali HA, Watson SP, Goodall AH. SIGNALLING VIA CLEC-2 GENERATES A PROCOAGULANT RESPONSE IN HUMAN PLATELETS. XXIV Congress of the International Society on Thrombosis and Haemostasis; Amsterdam, the Netherlands. June 29-July 4 2013. Abstract PB 2.26-3 (Poster presentation).

4- Popat BM, Watson C, Alsahli MA, Horner K, Hopkins B, Davidson S, Kitchen S, van Veen JJ, Pavord S, Swallow G and Goodall AH. DIAGNOSIS OF HEPARIN-INDUCED THROMBOCYTOPENIA USING HITALERT ASSAY. British Society for Haemostasis and Thrombosis and UK Platelet Group Joint Meeting; Bath, UK. 18-19th Oct 2012. BSTH Abstract P24, 2012 (Poster presentation).

Manuscripts in preparation

1- Alsahli MA, Appleby JA, Pollitt AY, Turnbull RE, Hughes CE, Hamali HA, Watson SP, Goodall AH. DIFFERENT CONTRIBUTION OF GPCRS AND ITAM-CONTAINING RECEPTORS IN PLATELET PROCOAGULANT ACTIVITY.

2- Alsahli MA, Popat BM, Watson C, Horner K, Hopkins B, Davidson S, Kitchen S, van Veen JJ, Pavord S, Swallow G, Goodall AH. EVALUATION OF TWO FUNCTIONAL ASSAYS FOR HEPARIN-INDUCED THROMBOCYTOPENIA DIAGNOSIS.

3- Alsahli MA, Hamali HA and Goodall AH. STANDARDISATION OF PRE- AND ANALYTICAL VARIABLES IN PROCOAGULANT MICROPARTICLE ANALYSIS AND CHARACTERISATION OF MICROPARTICLES DERIVED FROM DIFFERENT CELL TYPES.

Abbreviations

ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
AFM	Atomic force microscopy
APS	Antiphospholipid syndrome
aPTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CAT	Calibrated Automated Thrombogram
cDNA	Complementary deoxyribonucleic acid
CHO	Chinese Hamster Ovary
CLEC-2	C-type lectin-like receptor 2
COX-1	Cyclooxygenase-1
CRD-like	Carbohydrate-like recognition domain
CRP-XL	Collagen-related peptides cross-linked
C_T	Cycle threshold
CTI	Corn trypsin inhibitor
CV	Coefficient of variation
DAG	Diacylglycerol
Del-1	Developmental endothelial locus-1
DLS	Dynamic light scattering
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECDMPs	Endothelial cell-derived microparticles
ECL	Electrochemiluminescence
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbent assays
EM	Electron microscopy
ETP	Endogenous thrombin potential
FBS	Foetal bovine serum
FcRγ-chain	Fc receptor γ-chain
FITC	Fluoresceinisothiocyanate
FPP	Filtered pooled plasma
FS	Forward scatter
Gads	Grb2-related adaptor downstream of Shc
GAG	Glycosaminoglycan
GOI	Gene of interest
GP	Glycoprotein
GPCR	G protein-coupled receptor
GPRP	Gly-Pro-Arg-Pro peptide
GPVI	Glycoprotein VI
Grb2	Growth factor receptor bound protein 2
h.PDPN	Human podoplanin
HBS	HEPES-buffered saline
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIMA	HIT-induced microparticle assay
HIT	Heparin-induced thrombocytopenia
HK	Housekeeping
HMWK	High molecular weight kininogen
Ig	Immunoglobulin
IP3	Inositol-1,4,5-trisphosphate
ITAM	Immunoreceptor tyrosine-based activation motif
LAT	Linker for activation of T cells
LMWH	Low-molecular weight heparin
LPS	Lipopolysaccharide
MDMPs	Monocyte-derived microparticles
MP	Microparticle
mRNA	Messenger RNA
NPV	Negative predictive value
NTA	Nanoparticle tracking analysis
NTC	No template control
OCS	Open canalicular system
PAR	Protease-activated receptor
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDMPs	Platelet-derived microparticles
PDPN	Podoplanin
PE	Phycoerythrin
PF4	Platelet factor 4
PFP	Platelet-free plasma
PGI_2	Prostaglandin I_2
PI3K	Phosphatidylinositol 3-kinase
PIC	Protease inhibitor cocktail
PIP2	Phosphatidylinositol-4-5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PK	Prekallirein
PKC	Protein kinase C
PL	Phospholipid
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PPL	Procoagulant phospholipid
PPP	Platelet-poor plasma
PPV	Positive predictive value
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin glycoprotein ligand-1
RT	Room temperature
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SFK	Src family tyrosine kinase
SH2	Src homology domain 2
SLP-76	SH2-containing leuckcyte protein of 76 kDa
SNP	Single nucleotide polymorphism
Src	Nonrecptor tyrosine kinase
SS	Side scatter

Syk	Spleen tyrosine kinase
TBS	Tris-buffered saline
TEM	Transmission electron microscopy
TF	Tissue factor
TMEM16F	Transmembrane protein 16F
TNF-α	Tumour necrosis factor alpha
TRAP	Thrombin receptor agonist peptide
TxA_2	Thromboxane A_2
UFH	Unfractionated heparin
vWF	von Willebrand factor

CHAPTER 1: **General introduction**

1.1 **Haemostasis**

Haemostasis is the complex physiological process by which the body maintains blood in a fluid condition within the vascular system, stops bleeding in the event of vascular injury and removes excessive blood clots after vascular healing. This process is achieved by highly integrated and regulated interactions between the main four components of haemostasis; blood vessels, haematopoietic cells particularly platelets and microparticles (MPs), coagulation proteins and fibrinolysis. Upon vascular surface damage, blood becomes exposed to subendothelial extracellular matrix (ECM) components including von Willebrand factor (vWF), collagen, fibronectin, laminin and tissue factor (TF). Platelets interact and adhere to the ECM and subsequently they become activated, degranulated, leading to release soluble secondary mediators, which in turn recruit more platelets at the site of the injury. Platelets activation also shifts platelet integrins such as glycoprotein (GP) IIb/IIIa, to a high affinity state, leading to platelet aggregation through bridging with fibrinogen and subsequently formation of a haemostatic plug. Subendothelial TF initiates coagulation and generates trace amounts of thrombin and ultimately with multiple reinforcement loops in the coagulation system, large amounts of fibrin are formed from cleavage of fibrinogen, leading to stabilisation of the earlier formed platelet aggregate. Once a clot is formed and bleeding has stopped, the excessive clot is dissolved by fibrinolysis, which is mainly mediated by the action of plasmin. Imbalance in one or more of the components of these interactions leads to pathological conditions such as haemorrhage or thrombosis (Roberts, *et al* 2004, Versteeg, *et al* 2013, Vine 2009).

1.2 Coagulation

1.2.1 Cascade model of coagulation

The classical cascade model of coagulation consists of a sequential activation steps of serine protease enzymes that present in the circulation as zymogens (also known as proenzymes) to generate the next enzyme in the cascade, so-called waterfall model (Davie and Ratnoff 1964, Macfarlane 1964). This model is based on two independent cascade pathways, the extrinsic which requires subendothelial TF, and the intrinsic pathway in which all components are present in the circulation. Both pathways lead to the activation of Factor X (FX) in the common pathway and the subsequent generation of thrombin (Figure 1.1). The extrinsic pathway is thought to be initiated via the binding of FVIIa to TF at the site of vascular damage. The TF/FVIIa complex activates FX to FXa in the common pathway. In the intrinsic pathway or contact system, FXII is activated to FXIIa by negatively-charged surfaces and in the presence of prekallirein (PK) and high molecular weight kininogen (HMWK). FXIIa subsequently activates FXI to FXIa that in turn activates FIX to FIXa. FIXa in complex with FVIIIa activates FX to FXa in the common pathway. FXa then, with its cofactor FVa, converts prothrombin (FII) to thrombin, which in turn cleaves fibrinogen to fibrin monomers and ultimately forms a fibrin clot. Most of the proteolytic activity of the coagulation factors is dependent on the presence of calcium (Ca^{2+}) and anionic negatively-charged phospholipids (PLs), particularly phosphatidylserine (PS) (Bach, *et al* 1986, Thiagarajan and Tait 1990).

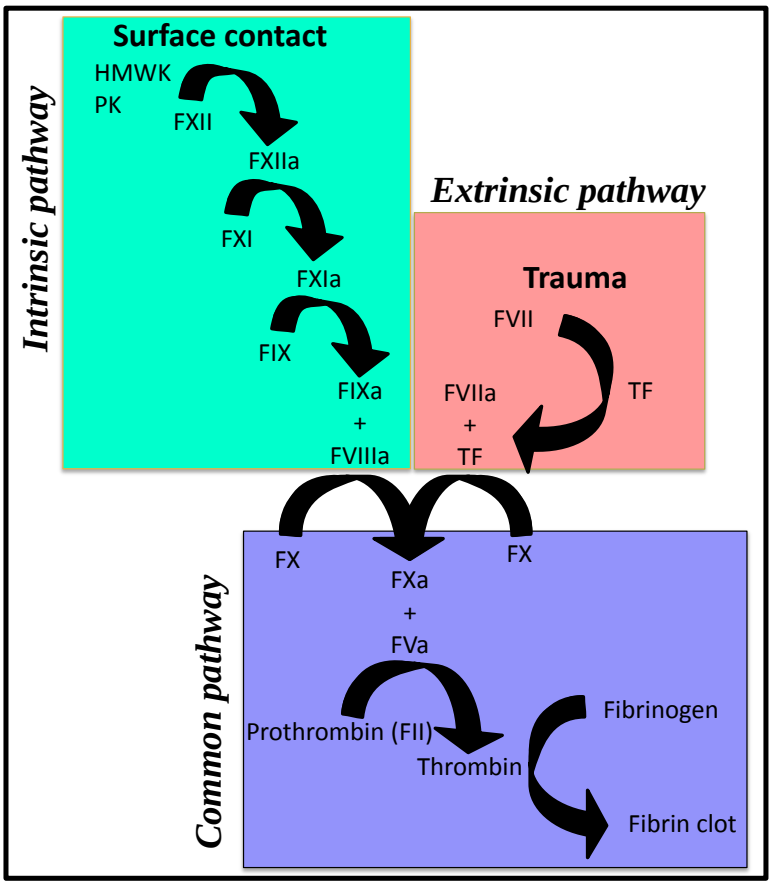


Figure 1.1. The cascade model of coagulation. Reactions in green-shaded box show the intrinsic pathway; in red, the extrinsic pathway; and in blue, the common pathway. HMWK: high molecular weight kininogen; PK: prekallirein; 'a' denotes the active form of the coagulation factor. Adapted from Roberts et al, 2004.

The development of the classical cascade model of coagulation was a major advance in the understanding of how coagulation process occurs *in vitro*, which provided useful interpretations of laboratory tests for coagulation system abnormalities. However, although this model supports laboratory investigations of coagulation disorders, it has inadequacies as a model of the *in vivo* haemostatic processes. According to this model, the intrinsic and the extrinsic pathways activation occur independent of each other, and both can result in the production of thrombin, while clinical observations of individual factor deficiencies contradict this concept. For instant, deficiency in FVII is associated with a very high risk of bleeding, despite the presence of an intact intrinsic pathway. Conversely, deficiency in FXI is

associated with mild bleeding tendency compared to deficiencies in FVIII or FIX that are associated with severe bleeding (Haemophilia A and B respectively), despite being in the next downstream step of the same pathway. Also FIX and FVIII deficiencies are not compensated by the intact extrinsic pathway. Additionally, although deficiencies in the contact pathway components PK, HK, and FXII, significantly prolong the activated partial thromboplastin time (aPTT), a test for the intrinsic pathway, they do not cause a bleeding disorder. Instead of the classical cascade model of coagulation, a cell-based model of coagulation has been recently proposed to replace the traditional cascade theory (Hoffman 2003, Hoffman and Monroe 2001, Roberts, *et al* 2004, Smith 2009, Vine 2009).

1.2.2 Cell-based model of coagulation

This model of coagulation places more emphasis on the interaction of clotting factors with cell surfaces, particularly TF and PS on cell surfaces, and provides a more realistic explanation of coagulation processes as they occur *in vivo*, rather than *in vitro*. According to a widely used model, the cell-based model can be divided into three overlapping phases, initiation, amplification and propagation. In the initiation phase, which is essentially the same as the classical extrinsic pathway, flowing blood becomes exposed to TF-bearing cells at the site of vascular injury. Circulating FVIIa immediately binds to TF forming TF/FVIIa complex, which subsequently cleaves small amounts of FIX and FX into FIXa and FXa, respectively (Figure 1.2). FXa slowly activates FV to FVa and forms the prothrombinase complex (FXa/FVa) on TF-positive cell surfaces, which subsequently forms a small amount of thrombin by cleaving FII. The small amount of thrombin generated at this stage is considered as a key factor for the amplification phase. During the amplification phase, platelets adhered to the site of the injury and become exposed to collagen and thrombin,

which in turn they become activated. At the same time, this low level of thrombin activates FV and FXI to FVa and FXIa respectively. In addition, thrombin cleaves FVIII from vWF and converts it into FVIIIa, which acts as a cofactor to FIXa to form the tenase complex. Platelets at this stage have FVa, FVIIIa and FXIa bound to their surfaces, which play a crucial role in the propagation stage. During the propagation stage, the intrinsic tenase complex (FIXa/FVIIIa) rapidly activates FX to FXa on the platelet, which in turn binds to its cofactor FVa and forms more prothrombinase complex. This complex converts large amount of FII to thrombin, which allows the subsequent cleavage of soluble fibrinogen to fibrin monomers and ultimately forms a fibrin clot (Hoffman 2003, Hoffman and Monroe 2001, Roberts, *et al* 2004, Smith 2009, Versteeg, *et al* 2013, Vine 2009).

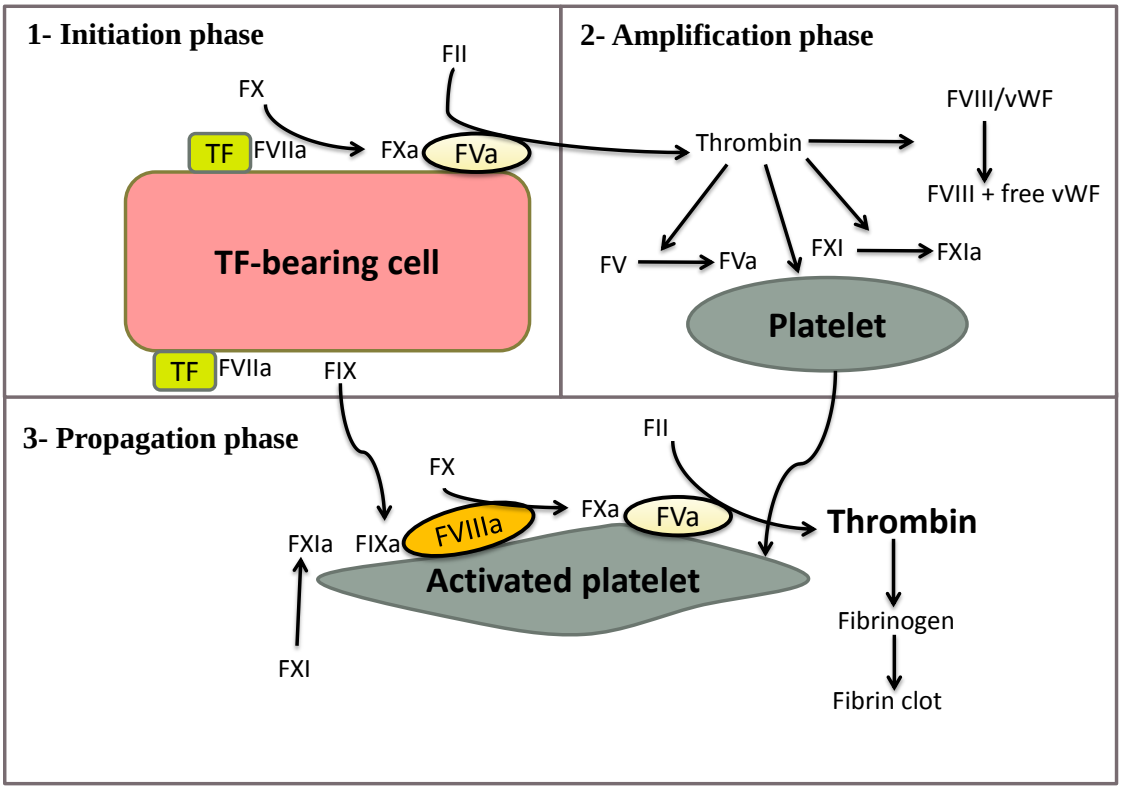


Figure 1.2. The cell-based model of coagulation. Illustration of the initiation, amplification and propagation phases of thrombin generation. Adapted from Hoffman (2003).

1.3 Role of PLs in haemostasis

It is well established that negatively-charged PL surfaces, plays a key role in several of the reactions of the coagulation process. The plasma membrane of normal cells is characterised by its asymmetrical distribution of phospholipids: negatively-charged phospholipids, such as PS and phosphatidyl-ethanolamine are located in the inner layer, whereas neutrally-charged such as phosphatidycholine and sphingomyelin are present in the external layer. This membrane asymmetry is tightly controlled under normal conditions. However, during cellular activation or apoptosis, there is loss of membrane asymmetry with the negatively-charged PLs being transferred to the external layer of the plasma membrane or MP (described in more details in section 1.6.2). The expression of negatively-charged PLs, particularly PS, on the cell or MP surfaces greatly enhances the assembly and the catalytic activity of the clotting cascade, in particular the TF/FVIIa, prothrombinase and tenase complexes. This enhancement is thought to be mediated via the electrostatic interaction between the negative charge of the PLs and Ca^{2+}, which in turn bridges with γ-carboxyglutamic acid (GLA) domains of some of the coagulation factors including FVII, FIX, FX and prothrombin (Heemskerk, *et al* 2002, Monroe, *et al* 2002, Owens and Mackman 2011, Zwaal, *et al* 2005). Conversely, the negatively-charged PLs have been also shown to be important for some of the anticoagulant factors. For instance, PLs are important for the inactivation of FVa by activated protein C (APC) (Ravanat, *et al* 1992).

1.4 Role of TF in haemostasis

TF, also known as thromboplastin or CD142, is the principle physiological initiator of coagulation. It is a 47 kDa transmembrane glycoprotein that acts as a cofactor for FVIIa, which greatly enhances its activity, particularly in the presence of negatively-charged PLs. TF is widely and constitutively expressed in cells of the adventitial and medial layers of the

vessel wall, serving as a haemostatic envelope that instantly triggers the clotting process upon vascular injury (Bach 1988, Drake, *et al* 1989, Khorana, *et al* 2008). For many years, it has been thought that TF is exclusively expressed in the extravascular tissues. However, the traditional concept fails to explain how coagulation is triggered within the bloodstream in the absence of vascular injury. Recent evidence has revealed that whole blood, even in healthy individuals, may already contain functionally active TF, so-called blood-borne TF (Giesen, *et al* 1999). It is thought that circulating blood-borne TF is associated with MPs and as a soluble form (Bogdanov, *et al* 2003). Additionally, vascular cells such as endothelial cells and monocytes express TF either at very low level or in inactive (also called encrypted) form. However, stimulation of these cells results in increase in the TF expression and activity (Breitenstein, *et al* 2010, Osterud 2012).

1.5 **Platelets**

1.5.1 Structure and function

Platelets, also called thrombocytes, are small anucleated subcellular fragments of megakaryocytes. They circulate in the blood for approximately eight to ten days before being removed from the circulation by macrophages mainly in the spleen. Platelets play a crucial role not only in haemostasis and thrombosis, but also contribute to several other physiological and pathological mechanisms such as inflammation, immunity, damaged tissue healing and tumour metastasis. Of these functions, the main physiological function is to minimize a possible blood loss and maintain the integrity of the vascular system (Gawaz, *et al* 2005, Leslie 2010, Ware and Suva 2011, Willoughby, *et al* 2002).

Platelets have a dynamic glycocalyx surface coating, plasma membrane, and membrane cytoskeleton. The cytoskeleton of platelets plays a crucial role in maintaining their discoid

shape through its network of microtubules, actin filaments and spectrin that are located proximal to the plasma membrane. Additionally, platelets have a surface-connected system of channels called the open canalicular system (OCS) that provides access to the interior of plasma components. There are also two types of specialised cytoplasmic granules: α-granules which are the most numerous and contain the greatest abundance of high molecular weight proteins such as cytokines, FV, vWF, P-selectin, thrombospondin, fibrinogen, fibronectin, plasminogen, and platelet factor 4 (PF4); and dense granules which are fewer in number and contain small molecules such as calcium, magnesium, phosphate, adenosine triphosphate (ATP), adenosine diphosphate (ADP) and histamine (Koseoglu and Flaumenhaft 2013, Menter, *et al* 2014, Thon and Italiano 2010). In their resting state, platelets are present in the circulation in a discoid shape with dimensions approximately 3 µm x 0.5 µm; however, platelet activation induces extensive cytoskeletal changes depending upon the nature of the stimuli leading to profound morphological changes. During the activation, microtubules and cytoskeleton actin filaments rapidly disassemble and collapse resulting in less rigid spheroid morphology and then platelets extend as filopodia and lamellipodia depending on the external force and physiological requirements. The OCS also contributes in the increasing plasma membrane surface area during platelet activation (Hartwig 1992, Menter, *et al* 2014, Nachmias 1980).

Platelet adhesion and activation at the site of vascular damage is induced by a multistep process, including multiple platelet receptor-ligand interactions. The first interaction of platelets with the ECM components under the high sheer rates is thought be mediated via the binding of vWF with its receptor on platelets membrane, GPIbα that forms part of the GP Ib-IX-V complex. This binding decelerates circulating platelets and retains them close to the vessel wall, which enables collagen in the ECM to interact with platelets through its receptor, glycoprotein VI (GPVI). The interaction between collagen and GPVI induces potent platelet

activation leading to actin polymerisation, which results in rapid changes in the morphological appearance from discoid to spherical shape followed by spreading on the damaged vascular surface. This activation also triggers intracellular signals, leading to a shift platelet integrins such as GPIIb/IIIa and GPIa/IIb from low to high affinity states. The interaction of activated integrins with the ECM promotes firm monolayer platelet adhesion at the site of vascular injury as well as inducing platelet aggregation through fibrinogen bridging with adjacent platelets via activated GPIIb/IIIa. At the same time, platelet activation induces the secretion of α-granule contents including adhesive proteins such as vWF and fibrinogen as well as the secretion of secondary platelet agonists, particularly ADP and thromboxane A_2 (TxA_2). Local accumulation of the secondary mediators, ADP and TxA_2, recruit and activate additional platelets from the circulation leading to formation of a platelet plug that seals the wound. Another important contribution of platelets at the site of vascular injury is the exposure of negatively-charged PLs, mainly PS, and the release of procoagulant MPs, which enhance the generation of thrombin through the coagulation process. Generated thrombin further activates platelets as well as cleaves fibrinogen to form fibrin, which in turn results in the formation of a fibrin mesh that further stabilises the early formed thrombus and occludes the wound (Heemskerk, *et al* 2005, Nieswandt, *et al* 2011, Offermanns 2006, Samara and Gurbel 2003, Varga-Szabo, *et al* 2008).

There has been a highly controversial debate whether platelets express TF or not. Initially, it was thought that TF is transferred to platelets following the interaction with TF-expressing cells since TF could not be isolated from megakaryocytes (Muller, *et al* 2003, Rauch, *et al* 2000). Indeed, it has been shown that platelets have the capacity to uptake TF from monocytes or TF-bearing MPs in the circulation, a process that is mainly mediated via the interaction of P-selectin glycoprotein ligand-1 (PSGL-1) and P-selectin on the surface of platelets (del Conde, *et al* 2005, Falati, *et al* 2003). On the other hand, it has been shown that

platelets have the ability to synthesize TF (Camera, *et al* 2003, Schwertz, *et al* 2006). In contrast, other studies failed to detect the presence or synthesis of TF in platelets (Bouchard, *et al* 2012, Bouchard, *et al* 2010, Osterud and Olsen 2013, Osterud, *et al* 2009).

1.5.2 Platelet agonists and receptors

Platelet activation initiates complex intracellular signalling pathways resulting in different platelet responses depending on the nature of the stimuli. Platelet agonists activate platelets via the interaction with a wide range of surface receptors that have distinct signalling pathways, all of which involve calcium signalling. There are two principle types of activating receptors and associated downstream signalling pathways in platelets. One group are the immunoreceptor tyrosine-based activation motif (ITAM)-containing receptors, GPVI, FcγRIIA and C-type lectin-like receptor 2 (CLEC-2); the other is via G protein-coupled receptors (GPCRs).

1.5.2.1 **ITAM-containing receptors**

1.5.2.1.1 **GPVI**

GPVI is platelet-specific transmembrane type I receptor, a member of immunoglobulin (Ig) superfamily that is non-covalently associated with the ITAM-containing Fc receptor γ-chain (FcRγ-chain) dimers. GPVI plays a critical role in platelet activation and thrombus formation at the site of vascular damage by interacting with collagen and laminin in the ECM. There are nine forms of collagen expressed in the vessel wall, but the fibrillar types I and III are the most common types in the ECM. Collagen binds with platelets through several receptors, however, the most significant interaction is with GPVI (Jandrot-Perrus, *et al* 2000, Nieswandt, *et al* 2011, Nieswandt and Watson 2003, Verkleij, *et al* 1998, Watson, *et al*

2010). There are other ligands that selectively interact with GPVI such as collagen-related peptides cross-linked (CRP-XL) (Asselin, *et al* 1997, Morton, *et al* 1995), snake venom toxin convulxin (Jandrot-Perrus, *et al* 1997, Polgar, *et al* 1997) and GPVI-specific antibodies such as JAQ1(Nieswandt, *et al* 2000).

GPVI signalling is dependent on sequential activation of Src and Syk family kinases. The FcRγ-chain dimers contain a typical ITAM with two cytosolic YxxL motifs, which is considered as docking sites for Syk phosphorylation (Figure 1.3). When GPVI is cross-linked either by collagen or by other ligands, this leads to bring the two Src family kinases, Fyn and Lyn, into contact with their substrate, the ITAM motif in the FcRγ-chain, leading to its phosphorylation. The phosphorylated ITAM provides a docking site for the Src homology domain 2 (SH2) domains of the Syk family tyrosine kinases. Upon binding of Syk to the phosphorylated ITAM, it undergoes auto-phosphorylation as well as phosphorylation by Src kinases. Syk requires two phosphorylated ITAMs. Subsequently, Syk initiates the phosphorylation and activation of downstream events, including the membrane scaffolding protein LAT. Among the proteins that bind to LAT are Grb2, Gads and SLP-76. These three proteins play a key role in regulating the major effector enzymes in the ITAM-containing receptor signalling mechanisms, PLCγ2. The activation of PLCγ2 leads to hydrolysis of its substrate PIP2 to liberate the second messengers IP3 and DAG; the former leading to the release Ca^{2+} from intracellular stores and the latter activating protein kinase C. PLCγ2 also binds to the product of phosphatidylinositol 3-kinase (PI3K), PIP3, via its PH domain, which further supports the PLCγ2 localisation to the membrane (Bergmeier and Stefanini 2013, Blake, *et al* 1994, Daniel, *et al* 1994, Gibbins, *et al* 1996, Suzuki-Inoue, *et al* 2002, Watson, *et al* 2005, Yanaga, *et al* 1995).

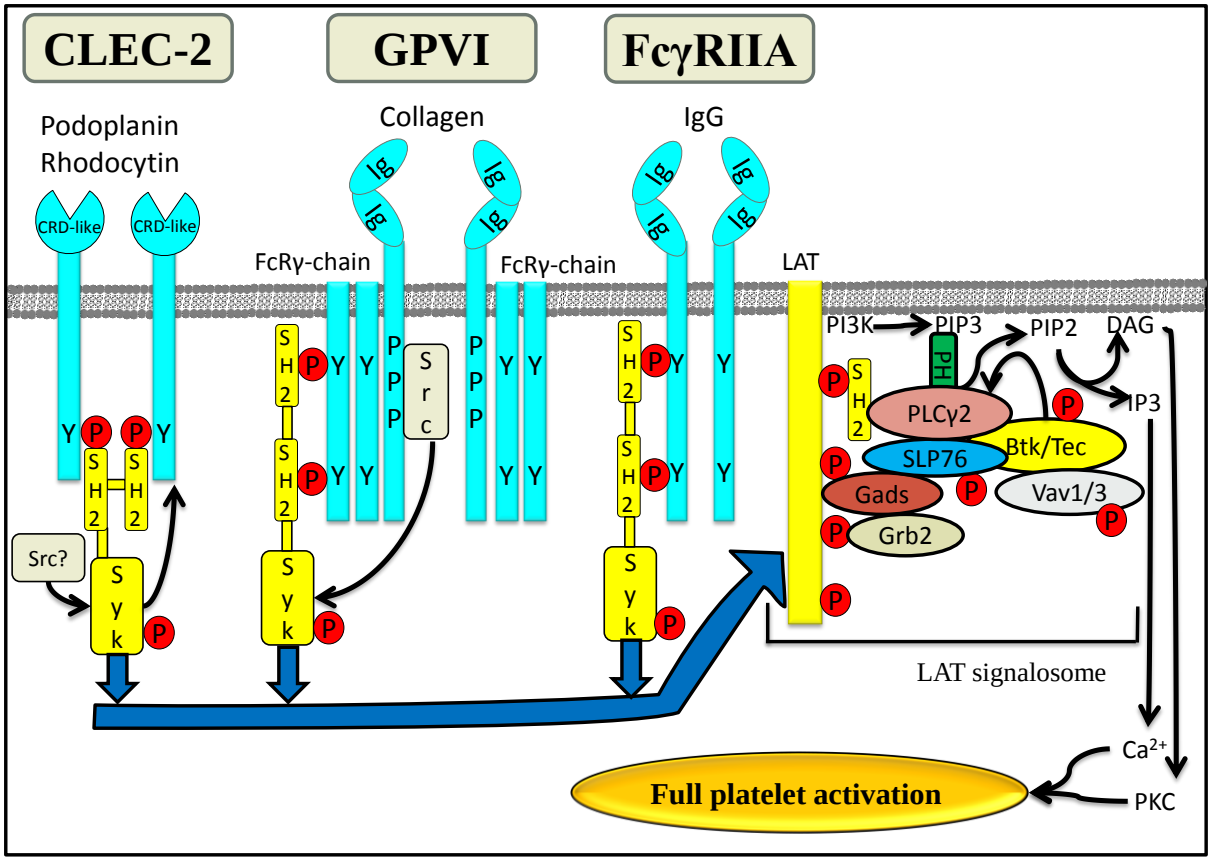


Figure 1.3. Platelet ITAM-containing receptors signalling pathways. Platelets express three known ITAM-containing receptors; GPVI, FcγRIIA and CLEC-2. Upon activation of these receptor by clustering, the YxxL motifs in the cytoplasmic domains of the receptors or the associated FcRγ-chain become tyrosine phosphorylated. The phosphorylated tyrosines provide docking sites for the recruitment and subsequent activation of Syk via its SH2 domains. Subsequently, Syk-dependent signalling cascades lead to formation of an LAT signalosome and activation of PLCγ2. CRD-like, carbohydrate-like recognition domain; Ig, immunoglobulin; Src, nonrecptor tyrosine kinase; SH2, Src homology domain 2; SLP-76, SH2-containing leuckcyte protein of 76 kDa; Syk, spleen tyrosine kinase; LAT, linker for activation T cells; PLC, phospholipase C; Grb2, growth factor receptor bound protein 2; Gads, Grb2-related adaptor downstream of Shc; Btk, Bruton's tyrosine kinase; Tec, tyrosine kinase expressed in hepatocellular carcinoma; Vav1/3, Rho-family guanine exchange factor; PH, pleckstrin homology; PI3K, phosphatidylinositol 3-kinase; PIP2, phosphatidylinositol-4-5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; IP3, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C. Adapted from Watson *et al* (2010) and Bergneier and Stefanini (2013).

1.5.2.1.2 **FcγRIIA**

A second ITAM-containing receptor on platelets is FcγRIIA, the platelet IgG receptor. FcγRIIA is also expressed on neutrophils, monocytes and macrophages. FcγRIIA plays a major role in the development of destructive inflammation and antibody-induced thrombocytopenia such as in heparin-induced thrombocytopenia (HIT). In HIT, anti-PF4/Heparin immune complexes activate platelets through FcγRIIA, leading to platelet activation, PS exposure, MP generation, thrombus formation and thrombocytopenia (Davoren and Aster 2006, Greinacher, *et al* 2007). However, the physiological role FcγRIIA in haemostasis remains unclear. Because FcγRIIA is not expressed on mouse platelets, it has been difficult to investigate its actual role *in vivo*. However, several studies suggested that FcγRIIA could play an important role haemostasis and thrombosis based on *in vitro* studies. It has been recently shown that FcγRIIA increases platelet sensitivity to thrombin (Berlacher, *et al* 2013). Additionally, it was observed that platelets that express low levels of FcγRIIA had low ability to spread on immobilised fibrinogen *in vitro*. The authors of that study demonstrated the downstream signalling pathway of FcγRIIA plays a key role in the outside-in signal amplification mediated by GPIIb/IIIa (Boylan, *et al* 2008). It was proposed that FcγRIIA is incorporated into GPIIb/IIIa clusters, bringing it close to the activated Src family kinases which then leads to phosphorylate its ITAM domains and subsequently provides a docking site for Syk (Boylan, *et al* 2008, Gao, *et al* 2009). A more recent study demonstrated that FcγRIIA expression in mouse platelets using transgenic mice with human FcγRIIA significantly enhances thrombus formation and stability *in vitro* as well as *in vivo*, further supporting the previous report of Boylan et al (2008) that FcγRIIA plays an important role in the outside-in signal amplification mediated by GPIIb/IIIa (Zhi, *et al* 2013).

Structurally, FcγRIIA is composed of two extracellular Ig domains similar to GPVI. Additionally, FcγRIIA has a typical ITAM with two cytosolic YxxL motifs (Figure 1.3).

However, the ITAM motifs of FcγRIIA are present in the cytoplasmic tail of the receptor, whereas in GPVI are in the FcRγ-chain dimers associated with GPVI receptor (Hibbs, *et al* 1988, Huang, *et al* 1992). The signalling pathway of FcγRIIA is believed to be similar to that of GPVI where cross-linking pf FcγRIIA with immune complexes or specific antibodies induces platelet activation through ITAM-dependent signalling mechanism initiated by tyrosine kinase cascades and ultimately results in PLCγ2 activation (Hulett and Hogarth 1994, Maxwell, *et al* 1999, Ozaki, *et al* 2000).

1.5.2.1.3 **CLEC-2**

CLEC-2 is type II transmembrane receptor that has been relatively recently identified to mediate platelet activation. CLEC-2 is also expressed in other cells such neutrophils, macrophages, liver sinusoidal endothelial cells and liver Kupffer cells. The only known physiological ligand of CLEC-2 is podoplanin, but can also be activated by the snake venom toxin, rhodocytin. Podoplanin is expressed on the surfaces of a wide range of cells such as lymphatic endothelial cells, kidney podocytes, fibroblastic reticular cells, lung type I alveolar cells, and some tumour cells, but not on vascular endothelial cells. Platelet activation via CLEC-2 has been shown to play a critical role in lymphatic/blood vessel separation in early development, in tumour metastasis and in HIV transmission (Navarro-Nunez, *et al* 2013, Nieswandt, *et al* 2011, Suzuki-Inoue, *et al* 2011, Watson, *et al* 2010).. However, because the only known natural ligand for CLEC-2, podoplanin, is absent in the cardiovascular system, its actual role in haemostasis and thrombosis is still poorly understood. Although podoplanin is not expressed in endothelial cells or in the ECM, a role of CLEC-2 in haemostasis has been demonstrated by the significant increase in the bleeding time in mice depleted of CLEC-2 using specific antibody (May, *et al* 2009). However, other studies showed only a mild increase in the bleeding time in CLEC-2-deficient mice, suggesting that CLEC-2 may play only a minor role in haemostasis (Hughes, *et al* 2010a, Suzuki-Inoue, *et al* 2010).

Interestingly, combined depletion in GPVI and CLEC-2 results in a dramatic haemostatic defect and significant impairment in thrombus stability (Bender, *et al* 2013), although GPVI-deficiency alone exhibits only a mild bleeding tendency (Dumont, *et al* 2009, Hermans, *et al* 2009). These findings suggest that GPVI and CLEC-2 could compensate for each other in preventing blood loss. At the level of platelet activation, CLEC-2 is considered as a strong inducer for platelet aggregation and secretion; however, its role in inducing platelet procoagulant activity remains to be established (Navarro-Nunez, *et al* 2013, Nieswandt, *et al* 2011, Suzuki-Inoue, *et al* 2011, Watson, *et al* 2010).

Unlike the typical ITAM-containing receptors, CLEC-2 has only a single YxxL motif in its cytoplasmic tail known as hemITAM (Figure 1.3). Signalling through CLEC-2 is similar, to some extent, to the signalling through GPVI (Suzuki-Inoue, *et al* 2011, Watson, *et al* 2010). However, the proximal events in the CLEC-2 signalling pathway are distinct from those in GPVI. The phosphorylation the hemITAM in CLEC-2 is mediated by both Src and Syk tyrosine kinases (Spalton, *et al* 2009), whereas in GPVI is dependent on Src kinases only. As mentioned in section 1.5.2.1.1 that GPVI signalling pathway is initiated by Src kinases leading to phosphorylate the YxxL motifs, which in turn recruit and activate Syk kinases. In contrast, the YxxL motif of CLEC-2 is believed to be phosphorylated by Syk kinases, which in turn activated by Src kinases. Since Syk kinases require two phosphorylated YxxL motifs, it has been suggested that occurs in two clustered CLEC-2 receptors. It has been shown that CLEC-2 forms monomers and dimers in resting platelets, which are active only through dimerization. Upon CLEC-2 dimerization, Syk molecules bind to two phosphorylated hemITAM in two CLEC-2 receptors, and this receptor clustering induces stronger signalling (Hughes, *et al* 2010b, Watson, *et al* 2009). Another important difference between CLEC-2 and GPVI was reported by Pollitt et al (2010), demonstrating that phosphorylation and activation of CLEC-2 is more dependent on actin polymerisation, secondary wave mediators

ADP and TxA_2, and activation of Rac (Pollitt, *et al* 2010). Nevertheless, the downstream signalling events of CLEC-2 are similar to those induced by GPVI (Suzuki-Inoue, *et al* 2011, Watson, *et al* 2010).

1.5.2.2 **GPCRs**

A variety of soluble platelet agonists are released from damaged or activated cells such as ADP and TxA_2, or produced during coagulation such as thrombin. These agonists activate platelets though GPCRs, a family of seven transmembrane domain receptors. GPCRs transmit signals via heterotrimeric G protein, which ultimately induce platelet activation (Figure 1.4) (Li, *et al* 2010, Offermanns 2006, Stegner and Nieswandt 2011).

1.5.2.2.1 **ADP and its receptors**

ADP has been shown to play a critical role in the positive-feedback mechanism during platelet activation. ADP is released from damaged endothelial cells and from the dense granules of platelets upon activation. Platelet activation via ADP is mediated by two purinergic GPCRs, $P2Y_1$ and $P2Y_{12}$, which are coupled to G_q and G_i respectively (Murugappa and Kunapuli 2006, Offermanns 2006). It has been shown that $P2Y_1$ receptor plays a key role in ADP-mediated Ca^{2+} mobilization required for shape change and aggregation (Hechler, *et al* 1998). On the other hand, $P2Y_{12}$ has been shown to be necessary for ADP-induced platelet aggregation and thrombus stability (Leon, *et al* 1999).

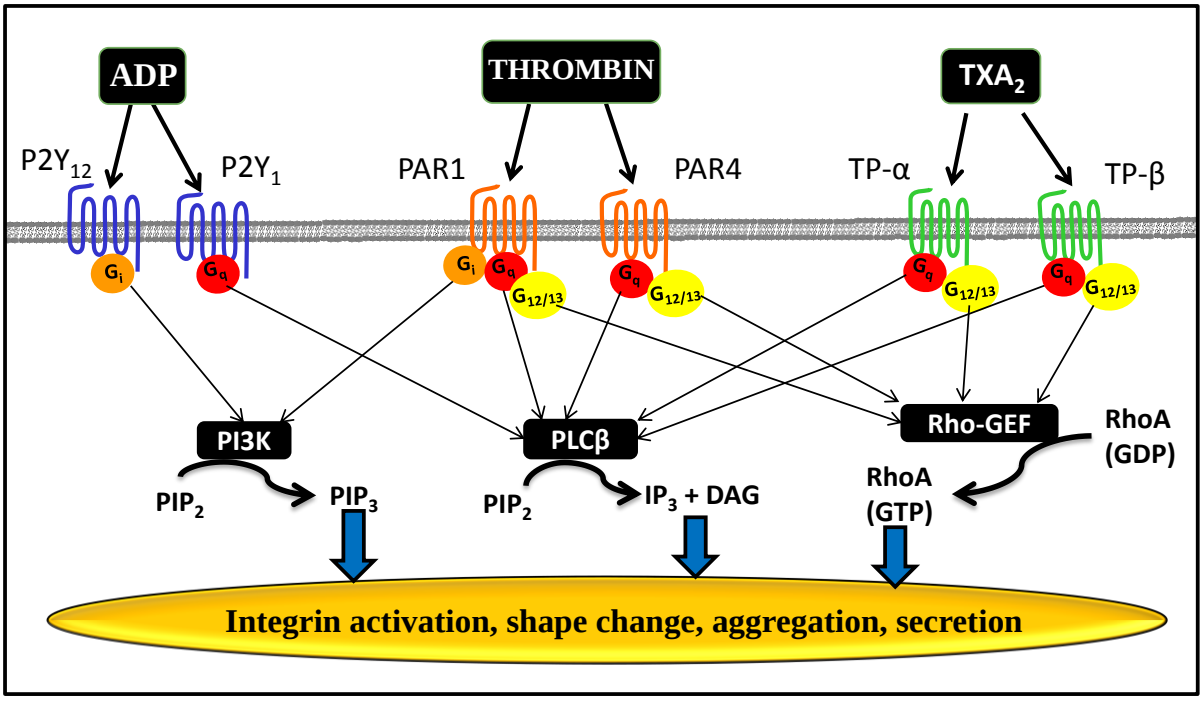


Figure 1.4. Platelet GPCRs signalling pathway. PI3K, phosphatidylinositol-3-kinase; PLC, phospholipase C; PIP2, phosphatidylinositol-4-5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; IP3, inositol-1,4,5-trisphosphate; DAG, diacylglycerol; RhoGEF, Rho-specific guanine nucleotide exchange factor. Adapted from Stegner and Nieswandt (2011).

1.5.2.2.2 **Thromboxane A_2 and its receptors**

TxA_2 is produced from its precursor arachidonic acid via conversion by cyclooxygenase-1 (COX-1). Released TxA_2 activates platelets through its platelet receptor, TP that is coupled to G_q and $G_{12/13}$. Human TP receptors exist in two variant isoforms, TPα and TPβ, which differ in their cytoplasmic domains. Similar to ADP, TxA_2 is an important second wave mediator that plays an important role in the positive-feedback mechanism during platelet activation (Offermanns 2006). It has been shown that the lack of the TxA_2 platelet receptors results in delayed platelet aggregation, prolonged bleeding time and low thrombus stability (Thomas, *et al* 1998).

1.5.2.2.3 **Thrombin and its receptors**

Thrombin, the most potent soluble platelet agonist, activates platelets through protease-activated receptors (PARs), which couple to G_q, $G_{12/13}$ and G_i. Of the four PARs, human platelets express PAR1 and PAR4. PAR receptors are activated when thrombin cleaves the extracellular domain, leading to unmasking of a new N terminus which in turn acts as a tethered ligand on itself. PAR1 is the primary receptor on human platelets that is activated at low concentrations of thrombin, whereas PAR4 contributes to platelet activation at high thrombin concentrations (Kahn, *et al* 1998, Offermanns 2006, Stegner and Nieswandt 2011). Additionally, thrombin has been shown to bind to GPIbα, which is thought to be involved in thrombin-induced signalling (Ramakrishnan, *et al* 2001).

1.6 **Microparticles**

1.6.1 Definition

Many cells release extracellular membrane vesicles in response to a variety of stimulation conditions. There are generally considered to be three main types of microvesicles; exosomes, MPs and apoptotic bodies. The best studied type of extracellular vesicles are exosomes (30-100 nm in diameter) released by exocytosis from multivesicular bodies when fused with cell membrane and exosomes are rich in microRNA. MPs (sometimes also called microvesicles; 100-1000 nm in diameter) are released from plasma membrane during budding and they are procoagulant. Apoptotic bodies (0.5-3 µm in diameter) are released by cells undergoing apoptosis, and they are procoagulant and contain nuclear materials (Hugel, *et al* 2005). Since the focus of this study is to evaluate the role of MPs in haemostasis and thrombosis, exosomes and apoptotic bodies will not be covered in this thesis.

MPs are generally defined as submicron, intact, anucleoid phospholipid particles that are derived from the budding of the cell membrane of virtually all body cell types upon activation (Hugel, *et al* 2005). MPs were first described in 1946 by Chargaff and West, when they reported a significant prolongation of plasma clotting time after high-speed centrifugation of plasma. Additionally, when the pelleted material was added back to the centrifuged plasma the clotting time was reduced, suggesting that some sedimentable procoagulant particles were present in the plasma which could be removed by high-speed centrifugation (Chargaff and West 1946). In 1967, Wolf reported the presence of lipid-rich, cell-membrane particles in platelet free plasma as shown by electron microscopy (EM), which could be pelleted by high-speed centrifugation. He further showed that activated platelets release what he called “platelet dust” that possessed procoagulant activities. He also reported a linear correlation between the platelet count of an individual and the levels of “platelet dust” in platelet free plasma from the same individual (Wolf 1967). In the past decades with the incremental improvements in the detection methods, it has become apparent that the “platelet dust” described by Wolf are released by most, if not all, cells and since then they have been called MPs.

MPs have been shown to be widely heterogeneous in their size and content. MP composition is highly dependent on the cellular origin and the nature of stimuli. When MPs bud off, they inherit some cytosolic and membranous components from their parental cells. These components such as proteins, lipids and glycoproteins, can be also used to classify and identify MPs cellular origin (Boulanger, *et al* 2006, Jimenez, *et al* 2003). Additionally, these markers are useful tools to determine the biological functions of MPs. For instance, MPs that express PLs and TF on their surface show high procoagulant activity (del Conde, *et al* 2005).

Circulating MPs are present in healthy people, however their levels increase in certain physiological and pathological conditions such as cardiovascular diseases, atherosclerosis,

hypertension, infections, diabetes and sickle cell anaemia (Hugel, *et al* 2005, Morel, *et al* 2008, Piccin, *et al* 2007). Therefore, studying MPs may contribute new patho-physiological insights into the mechanisms of several conditions. Moreover, MPs show a great promise to be used as surrogate diagnostic and prognostic biomarkers for many pathological conditions, as well as to monitor therapeutic response.

1.6.2 MP formation

MPs shedding involves local disruption of the asymmetric membrane phospholipids as well as the cytoskeleton. Although the process that takes place during MPs formation *in vivo* is not fully understood, there are two well-studied cellular processes that can cause the release of MPs *in vitro*: 1) cell activation, either chemically by agonists, or physically by shear stress, and 2) apoptosis. The mechanism of MP generation appears to be tightly regulated despite the heterogeneity of MPs in their sizes and phenotypes. In a resting-state, cells maintain their membrane asymmetrical distribution of PLs mainly by the activities of the transmembrane enzymes: flippase, floppase and scramblase (Bevers, *et al* 1999, Morel, *et al* 2011, Zwaal and Bevers 1983, Zwaal, *et al* 2005). It has been recently shown that transmembrane protein 16F (TMEM16F), an eight-transmembrane protein, is a calcium-dependent PL scramblase (Suzuki, *et al* 2010).

Flippase activity is dependent on the presence of ATP and it specifically transports the negatively-charged PLs PS and PE from the external layer of the cell membrane to the internal layer. Similarly, floppase is ATP-dependent, but it transfers PLs from the internal layer to the external layer. Scramblase is ATP-independent and its bi-directional non-specific PLs transporter that allows all PLs to move according to their concentration gradients. During cell activation or apoptosis, the cytosolic Ca^{2+} concentrations increase dramatically leading to

inactivation of flippase and activation of floppase and scramblase (Figure 1.5). This activation/inhibition mechanism of the transmembrane enzymes results in collapse of the membrane phospholipid asymmetry distribution and subsequently the negatively-charged PLs PS and phosphatidyl-ethanolamine are exposed in the outer layer of the cell membrane (Diaz and Schroit 1996, Fox, *et al* 1990, Morel, *et al* 2011, Zwaal, *et al* 2005, Zwaal, *et al* 1993). In addition, the increase in the cytosolic Ca^{2+} levels leads to activate two cytoplasmic enzymes, calpain and gelsolin, although the latter is only identified in platelets (McLaughlin, *et al* 1993). Both calpain and gelsolin are involved in the cleavage of cytoskeleton actin filaments and spectrin, which subsequently leads to disrupting the cytoskeleton. The remodelling of both membrane and cytoskeleton results in membrane budding, and MPs are shed into the extracellular fluid (Burnier, *et al* 2009, Fox, *et al* 1990, Morel, *et al* 2011).

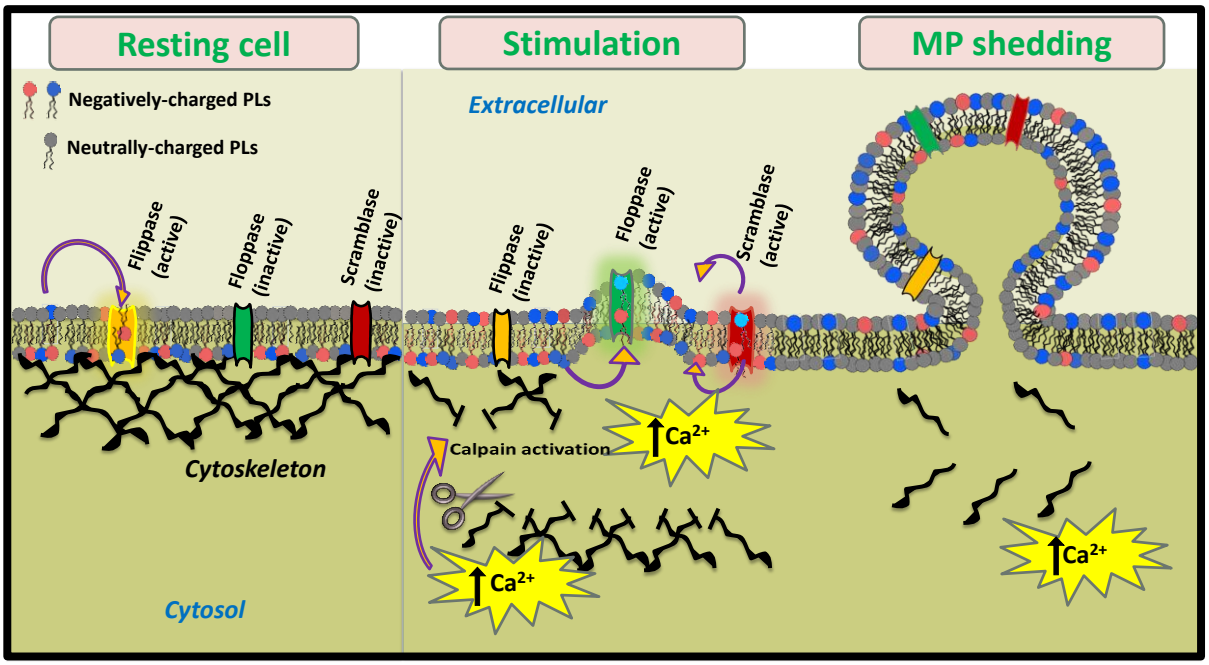


Figure 1.5. Membrane phospholipid disruption and MP formation during activation. Adapted from Hugel *et al* (2005) and Morel *et al* (2011).

1.6.3 MP function in haemostasis and thrombosis

MPs have been demonstrated to play important roles in haemostasis and thrombosis, vascular reactivity, inflammation, angiogenesis, immunity and tumour progression (Hugel, *et al* 2005, Morel, *et al* 2008, Piccin, *et al* 2007). Circulating MPs are mainly derived from platelets or megakaryocytes, but also from endothelial cells and leukocytes (Flaumenhaft, *et al* 2009, Shantsila, *et al* 2010). Both the negatively-charged PLs and the other component proteins play different physiological and pathophysiological roles via several mechanisms such as inflammation, cell-cell communication and angiogenesis. For instance, platelet-derived microparticles (PDMPs) have been shown to enhance leucocyte aggregation and monocyte binding to endothelial cells, which in turn promote vascular inflammation (Nomura, *et al* 2001). Furthermore, it has been shown that MPs isolated form atherosclerotic plaques are involved in neovessel formation (Leroyer, *et al* 2008). However, since the focus of this study is to evaluate the role of MPs in haemostasis and thrombosis, other aspects of MP functions will not be covered in this section.

The fact that MPs are present in the circulation of healthy individuals would imply a role for MPs in maintaining normal haemostasis. The central role of MPs in haemostasis and thrombosis can be clearly illustrated in two examples: 1) the high bleeding risk in Scott syndrome, which is associated with reduced PS exposure on activated platelets and low levels of circulating MPs (Zwaal, *et al* 2004) and 2) the high levels of MPs found in thrombotic diseases (Biro, *et al* 2003, VanWijk, *et al* 2003). It has been shown that the largest proportion of circulating MPs in healthy individuals are derived from platelets or megakaryocytes (Berckmans, *et al* 2001, Flaumenhaft, *et al* 2009), indicating that they play a role in maintaining haemostasis. On the other hand, under pathological conditions, the levels of MPs derived from other cells such as leucocytes, endothelial cells, red blood cells and vascular

smooth muscle cells as well as from platelets are elevated in the circulation (Hugel, *et al* 2005, Morel, *et al* 2008, Piccin, *et al* 2007).

It is well established that MP are highly procoagulant and their procoagulant activities rely mainly on the PS exposure and/or TF expression on the outer surface of their membrane. As discussed earlier, the presence of PS on the outer surface of MPs provides a catalytic surface promoting the binding and assembly of several coagulation factors including the tenase and prothrombinase complexes. It has been reported that the surface of MP possesses approximately 50- to 100-fold higher procoagulant activity per unit area than an equal surface area of activated platelets (Sinauridze, *et al* 2007).

The role of MPs in haemostasis is not restricted to PS and TF expression. Endothelial cell-derived microparticles (ECDMPs) have been shown to express ultra-large vWF multimers that promote platelet aggregation and increase clot stability (Jy, *et al* 2005). It has been reported that vWF bound to ECDMPs has more affinity to bind platelet vWF receptor than soluble vWF. Moreover, other procoagulant properties include the expression of P-selectin on the surface of PDMPs and P-selectin glycoprotein ligand 1 (PSGL-1) on monocyte-derived microparticles (MDMPs). The expression of PSGL-1 on monocytes or MDMPs has been shown to play a key role in transferring TF from monocytes or their MPs to activated platelets via its receptor P-selectin (del Conde, *et al* 2005, Rauch, *et al* 2000). Additionally, it has been shown that the expression of PSGL-1 in MDMPs is important to recruit these MPs to the site of vascular injury, which is mediated through the interaction with P-selectin on the surface of platelets at the site of the injury (Falati, *et al* 2003). On the other hand, the contribution of MPs to haemostasis and thrombosis is not limited to providing procoagulant activities, as they also carry anti-coagulant proteins such as TFPI and protein C (Perez-Casal, *et al* 2005, Steppich, *et al* 2005).

1.6.4 Laboratory methods for MP analysis

Several methods can be used to quantify, measure and/or phenotype MPs from different biological samples. These methods include flow cytometry, electron microscopy (EM), enzyme linked immunosorbent assays (ELISA) and functional assays such as the thrombin generation test. In addition, new approaches are being developed and evaluated such as nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) and atomic force microscopy (AFM). Unfortunately, there is no single method that can be used as a reliable technique to determine MPs quantity, size, function and cellular origin, but combinations of different methods may enable this to be achieved (Freyssinet and Toti 2010). Despite the incremental improvements in the analysis approaches of MPs, the detection and quantification of MPs remains extremely challenging because of the MP biological complexity, small size, low refractive index and high heterogeneity.

1.6.4.1 **Flow cytometry**

Flow cytometric analysis has, to date, been the most commonly used approach to detect, count and/or phenotype MPs. It is well known for its multi-parameter measurements of single cell or MP at a rate of thousands of events per second. Samples pass through a flow chamber where one or several laser beams (depending on the model of the machine) hit the cells or MPs and results in light scattering. The amount of scattered light deflected by the surface of the particles is called forward scatter (FS) and it reflects the size of the particles. Granularity can be determined by measuring the amount of light scattered at 90 degrees to the axis of the laser beam, termed side scatter (SS). Therefore, cells and MPs can be discriminated by their size and granularity. In addition to the FS and SS measurements, surface and cytosolic antigens can be identified using antibodies that are labelled with fluorescent dyes. The laser

beam excites the dyes at higher energy state, which in turn they emit a coloured light that can be reflected by certain fluorescent intensity filters at different wavelengths.

Annexin V is commonly used to measure the total number of MPs with flow cytometry. It is a phospholipid-binding protein that binds with high affinity to PS and strongly dependent on the presence of Ca^{2+} (Gerke and Moss 2002). It was originally thought that all MPs expose PS on their surfaces; however, it was reported that large proportion of MPs are not Annexin V-positive (Horstman, *et al* 2004, Piccin, *et al* 2007). Lactadherin is another marker for PS exposure, which has been claimed to be more sensitive than Annexin V (Dasgupta, *et al* 2006).

Flow cytometry has many benefits including its ability to use antibodies to identify MPs of different cellular sources. Furthermore, the flow cytometry technique not only uses a small volume of samples, but also two or more antibodies conjugated with different dyes can be simultaneously used to detect MPs from different cellular source in the same sample (Lynch and Ludlam 2007, Redman and Sargent 2007).

This conventional flow cytometry approach is, however, subject to criticisms. Because of the small size of MPs, most of the MPs are below the detection limit (around 200-400 nm for most of flow cytometers) and cannot be discriminated from the electronic noise or other cellular debris (Burnier, *et al* 2009, van der Pol, *et al* 2013). The vast majority of MPs are below the detection limit of the currently available flow cytometers, therefore they can only detect the “tip of the iceberg” with much of MP population being undetectable. There are ongoing efforts to overcome the technical limitations such as using beads of known size to discriminate between MPs and electronic noise (Lacroix, *et al* 2010, Robert, *et al* 2009). Additionally, some of the new generation flow cytometers have been designed to address the issue of the size-limit by increasing the FS collection angle (Nolte-'t Hoen, *et al* 2012).

Another limitation is associated with lack of sensitivity and specificity of Annexin V-binding, although it remains the most widely used marker for MP detection. Furthermore, recent reports showed that the presence of multiple undetectable MPs "swarm" within the analysed sample, which could be counted as single event, leading to underestimates of the MP count in the sample (Harrison and Gardiner 2012, van der Pol, *et al* 2012).

Despite these limitations of flow cytometry, it remains the most powerful and widely used method to analyse MPs. Several studies have revealed that a conventional flow cytometry approach to determine MP concentration could provide clinically significant results (Amabile, *et al* 2012, Amabile, *et al* 2008, Lee, *et al* 2012b). Additionally, a very recent report demonstrated that MP concentrations measured by conventional flow cytometry using a BD FACSCalibur machine correlate with their functional activities using three functional assays; thrombin generation, Zymuphen MP-activity and STA Phospholipid Procoag Assay (Ayers, *et al* 2014). This finding suggests that the MP count obtained from conventional flow cytometry could be clinically relevant.

1.6.4.2 **Electron microscopy**

Transmission electron microscopy (TEM) is considered the gold standard for MP analysis providing direct evidence for their presence with great details about size, morphology and composition of MPs (van der Pol, *et al* 2010). Additionally, immunogold-labelled antibodies can be used to investigate the antigenic composition of MPs. As the wavelength of the beam of electrons is very short, the resolution of TEM can be lower than 1 nm. For TEM studies, samples undergo several pre-analytical steps including pelleting by centrifugation, dehydration, and fixation, and there are some concerns about the effect of these procedures of TEM that may alter the size and morphology of MPs (van der Pol, *et al* 2010).

1.6.4.3 **Nanoparticle tracking analysis**

NTA provides the absolute count and size distribution of nanoparticles. NTA is a direct, real-time visualisation and analysis of nanoparticles in solution relying on laser light scattering and Brownian motion. A finely focussed laser beam passes through a prism-edge glass and refracts at a low angle into the sample suspension (Figure 1.6). The laser beam illuminates particles that are moving rapidly under Brownian motion, which can be visualised using a 20X optical microscope objective lens. Illuminated particles are recorded by a video camera, which captures multiple frames that can be analysed by the NTA software, which simultaneously identifies and tracks each particle on a frame-by-frame basis. The velocity of particle movement reflects its size. However, particle size is dependent on the type of the particle and its refractive index. NTA can be used to count and measure the size of nanoparticles between 30 and 1000 nm (Dragovic, *et al* 2011, van der Pol, *et al* 2010, Yuana, *et al* 2011).

NTA has a great potential for MP analysis as it provides a rapid detection method with absolute count and actual size of MPs. Samples are analysed in suspension with avoiding the effects of fixation. It also requires relatively small sample sizes. However, NTA with the current settings does not provide information on the biochemical composition or cellular origin (van der Pol, *et al* 2013).

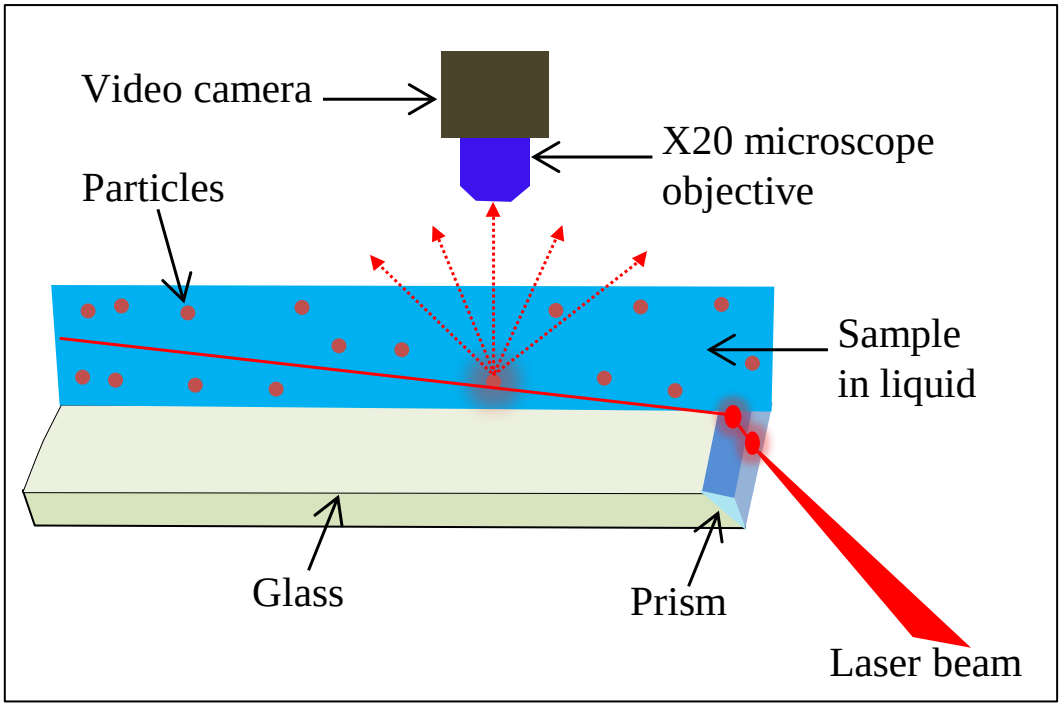


Figure 1.6. Schematic showing the principle of NTA.

1.6.4.4 **Dynamic light scattering**

DLS is used to determine the size distribution of suspended particles ranging in size between 1 nm and 6 µm. DLS uses the similar principle of NTA where a laser beam passes through to illuminate the particles in sample suspension that are moving in Brownian motion. The intensity fluctuations of the scattered light reflected by the particles determine their size distribution. However, whereas accurate size distribution can be obtained by DLS from monodisperse samples, it is less accurate in polydisperse samples because of the presence of small numbers of larger particles that scatter more light than small particles that in turn influences the size distribution. Furthermore, similar to the NTA, DLS does not allow for identification of the biochemical composition or cellular source of the particles (van der Pol, *et al* 2013, van der Pol, *et al* 2010, Yuana, *et al* 2011).

1.6.4.5 **Atomic force microscopy**

AFM provides very high resolution to a horizontal of 3 nm and a vertical of <0.1 nm of particles. It consists of a mechanical cantilever, which has a very sharp tip at the end that scans a sample surface. The interaction force between the tip and the sample relays on their distance. A laser beam measures the cantilever deflection when the tip moves across the sample. The measured cantilever deflections are calculated, providing a three-dimensional profile of the surface. Specific antibodies can be used to bind MP to the surface, so the phenotype can be provided (van der Pol, *et al* 2010, Yuana, *et al* 2011, Yuana, *et al* 2010). However, the AFM studies are time- and labour-consuming which make it not suitable for routine use or for analysing large scale of samples (Yuana, *et al* 2010).

1.6.4.6 **Capture-based assay**

In capture-based assays, MPs are captured onto ELISA plates coated with a primary probe that binds the MPs from samples. The captured MPs can be counted and characterised using either a secondary probe, or by measuring their functional properties. Several approaches have been described using monoclonal antibodies that are specific for a subset of MPs as primary probes in the first step combined with detection approach such as prothrombinase or peroxidase activity (Habib, *et al* 2008, Jy, *et al* 2004, Nomura, *et al* 2003). For instant, two independent studies described a method to detect PDMPs using platelet-specific CD42a (anti-GPIX) as a primary probe and CD42b (anti-GPIbα) as a secondary probe (Figure 1.7 A) (Nomura, *et al* 2009, Ueba, *et al* 2008). Another study used Annexin V as a primary probe and prothrombinase activity was used to evaluate the functional properties of the captured MPs (Figure 1.7 B) (Aupeix, *et al* 1997). Moreover, TF-bearing MPs can be also quantified using a FXa generation assay; capturing the MPs using immobilised monoclonal antibodies to

cellular antigens on a plate. TF activity associated with the captured MPs can be then evaluated by the activation of FX by FVIIa-TF complex (Aras, *et al* 2004).

Capture-based assays have several advantages, including their ability to semi-quantify MPs directly in plasma, wide availablility and relative ease of use. They are not restricted by the small size of MPs and are able to measure the cellular origin and/or procoagulant activities of MPs. However, samples usually are analysed in bulk rather than individual sample, which is considered one of the drawback of this approach (Hayasaka, *et al* 2006, Jy, *et al* 2004, Shet, *et al* 2003, Yuana, *et al* 2011).

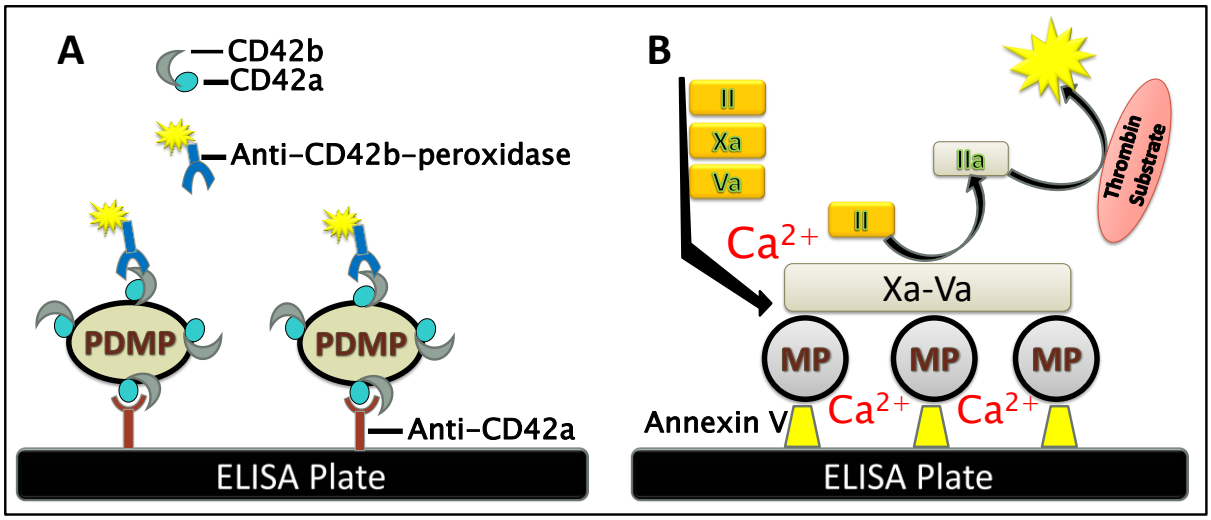


Figure 1.7. Representative examples of capture-based assay for MP analysis. Panel A: Representative example of cell-specific ELISA. ELISA plate is coated with anti-CD42a antibodies to capture the PDMPs from the plasma. Captured MPs are detected by the anti-CD42b antibodies labelled with peroxidase. Panel B: Representative example of procoagulant activity-based ELISA. ELISA plate is coated with Annexin V to capture the MPs in the sample. FXa and FVa are then added, which form prothrombinase complexes on MP surfaces. Prothrombin is added and the amount of generated thrombin is measured, which reflects the procoagulant activity of MPs in the plasma.

1.6.4.7 **Calibrated Automated Thrombogram**

The thrombin generation assay reflects the overall capacity of the haemostatic response, including the contribution of the procoagulant factors (TF, procoagulant phospholipids (PPLs) and coagulation factors) and anticoagulant systems. Hemker and colleagues have developed the thrombin generation assay into a semi-automated procedure that is called the Calibrated Automated Thrombogram (CAT) assay (Hemker, *et al* 2003). This development has led to this approach being used as a tool to evaluate the hypo- and hyper-coagulability activity of plasma samples including MP-rich samples. The CAT assay uses a slow fluorogenic thrombin substrate and measures the amount of thrombin converted from prothrombin. The splitting of the fluorogenic thrombin substrate reflects the amount of thrombin generated in the sample (Hemker, *et al* 1993).

The strengths of the CAT in MP analysis include high sensitivity, use of well-defined reagents, reflects the functional properties of MPs including pro-and anti-coagulant activities and is not limited by the small size of MPs. On the other hand, the CAT assay, suffers from some drawbacks as it fails to determine MP cellular origin, actual size, absolute concentration and physical properties. Additionally, when MPs are analysed directly in patient plasma samples, the CAT assay result becomes heavily influenced by the changes in the levels of coagulation factors or by the presence of anti-coagulant effects in patient samples who are taking anti-coagulant therapy such as heparin or warfarin (Bidot, *et al* 2008, Freyssinet and Toti 2010, Owens and Mackman 2011).

1.7 Aims of the study

There is growing interests in the recent years in MPs as diagnostic and prognostic biomarkers for many pathological conditions, but this is hindered by the wide variety of methodological approaches of detection, counting and/or phenotyping. The current variability, inconsistency and/or conflict in the literature between different studies reveal the importance of standardising the pre-analytical and analytical variables for MP analysis. Additionally, it has been well established that different cell types generate different kinds of MPs, which show wide variations in their characteristics. However, at the time that this study was begun, the functional properties of MPs from different cells were poorly understood. Furthermore, the contribution of different platelet receptors in inducing platelet procoagulant response and MP formation remain highly controversial.

The specific aims were:

- To optimise the pre-analytical and analytical variables in the analysis of MP procoagulant activity.
- To characterise the procoagulant activity of MPs derived from different cellular sources.
- To investigate the role played by GPCRs and ITAM-containing receptors in generating the platelet procoagulant response and the release of procoagulant MPs.
- To characterise the mechanisms by which CLEC-2 induces the platelet procoagulant response and MP formation.
- To evaluate the procoagulant properties of PDMPs generated via FcγRIIA.
- To develop a novel functional assay for HIT diagnosis using the CAT assay.

CHAPTER 2: **Materials and methods**

2.1 **Materials**

The agonists and inhibitors used in this project are listed in Table 2.1 and Table 2.2. Unless otherwise stated, reagents and materials were obtained from Sigma-Aldrich Ltd (Dorset, UK).

Agonist	**Target**	**Source**
CRP-XL	GPVI	Professor Richard Farndale, University of Cambridge, UK
Rhodocytin Podoplanin	CLEC-2	Professor Steve Watson, University of Birmingham, UK. (Eble, *et al* 2001)
Thrombin	PAR-1 PAR-4	Sigma-Aldrich
Thrombin receptor agonist peptide (TRAP)	PAR-1	Calbiochem
ADP	$P2Y_1$ $P2Y_{12}$	Sigma-Aldrich
U-46619	TxA_2 (TP) receptor agonist	Sigma-Aldrich
Calcium ionophore A23187	Ca^{2+}	Sigma-Aldrich
Lipopolysaccharide (LPS)	CD14	Sigma-Aldrich
Phorbol myristate acetate (PMA)	Protein kinase C (PKC)	Sigma-Aldrich
Tumour necrosis factor alpha (TNF-α)	TNF-R55 TNF-R75	Enzo Life Sciences Ltd

Table 2.1. Agonists and activators used in the project.

Inhibitor	**Target**	**Source**
Prostaglandin I_2 (PGI_2)	IP	Sigma-Aldrich
Apyrase	ADP	Sigma-Aldrich
MRS 2179	$P2Y_1$	AstraZeneca
AR-C69931MX	$P2Y_{12}$	AstraZeneca
Aspirin	COX-1	Sigma-Aldrich
LY294002	PI3-K	Alomone Labs
Wortmannin	PI3-K	Alomone Labs
R406	Syk kinases	Selleckchem
PRT-318	Syk kinases	Professor Steve Watson
Dasatinib	Src kinases	Professor Steve Watson
PP2	Src kinases	Professor Steve Watson
Corn trypsin inhibitor (CTI)	Contact factor pathway	Cambridge Biosciences
Gly-Pro-Arg-Pro peptide (GPRP)	Fibrin polymerisation	Peptide Protein Research Ltd

Table 2.2. Inhibitors used in the project.

2.2 Methods

2.2.1 Blood collection and sample preparation

Peripheral blood was collected from healthy subjects who had not taken any medication for the last two weeks prior to donation, with the approval of the Ethical Practices Committee of the University of Leicester. All participating blood donors provided informed written consent.

2.2.1.1 Blood collection from normal healthy donors

Blood was collected using a standardised phlebotomy technique in which blood was drawn using a 21 gauge butterfly needle with a light tourniquet applied to obtain venous access, which was released prior to the collection of the blood to avoid platelet activation. The first 3 mL of blood, were collected into a 3 mL ethylenediaminetetraacetic acid (EDTA) Vacutainer tube (Becton Dickinson Vacutainer®, Oxford, UK) and used to obtain a full blood count using a Coulter A^{C}T diff™ haematology analyser (Beckman Coulter, High Wycombe, UK). All subsequent blood was collected into 4.5 mL sodium citrate tubes (Vacutainer containing 0.105 M sodium citrate with a 1:9 v/v proportional volume to blood). For some studies, corn trypsin inhibitor (CTI) was immediately added to citrated blood to a final concentration of 18.3 µg/mL and mixed by gentle inversion to inhibit the contact factor pathway. For all studies the blood was processed within 10 minutes of collection.

2.2.1.2 Platelet-poor and platelet-free plasma

Two centrifugation protocols were used throughout the project to prepare plasma from whole blood. Platelet-poor plasma (PPP) was prepared by centrifugation of the blood tubes at 1800g

for 30 minutes at room temperature (RT). Approximately 90% of the supernatant was carefully transferred into new tubes. For Platelet-free plasma (PFP), whole blood was centrifuged at 1500g for 15 minutes, the supernatant removed into new tubes and centrifuged again at 13000g for 2 minutes.

To obtain MP-depleted plasma, PFP was either further centrifuged at 20000g for 30 minutes to pellet MPs or passed through 0.2 µm pore size filters using either a 96-filter plate attached to Ceveron MFU 500 vacuum device (Technoclone, Dorking, UK) or an Acrodisc syringe-driven filter units (Pall Corporation, Portsmouth, UK). The former filtration method was used for small plasma volumes, whereas the latter for large volumes.

2.2.1.3 **Pooled plasma**

Pooled plasma preparations were used as a standard material for many of the assays, or to resuspend isolated MPs. To prepare this, 6 citrated blood tubes were collected from 22 to 25 healthy donors as described in section 2.2.1.1. CTI was immediately added to the blood tubes, which were centrifuged at 1800g for 30 minutes at RT. Equal volumes of plasma from each donor were pooled, aliquoted into 1.5 mL tubes and stored at -80ºC. Blood from all of the donors was collected on the same day, within a 30 minute time period, and aliquots of pooled plasma were frozen within 2 hours of collection.

2.2.1.4 **Platelet-rich plasma**

Platelet-rich plasma (PRP) was obtained by centrifuging fresh citrated whole blood at 160 g for 20 minutes at RT with the brake off. Approximately 80% of the platelet supernatant was gently aspirated without disturbing the buffy coat or causing the formation of air bubbles. Except where stated, the platelet count was adjusted with autologous PPP or PFP to 150 x 10^3

platelets/µL. For some studies, Gly-Pro-Arg-Pro peptide (GPRP) was added to a final concentration of 0.5 mg/mL prior to platelet activation to prevent fibrin polymerisation.

2.2.1.5 **Washed platelets**

Washed platelets were prepared from freshly collected whole blood, taken into 10% (w/v) sodium citrate. The blood was further anti-coagulated by addition of acid citrate dextrose (ACD: 0.085 M tri-sodium citrate, 0.071 M citric acid, 0.11 M glucose, pH 6.0) (in a ratio of 6:1 (v/v) blood:ACD and PRP was prepared as above. Platelets were pelleted by centrifugation of PRP at 600g for 15 minutes in the presence of 200 ng/mL freshly prepared prostaglandin I_2 (PGI_2) to prevent platelet activation. The platelet pellet was resuspended in HEPES-buffered saline (HBS) pH 6.0 (HBS: 10 mM HEPES, 150 mM NaCL, 5 mM KCl, 1 mM $MgSO_4$, pH 6.0) containing freshly prepared 200 ng/mL PGI_2. Platelets were pelleted again, resuspended into HBS pH 7.4 containing 2 mM $CaCl_2$ at a platelet density of 1.5 x 10^8/mL. Washed platelets were left for 30 minutes at RT prior to use to allow the decay of the effects of the PGI_2.

2.2.1.6 **MP isolation**

MPs from whole blood, PRP or cell culture media supernatants were separated from cells and cell debris by centrifugation at either 1800g for 30 minutes or (for the majority of the studies) at 1500g for 15 minutes, followed by 13000g for 2 minutes. The resulting supernatant was collected into clean tubes, and is referred to as MP-rich supernatant hereafter. To prepare washed MPs these were pelleted by centrifugation of MP-rich supernatant at 20000g for 30 minutes. The supernatant was discarded and the MPs were resuspended in HBS buffer. The MPs were again centrifuged at 20000g for 30 minutes and resuspended in appropriate volume

of HBS or filtered pooled plasma, depending on the study design. When MPs were not used immediately MP pellets were resuspended into filtered pooled plasma and stored at -80ºC.

2.2.2 Cell culture

EA.hy926 cells (derived by fusion of human umbilical vein endothelial cells with the human lung adenocarcinoma permanent cell line A549 (Edgell, *et al* 1983) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) (Gibco, Invitrogen Ltd., Paisley, UK), 2mM L-glutamine, 100 unit/mL penicillin and 0.1mg/mL streptomycin. For endothelial cell-derived MP generation, 5 x10^5 cells were cultured in T25 (25 cm^2) flasks and maintained for four days. At day four when the cells reached 90% confluence, 10 ng/mL TNF-α in fresh media was added and incubated for 24 hours to generate MPs. The MP-rich cell culture supernatants were then collected and centrifuged at 160g for 20 minutes to remove cell debris. The resulting supernatant was collected into clean tubes, centrifuged at 1500g for 15 minutes and the supernatant containing the MPs was collected. The MPs were then pelleted and washed as described in section 2.2.1.6.

THP-1 cells (derived from one-year old male with acute monocytic leukaemia (Tsuchiya, *et al* 1980)) were grown in RPMI-1640 media supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/mL penicillin and 0.1 mg/mL streptomycin. In order to transform THP-1 cells into monocyte-derived macrophages, cells were incubated for 72 hours in fresh media containing 100 ng/mL phorbol myristate acetate (PMA). For macrophage-derived MP generation, PMA-treated THP-1 cells were stimulated with 20 ng/mL LPS for 6 hours. Media was collected and MPs were isolated and washed as described in section 2.2.1.6.

Chinese Hamster Ovary (CHO) cells expressing human podoplanin (h.PDPN) were provided by Alice Y. Pollitt (School of Clinical and Experimental Medicine, University of Birmingham). CHO cells has been transfected with full-length human podoplanin (h.PDPN) as previously described (Pollitt, *et al* 2010). Both control- and h.PDPN-CHO cells were grown in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 unit/mL penicillin and 0.1 mg/mL streptomycin. Surface expression of podoplanin was regularly checked by flow cytometry.

Routine culture of the different cells was carried out by seeding them at appropriate concentrations and maintaining them in a 37°C humidified incubator containing 5% CO_2. Culture medium was exchanged every 2-3 days and cells were passaged when they were 90-95% confluent. The THP-1 cells grew in suspension. The adherent EA.hy926 and CHO cells were detached from the plate by incubating with 2 mL 0.25 µg/mL trypsin for approximately 2 minutes at 37°C. Subsequently, DMEM containing 10% FBS was added to inactivate the trypsin and the cell suspension was centrifuged at 200g for 5 minutes at 20°C. The supernatant was discarded and the cell pellet was re-suspended in DMEM.

2.2.3 Caspase-3 activity

Caspase-3 activity was measured to assess cellular apoptosis in EA.hy926 cells treated with TNF-α for 24 hours. Washed cells were resuspended into cell lysis buffer (50 nM HEPES, 0.1% CHAPS, 1 mM Dithiothreitol (DTT), 0.1 mM EDTA, 0.1% Triton X-100, pH 7.4) at cell density of 1 x 10^7 cells/mL and incubated for 5 minutes in ice bath. Cells lysates were centrifuged at 10000g for 10 minutes at 4°C and the supernatants were collected. In a 96-well plate, 10 µL of the cell extracts was added to 80µL assay buffer (50 nM HEPES, 100 nM NaCl, 0.1% CHAPS, 1 mM DTT, 1 mM EDTA, 10% glycerol, pH 7.4) and incubated at

37°C for 10 minutes. 10 µL of 2 mM caspase-3 substrate, Ac-DEVD-AMC (Enzo Life Sciences Ltd) was added to each well. Where indicated, 20 nM caspase-3 inhibitor, Z-DEVD-FMK (Enzo Life Sciences Ltd) was included in the assay buffer. Ac-DEVD-AMC cleavage was monitored by measuring excitation at 360 nm and emission at 460 nm on a Fluoroskan Ascent plate-reader for 120 minutes at 37°C with 10 minutes interval between each reading. Samples were analysed on triplicate and results were corrected for background readings (samples without caspase-3 substrate).

2.2.4 Calibrated Automated Thrombogram (CAT assay)

All reagents used in CAT assay were purchased from Stago Diagnostic, Asnieres sur Seine, France unless stated otherwise.

The assay was performed essentially as described by Hemker *et al*, (2003), with some modifications. Thrombin generation was measured in PRP, PPP, PFP and MP-rich plasma samples. To prevent fibrin polymerisation, 0.5 mg/mL GPRP was added to samples prior to activation. In order to restore coagulation in washed cells or MP-rich supernatants, samples were mixed into CTI-treated, pre-filtered pooled plasma. Different thrombin generation reagents that contain certain concentrations of exogenous TF and/or PLs were used to support thrombin generation according to purpose of the study (Table 2.3).

Reagent	Composition	Applications	
		Platelet Poor Plasma (PPP)	**Platelet Rich Plasma (PRP)**
PPP	5 pM TF 4 µM PL	Screening hypercoagulablity	
PPP low	1 pM TF 4 µM PL	Screening hypocoagulablity Sensitive to FVIII, FIX & FXI	
PRP	1 pM TF	MPs	1- anticoagulant 2- hypercoagulable 3- hypocoagulable
MP	4 µM PL	TF-bearing MPs	

Table 2.3. Thrombin generation reagents and their constitutions and applications.

In 96-well round bottom plate (Immulon 2HB), 80 µL of test sample was mixed with 20 µL of the appropriate thrombin generation reagent. Plates were loaded in Fluoroskan Ascent plate reader (Thermo-Fisher Scientific, Cramlington, UK) equipped with Thrombinoscope software (Thrombinoscope, Synapse b.v., Maastricht, The Netherlands) version 3.0.0.26. Thrombin generation was triggered by automatically dispensing 20 µL fluorogenic substrate (2.5 mM of Z-Gly-Gly-Arg-AMC, in 0.1 M $CaCl_2$). Sample mixtures were excited at a wavelength of 390 nm and the emission was measured at 460 nm. Each thrombin generation measurement was corrected against thrombin calibrator containing α2-macroglobulin/thrombin complex. Samples were analysed in duplicate and continuously monitored for 60 minutes. In some experiments, pooled plasma was included on each plate to serve as an internal control.

At the end of the assay, a curve of thrombin generation was formed with the four CAT assay parameters (Figure 2.1); (1) the lag time, which reflects the time between the addition of the $CaCl_2$ and fluorogenic thrombin substrate until the thrombin generation starts, (2) time to peak (ttPeak) which measures the time required for the thrombin generation to reach its peak, (3) peak thrombin which is the maximum amount of thrombin generated and (4) endogenous thrombin potential (ETP), also called area under the curve (AUC), which is a measurement of the total amount of thrombin generated.

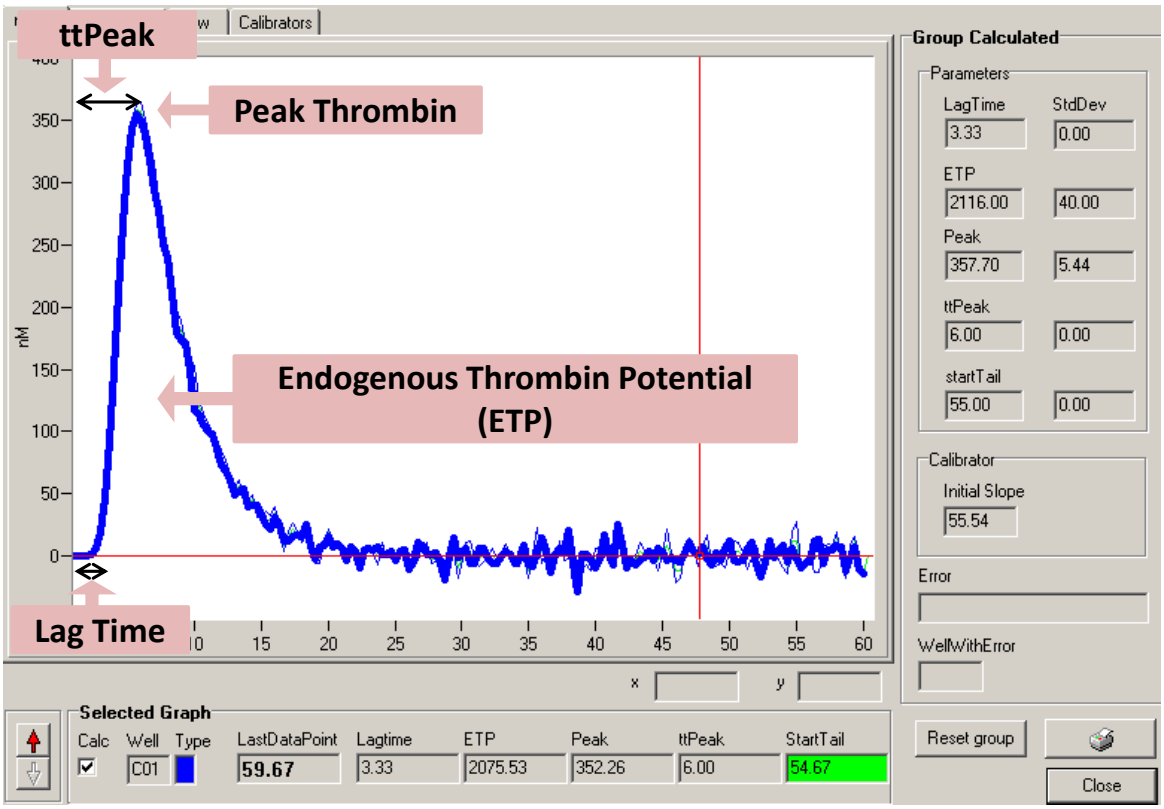


Figure 2.1. A representative example of TG curve on CAT assay shows all TG parameters. Screen picture of Thrombinoscope software (Thrombinoscope, Synapse b.v., Maastricht, The Netherlands, version 3.0.0.26).

2.2.5 Flow cytometry

2.2.5.1 General methods

Flow cytometry was used to count, identify cellular origin, and measure the size and granularity of cells and MPs as well as surface expression of antigens. Flow cytometry experiments were carried out using either a Coulter EPICS XL-MCL or a CyAn-ADP flow cytometers (Beckman Coulter). The machines were aligned daily using Flow-check beads (Beckman Coulter). In order to minimise the background noise in the machines, buffers were filtered using 0.2 µm filters prior to experiments. The optimum cell and MP concentrations

for all samples suspensions were determined by running samples at different concentrations to get the appropriate event rates. Additionally, all antibodies used were titrated to determine the optimum concentrations. When two or more fluorescent dyes were used, colour correction was carried out using single labelled samples, to set up the colour compensation. Negative controls were included in each experiment and they were set at 2% positive. Unless otherwise stated, these negative controls were nonspecific isotype control antibodies.

Cell and MP populations were identified using the logarithmic FS and SS scales with the help of 3, 2, 1 and 0.4 µm latex beads (Spherotech, USA). Additionally, cell-type specific antibodies were used to confirm the target populations. Electronic gates were set around the appropriate populations to select events for further analysis. Where whole blood or PRP samples were used, a final concentration of 10 U/mL of hirudin or 0.5 mg/mL GPRP was added prior to activation to prevent the formation of clot. All samples were analysed in duplicate.

2.2.5.2 **Annexin-V binding assay**

This assay was developed to measure the platelet procoagulant surface and MP formation. It is a two-colour flow cytometry protocol using Annexin V-FITC binding (Pharmingen, BD Biosciences, Oxford, UK) to detect PS exposure. CD42b-PE antibody (Biolegend, San Diego, USA) was used as platelet generic marker.

5 µL of PRP or washed platelet preparation was added to 45 µL HBS containing 1µL of CD42b-PE (Biolegend, San Diego, USA), 5 µL of Annexin V-FITC (Pharmingen, BD Biosciences, Oxford, UK) and 2 mM $CaCl_2$, and mixed gently. Mouse IgG-PE isotype (Biolegend) was used as a negative control for CD42b-PE, whereas HBS without $CaCl_2$ was used as a negative control for Annexin V-FITC. Mixtures were incubated in the dark for 20

minutes at RT. The reaction was then stopped by diluting the mixtures 1:100 by sub-aliquotting with HBS/Ca^{2+} or HBS without $CaCl_2$ (for Annexin V binding experiments). Samples were analysed in flow cytometry within one hour.

Intact platelets and MP populations were discriminated by their sizes, using latex beads of known size (3, 2, 1 and 0.4 µm), a MP gate region was set at events below 1 µm in size, as illustrated in Figure 2.2.

2.2.5.3 **Platelet P-selectin surface expression**

Platelet degranulation was assessed using surface expression of P-selectin by flow cytometry. 5µL of sample was incubated for 20 minutes at RT in the dark with 2µL of anti-human P-selectin-FITC antibody or IgG-FITC isotype control (R&D Systems Abingdon, UK) in a total volume of 50µL HBS. Samples were then diluted 1:10 with 0.2% formyl saline and incubated for 10 minute to fix the cells. A further 1:10 dilution was made in 0.2% formyl saline and the samples were analysed in the flow cytometer within 1 hour of preparation.

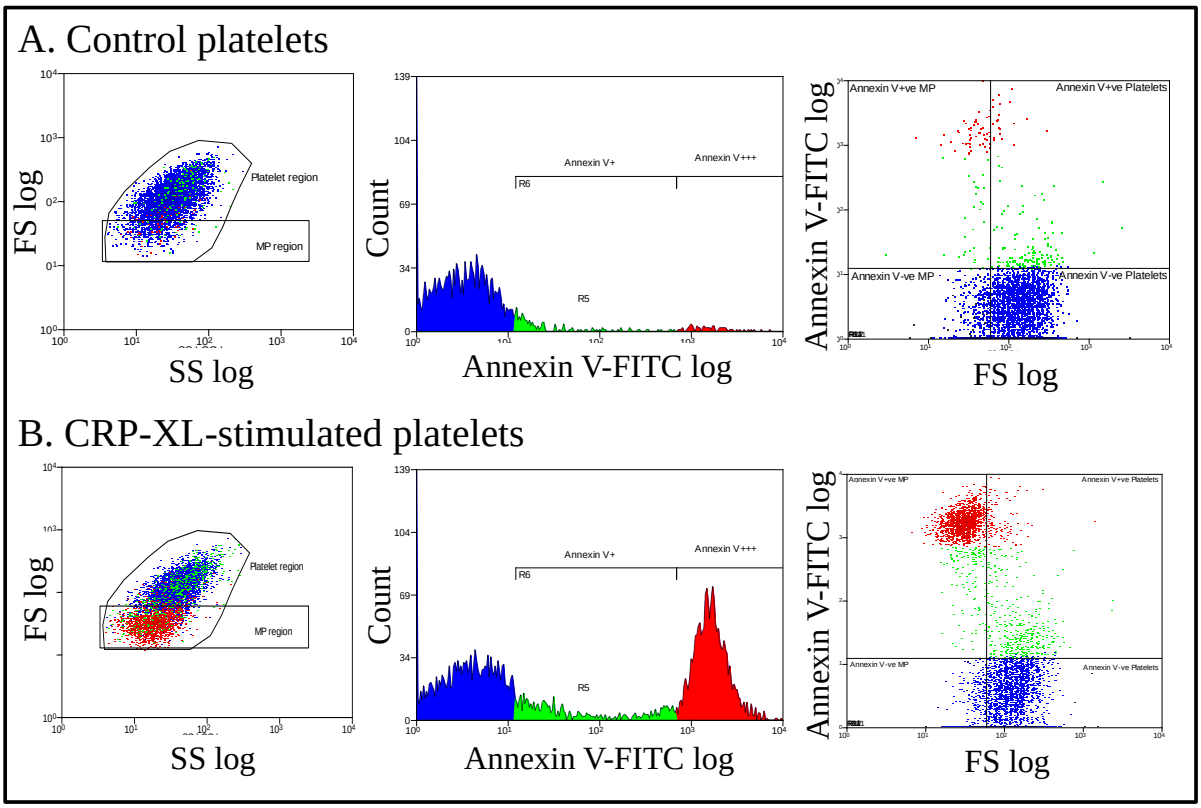


Figure 2.2. Representative flow cytometric analysis on Annexin V binding assay. In the FS and SS scatter plot (left panels), the platelet region gate was determined for unstimulated platelets on the basis of logarithmic FS and SS. This was further confirmed by binding of the platelet generic CD42b-PE antibody. The MP region gate was set on the basis of the latex bead as any events below 1 µm in the size. In Annexin V-FITC histogram (middle panels), the bar was set to include only 2% of fluorescent of negative control platelets (Annexin V-FITC in HBS without $CaCl_2$). The bar was divided into weak Annexin V^+ (green) or strong Annexin V^{+++} (red) depending on the intensity of the fluorescence. The right panels show the relationship between size and Annexin V-FITC positive events; the upper left-hand quadrant shows Annexin V^{+ve} events in MP-size gate, the lower left-hand quadrant shows Annexin V^{-ve} events in MP-size gate, the upper right-hand quadrant shows Annexin V^{+ve} events in platelet-size gate, the lower right-hand quadrant shows Annexin V^{-ve} events in platelet-size gate. (A) shows control (un-activated) platelets, which are seen as a single population that are mostly Annexin V^{-ve}. (B) shows a typical example of CRP-XL-stimulated platelets with two distinct cell populations, which of the smaller population is falling in the MP region (below 1 µm) and they are strongly Annexin V^{+ve}.

2.2.6 Nanoparticle tracking analysis

The NTA analysis was carried out using a NanoSight NS500 instrument (Malvern Instruments Ltd., UK). A standardised protocol of NTA for MP and exosomes analysis has been recently introduced (Dragovic, *et al* 2011, Gardiner, *et al* 2013). For optimum particle concentration, samples were diluted in filtered HBS (that had been passed through a 0.1 µm filter), to a range of concentrations between 1 and 9 x 10^8 particles/mL, mixing before loading onto the instrument. In order to standardise NTA analysis and maximise its reproducibility, analysis were carried out at 25°C and camera gain was fixed at level 8 throughout the study, which was pre-determined on the basis of a preliminary analysis of different concentrations of MP preparations. Samples were loaded into the machine using an automatic pump, left for 10 seconds to settle, and the focus was adjusted manually prior to initiating video acquisition. Two 60-second videos were acquired for each sample using different fields. Recorded videos were analysed on NTA software, which provides the count, and the mean and mode of particle size.

2.2.7 Platelet aggregometry

Platelet aggregation was measured in PRP using a Platelet Aggregation Profile PAP-8E (Bio-Data Corporation, UK), which is based on the turbidmetric method developed by Gustav Born (Born 1962). Siliconised glass tubes containing 250 µL PRP and a magnetic stirrer were pre-warmed at 37°C for 2 minutes before being transferred to test wells and stirred at 1200rpm. Autologous PPP was used to set up the 100% light transmission (blank) and control PRP to set up the platelet aggregation baseline. Aggregation was initiated by addition of agonists and measured for 6 minutes.

2.2.8 MP procoagulant activity ELISA

The procoagulant activity of the PL and TF content of MPs were assessed using Zymuphen MP-activity and MP-TF ELISA kits respectively (Hyphen BioMed, Quadratech Diagnostics Ltd, Surrey, UK). Washing steps were performed using a microplate auto-washer (Tecan UK Ltd, Reading, UK). In order to confirm the accuracy of measurements, two samples of known concentration controls were included in each run. All samples and standards were analysed in duplicate.

2.2.8.1 **Chromogenic assay for PS on MP surfaces**

Zymuphen MP-activity assay is a chromogenic functional assay based on Annexin V binding to MPs (Figure 2.3 A). Briefly, samples and standards were diluted with sample diluent before being added to 96-well plate strips coated with Annexin V-streptavidin. Samples were incubated for 60 minutes at 37°C to allow MPs to bind to Annexin V, then washed 4 times. Bovine FVa-FXa and human prothrombin were added to wells and incubated for 10 minutes at 37°C to form prothrombinase complex on the negatively-charged PLs of the captured MPs, which in turn converts prothrombin to thrombin. Thrombin chromogenic substrate was added and incubated for 3 minutes at 37°C to develop the colour before the addition of the stop solution. The absorbance was measured 405 nm and samples readings were plotted against the standard curve. Data were expressed as nM PS equivalents.

2.2.8.2 **TF activity ELISA**

Zymuphen MP-TF ELISA kit uses plates coated with anti-TF antibodies that recognise the extracellular domain of human TF to capture TF-bearing MPs in samples (Figure 2.3 B). Samples and standards were added to 96-well plate strips coated with anti-TF antibodies and

incubated overnight at RT to allow the antibodies to interact with TF-bearing MPs. Samples were washed five times before the addition of FVIIa and FX and incubated for 2 hours at 37°C. FVIIa forms a complex with TF on the surface of the captured MPs, which in turn converts FX to FXa. FXa chromogenic substrate was added and incubated for 2 hours at 37°C. The reaction was stopped by adding stop solution. The absorbance was measured 405 nm and samples readings were plotted against the standard curve. MP-TF concentrations were expressed as pg/mL.

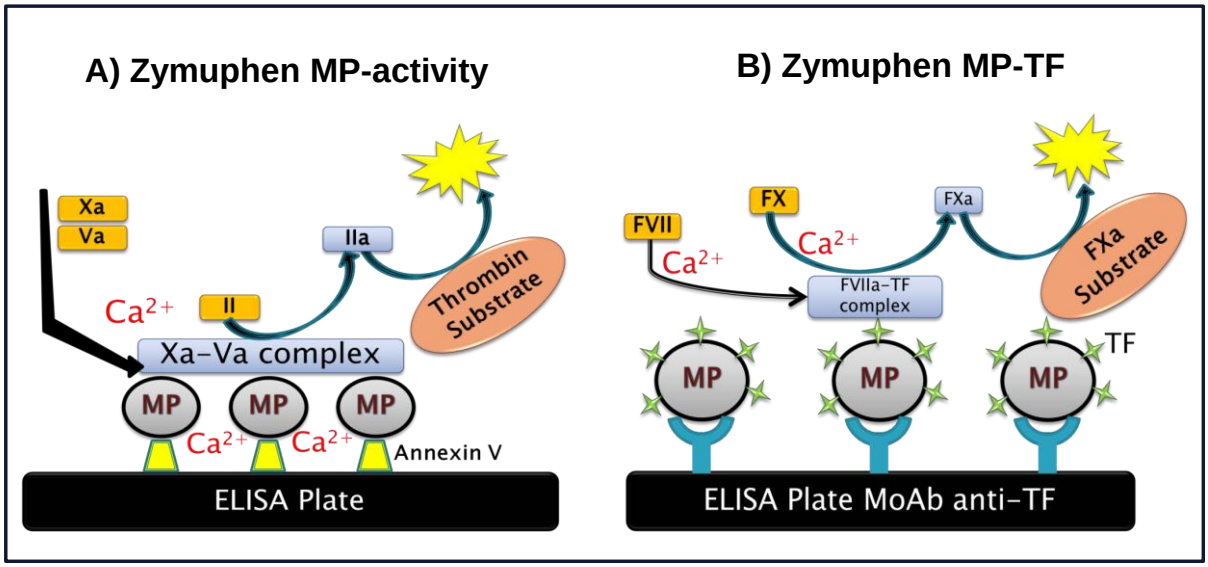


Figure 2.3. Schematic showing the principle of Zymuphen ELISA kits.

2.2.9 Western blotting

The relative podoplanin protein expression in monocytes was assessed by Western blotting. Isolated cells were resuspended into 1 mL RIPA buffer (150 mM NaCl, 50 mM TRIS, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) sodium dodecyl sulphate (SDS), 1% (w/v) IGEPAL

CA630, pH 7.4) containing protease inhibitor cocktail (PIC) (Roche Diagnostics Ltd, West Sussex, UK) and left in an ice bath for 30 minutes. Cell lysates were centrifuged at 1300rpm for 10 minute and supernatant was collected and stored at -80°C.

Protein concentrations in cell extracts were measured using the DC protein assay (Bio-Rad Laboratories Ltd, Hertfordshire, UK) according to the manufacturer's instructions. Briefly, samples were diluted 1:3 in the supplied assay buffer and 5 µL was loaded into 96-well plates alongside serial dilutions of standard bovine serum albumin (BSA). 25 µL of supplied assay reagent was added to each well and incubated for 15 minutes. Absorbance was measured at 650 nm using the NOVOstar microplate reader (BMG LABTECH Ltd, Buckinghamshire, UK) and sample readings were plotted against standard BSA. Samples and standards were analysed in triplicates and protein concentration was expressed as µg/mL.

Proteins were separated on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (0.375 M Tris/HCl pH 8.8, 33.35% Bis/Acrylamide, 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulphate, 0.05% (v/v) TEMED). Total of 20 µg/mL protein in reducing 5X loading buffer (10% SDS, 250 mM Tris HCl pH 6.8, 20% (v/v) glycerol, 0.02% (v/v) bromophenol blue, 10 mM DTT) was boiled for 5 minutes before being loaded on SDS-PAGE and run at 100V for 15 minutes followed by 120V for 90 minutes in running buffer (20.5 mM Tris pH 8.6, 0.192 M glycine, 0.1% (w/v) SDS). Proteins were then transferred to a polyvinylidene fluoride (PVDF) membrane at 115V for 72 minutes in transfer buffer (20.5 mM Tris, 150 mM glycine, 20% (v/v) methanol).

Non-specific binding was blocked by incubating the membranes in tris-buffered saline (TBS) (137 mM NaCl, 25 mM Tris, 2.7 mM KCl, pH 7.4) containing 1% Tween-20 (TBST) and 5% (w/v) non-fat milk powder for 1 hour at RT, followed by overnight incubation at 4°C with the primary antibody (diluted in TBST with 5% (w/v) milk powder) (Table 2.4). Membranes

were then washed 3 times in TBST and incubated for 1 hour at RT with the horseradish peroxidase (HRP) conjugated secondary antibody diluted in TBST with 5% milk powder, then washed in TBST. Membranes were developed using an electrochemiluminescence (ECL) kit (GE Healthcare Life Sciences, Buckinghamshire, UK) and visualised using a ImageQuant Las4000 (GE Healthcare). Pre-stained molecular weight ladders (10-170 kDa; Thermo Scientific) were included alongside samples.

Antibody	**Clone**	**Dilution**	**Source**	**secondary**	**Dilution**	**Source**
Anti-podoplanin	18H5	1:1000	Abcam	Anti-mouse	1:5000	Abcam
Anti-podoplanin	NZ-1	1:1000	eBioscienece	Anti-rat	1:3000	Thermo Scientific
Anti-actin	AC-15	1:5000	Abcam	Anti-mouse	1:10000	Abcam
Anti-TF	D3	1:2000	Dr. John McVey University of Surrey	Anti-mouse	1:2500	Abcam

Table 2.4. Primary and secondary antibodies used in Western blotting experiments.

2.2.10 Immunofluorescence microscopy

Immunofluorescence experiments were performed to examine the nature of the interaction between podoplanin-expressing cells and platelets. Control or h.PDPN-CHO cells were cultured for 24 hours at 1.2 x 10^3 on 8-well chamber slides (BD Falcon CultureSlides, BD Biosciences). Cells were washed 3 times with 300 µL PBS/BSA (phosphate buffered saline: PBS containing 1% (w/v) BSA). 200 µL washed platelets at a density of 200 x 10^3/µL was added to the chambers and incubated for 10 minutes at 37°C. The chambers were again washed 3 times with PBS/BSA. Platelets were labelled by adding 100 µL PBS/BSA containing 10 µL anti-CD42b-Cy5 (Pharmingen) and 2 mM $CaCl_2$. To analyse platelet degranulation and the platelet procoagulant response, 4 µL anti-human P-selectin-FITC

(R&D Systems) or 10 µL Annexin V-FITC (Pharmingen) were added respectively. Antibodies were incubated for 20 minutes then the slides were washed 3 times with PBS/BSA containing $CaCl_2$. Nuclei were counter stained using Hoescht stain at a final concentration of 100 nM in PBS/BSA containing $CaCl_2$ and incubated for 20 minutes. Nuclear stain was washed off using 0.2% formyl saline for P-selectin and HBS with $CaCl_2$ for Annexin V. Following washing, slides were mounted in Prolong Gold antifade reagent (Molecular Probes) and examined using an EVOS AMG fl LED fluorescent microscope (Washington, USA).

2.2.11 Molecular biology methods

2.2.11.1 **Detection of single nucleotide polymorphism (SNP)**

2.2.11.1.1 **DNA extraction**

Deoxyribonucleic acid (DNA) was extracted from the buffy coat of healthy subjects. Whole blood samples were drawn via 21-gauge butterfly needles into 10 mL EDTA vaccutainer tubes and centrifuged at 1800g for 30 minutes. The buffy coat was carefully isolated from the whole blood and stored in -80ºC. DNA was extracted using a QIA cube® (Qiagen Ltd., West Sussex, UK) according to the manufacturer's instructions. Briefly, 200 µL of the buffy coat was lysed by adding lysis buffer (20 µL proteinase K and 200 µL AL buffer). For efficient lysis, samples were incubated at 56ºC for 10 minutes. 200 µL ethanol was then added and the samples centrifuged at 6000g for 1 minute. The purity of the DNA bound to the membrane was improved by two washing steps using supplied buffers. In the first step, 500 µL AW1 buffer (Qiagen) was added and centrifuged at 6000g for 1 minute. In the second step, 500 µL AW2 buffer (Qiagen) was added and centrifuged at 20,000g for 3 minutes. The used lysate

buffering conditions only allowed DNA to bind to the silica membrane of the QIAamp (Qiagen), whereas other contaminants were not retained on the membrane. The purified DNA was finally eluted by adding 100 µL elution buffer (AE buffer, Qiagen) and centrifuged at 6000g for 1 minute. DNA concentration was measured using a nano-drop spectrophotometer (Labtech International, East Sussex, UK) then adjusted at 20 ng/µl using nuclease-free water.

2.2.11.1.2 **TaqMan assay for single nucleotide polymorphism (SNP) detection**

In a 384-well plate, 1 µl of 20 ng/mL DNA was added to a 6 µL reaction volume containing X2 TaqMan universal master mix, sequence-specific primer and TaqMan FAM probe (Applied Biosystems, California, USA) and nuclease-free water. Samples were amplified using a Gene Amp PCR System 9700 (Applied Biosystems) with cycling conditions set at: 95ºC for 10 minutes followed by 40 cycles of 92ºC for 15 seconds for DNA denaturation, and 60ºC for 1 minute for annealing, followed by a final extension step of 72ºC for 10 minutes. The ABI PRISM 7900HT sequence detection system (Applied Biosystems) was used to determine allelic discrimination by reading the reaction products. 96 DNA samples from the Bloodomics cohort (Jones, *et al* 2009) of known GPVI genotye for both SNPs were included into the genotyping run as a quality control, as well as to improve the fluorescence clustering plot and to adjust plotting axis. No template control (NTC) wells were also included as negative controls.

2.2.11.2 **Podoplanin gene upregulation**

Podoplanin gene (*PDPN*) expression was assessed by RT-qPCR in (1) monocytes isolated from whole blood before and after stimulation with platelets and (2) thrombi formed from whole blood using the Chandler loop method. Sample preparation, RNA extraction and

reverse transcription were performed by Robert Turnbull (Department of Cardiovascular Sciences, University of Leicester).

2.2.11.2.1 **Total RNA isolation**

Monocytes were isolated from citrated whole blood using CD14 Dynabeads (Life Technologies Ltd). Thrombi were formed in a Chandler loop (Chandler 1958) by rotating 1 mL re-calcified citrated whole blood into PVC tubing (45 cm long, 3 mm internal circumference) at 36rpm for 6 hours either with or without monocytes (depleted using CD14 Dynabeads). Resulting thrombi were carefully collected and washed in Hank's buffered salt solution (Jones and Goodall 2003). Isolated monocytes or thrombi were lysed in TRIzol reagent and incubated for 5 minutes. Chloroform was immediately added at 200 µL per 1 mL TRIzol, vortexed vigorously and left to stand for 3 minutes at RT. Following centrifugation at 13400rpm for 15 minutes at 4ºC, the colourless upper aqueous phase (containing RNA) was collected and diluted with an equal volume on 70% (v/v) ethanol. Total RNA was extracted using RNeasy mini-kit (Qiagen) following the manufacturer's instructions. Briefly, 700µl of cell lysate was loaded into RNeasy spin column and washed with RWI buffer by centrifugation at 10000rpm for 30 seconds. DNA was removed from the samples by adding 80 µl DNase 1 (Qiagen) and incubated for 25 minutes at RT. The column was again washed with RWI buffer (Qiagen) followed by 2 washes in RPE buffer (Qiagen). Any remaining ethanol in the column was removed by centrifugation at 13400rpm for 3 minutes. The remaining RNA was dissolved and collected into sterile tubes by adding 25 µl RNase/DNase free water to the columns and centrifuged at 10000rpm for 2 minutes. RNA quantity and quality was measured using the NanoDrop™8000 (ThermoScientific).

2.2.11.2.2 **Reverse transcription**

All reagents were obtained from Life Technologies Ltd unless otherwise stated. DNA was further eliminated from samples using DNase1 (Sigma) before reverse transcription. Reverse transcription was performed using avian myeloblastoma virus (AMV) reverse transcriptase (RT). Briefly, final concentration of 2.5 µM oligo$(dT)_{20}$ primers and 1 mM deoxyribonucleotide triphosphate (dNTP) were added to samples, mixed and incubated at 65ºC for 5 minutes. 4 µl 5X cDNA synthesis buffer (250 mM Tris-Acetate pH 8.4, 375 mM Potassium acetate, 40 mM Magnesium acetate), 1 µL 0.1 mM DTT, 1 µl AMV-RT (15 units/µL) and 1 µl Riboblock RNase inhibitor (40 units/µL) (ThermoScientific) were added to the RNA samples. The mixtures were incubated at 45ºC for 60 minutes in a thermal cycler then heated to 85ºC for 5 minutes. cDNA samples were stored at -80ºC until they were used.

2.2.11.2.3 **Real-time PCR (RT-qPCR)**

Levels of mRNA were measured using ViiA™ 7 Real-Time PCR machine (Applied Biosystems), and the QuantiFast SYBR Green PCR kit (Qiagen) according to manufacturer's protocols. In a 96-well plate, a 25 µL reaction volume for each sample containing 12.5 µL of 2x QuantiFast SYBR green master mix, 2.5 µL specific primer (Table 2.5), 8 µL DNase/RNase free water and 2 µL cDNA sample were added together and loaded in the ViiA™ 7 Real-Time PCR machine. The PCR cycling conditions were set at: 50ºC for 2 minutes then 95ºC for 5 minutes followed by 40 cycles of 95ºC for 10 seconds and 60ºC for 30 seconds. Finally, a melt curve was produced by incubation at 95ºC for 15 seconds, 60ºC for 15 seconds and 95ºC for 15 seconds. NTC samples were also included in each plate. Reactions were carried out in triplicate to assess the reproducibility and accuracy of the PCR reaction.

The mean cycle threshold (C_T) values were used to calculate the relative gene expression of using $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008). C_T values of gene of interest (GOI), *PDPN*, were normalised against the control or time 0 C_T values of housekeeping (HK) gene, β-2 microglobulin (*B2M*). Gene upregulation fold change was then calculated using the following equation: $2^{\wedge-((CT(GOI)-CT(HK))TimeX-(CT(GOI)-CT(HK))Time0)}$. For samples that failed to show C_T values, results were confirmed by running the PCR products on a 2% agarose gel, stained with GelRed and visualised under UV illumination.

Primer	Detected transcript	Ensembl transcript ID	Amplified exons	Product size
PDPN	NM_001006624 NM_006474	ENST00000294489	5/6	108 bp
B2M	NM004048	ENST00000349264	1/2	98 bp

Table 2.5. QuantiTect primers used in SYBR green RT-qPCR.

2.2.12 Statistical analysis

Unless otherwise stated statistical analysis was performed using Graph Pad Prism version 6 (GraphPad Software Inc., San Diego, CA, USA) and data are expressed as mean ± standard deviation (SD). Comparison between two normally distributed variables was performed using the paired *t* test. For multiple variables, one-way ANOVA and Bonferroni multiple comparison test were used. A p-value of <0.05 was considered significant.

CHAPTER 3: **Measurement and characterisation of the procoagulant activity of microparticles derived from vascular cells**

3.1 Introduction

MPs have been associated with various physiological and pathological conditions such as cardiovascular disease, diabetes, cancer and infection (Morel, *et al* 2008, Piccin, *et al* 2007). Circulating MPs are mainly derived from platelets or megakaryocytes, but also from endothelial cells and leukocytes (Flaumenhaft, *et al* 2009, Shantsila, *et al* 2010). As already described (section 1.3) the presence of PS on the outer surface of MPs provides a binding site for a number of activated clotting factors including those forming the tenase (FIXa/FVIIIa) and prothrombinase (FXa/FVa) complexes, leading to acceleration and enhancement of thrombin generation (Berckmans, *et al* 2001, Sims, *et al* 1989). The PL content of MPs may vary depending on the cellular of origin and the nature of stimuli. Additionally, it has been shown that some MPs express TF, the principle activator of the coagulation cascade. The exact cellular origin of TF-bearing MPs in the circulation is still subject to debate, although several studies showed that the majority are derived from monocytes (Owens and Mackman 2011). MPs containing both PS and TF are thought to have the highest level of procoagulant activity, as they may independently initiate and promote coagulation via the TF/FVIIa complex, as well as facilitate the assembly of components of the clotting cascade (Owens and Mackman 2011). However, the relative contribution of the PL and TF composition of MPs on haemostasis and thrombosis are not fully understood.

The presence of circulating MPs in the circulation of healthy individuals would imply a role for MPs in the maintenance of normal haemostasis. This is well supported by the high bleeding tendency in individuals with Scott syndrome, who have low levels of circulating

MPs due to a defect in the ability to "flip" PS to the outer plasma membrane leaflet (Zwaal, *et al* 2004), while the increased levels of MPs in thrombotic disease suggest that MPs play a key role in promoting thrombosis. However, despite the accumulating evidence that supports their critical role in haemostasis and thrombosis; further studies are needed to understand how MPs of different cellular origin contribute to haemostasis and thrombosis. The largest proportion of circulating MPs in healthy individuals are derived from platelets or megakaryocytes (Berckmans, *et al* 2001, Flaumenhaft, *et al* 2009), while the levels of MPs derived from other cells such as leucocytes, endothelial cells, red blood cells and vascular smooth muscle cells as well as from platelets are elevated in the circulation in different disease conditions.

Several methods are being used to detect, count, size and phenotype MPs from different biological samples. As detailed in Chapter 1 (section 1.6.4) these include flow cytometry, EM, ELISA, NTA, DLS, AFM, proteomics analysis and functional assays such as measuring their procoagulant activity in the CAT assay. However, there is no uniform consensus regarding the definition and detection of MPs, which make it difficult to compare the outcomes across different studies (Freyssinet and Toti 2010, van der Pol, *et al* 2013). Additionally, individual studies use a wide range of different pre-analytical conditions prior to MP analysis that include differences in the method of blood sampling, anticoagulants and types of collection tubes used, needle size, isolation protocols, freezing and thawing temperatures, and conditions and duration of storage. MP analysis has been shown to be heavily influenced by these pre-analytical variables, which form the major source of inter-laboratory variation and potential artefacts in MP analysis (Freyssinet and Toti 2010, Piccin, *et al* 2007). Therefore, it is vital to optimise and standardise the pre-analytical variables of MP measurement methods before they become truly useful as clinical tools.

In 2003, the Scientific and Standardisation Committee (SSC) of the International Society on Thrombosis and Haemostasis (ISTH) decided, in their meeting, to address issues related to the methodology of MP detection and characterisation (Jy, *et al* 2004). In the intervening period, several groups have investigated the impact of pre-analytical and analytical conditions on the levels on MPs (Dey-Hazra, *et al* 2010, Lacroix, *et al* 2012, Lee, *et al* 2012a, Robert, *et al* 2009, Shah, *et al* 2008, Trummer, *et al* 2008, Trummer, *et al* 2009, Yuana, *et al* 2011). However, the majority of these studies examined the effect of pre-analytical variables on MP count and PS exposure using flow cytometry. Only a few studies have briefly explored the impact of pre-analytical and analytical conditions on the procoagulant activity of MPs (Keuren, *et al* 2006, Lacroix, *et al* 2012, Lee, *et al* 2012a). Procoagulant activity of MPs measured using the CAT assay has been shown as a promising tool for MP detection and characterisation (Aleman, *et al* 2011, Bidot, *et al* 2008, Lee, *et al* 2012a). Therefore, there is a need to evaluate the impact of the pre-analytical variables on the analysis of the procoagulant activity of MPs.

The first aim of this study was to investigate the effect of pre-analytical and analytical variables on the procoagulant activity of MPs in plasma; either taken from healthy individuals, or containing MPs generated from platelets (PDMP-rich plasma) using the CAT assay, and initiating the reaction with several commercially available reagents containing different concentrations of TF and/or PL that have been designed for different purposes.

The second aim was to examine how MPs derived from different cellular origins contribute in plasma procoagulant activity, and how these are affected by different pre-analytical conditions. This was achieved by evaluating the procoagulant activity of MPs generated from washed platelets, endothelial cells and monocyte-derived macrophages. The CAT assay was used to evaluate the ability of these MPs to support thrombin generation in a PL-dependent and/or TF-dependent manner. In addition, MP activity was analysed using the Zymuphen

Activity and Zymuphen MP-TF ELISA kits to measure the PS and TF concentrations respectively.

3.2 Results

3.2.1 CAT assay normal ranges

In order to establish normal ranges for CAT assay parameters blood samples were taken from 22 healthy individuals who had no history of bleeding or thrombosis, and who had not taken any medication in the 10 days prior to blood sampling. Blood was collected into tri-sodium citrate (3.2%) tubes, CTI (at final concentration of 18.3 µg/mL) was immediately added, and plasma was obtained by centrifugation at 1800g for 30 minutes, and was rapidly frozen and stored in aliquots at -80°C. Plasma samples were analysed in the CAT assay using the following reagents containing different concentrations of TF and/or PL:

- Stago PPP (5 pM TF/4 µM PL)
- Stago PPP low (1 pM TF/4 µM PL)
- Stago PRP (1 pM TF)
- Stago MP (4 µM PL)
- No reagent, without any exogenous TF or PL.

The different reagents produced markedly different thrombin generation curves, as illustrated in Figure 3.1, which shows representative traces from a single sample of normal plasma. It can be clearly seen that the lag time and ttPeak are mainly affected by the concentrations of TF, whereas ETP and peak thrombin are more influenced by the concentrations of PL.

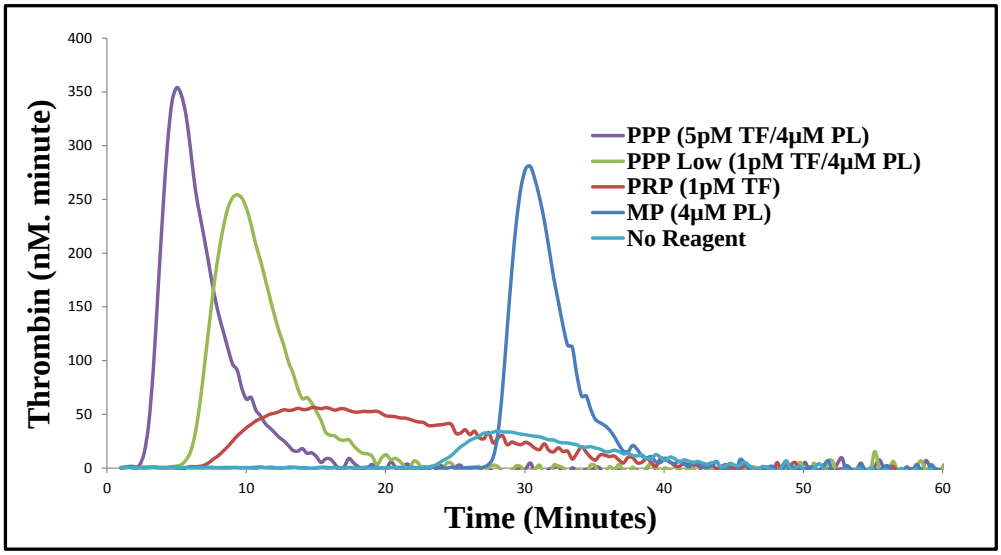


Figure 3.1. Representative thrombin generation curves using different reagents.

Thrombin generation results showed significant variations between individuals in all parameters (Figure 3.2 and Table 3.1). This was most marked when the plasma samples were analysed in the absence of exogenous TF and/or PL. Five of the 22 samples completely failed to support thrombin generation when analysed with the MP reagent or without exogenous PL or TF, suggesting that these samples lack sufficient levels of endogenous TF activity to initiate coagulation. These five samples were excluded from the analysis of the normal ranges with the MP reagent and without no reagent assays. It should be noted that these thrombin generation ranges from normal individuals were performed in the presence of CTI, a selective FXIIa inhibitor (Rand, *et al* 1996) as it has been demonstrated that the intrinsic coagulation pathway can cause an overestimation of thrombin generation capacity, particularly when low TF concentration is used in the assay (Luddington and Baglin 2004, Rand, *et al* 1996, Van Veen, *et al* 2008), a finding that was later supported by Ollivier *et al* (Ollivier, *et al* 2010). Therefore, the present normal range values of thrombin generation are with the inhibition of contact pathway contribution.

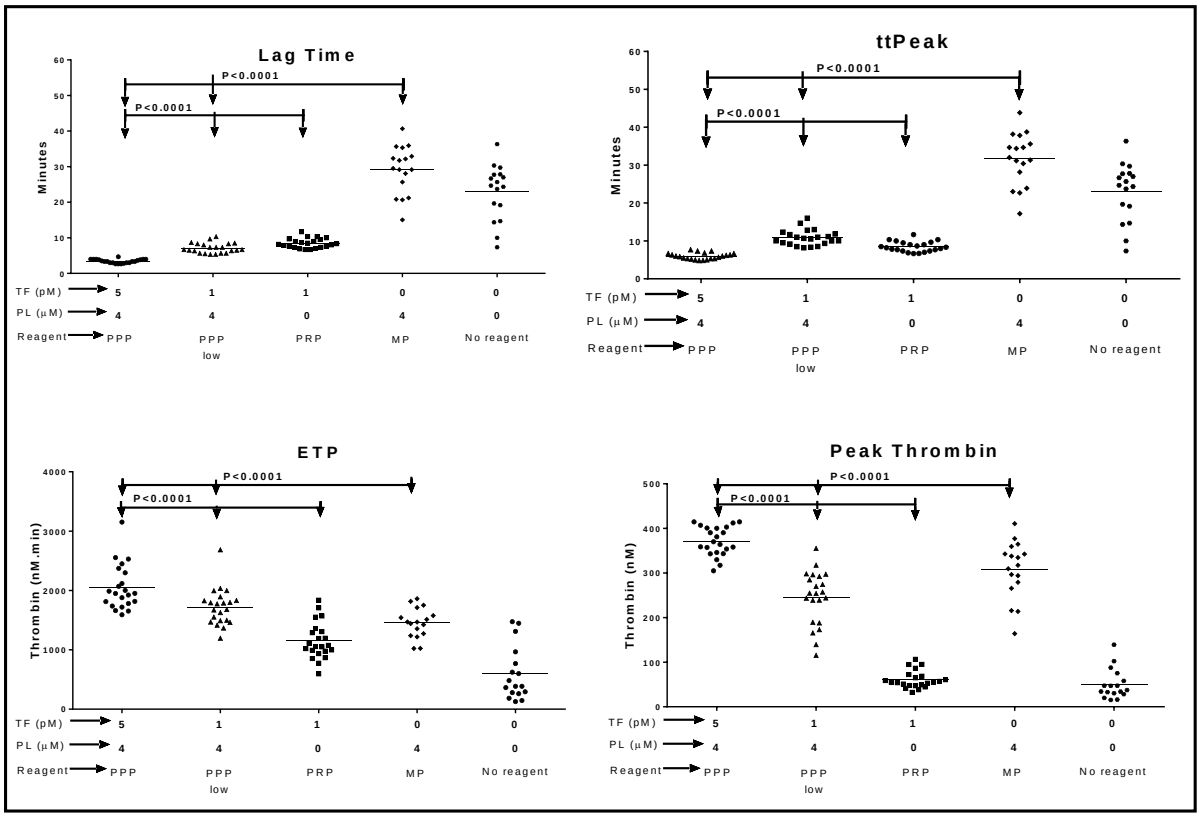


Figure 3.2. CAT assay results from normal plasma using different reagents. CTI-treated plasma was collected from 22 healthy individuals and stored at -80°C. Thrombin generation was evaluated in the CAT assay using the following reagents: PPP (n=22), PPP low (n=22), PRP (n=22), MP (n=17) and no reagent (n=17).

PPP reagent (n=22)	Mean±SD	Minimum	Maximum	%CV
Lag time (Minutes)	3.44±0.55	2.67	4.67	16.07
ttPeak (Minutes)	5.96±0.85	4.83	7.67	14.27
ETP (nM.min)	2046±378.7	1590	3153	18.51
Peak Thrombin (nM)	371.0±32.68	305.0	414.7	8.81

PPP low reagent (n=22)	Mean±SD	Minimum	Maximum	%CV
Lag time (Minutes)	7.09±1.42	5.33	10.33	20.07
ttPeak (Minutes)	10.83±2.04	8.18	16.00	18.81
ETP (nM.min)	1720±310.2	1195	2685	18.03
Peak Thrombin (nM)	244.9±60.55	115.4	355.1	24.73

PRP reagent (n=22)	Mean±SD	Minimum	Maximum	%CV
Lag time (Minutes)	8.47±1.35	6.67	11.67	15.96
ttPeak (Minutes)	17.02±2.27	13.53	22.83	13.33
ETP (nM.min)	1151±308.2	596.0	1838	26.77
Peak Thrombin (nM)	60.65±19.53	32.19	106.3	32.21

MP reagent (n=17)	Mean±SD	Minimum	Maximum	%CV
Lag time (Minutes)	29.18±6.69	15.03	40.67	22.93
ttPeak (Minutes)	31.63±6.89	17.20	43.83	21.78
ETP (nM.min)	1453±249.3	1023	1860	17.15
Peak Thrombin (nM)	274.3±64.4	164	410.6	20.95

No reagent (n=17)	Mean±SD	Minimum	Maximum	%CV
Lag time (Minutes)	22.90±7.69	7.35	36.33	33.60
ttPeak (Minutes)	27.66±7.02	13.03	40.83	25.40
ETP (nM.min)	594±108.9	127	1475	75.61
Peak Thrombin (nM)	50.11±33.49	15.3	139.2	66.82

Table 3.1. CAT assay normal ranges and thrombin generation inter-individual variability. CTI-treated plasma was collected from 22 healthy individuals and stored at -80°C. Thrombin generation was evaluated on CAT assay using the PPP (n=22), PPP low (n=22), PRP (n=22), MP (n=17) and no reagent (n=17) assays.SD: standard deviation, %CV: coefficient of variation.

3.2.2 Inter-assay variability of CAT assay

Table 3.1 shows the inter-sample %CV for the CAT assay with the different reagents. To determine the inter-assay %CV, thrombin generation was measured in the CAT assay with each of the 5 reagents; (1) PPP, (2) PPP low, (3) PRP, (4) MP and (5) no reagent using CTI-inhibited pooled normal plasma measured on different occasions over a one year period. Table 3.2 clearly shows that lag time and ttPeak were more reproducible than ETP and peak thrombin. For the majority of reagents the ETP and peak thrombin had more than 10% CV, which is considered marginally high. Because the samples were analysed over a long period it is unclear at this stage whether the high %CV of the ETP and peak thrombin values were due to truly low inter-run precision or to plasma sample deterioration during the storage. Therefore, it was decided to re-evaluate the inter-assay variability of CAT assay using a different batch of CTI-inhibited pooled normal plasma, but analysed within 30 days. It can be clearly seen from Table 3.3 that ETP and peak thrombin reproducibility was significantly improved after minimising the effect of storage duration.

PPP reagent (n=14)	Mean±SD	Minimum	Maximum	%CV
Lag Time (Minutes)	3.37±0.21	2.95	3.67	6.1
ttPeak (Minutes)	5.75±0.26	5.28	6.17	4.5
ETP (nM.min)	2233±341	1702	2844	15.3
Peak Thrombin (nM)	410±36.7	348	470	8.9

PPP low reagent (n=14)	Mean±SD	Minimum	Maximum	%CV
Lag Time (Minutes)	6.90±0.41	6.12	7.67	5.9
ttPeak (Minutes)	10.24±0.46	9.46	11.33	4.5
ETP (nM.min)	1812±300	1411	2399	16.6
Peak Thrombin (nM)	279±31.4	240	336	11.3

PRP reagent (n=14)	Mean±SD	Minimum	Maximum	%CV
Lag Time (Minutes)	8.13±0.79	7.29	9.67	9.7
ttPeak (Minutes)	15.92±0.55	15.30	16.83	3.5
ETP (nM.min)	1395±167	1148	1719	12.0
Peak Thrombin (nM)	71±7.40	61	88	10.4

MP reagent (n=14)	Mean±SD	Minimum	Maximum	%CV
Lag Time (Minutes)	25.7±1.43	23.39	27.67	5.6
ttPeak (Minutes)	28.09±1.45	25.73	30.17	5.1
ETP (nM.min)	1574±158	1358	1880	10.1
Peak Thrombin (nM)	321±29.9	282	392	9.3

No reagent (n=14)	Mean±SD	Minimum	Maximum	%CV
Lag Time (Minutes)	26.5±1.69	23.33	28.33	6.4
ttPeak (Minutes)	30.85±1.33	28.33	32.67	4.3
ETP (nM.min)	637±98.9	457	839	15.5
Peak Thrombin (nM)	49±7.4	37	66	15.1

Table 3.2. Inter-assay variability of CAT assay (first analysis). Stored CTI-treated pooled plasma was used to assess CAT assay precession using the indicated reagents. Samples were analysed at different occasions over one year. SD: standard deviation, CV: coefficient of variation.

PPP reagent (n=22)	**Mean±SD**	**Minimum**	**Maximum**	**%CV**
Lag Time (Minutes)	3.89±0.23	3.66	4.50	6.0
ttPeak (Minutes)	6.79±0.40	6.00	7.83	5.9
ETP (nM.min)	1948±152	1697	2315	7.8
Peak Thrombin (nM)	321±32	275	381	9.8

PRP reagent (n=22)	**Mean±SD**	**Minimum**	**Maximum**	**%CV**
Lag Time (Minutes)	8.03±0.31	7.50	8.69	3.8
ttPeak (Minutes)	16.7±0.85	14.83	18.16	5.1
ETP (nM.min)	1527±111	1242	1728	7.3
Peak Thrombin (nM)	83±7.7	68	101	9.2

MP reagent (n=22)	**Mean±SD**	**Minimum**	**Maximum**	**%CV**
Lag Time (Minutes)	29.5±2.8	24.37	34.54	9.4
ttPeak (Minutes)	32.1±2.8	27.04	37.38	8.7
ETP (nM.min)	1432±91	1235	1567	6.4
Peak Thrombin (nM)	267±26	221	296	9.8

Table 3.3. Inter-assay variability of CAT assays (second analysis). Stored CTI-treated pooled plasma was used to assess CAT assay precession using the indicated reagents. Samples were analysed at different occasions over one month. SD: standard deviation, CV: coefficient of variation.

3.2.3 Plasma stability and thrombin generation

It is common that plasma samples are stored at -80°C for long periods. Therefore, it was decided to examine the effect of sample age on the procoagulant activity of plasma. This was achieved by continuing monitoring the procoagulant activity of the same pooled plasma used in Table 3.3 for a longer period, up to 638 days, using the PPP, MP and PRP reagent assays. Figure 3.3 shows that with all three reagents, the lag time and ttPeak tend to be stable and were not significantly affected by the duration of storage. On the other hand, the ETP and peak thrombin values showed a trend to a slight decrease in the thrombin generation capacity, probably affecting a decay of coagulation factors over the time. This was most notably with the PPP reagent, which is the assay most sensitive to levels of clotting factors in the plasma (ETP slope values: -1.2±0.20, -0.28±0.14 and -0.17±0.11 for the PPP, MP and PRP reagent assays respectively). It should, however, be noted that these data were obtained from a single preparation of pooled plasma and further investigations with larger number of samples are required to confirm this finding.

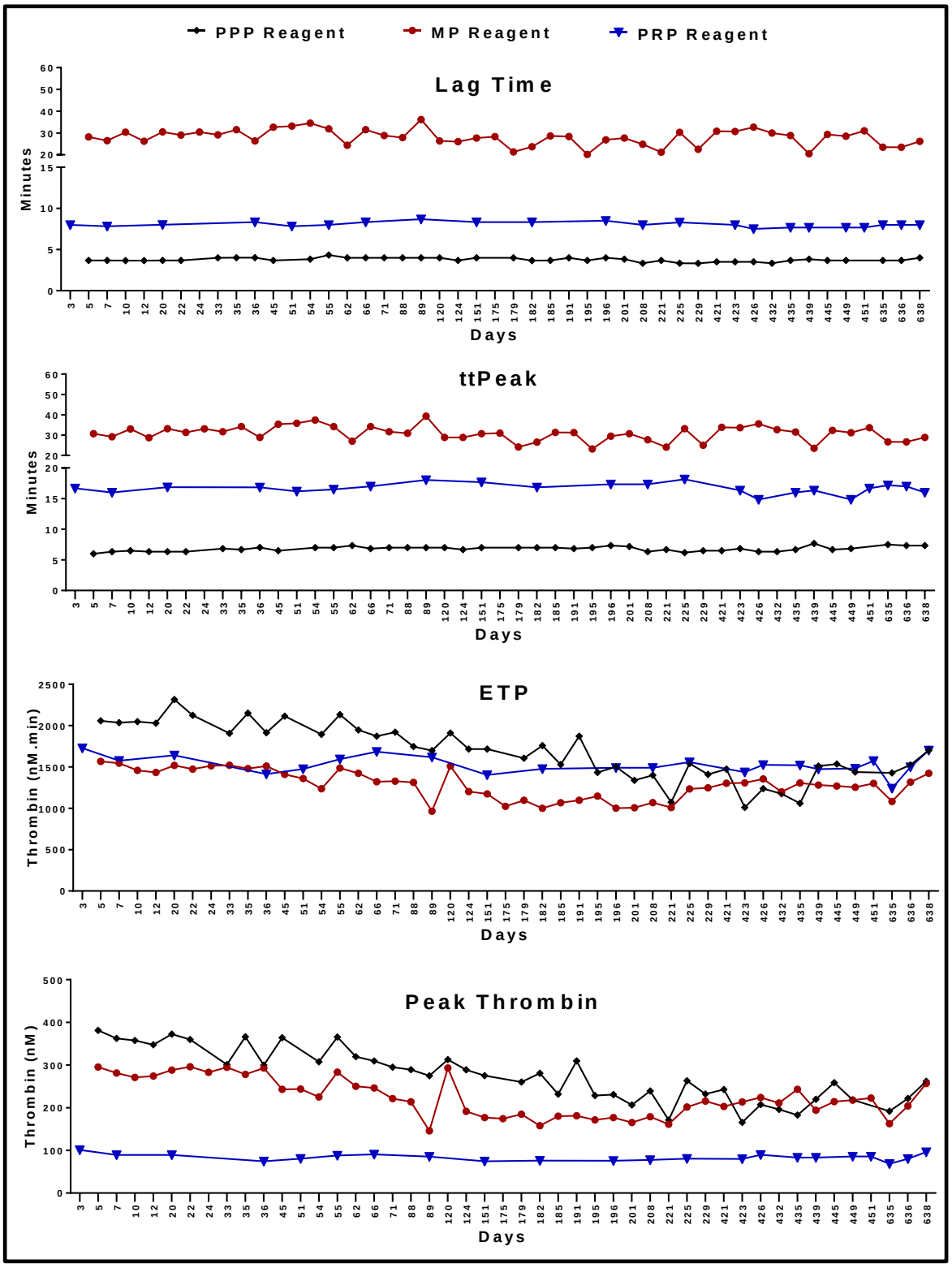


Figure 3.3. Effect of plasma storage on thrombin generation. Frozen pooled plasma was analysed on CAT assay assayed using the PPP (black line), MP (red line) and PRP (blue line) reagents at different time points.

3.2.4 The effect of PDMPs on thrombin generation measured with different reagents

3.2.4.1 Microparticle generation from platelets

3.2.4.1.1 Selection of donors based on GPVI haplotype

MPs were generated from platelets by activation through GPVI, which is well known to be able to induce platelet PS exposure and MPs formation by its sole action (Nieswandt and Watson 2003). A number of SNPs have been reported in the GPVI gene that are associated with variation in GPVI expression and function. Joutsi-Korhonen et al (2003) reported two common GPVI haplotypes, GPVIa and GPVIb, which differ by five amino acid substitutions produced by five non synonymous SNPs; rs1613662, rs1654416, rs2304167, rs1654413 and rs1671152. Significant differences were reported between these haplotypes in terms of the levels of platelet GPVI expression as well as functional responses to CRP-XL such as platelet aggregation, degranulation and the ability to support thrombin generation, with GPVIa associated with a 3-fold higher response to CRP-XL. Two of the 5 SNPs; rs1613662 and rs1654413, can give up to 99% confidence of the GPVI haplotypes (Joutsi-Korhonen, *et al* 2003), however, rs1613662 has been shown to have the strongest effect, which in a large cohort study was associated with 38.6% of variation in platelet response to CRP-XL (Jones, *et al* 2009). In order to minimise the inter-individual variability in platelet response induced via GPVI, it was decided to study only healthy donors with the GPVIa haplotype and exclude subjects who carried the GPVIb variant. Therefore, all the subjects in this project were genotyped for the two most common alleles, rs1613662 and rs1654413. Genotyping showed that 70.4% of the donors were homozygotes AA for rs1613662 and 74.1% TT homozygous for rs1654413 (Figure 3.4), indicating that around 70% of the healthy participants were

GPVIa. This allele frequency is similar to those previously reported by Joutsi-Korhonen *et al* and Jones *et al* (65% and 69% respectively).

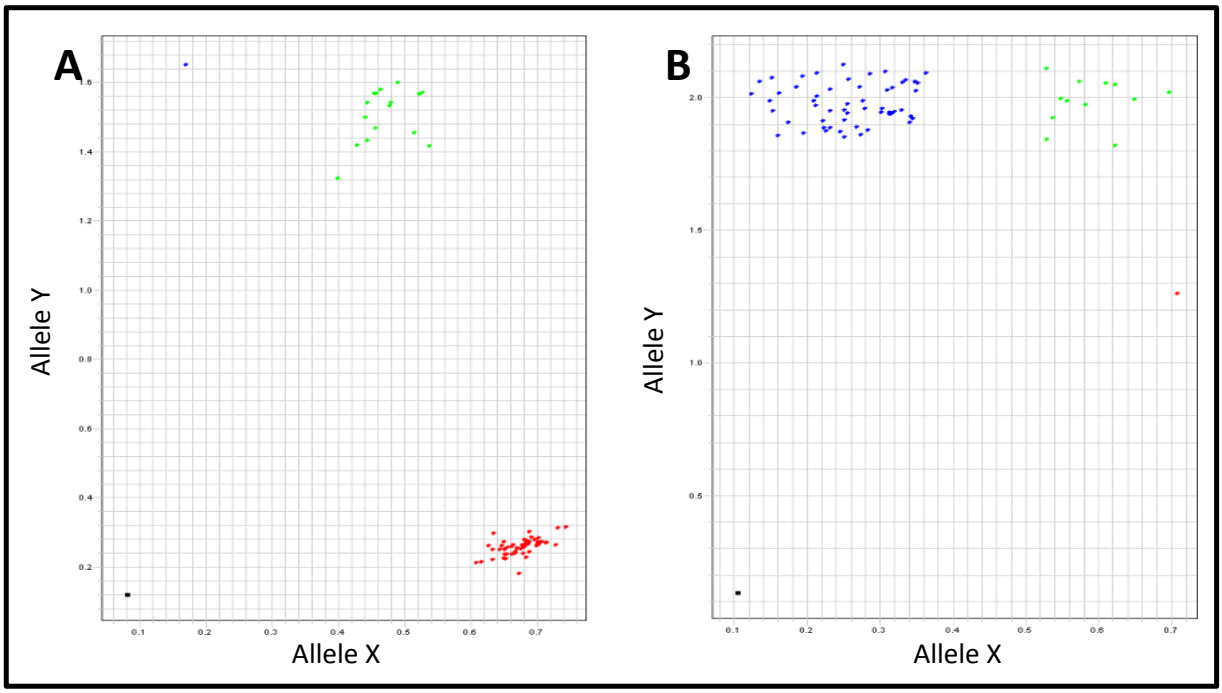
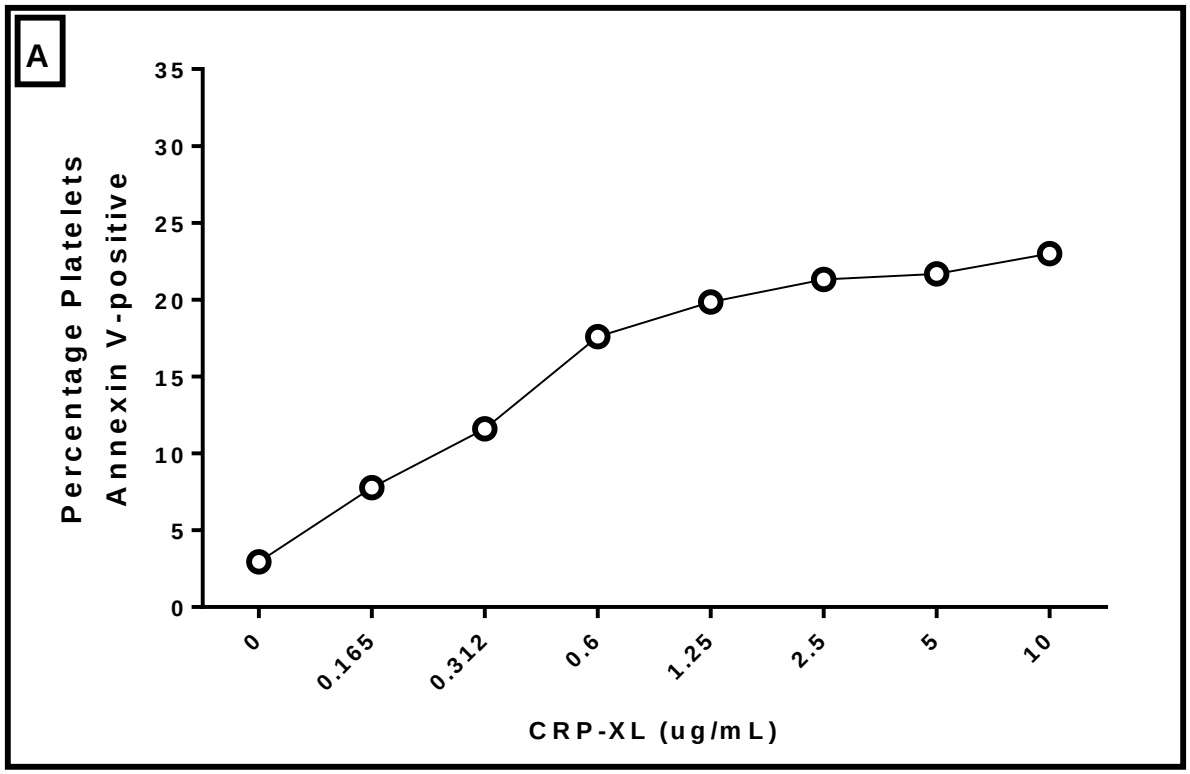


Figure 3.4. Fluorescence plots of allelic discrimination of rs1613662 and rs1654413. A) cluster plots for rs1613662, whereas B) cluster plots for rs1654413. Additional DNA samples of known genotype were selected from the Bloodomics cohort and added into the assay as controls, and to enhance clustering. Homozygous A alleles are shown by the red dots, homozygous T alleles are shown by the blue dots, heterozygotes are shown by the green dots and no-template controls (NTC) are shown by the small black squares.

3.2.4.1.2 **Optimal dose of CRP-XL to induce maximum platelet stimulation**

CRP-XL was used as the main agonist to induce procoagulant platelets and MP generation but as batches of this peptide can vary in their activity the CRP-XL was titrated to establish the optimum concentration to use. Blood from the genotyped, GPVIa haplotype healthy donors was collected into tri-sodium citrate (3.2%) tubes containing CTI. PRP samples were prepared from fresh blood and the platelet count was adjusted at 150 x 10^3/µL with autologous PPP. GPRP peptide was added to a final concentration of 0.5 mg/mL prior to platelet activation to prevent fibrin cross-linking and therefore prevent clotting of the plasma. Platelets were then stimulated for 10 minutes at 37°C with serial doses of CRP-XL (0-10 µg/mL) and analysed by flow cytometry for Annexin V binding and MP formation. Figure 3.5 A shows that CRP-XL induces platelet Annexin V binding in a dose-dependent manner, reaching a maximum at a CRP-XL concentration around 2µg/mL. Platelet Annexin V binding induced by the serial doses of CRP-XL was accompanied by the release of strongly Annexin V^{+ve} MPs that were smaller than platelets (Figure 3.5 B).



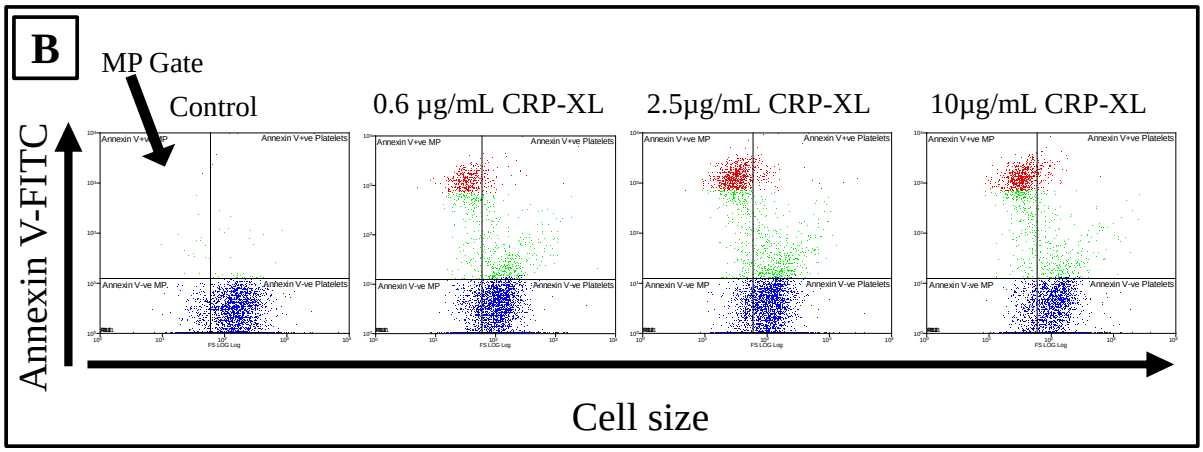


Figure 3.5. Platelet Annexin V binding and MP formation induced by CRP-XL platelet activation. PRP samples were stimulated with increasing concentrations of CRP-XL for 10 minutes at 37°C and analysed on flow cytometry for Annexin V binding and MP formation. A) shows total platelet Annexin V binding (n=2). B) shows representative flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter of platelets stimulated with indicated CRP-XL doses. In the dot-plot graphs; the upper left-hand quadrant shows Annexin V^{+ve} events in MP-size gate, the lower left-hand quadrant shows Annexin V^{-ve} events in MP-size gate, the upper right-hand quadrant shows Annexin V^{+ve} events in platelet-size gate, the lower right-hand quadrant shows Annexin V^{-ve} events in platelet-size gate; blue dots are Annexin V^{-}; green dots are weak Annexin V^{+}; red dots are strong Annexin V^{+++}.

The release of MP from platelets in response to CRP-XL activation was also assessed in the CAT assay (Figure 3.6). PRP samples were prepared as described above and stimulated for 10 minutes at 37°C with increasing doses of CRP-XL. PDMPs were separated from their parent platelets by centrifugation at 1800g for 30 minutes. PDMP-rich plasma was collected and the procoagulant activity of the PDMPs was evaluated in the CAT assay using the 1pM TF reagent assay (Stago PRP reagent). To demonstrate that the procoagulant activity in these samples was due to the effect of MPs, samples were analysed before and after filtration by passing the samples through 0.2 µm pore size filters. Thrombin generation data, particularly ETP and peak thrombin, clearly showed that CRP-XL platelet stimulation induces MP formation. There was a marked increase in the amount of ETP and peak thrombin and slight decrease in lag time and ttPeak. On the other hand, the enhancement in thrombin generation induced by PDMPs was completely abolished after filtration, confirming that the effect is due to the presence of PDMPs in CRP-XL stimulated samples.

Based on flow cytometry and CAT assay results, 2 µg/mL CRP-XL was selected as the concentration for subsequent experiments investigating the procoagulant activity of PDMPs.

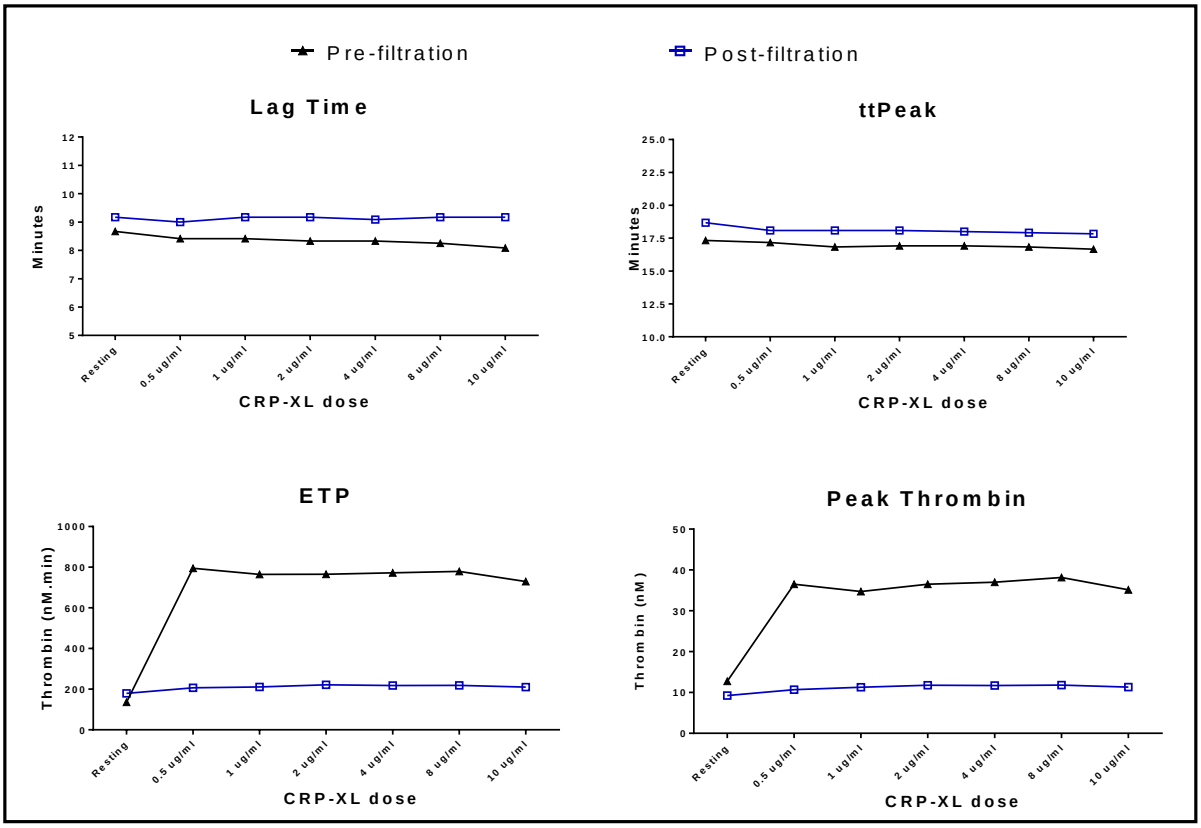


Figure 3.6. Thrombin generation of PDMPs induced by serial concentration of CRP-XL. PRP samples were stimulated with serial doses of CRP-XL for 10 minutes at 37°C, then MP-rich plasma was obtained by centrifuging the PRP samples at 1800g for 30 minutes. Thrombin generation was triggered using the 1 pM TF reagent (Stago PRP reagent). Samples were analysed before (black lines) and after (blue lines) filtration through a 0.2 µm filter using the Ceveron unit. Data are shown as mean of two separate experiments using blood from different donors.

3.2.4.2 **Analysis of the impact of PDMPs on thrombin generation**

To study the effect of the increased levels of PDMPs in plasma on thrombin generation, PDMP-rich plasma was analysed alongside the autologous normal plasma in the CAT assay using the same five reagents: (1) PPP, (2) PPP low, (3) PRP, (4) MP and (5) no reagent. PRP samples were prepared from fresh blood collected from healthy donors into sodium citrate tubes containing CTI. As previously the platelet count was adjusted at 150 x 10^3/µL with autologous PPP, and GPRP was added to prevent fibrin cross-linking and therefore prevent clotting of the plasma. Platelets were then stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C. Normal plasma and PDMP-rich plasma were separated from the platelets by centrifugation at 1800g for 30 minutes and analysed immediately in the CAT assay. To demonstrate the effect of PDMPs in the plasma, samples were analysed before and after filtration using the Ceveron unit to remove the MPs.

Interestingly, the presence of PDMPs failed to show any enhancement in thrombin generation capacity when the PPP or PPP low reagents were used (Figure 3.7). There were no significant differences in any of the measured thrombin generation parameters between PDMP-rich plasma and autologous normal plasma. Additionally, samples showed no significant difference in the thrombin generation after removing the MPs by filtration from both PDMP-rich plasma as well as from normal plasma. This finding clearly indicates that the PPP and PPP low reagents lack the sensitivity to detect the procoagulant activity of PDMPs in plasma samples.

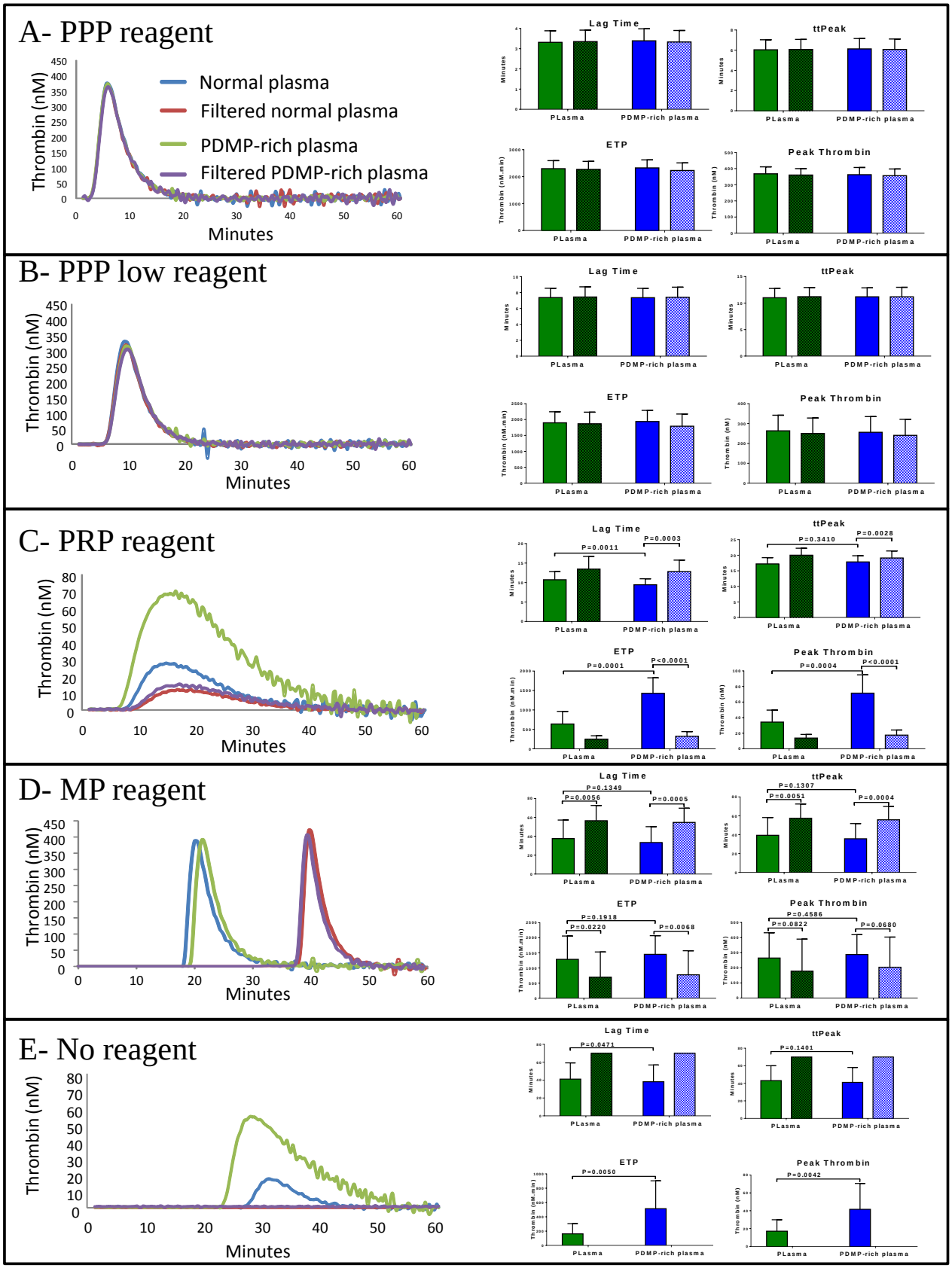

A- PPP reagent
Thrombin (nM)
Minutes
Normal plasma
Filtered normal plasma
PDMP-rich plasma
Filtered PDMP-rich plasma
Lag Time
ttPeak
ETP
Peak Thrombin
PLasma
PDMP-rich plasma
B- PPP low reagent
C- PRP reagent
P=0.0011
P=0.0003
P=0.3410
P=0.0028
P=0.0001
P<0.0001
P=0.0004
D- MP reagent
P=0.1349
P=0.0056
P=0.0005
P=0.1307
P=0.0051
P=0.0004
P=0.1918
P=0.0220
P=0.0068
P=0.4586
P=0.0822
P=0.0680
E- No reagent
P=0.0471
P=0.1401
P=0.0050
P=0.0042

Figure 3.7. Effect of PDMPs on thrombin generation measured with different reagents. PRP samples were prepared from healthy donors and stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C. Normal plasma and PDMP-rich plasma samples were obtained by centrifuging the PRP samples at 1800g for 30 minutes. Thrombin generation was promoted with the following reagents: (A) Stago PPP (5pM TF/4µM PL), (B) Stago PPP low (1pM TF/4µM PL), (C) Stago PRP (1pM TF), (D) Stago MP (4µM PL) and (E) no reagent, without any exogenous TF or PL. Samples were analysed before (solid bars) and after (hatched bars) filtration using the Ceveron unit. Left panels are representative examples of thrombin generation curves from single donor produced by the indicated sample types, whereas right panels are numerical data of nine separate experiments using blood from different donors. Data are shown as mean±SD.

In contrast, the presence of PDMPs in plasma showed a marked enhancement in the amount of thrombin generation when the 1 pM TF reagent was used (Stago PRP reagent) (Figure 3.7 C), giving a significant increase in the ETP and peak thrombin values compared to normal plasma (ETP and peak thrombin increased from 640±322 and 32±15.5 to 1428±398 and 71.4±23.5 respectively; p values 0.0001 and 0.0004 respectively, n=9). However, the lag time was less influenced by the presence of PDMPs, showing only a slight, but statically significant decrease compared to the normal plasma (from 10.7±2.7 to 9.4±1.5; p=0.0011, n=9). This finding supports the view that PDMPs promote thrombin generation mainly via PL-dependent manner. The removal of the MPs by filtration reduced thrombin generation to a level below that seen in the normal plasma. This finding not only confirms that the increased thrombin generation capacity is due to the presence of PDMPs in plasma, but also shows that plasma from normal individuals contains low levels of MP activity.

The effect of PDMPs on thrombin generation was also investigated with using the 4 µM PL reagent (Stago MP reagent), which is sensitive the levels of TF-bearing MPs in plasma. As previously, some samples completely failed to support thrombin generation (two in the pre-filtered and five in the post-filtered samples). For those samples that failed to support

thrombin generation, lag time and ttPeak were considered as 70 minutes as an arbitrary value, whereas ETP and peak thrombin were recorded as zero value. Interestingly, all measured thrombin generation parameters failed to show a significant difference between the PDMP-rich and normal plasma samples (Figure 3.7 D), suggesting they lack TF activity. Even more interestingly, there was a significant difference between pre- and post-filtered samples in both PDMP-rich as well as normal plasma. This difference was more pronounced in the lag time and ttPeak values, which are the most sensitive thrombin generation parameters to the levels of TF in plasma, suggesting that some individuals' plasma contains low levels of TF-bearing MPs.

The effect of PDMPs on thrombin generation was investigated in the absence of exogenous TF and PL (no reagent assay), making the assay dependent on the levels of endogenous TF and PL. This was simply achieved by replacing the Stago reagents with the same volume of HBS. As with the MP reagent assay, the same two pre-filtered plasma samples completely failed to support thrombin generation. However, in the absence of added TF or PL there was no thrombin generation in the post-filtered samples from any of the nine donors. When plasma was enriched with PDMPs these significantly promoted thrombin generation giving higher values of the ETP and peak thrombin compared to normal plasma (ETP and peak thrombin increased from 162±47 and 17.1±4.3 to 514±129 and 41.6±9.6; p values 0.0050 and 0.0042 respectively, n=9) (Figure 3.7 E). Additionally, PDMPs slightly shortened the lag time (from 41.1±18.2 to 38.2±18.8; p value 0.0471, n=9). This finding further supports the previous observation, that PDMPs promote thrombin generation mainly through a PL-dependent manner.

It should be noted that some samples of PDMP-rich plasma supported thrombin generation in the absence of exogenous TF, suggesting that they carry functional TF activity. However, this does not necessarily prove that PDMPs carry TF. These were generated in plasma, which

may already contain TF-positive MPs. It is notable that the plasma samples that failed to support thrombin generation using the MP reagent also failed to support thrombin generation with no reagent, and the addition of PDMPs to the plasma of these donors was not enough to initiate thrombin generation. These findings support the idea that that platelets do not, by themselves, generate TF-bearing MPs. Overall, these findings suggest that the thrombin generation enhancement induced by PDMPs that was seen in the no reagent assay was initiated by the endogenous TF-bearing MPs in the plasma, whereas PDMPs propagate it.

3.2.5 CAT assay sensitivity to PDMP activity

To investigate the sensitivity of the CAT assay for PDMPs these were generated from PRP samples as described in section 3.2.4 and then diluted into autologous pre-filtered plasma up to 1000 times. Samples were analysed in the CAT assay using the 1 pM TF reagent (as deemed to be the most sensitive reagent to PPL activity) before and after passing through a 0.2 µm filter. Two different filtration methods were also compared: (1) Ceveron unit and (2) Pall acrodisc. Figure 3.8 clearly shows that the 1 pM TF reagent is sensitive to the levels of PDMPs in plasma down to a dilution of at least 1:10. Filtration by either method significantly removed the procoagulant activity of the MPs from plasma. However, the Pall acrodisc filter appears to be slightly more efficient than the Ceveron unit in removing MPs from plasma, particularly when MPs are present at high levels in plasma.

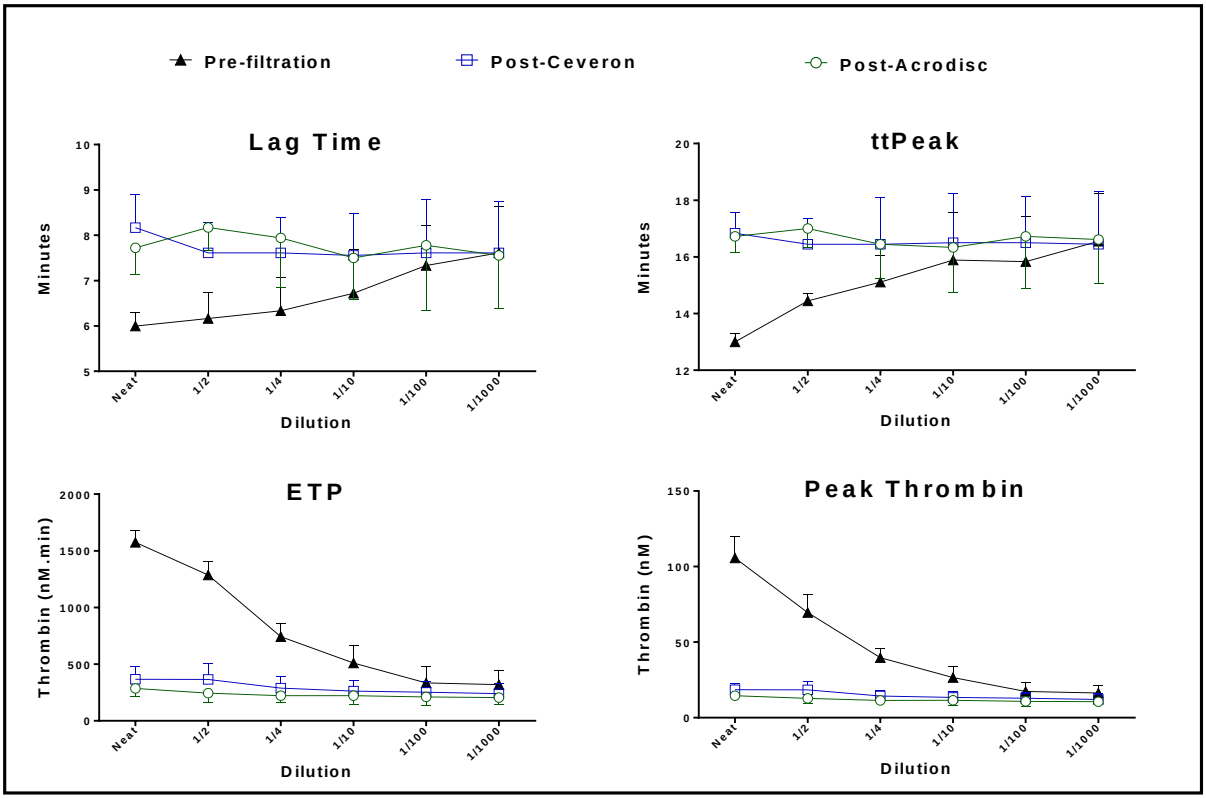


Figure 3.8. The 1pM TF reagent CAT assay sensitivity to MPs prepared from PRP samples. PRP samples were prepared from healthy donors and stimulated with 2µg/mL CRP-XL for 10 minutes at 37°C. PDMP-rich plasma was obtained by centrifuging the PRP samples at 1800g for 30 minutes. PDMP-rich plasma was diluted with autologous pre-filtered plasma. Samples were analysed in CAT assay using Stago PRP reagent (1pM TF). Samples were analysed before filtration (black lines) or after filtration using either Ceveron unit (blue lines) or Pall acrodisc (green lines). Data are from three separate experiments using blood from different donors and shown as mean±SD.

3.2.6 Impact of freezing and thawing on the procoagulant activity of normal and PDMP-rich plasma samples

Ideally, the procoagulant activity of plasma should be investigated immediately after collection and preparation. However, for practical reasons it is common that patients' samples are stored at -80°C until the analysis. Additionally, on some occasions samples are used several times, therefore they are exposed to repeated freezing and thawing. It was therefore decided to examine the effect of freezing and thawing on the procoagulant activity of: (1) coagulation factors using the PPP and PPP low reagent assays and (2) MPs using the PRP, MP and no reagent assays. Samples were analysed fresh and after each of two freeze/thaw cycles. These studies were carried out on the same samples described in section 3.2.4.

3.2.6.1 Impact of freezing and thawing on the procoagulant activity of coagulation factors

The process of freezing and thawing caused a slight reduction in thrombin generation capacity, particularly in the ETP and peak thrombin values, in all types of sample (i.e. normal plasma, filtered normal plasma, PDMP-rich plasma and filtered PDMP-rich plasma) (Figure 3.9 A and B). Since these two assays are sensitive to the procoagulant capacity of coagulation factors and since all used different sample types showed the similar trends of reduction in the ETP and peak thrombin values, it can be concluded that freezing and thawing plasma results in a small deterioration of the coagulation factors.

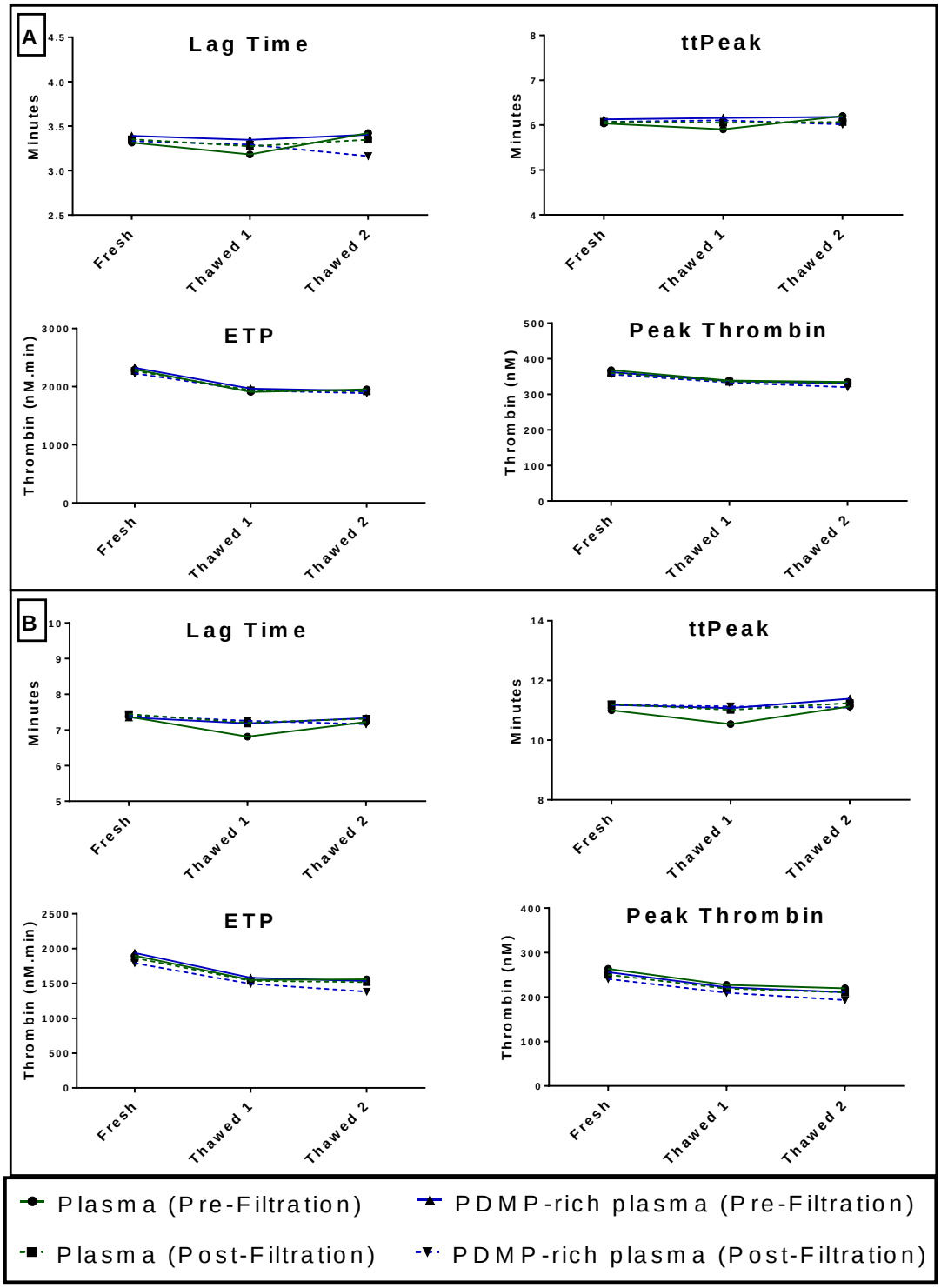
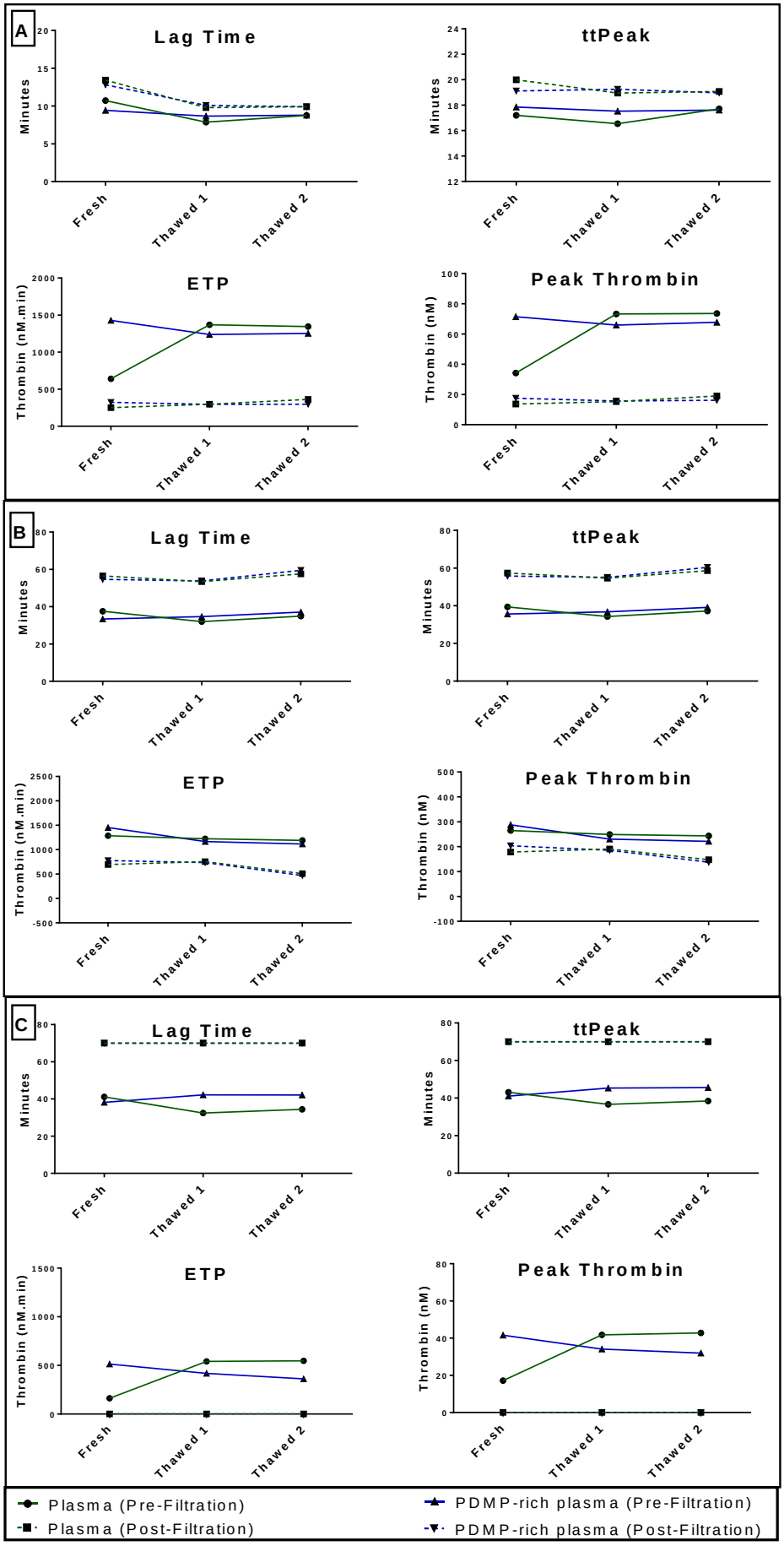


Figure 3.9. Effect of freezing and thawing on the procoagulant activity of coagulation factors using PPP and PPP low reagents. PRP samples were prepared from healthy donors and stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C. Normal plasma and PDMP-rich plasma samples were obtained by centrifuging the samples at 1800g for 30 minutes. Thrombin generation was measured on CAT assay using (A) the 5 pM TF/4 µM PL reagent (Stago PPP reagent) and (B) the 1 pM TF/4 µM PL reagent (Stago PPP low reagent). Samples were analysed before and after filtration using the Ceveron unit. Samples were analysed as fresh and after subjecting to two freeze/thaw cycles. Data are from nine separate experiments using blood from different donors. Data are shown as mean values (n=9).

3.2.6.2 Impact of freezing and thawing on the procoagulant activity of endogenous and PDMPs in plasma

Most previous studies have investigated the effect of freezing on MPs detection by flow cytometry, measuring Annexin V binding. However, this study investigated the effect of freezing and thawing on the total procoagulant activity of MPs in plasma samples. PDMPs were generated from PRP samples using the same samples described in section3.2.6.1. Aliquots of the normal and PDMP-rich plasma were analysed on CAT assay fresh and after being thawed once or twice. The PRP, MP and no reagent assays revealed that repeat freezing and thawing of PDMP-rich plasma samples had similar trends in reducing the ETP and peak thrombin values to those seen with the PPP and PPP low assays (Figure 3.10 A, B and C). These findings suggest that freezing and thawing have no significant impact on the procoagulant activity of PDMPs, whereas the modest decrease in the procoagulant activity was seen could be due to coagulation factor degradation.

Conversely normal plasma samples that had been frozen and thawed showed a significant and marked increase in the procoagulant activity (more than the PDMP-rich plasma samples) with both the PRP and no reagent assays, but not with the MP reagent. These data suggest that the increased levels of procoagulant activity of normal plasma that was seen in the PRP and no reagent assays is a PL-dependent rather than TF-dependent activity. Additionally, sample filtration completely eliminated the increase levels of the PPL activity of frozen normal plasma, suggesting that the cause of this increase in the procoagulant activity is present in a particulate form.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Thawed 2
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Plasma (Post-Filtration)
PDMP-rich plasma (Pre-Filtration)
PDMP-rich plasma (Post-Filtration)

Figure 3.10. Effect of freezing and thawing on the procoagulant activity of PDMPs. PRP samples were prepared from healthy donors and stimulated with 2µg/mL CRP-XL for 10 minutes at 37°C. Normal and PDMP-rich plasma samples were obtained by centrifuging the samples at 1800g for 30 minutes. Thrombin generation was measured on CAT assay using (A) the 1pM TF reagent (Stago PRP reagent), (B) the 4µM PL reagent (Stago MP reagent) and (C) without the addition of TF or PL to the assay (no reagent assay). Samples were analysed before and after filtration using the Ceveron unit. Samples were analysed as fresh and after subjecting to two freeze/thaw cycles. Data are from nine separate experiments using blood from different donors. Data are shown as mean.

A number of possibilities can be put forward for the increased procoagulant activity in the frozen normal plasma, such as the presence of platelet contamination, apoptotic bodies or loss of CTI activity during the sample storage. To address these possibilities, separate experiments were carried out by Briana M Maffessanti, an MSc student under my supervision, to rule out the platelet contamination in normal plasma that was prepared using different isolation protocols.

As previously, PRP samples were prepared from fresh blood collected from healthy donors into sodium citrate tubes containing CTI. The platelet count was adjusted at 150 x 10^3/µL with autologous PPP and GPRP was added prior to platelet activation by stimulation with 2 µg/mL CRP-XL for 10 minutes at 37°C. Normal and PDMP-rich plasma samples were prepared using four different centrifugation protocols, selected based on current practice and published protocols: (1) double centrifugation, each at 2500g for 15 minutes (called protocol A hereafter), (2) double-step centrifugation first at 1500g for 15 minutes followed by 13000g for 2 minutes (called protocol B hereafter), (3) single-step centrifugation at 1800g for 30 minutes (called protocol C hereafter) and (4) single-step centrifugation at 2000g for 20 minutes (called protocol D hereafter). All samples were analysed in the CAT assay fresh or after a single freeze-thaw cycle, using the 1 pM TF reagent assay as this is the most sensitive assay to the levels of PPL in plasma. Additionally, to examine the presence of platelet

fragment contamination, samples were labelled with phalloidin and analysed on flow cytometry using a method adapted from Mobarraz et al (Mobarrez, et al 2010). Phalloidin is a phallotoxin isolated from the toxic death cap mushroom (*Amanita phalloides*), and binds with high affinity to filamentous actin (Faulstich, et al 1993), which is present in platelet fragments, but not PDMPs. A two-colour flow cytometry protocol was used to detect platelet fragments before and after freezing. Samples were stained with CD42a-PE and phalloidin-FITC as pan-platelet and cell fragment markers respectively. The identity of the events of phalloidin-positive platelet fragments was confirmed by their binding to the pan-platelet marker, CD42a-PE. To determine the absolute count of the events, 2 µm Sphero beads (Sphero AccuCount Blank Particles; Spherotech Inc., Illinois, USA) of known concentration were included in the assay.

PDMP-rich plasma showed a slight decrease in thrombin generation capacity after freezing with all tested centrifugation protocols (Figure 3.11 A). This finding is in the line with the early findings that freezing and thawing had no significant impact on the procoagulant activity of PDMPs in plasma samples. In sharp contrast, significant differences between the isolation protocols were seen in the procoagulant activity of the normal plasma after freezing and thawing (Figure 3.11 B). Protocols A and B showed no significant difference between the fresh and frozen samples, indicating that these protocol are suitable for normal and MP-rich plasma preparation. However, there was a marked increase in the procoagulant activity of the normal plasma after freezing when it was prepared using either protocol C or D. This finding indicates that the plasma prepared by these protocols might carry some platelet contamination that leads to the false enhancement in thrombin generation after freezing.

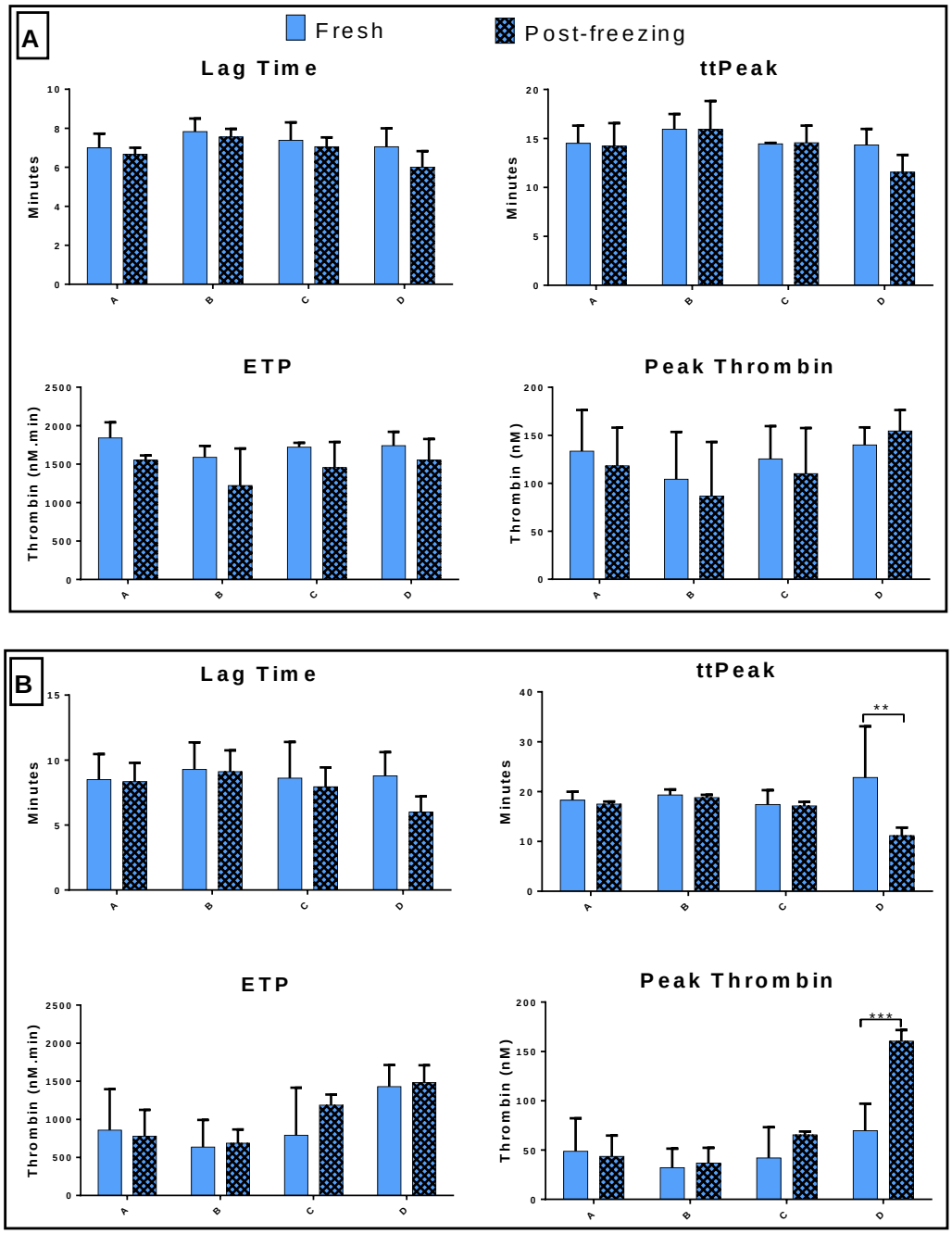


Figure 3.11. Procoagulant activity of normal and PDMP-rich plasma samples prepared using different centrifugation protocols. PRP samples were prepared from healthy donors and either stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C (Panel A) or without stimulation (Panel B). Samples were then centrifuged using: (A) double centrifugation, each at 2500g for 15 minutes, (B) double-step centrifugation as 1500g for 15 minutes followed by 13000g for 2 minutes, (C) single-step centrifugation at 1800g for 30 minutes and (D) single-step centrifugation at 2000g for 20 minutes. Thrombin generation was measured in the CAT assay using the 1 pM TF reagent (Stago PRP reagent). Samples were analysed as fresh and after a single-cycle freezing. Data are from three separate experiments using blood from different donors and presented as mean±SD. P-value between the fresh and frozen samples is indicated when it was significant; **p<0.001; ***p<0.0001. These data are from Briana M Maffessanti (MSc dissertation, 2013, University of Leicester).

Analysis of platelet fragments by flow cytometry also revealed that PDMP-rich plasma samples had low platelet contamination in both pre- and post-freezing preparations (*Figure 3.12* A). On the other hand, protocols A and B had the lowest platelet contamination in normal plasma, whereas protocol C and to a larger extent the protocol D had relatively high platelet contamination (*Figure 3.12* B). This finding explains that the source of the enhancement of the procoagulant activity of the stored normal plasma is due to the presence of platelet fragments when protocols C and D were used. Overall, it appears that protocols A and B remove platelet contamination from blood samples, whilst retaining relatively high yield of MPs in plasma.

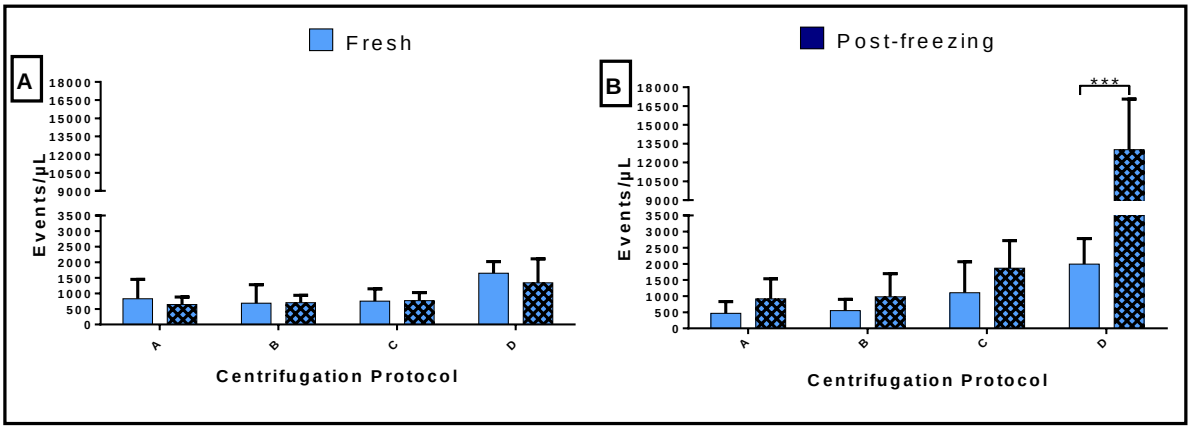
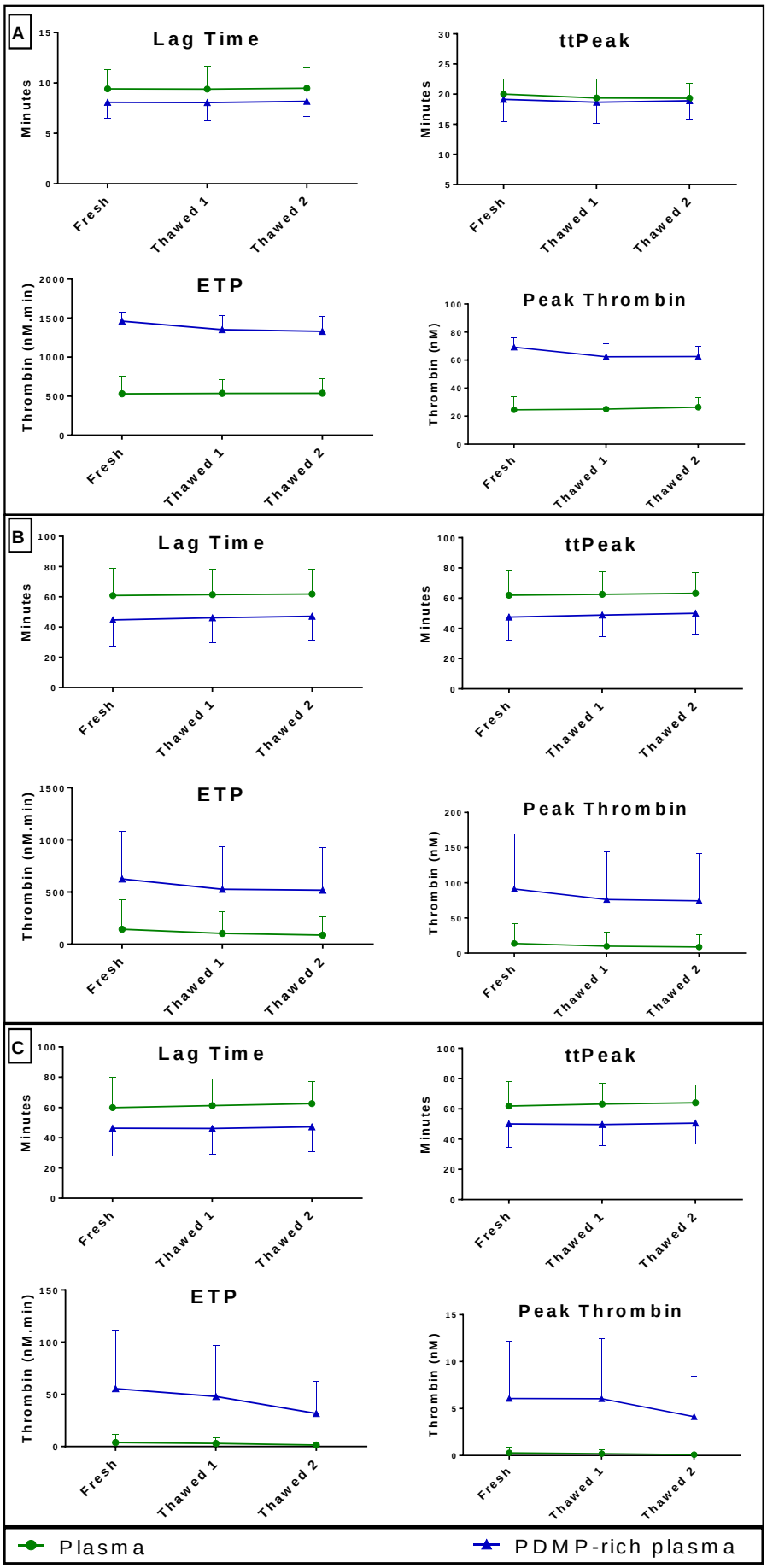


Figure 3.12. Platelet contamination in fresh and stored samples. Samples were prepared as described in Figure 3.11 and analysed for phalloidin binding on flow cytometry. Panel A shows PDMP-rich plasma and Panel B shows normal plasma. Data are from three separate experiments using blood from different donors and shown as mean±SD. P-value between the fresh and frozen samples is indicated when it was significant; ***p<0.0001.These data are from Briana M Maffessanti (MSc dissertation, 2013, University of Leicester).

Having established that the increased levels of the procoagulant activity of normal plasma prepared using protocol C is due to the presence of platelet contamination, it was decided to re-investigate the effect of freezing and thawing on PDMPs but with samples prepared using protocol A (double centrifugation, each at 2500g for 15 minutes) to avoid the contribution of platelet fragments. This finding corresponds to the current recommendation for MP isolation protocol by the ISTH (Lacroix, *et al* 2012).

The procoagulant activity of these samples was evaluated without thawing and after each of the two freeze/thaw cycles in the CAT assay, using the PRP, MP and no reagent assays. Freezing and thawing process showed similar slight reductions in thrombin generation capacity of both normal and PDMP-rich plasma with all three assays (Figure 3.13), further confirming that the reduction in the samples activity after freezing could be due to coagulation factor degradation.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Thawed 2
Plasma
PDMP-rich plasma

Figure 3.13. Effect of freezing and thawing on PDMPs isolated using centrifugation protocol A. PRP samples were prepared from healthy donors and stimulated with 2µg/mL CRP-XL for 10 minutes at 37°C. Normal and PDMP-rich plasma samples were obtained by two successive centrifugations of 2500g for 15 minutes at RT. Thrombin generation was measured on CAT assay using (A) the 1pM TF reagent (Stago PRP reagent), (B) the 4µM PL reagent (Stago MP reagent) and (C) without the addition of TF or PL to the assay (no reagent assay). Samples were analysed as fresh and after subjecting to two freeze/thaw cycles. Data are from four separate experiments using blood from different donors. Data are shown as mean±SD.

3.2.7 Functional properties of MPs derived from platelets, endothelial cells and macrophages

The functional properties of MPs can be argued to be a more relevant biomarker than their count as this reflects the magnitude of their contribution into the procoagulant status of plasma. MPs found in different disease states can be derived from a range of different cell types. To test whether they all behaved the same in respect of their procoagulant activity, and after freezing and thawing, the procoagulant activity of MPs derived from washed platelets, endothelial cells and macrophages in terms of their PPL and TF activities. To avoid variations due to plasma these experiments were carried out by generating MPs from washed cells that were then reconstituted into pooled, filtered (i.e. MP-free) normal plasma.

3.2.7.1 **Preparation of MPs from isolated cells**

MPs from individual cell types were generated from either washed platelets, cultured endothelial cell line EA.hy926 or cultured monocytic cell line THP-1. Some preliminary experiments were carried out to ensure optimal MP generation from these three cell types.

3.2.7.1.1 **Preparation of washed platelets**

To ensure that the process of washing platelets did not induce platelet activation, washed platelets were examined by flow cytometry for markers of activation before and after stimulation with CRP-XL. Washed platelets were prepared as described in section 2.2.1.5 then stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C. Platelet Annexin V binding and P-selectin expression were assessed by flow cytometry as markers of degranulation and PS exposure respectively. Figure 3.14 shows that unstimulated washed platelets had only negligible percentages of Annexin V binding and P-selectin expression, whereas they showed full activation following CRP-XL stimulation. These data clearly indicate that washing the platelets did not cause non-specific platelet activation, whilst they remain responsive to CRP-XL.

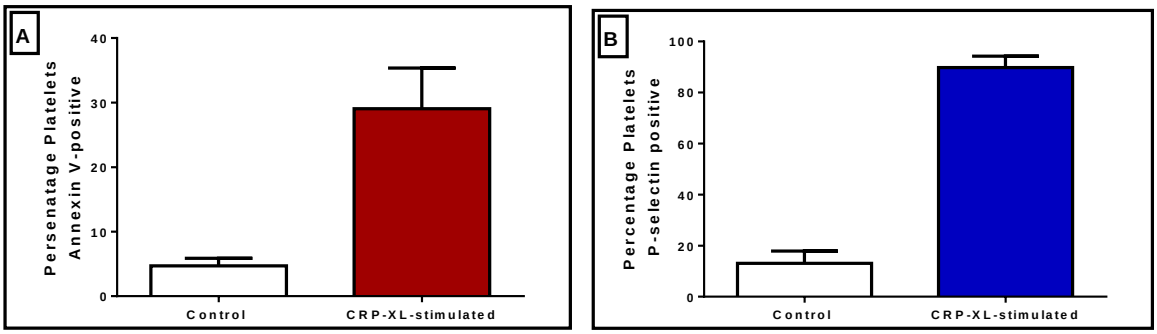


Figure 3.14. Washed platelet preparation does not induce non-specific platelet activation. Washed platelets were prepared from fresh whole blood and resuspended into HBS pH 7.4 containing 2 mM $CaCl_2$ at platelet density of 1.5 X 10^8/mL. Washed platelets were then stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C. Sub-samples of platelet preparations were analysed on flow cytometry for Annexin V binding (graph A, n=10) and P-selectin expression (graph B, n=8) before they were used in the study. Data are shown as mean±SD.

3.2.7.1.2 **Microparticle generation from endothelial cells**

ECDMPs were generated as previously described in several studies using different endothelial cell lines (Combes, *et al* 1999, Jimenez, *et al* 2003, Lacroix, *et al* 2007, Peterson, *et al* 2008, Sabatier, *et al* 2002), but with some modifications. Briefly, EA.hy926 cells were cultured at a concentration of 5 x10^5 cells in T25 flasks and maintained for four days prior to the assays. The cell count and time in culture were selected based on preliminary experiments (data not shown). Fresh media was added with or without 10 ng/mL TNF-α to generate ECDMPs and the flasks maintained for a further 24 hours at 37°C. Culture supernatants were then collected, cell fragments were removed by centrifugation at 160g for 5 minutes followed by 1500g for 15 minutes. ECDMPs were then isolated by centrifuging at 20000g for 30 minutes and washed two times in HBS. MP preparations were pelleted and resuspended into CTI-inhibited pre-filtered pooled plasma to restore the coagulation system. Normal pooled plasma was pre-filtered using 0.2 µm pore size Pall acrodisc syringe-driven filter units to remove endogenous MPs (section 2.2.1.3).

To determine the optimal TNF-α concentration, EA.hy926 cells were exposed to serial doses of TNF-α between 0 and 20 ng/mL, for 24 hours at 37°C. ECDMPs were isolated, washed, resuspended into CTI-inhibited pre-filtered pooled plasma and analysed fresh (i.e. without freezing) in the CAT assay, using the 1pM TF reagent assay. Figure 3.15 shows that TNF-α induces ECDMPs formation in dose-dependent manner as shown by the reduction in LT and ttPeak and, more markedly, in the rise in ETP and peak thrombin values. The post-filtered samples further confirmed that the enhanced thrombin generation was due to the presence of ECDMPs in the samples. TNF-α reached a maximum effect at 10 ng/mL. Based on this finding, 10 ng/mL TNF-α was chosen as the dose for subsequent experiments to generate ECDMPs from EA.hy926 cells.

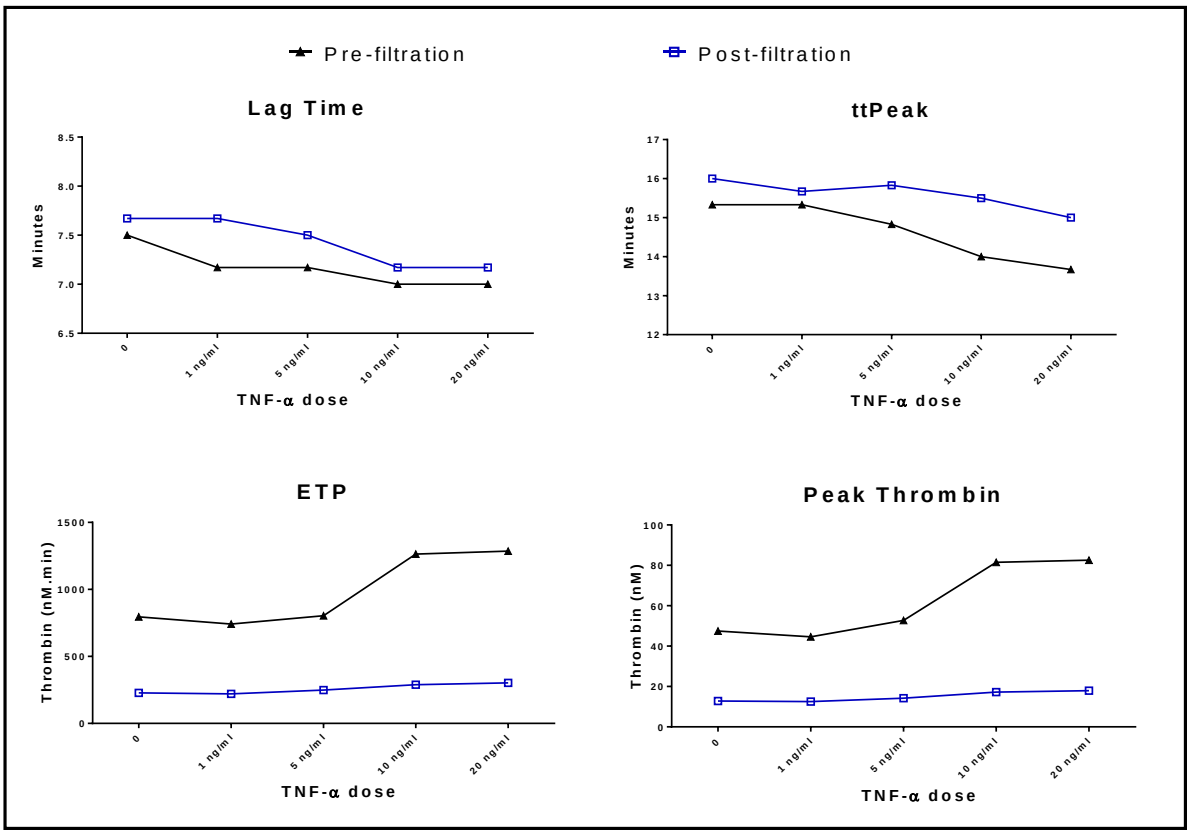


Figure 3.15. Thrombin generation of ECDMPs induced by serial concentrations of TNF-α. EA.hy926 cells were stimulated with increasing concentrations of TNF-α for 24 hours then media supernatant containing ECDMPs was collected. ECDMPs were purified from cell debris by centrifugation at 160g for 20 minutes followed by 1500g for 15 minutes. ECDMPs were washed two times in HBS and resuspended into CTI-inhibited pre-filtered pooled plasma. ECDMPs generation was evaluated by measuring their procoagulant activity on CAT assay using the 1 pM TF reagent assay (Stago PRP reagent). Samples were analysed before (black lines) and after (blue lines) filtration using the Ceveron unit. Data are presented as mean (n=2).

To confirm that TNF-α treatment for 24 hours was not inducing apoptosis of EA.hy926 cells separate experiments were carried out to test for activation of caspase 3, an early stage apoptosis marker (Hengartner 2000). Cultured EA.hy926 cell flasks were incubated with 10 ng/mL TNF-α for 24 hours along with a control. For a positive control of apoptosis, cultured cells were exposed to staurosporine, which is a potent trigger for cell apoptosis (Bertrand, *et al* 1994). Three doses of staurosporine were used; 250, 125 and 500 nM, and incubated with the cells for 24, 12 and 2 hours respectively. Caspase 3 activity was measured as described in section 2.2.3 using the fluorogenic assay. Figure 3.16 shows that stimulation of EA.hy926 cells with 10 ng/mL TNF-α gave low levels of caspase 3 activity, similar to that seen in the control, unstimulated EA.hy926 cells. This finding indicates that the dose of TNF-α employed here does not induce early stage apoptosis. In sharp contrast, there was a marked increase in the caspase 3 activity in the staurosporine-treated EA.hy926 cells, particularly with the highest concentration (500 nM staurosporine for 2 hours) where there was more than 27-fold increase in the Ac-DEVD-AMC cleavage. The specificity of this assay was confirmed by the ability of the specific caspase 3 inhibitor Z-DEVD-FMK to completely abrogate caspase 3 activity, which was not seen in the vehicle control dimethylsulfoxide (DMSO). The caspase 3 activity data was further supported by the morphological characteristics of the cells; where the TNF-α treated EA.hy926 cells, unlike staurosporine–treated cells, showed similar morphology to the control cells (Figure 3.17). This finding is consistent with previous reports showing that TNF-α treatment in similar conditions to those used here does not induce apoptosis in endothelial cells (Combes, *et al* 1999, Jimenez, *et al* 2001, Jy, *et al* 2002).

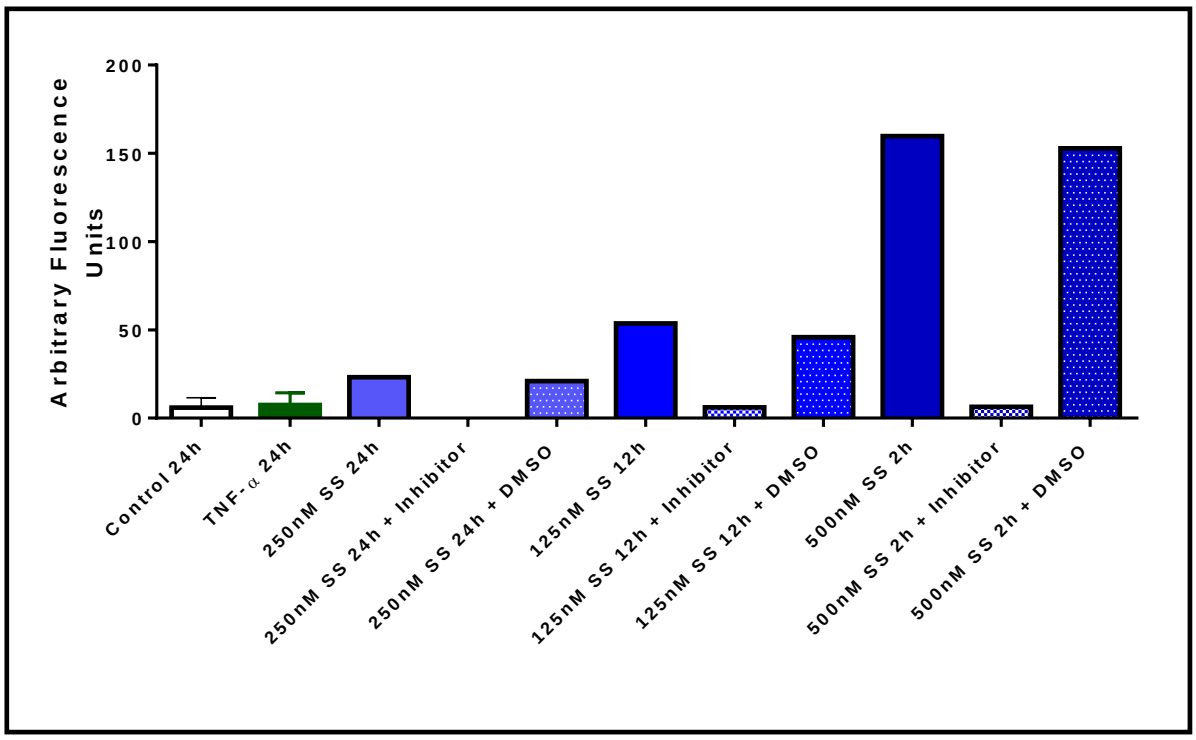


Figure 3.16. Analysis of apoptosis of EA.hy926 cells by fluorometric caspase 3 activity assay. EA.hy926 cells were cultured and maintained for four days. In day four, the flasks were divided into three groups and fresh media was added supplemented with the following: (1) control, where no agonist was added (n=3), (2) TNF-α treated, where 10 ng/mL TNF-α was added (n=3) and (3) apoptosis positive controls where indicated SS doses were added (n=1 for each dose). Flasks were then incubated in culture incubator for the indicated periods. Caspase 3 activity was measured using the fluorometic assay in the presence of a specific caspase 3 inhibitor and control vehicle, DMSO. The cleavage of the caspase-3 substrate, Ac-DEVD-AMC, was monitored for 120 minutes. Data represent the reading at 120 minute only.

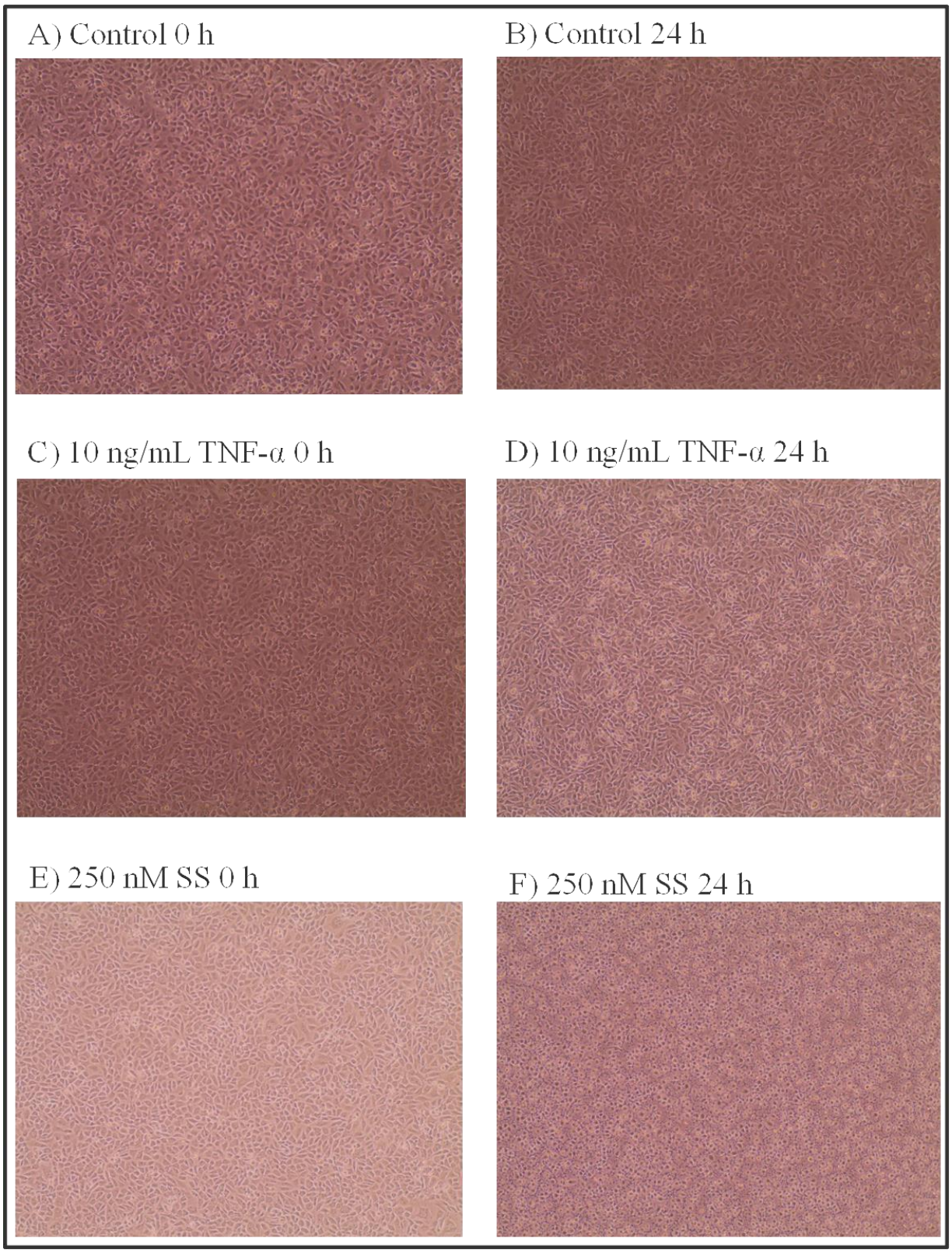


Figure 3.17. Morphology of EA.hy926 cells. Microscopic pictures of EA.hy926 cells before and after stimulation with TNF-α or staurosporine (SS). Images were taken at 10X magnification.

3.2.7.1.3 **Microparticle generation from monocytes**

THP-1 cells are pre-monocytes that grow in suspension and lack the expression of CD14, the main receptor for LPS (Abrink, *et al* 1994, Schwende, *et al* 1996, Wright, *et al* 1990). To induce THP-1 cell differentiation into a more-macrophage-like cell that expresses CD14 they were cultured in the presence of 100 ng/mL PMA for 72 hours, a method that is well established to differentiate THP-1 cells into macrophages (Gantner, *et al* 1997, Schwende, *et al* 1996, Takashiba, *et al* 1999). Preliminary experiments showed that the unstimulated THP-1 cells lacked CD14 and did not respond to LPS (data not shown). After incubation with PMA, and removal of non-attached cells, the adherent cells were washed in RPMI prior to stimulation with LPS. Microscopic examinations revealed significant changes in the morphology of PMA-treated cells; the THP-1 cells became adherent and showed the morphological phenotypes of macrophages (Figure 3.18 A and B). Flow cytometry analysis showed that PMA-treated cells, unlike the native THP-1 cells, express high levels of CD14, a marker of macrophage-specific differentiation (Figure 3.18 C). The PMA-treated THP-1 cells are called macrophages hereafter.

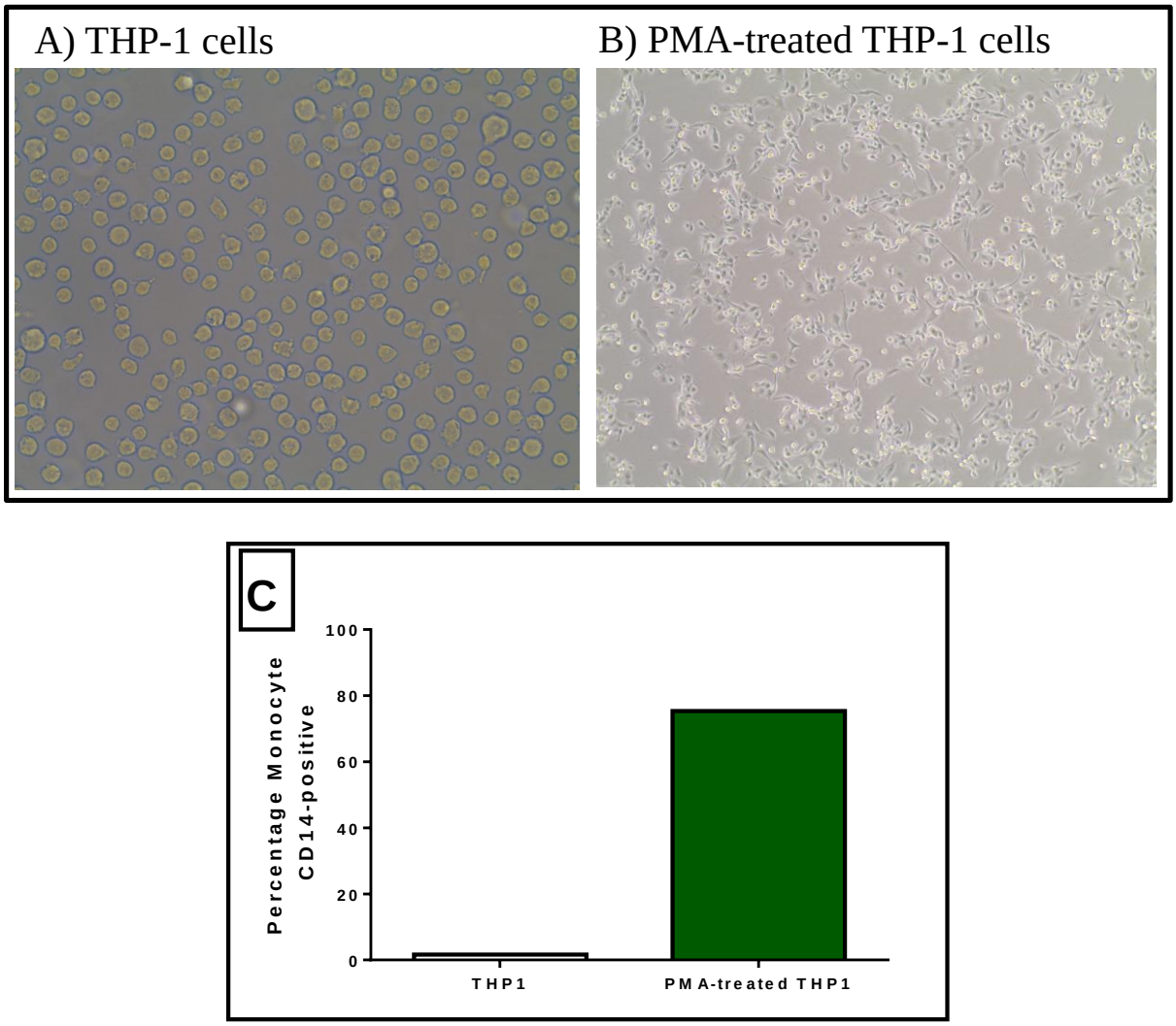


Figure 3.18. Induction of differentiation in THP-1 cells by PMA. THP-1 cells were incubated for 72 hours with or without PMA (100ng/mL). A) shows a microscopic picture of THP-1 cells in suspension before the incubation with PMA, whereas B) after the incubation. C) shows flow cytometry analysis of CD14 expression in THP-1 cells before and after incubation with PMA (data are shown as mean; n=2).

To generate MPs from macrophages, PMA-transformed THP-1 cells were first exposed to 100 ng/mL PMA for 72 hours, non-adherent cells were removed and the adherent cells were washed in RPMI prior to stimulation for 6 hours at 37°C with LPS at serial doses of 0 to 100 ng/mL. MP-containing supernatant was collected and the MPs were isolated from cell debris by serial centrifugation steps; 160g for 5 minutes, 1500g for 15 minutes and 13000g for 2 minutes. Isolated MDMPs were washed two times in HBS by centrifugation at 20000g for 30 minutes, then resuspended into CTI-inhibited pre-filtered pooled plasma and their procoagulant activity was assessed in in the CAT assay using the 1 pM TF reagent assay.

Thrombin generation data revealed that LPS induces MP shedding from macrophages. Figure 3.19 clearly shows that LPS caused a marked enhancement in the amount of thrombin generation with shorter lag time and ttPeak and higher ETP and peak thrombin, indicating the presence of higher number of MPs. The procoagulant activity of MPs was markedly reduced in the post-filtered samples, confirming that the effect is due to the presence of higher concentration of MDMPs in LPS-stimulated macrophages. LPS reached the maximum activation at 10 ng/mL, after which there was no further increase with higher doses of LPS. Based on this finding, 10 ng/mL LPS was chosen as the dose for further experiments using to generate MDMPs from macrophages.

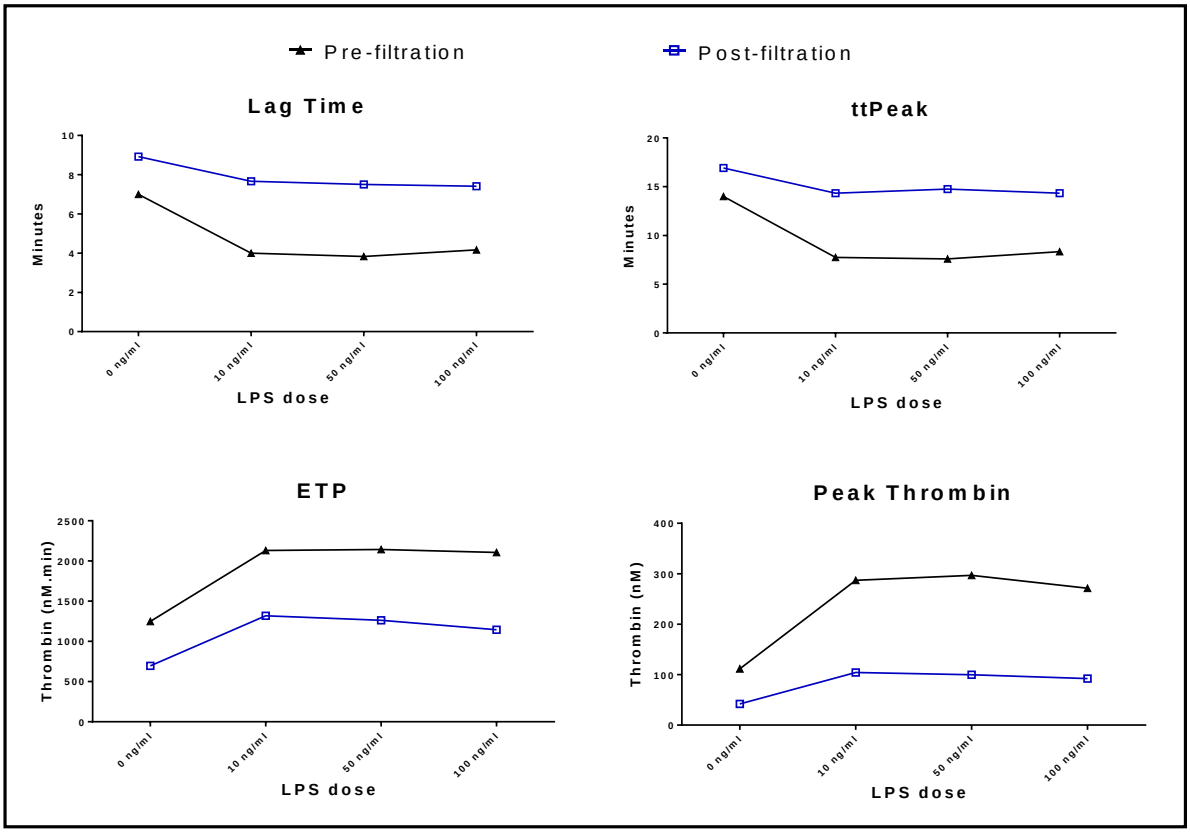


Figure 3.19. LPS induces MP formation in macrophages. THP-1 cells were transformed into macrophages using 100 ng/mL PMA for 72 hours. Macrophages were exposed to increasing concentrations of LPS for 6 hours at 37°C. MDMPs were isolated, washed and resuspended into CTI-inhibited pre-filtered pooled plasma. The activity of MDMPs was evaluated by measuring their procoagulant activity on CAT assay using the 1 pM TF reagent (Stago PRP reagent). Samples were analysed before (black lines) and after (blue lines) filtration using the Ceveron unit. Data are presented as mean (n=2).

3.2.7.2 **Characterisation of MPs derived from platelets, endothelial cells and macrophages**

Based on the findings in the preliminary experiments, washed platelets were prepared and suspended in HBS containing 2 mM $CaCl_2$ at platelet density of 1.5 x 10^3/µL. MPs generation from washed platelets was achieved by stimulation with 2 µg/mL CRP-XL for 10 minutes at 37°C. PDMPs were purified from platelets by centrifugation at 1500g for 15 minutes followed by 13000g for 2 minutes. ECDMPs were generated from EA.hy926 cells by stimulation with 10 ng/mL TNF-α as described in section 3.2.7.1.2, and MDMPs were generated from macrophages using LPS stimulation as described in section 3.2.7.1.3. All MP preparations were pelleted and resuspended into CTI-inhibited pre-filtered pooled plasma to restore the coagulation system.

For CAT assay experiments, samples were analysed using two conditions: the 1 pM TF reagent assay and the 4 µM PL reagent assay. PS and TF exposure on MPs were also evaluated using the Zymuphen MP activity and Zymuphen MP-TF ELISA kits respectively as described in section 2.2.8. Of note, for the Zymuphen MP activity ELISA it is recommended by the manufacturer to dilute 5 µL of MP preparation into 100 µL sample diluent, however, for the PDMP sample preparation 10 µL was diluted into 100 µL sample diluent. MP count was performed using NTA to allow normalisation of the data from the CAT assays and ELISAs for MP numbers. NTA analysis showed that MP concentrations in sample suspensions were 0.31±0.05, 0.46±0.08 and 0.50±0.07 x 10^{12} MP/mL for PDMPs, ECDMPs and MDMPs respectively.

3.2.7.2.1 **PPL activity of MPs from different cell types**

Using the 1 pM TF reagent, which is sensitive to the level of PPL activity in plasma, the presence of PDMPs, ECDMPs or MDMPs to normal plasma markedly enhanced thrombin generation (Figure 3.20 A). The ETP and peak thrombin values, which are the thrombin generation parameters most sensitive to the levels of PPL activity, revealed that PDMPs and MDMPs had the highest activity, whereas ECDMPs had the least activity. In contrast, Zymuphen MP activity ELISA indicated that PDMPs had a low level of PPL, whereas ECDMPs and MDMPs showed PS concentrations that are consistent with their PPL activity on thrombin generation (Figure 3.20 B).

A number of possibilities can be put forward for the discrepancy in the phospholipid-mediated activity of PDMPs in the CAT assay and ELISA. Because 10 µL PDMP preparation was used in the Zymuphen MP activity ELISA instead of the recommended volume by the manufacturer, 5 µL, this may have caused slight decrease in calcium concentration in the assay. It is well known that calcium concentration is critical for the Annexin V binding with PS. In addition, it may be that MPs generated from washed platelets are less able to bind to Annexin V in the ELISA. To address these possibilities, it was decided to conduct subsequent experiments using the recommended sample volume, 5 µL, of PDMPs generated in PRP than from washed platelets. For this, subsamples of the PDMP preparations used in Figure 3.7 were analysed in the Zymuphen MP activity ELISA. Interestingly and consistent with the PDMPs generated from washed platelets, the PDMPs from PRP samples also showed similar, contradictory results between the CAT assay and ELISA. MPs generated from PRP samples showed a significant PPL activity in the CAT assay (peak thrombin increased from 34.2±5.1 in the normal plasma to 71.4±7.8 in the PDMP-rich plasma; $p=0.0004$, $n=9$; Figure 3.7 C). In contrast, MPs generated from PRP samples failed to show a significant increase in the PS

concentration in the Zymuphen MP activity ELISA (PS concentration of 1.97±0.16 and 2.24±0.20 in normal and PDMP-rich plasma respectively, p=0.0507, n=9; Figure 3.21). Therefore, it appears that the Zymuphen MP activity assay lacks the sensitivity to detect PDMPs. Interestingly, a recent report showed that the Zymuphen MP activity had weak correlation with MP concentration measured by flow cytometry compared to the correlation of thrombin generation and STA Phospholipid Procoag assays with the MP concentration (Ayers, *et al* 2014). This finding supports the present finding that the Zymuphen MP activity assay lacks the sensitivity to detect PDMPs. However, it has to be noticed that the MP samples in Ayres *et al* report were obtained directly from healthy individuals and patients with obstructive sleep apnoea, which may contain a mixture of MPs as well as apoptotic bodies derived from different cellular sources. The question arises here as why the Zymuphen MP activity assay has less sensitivity to PDMPs compared to MPs derived from other cell types. One possibility is that PDMPs are less able to bind to Annexin V in this assay due to calcium concentration, steric hindrance, different PL composition or the exposed PS is already bound to other molecule preventing it from binding to Annexin V. Other possibility is that the PDMPs weakly bind to Annexin V compared to MPs derived from other cells, which in turn they were washed out from the plate during washing steps. In addition, it could be the concentration of PDMPs was below the detection limit of the assay, although this could be argued as both the CAT assay and NTA analysis suggested that the PDMPs were in a similar concentration to the other MP preparations. Further investigation, however, is required to clarify the reasons for this discrepancy.

Despite the discrepant result of PS concentration of PDMPs, all the three types of MPs significantly promoted thrombin generation in a PPL-dependent manner; a finding that is supported by other reports, some of which were published at the time this study was being conducted (Aleman, *et al* 2011, Combes, *et al* 1999, Van Der Meijden, *et al* 2012).

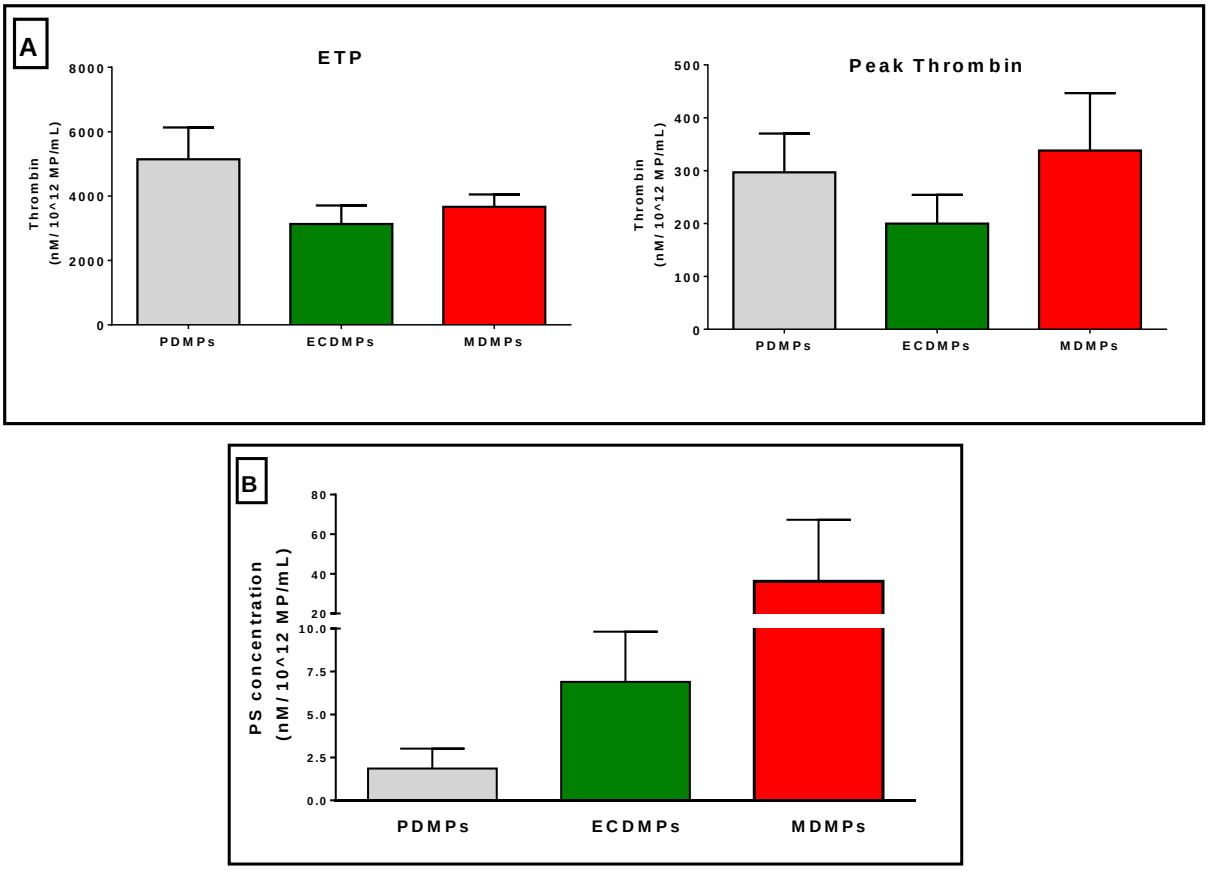


Figure 3.20. PPL activity and concentration of PDMPs, ECDMPs and MDMPs. MPs were generated from washed platelets (grey bars; n=5), endothelial cells (green bars; n=4) and macrophages (red bars; n=3), added to CTI-inhibited pre-filtered pooled plasma. MP count was performed using NTA. Panel A: PPL activity of MPs was evaluated on CAT assay using the PPL-dependent assay (1pM TF reagent). Panel B: PS concentration was measured using Zymuphen MP activity ELISA kit. Data are normalised to MP count and shown as mean±SD.

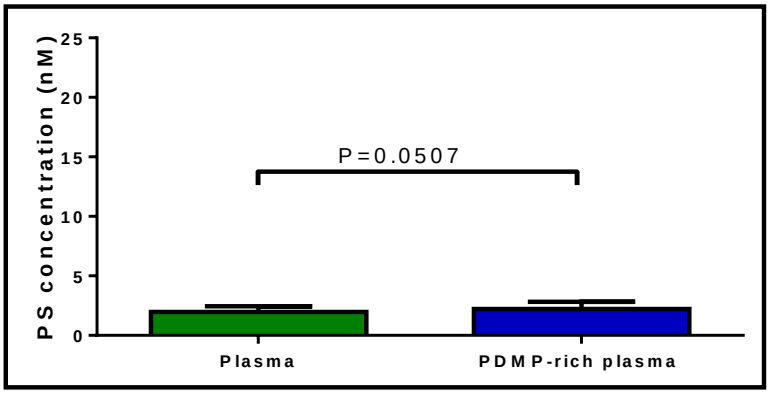


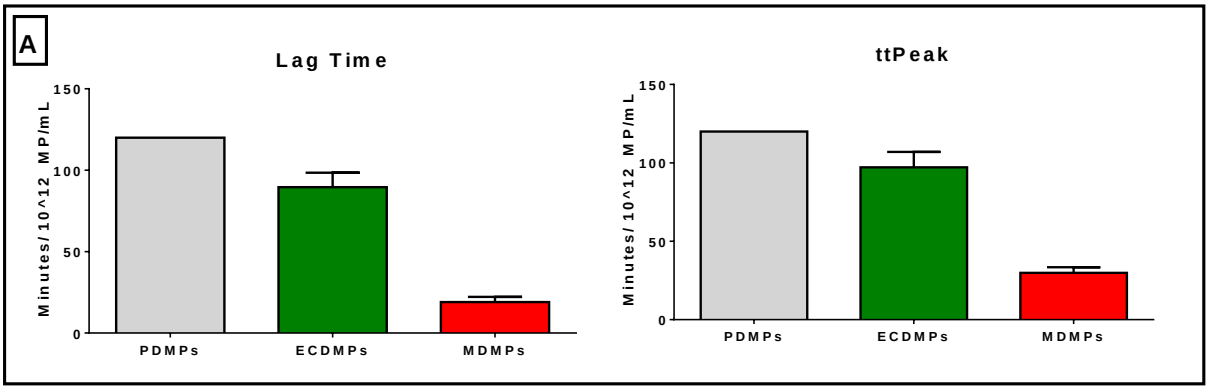
Figure 3.21. PS concentration of PDMPs generated from PRP samples.PRP samples were prepared from healthy donors and stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C. Normal plasma and PDMP-rich plasma samples were obtained by centrifuging the PRP samples at 1800g for 30 minutes. PS concentration was measured using Zymuphen MP activity ELISA kit. Data are nine separate experiments using blood from different donors and presented as mean±SD**.**

3.2.7.2.2 **TF activity of MPs from different cell types**

The CAT assay was also used to compare the TF activity of PDMPs, ECDMPs and MDMPs. This was achieved using the 4 µM PL reagent (Stago MP reagent), which is sensitive to the level of TF-positive MPs in plasma. These assays were carried out in plasma containing CTI to inhibit the intrinsic coagulation pathway, thus makes the assay TF-dependent. Interestingly, PDMPs generated from washed platelets failed to support thrombin generation in TF-dependent manner, indicating that they inherently do not have functional TF activity (Figure 3.22 A). Consistently, TF concentration experiments using Zymuphen MP-TF ELISA assay also failed to detect any TF antigen on the PDMPs from washed platelets (Figure 3.22 B). In contrast, ECDMPs showed moderate TF activity as evidenced by a relatively shorter lag time and ttPeak and moderate TF concentration in the ELISA. Additionally, MDMPs had the highest relative TF activity as they generated thrombin with the shortest lag time and ttPeak and showed the highest TF concentration in the ELISA compared to other types of MPs. These findings are consistent with previous reports showing that PDMPs, unlike ECDMPS and MDMPs, do not inherently express TF (Aleman, *et al* 2011, Combes, *et al* 1999, Van Der Meijden, *et al* 2012). However, there is a controversial debate in the literature about the TF activity on platelets and PDMPs, which will be considered in more detail in the discussion section of this chapter.

Additionally, the presence of TF protein in the ECDMPs and MDMPs was also examined by Western blotting using the same MP preparations as analysed above. The Western blots were prepared by Dr Mary Collier (Department of Cardiovascular Sciences, University of Leicester). Figure 3.23 shows that even unstimulated monocyte-derived macrophages release a baseline of MDMPs that contain TF, which increases after stimulation with LPS. This finding is in agreement with preliminary experiments demonstrating low TF-bearing MP

activity in unstimulated monocyte-derived macrophages (data not shown). In contrast, Western blotting failed to detect TF in ECDMPs. This is unsurprising knowing that ECDMPs exert only moderate TF activity, which may be below the detection limit of Western blotting.



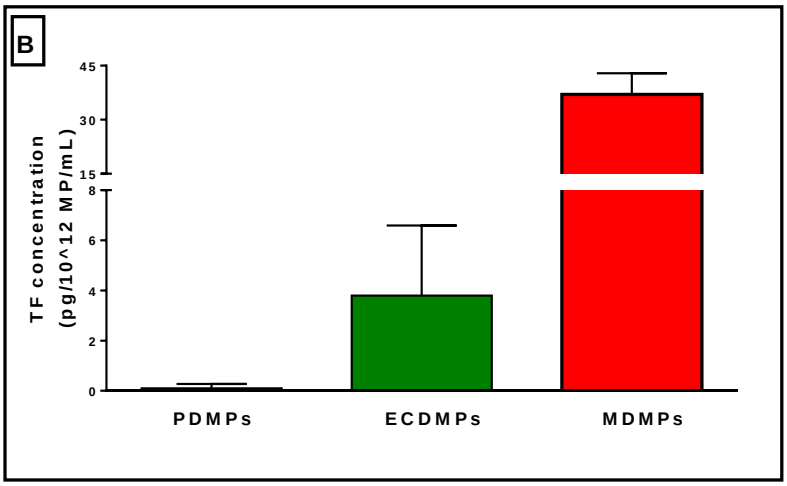


Figure 3.22. TF activity and concentration of PDMPs, ECDMPs and MDMPs. MPs were generated from washed platelets (grey bars; n=5), endothelial cells (green bars; n=4) and macrophages (red bars; n=3), added to CTI-inhibited pre-filtered pooled plasma. MP count has been normalised using the MP count analysed using NTA. Panel A: TF activity of MPs was evaluated on CAT assay using the TF-dependent assay (4µM PL reagent). Panel B: TF concentration was measured using Zymuphen MP-TF ELISA kit. Data are normalised to MP count and shown as mean±SD.

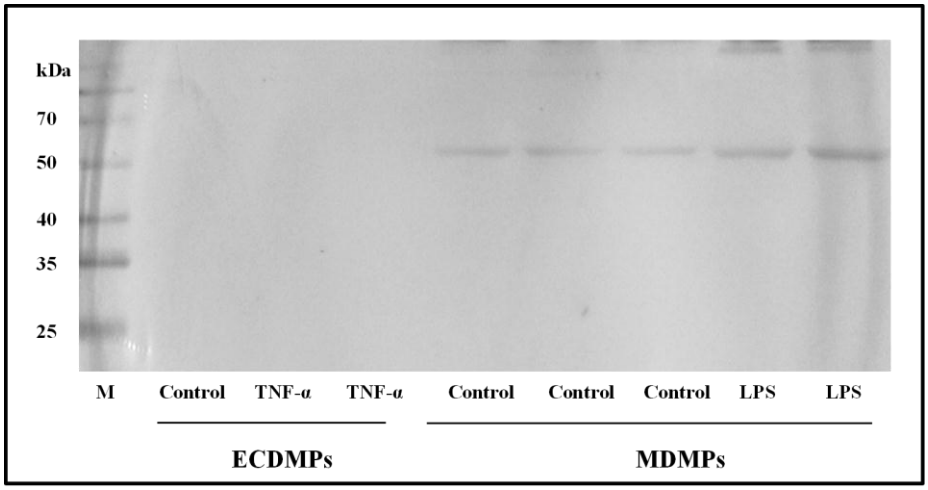


Figure 3.23. Western blotting analysis of TF in ECDMPs and MDMPs. Equal volumes (50µl) of ECDMP and MDMP lysates were loaded in SDS-PAGE and analysed for the presence of TF protein using D3 antibody at 1:2000 dilution (D3 antibody is mouse anti-TF was obtained from Dr. John McVey, University of Surrey). Using the same MP preparations that were used in the CAT and ELISA assays showed that there was no TF protein detected in the ECDMP samples, whereas the MDMP preparations showed bands at the expected position for TF protein in unstimulated samples, which increased after LPS stimulation.

3.2.8 CAT assay sensitivity to MP derived from different cells

Since MPs derived from different parent cells showed unique PL and/or TF activities, it was sensible to examine the CAT assay sensitivity to these MPs. PDMPs were used as MPs that have PL^{+++}/TF^{-} activity, ECDMPs as PL^{+}/TF^{+} activity and MDMP as PL^{+++}/TF^{+++} activity. MPs generated from the isolated cells were resuspended into CTI-inhibited pre-filtered pooled plasma and then diluted with the same plasma to a range of dilutions up to 1:20. MP suspensions were analysed in the CAT assay using the PRP, MP and no reagent assays before and after filtration using Ceveron unit to account for the presence of MPs.

As seen previously with PDMP-rich plasma the 1 pM TF reagent showed high sensitivity to the procoagulant activity of PDMPs from washed platelets in a dose-dependent manner, with

the greatest effect seen for the ETP and peak thrombin values (Figure 3.24 A). In sharp contrast, the MP and no reagent assays, where no exogenous TF was added, failed to show any thrombin generation (data not shown). This finding is consistent with the early findings and it is unsurprising since PDMPs have been shown to lack functional TF activity.

The 1 pM TF reagent also showed a linear relationship between the concentration of ECDMPs and the enhancement of thrombin generation capacity (Figure 3.24 B). On the other hand, despite the high procoagulant activity of ECDMPs seen with the 1 pM TF reagent assay, the 4 µM PL reagent assay showed thrombin generation curves only with the undiluted ECDMPs preparations (data not shown). In contrast, ECDMPs failed to support thrombin generation even with the highest concentration of ECDMPs, when no reagent assay was used (data not shown). This finding suggests that the MP reagent enhances the sensitivity of the CAT assay towards the levels of TF-bearing MPs in plasma.

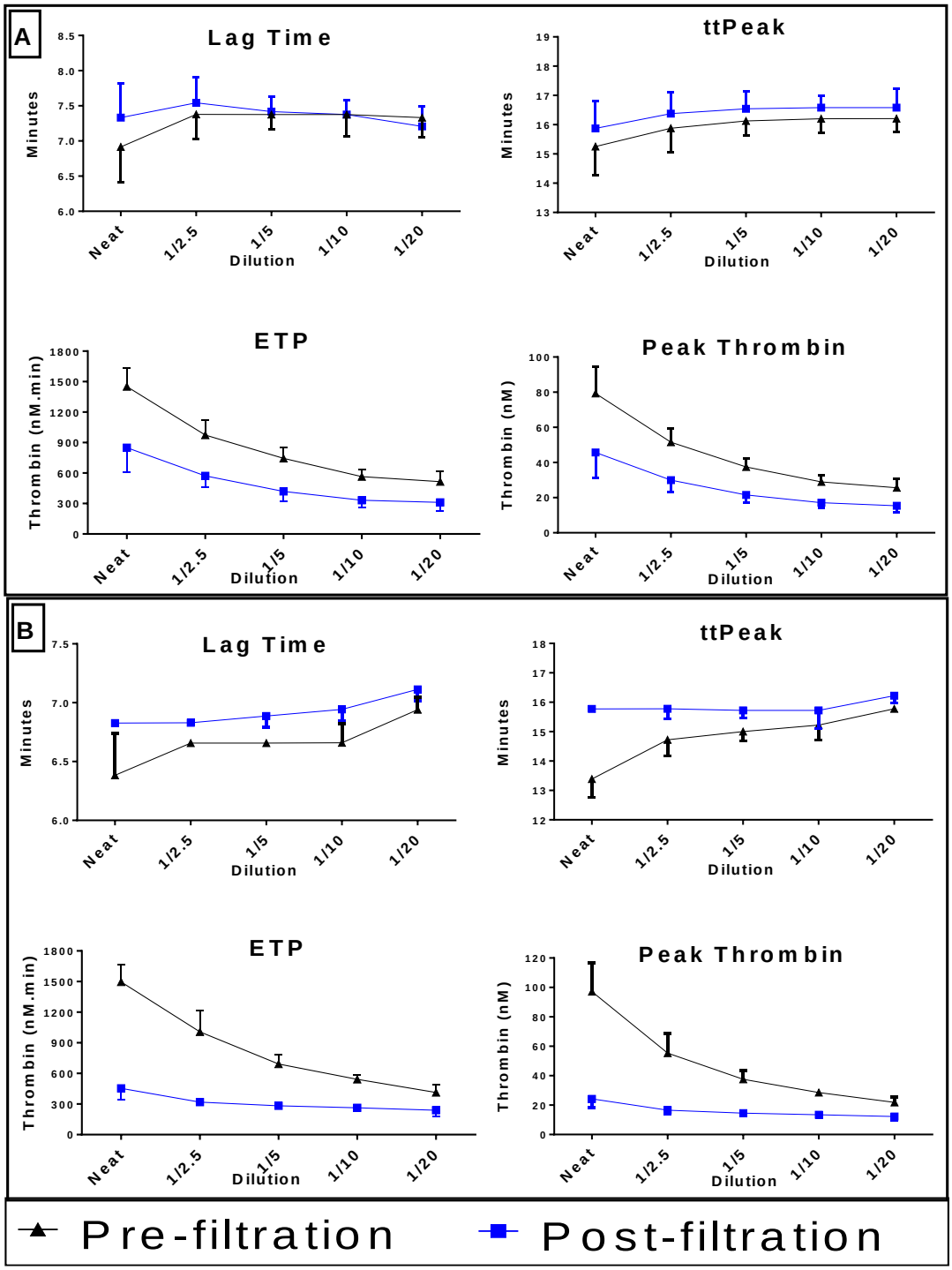
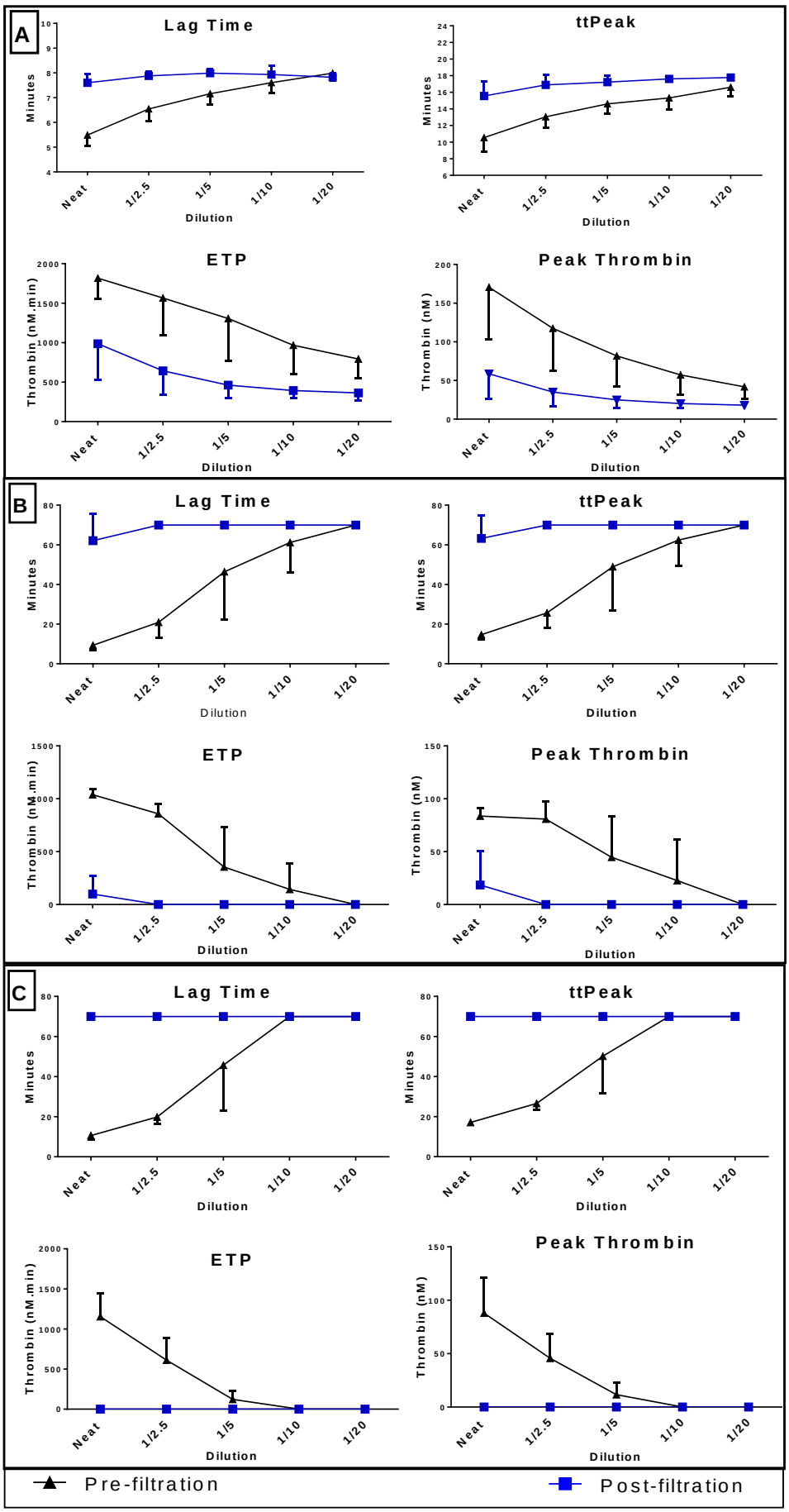


Figure 3.24. CAT assay sensitivity to MPs prepared from washed platelets and endothelial cells using the 1 pM TF reagent. Panel A: PDMPs were generated from washed platelets by stimulation with 2 µg/mL CRP-XL for 10 minutes at 37°C (n=4). Panel B: EA.hy926 cells were stimulated with 10 ng/mL TNF-α for 24 hours 37°C (n=3). ECDMPs were isolated from media suspension and purified from cell debris by centrifugation at 160g for 20 minutes followed by 1500g for 15 minutes. ECDMPs were washed two times in HBS. MP preparations were resuspended into CTI-inhibited pre-filtered pooled plasma at the indicated concentrations. Samples were analysed in CAT assay using Stago PRP reagent (1 pM TF). Samples were analysed before filtration and after filtration using the Ceveron unit. Data are shown as mean±SD.

Consistently, the 1 pM TF reagent showed a linear relationship between the plasma concentration of MDMPs and the amount of thrombin generation (Figure 3.25 A). Interestingly, the effect of the presence of MDMPs on the plasma on lag time and ttPeak was more pronounced compared to PDMPs and ECDMPs. Lag time and ttPeak were markedly shortened by the presence of MDMPs in a dose-dependent manner even with the addition of exogenous 1 pM TF in the assay, reflecting the relative potency of TF activity in MDMPs compared to other types of MPs. Furthermore, the 4 µM PL reagent showed a liner relationship between the plasma concentration of MDMPs and the amount of thrombin generation, particularly in the lag time and ttPeak values (Figure 3.25 B). At the lowest dilution (1/20), MP reagent failed detect MDMPs in the samples, although they showed PPL activity with the PRP reagent at this particular concentration. Finally, the no reagent assay was less sensitive to MDMPs as it failed to detect MDMPs at the 1/10 dilution (Figure 3.25 C), whereas the MP reagent assay was able to detect MDMPs at this particular concentration. This finding supports the early observation that the MP reagent assay slightly enhances the sensitivity of CAT assay towards the levels of TF-bearing MPs in plasma.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1/10
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Pre-filtration
Post-filtration

Figure 3.25. CAT assay sensitivity to MDMPs using different reagents. THP-1 cells were transformed into macrophages using 100 ng/mL PMA for 72 hours. Macrophages were stimulated with 10 ng/mL LPS for 6 hours at 37°C. MDMPs were purified from cell debris by sequential centrifugation steps: 160g for 20 minutes, 1500g for 15 minutes and 13000g for 2 minutes. MDMPs were washed two times in HBS and resuspended into CTI-inhibited pre-filtered pooled plasma at the indicated concentrations. Samples were analysed in CAT assay using (A) Stago PRP reagent (1 pM TF), (B) Stago MP reagent (4 µM PL) and (C) without the addition of TF or PL to the assay (no reagent assay). Samples were analysed before filtration and after filtration using the Ceveron unit. Data are from three separate experiments and shown as mean±SD.

3.2.9 Impact of freezing and thawing on the procoagulant activity of MPs derived from different cell types

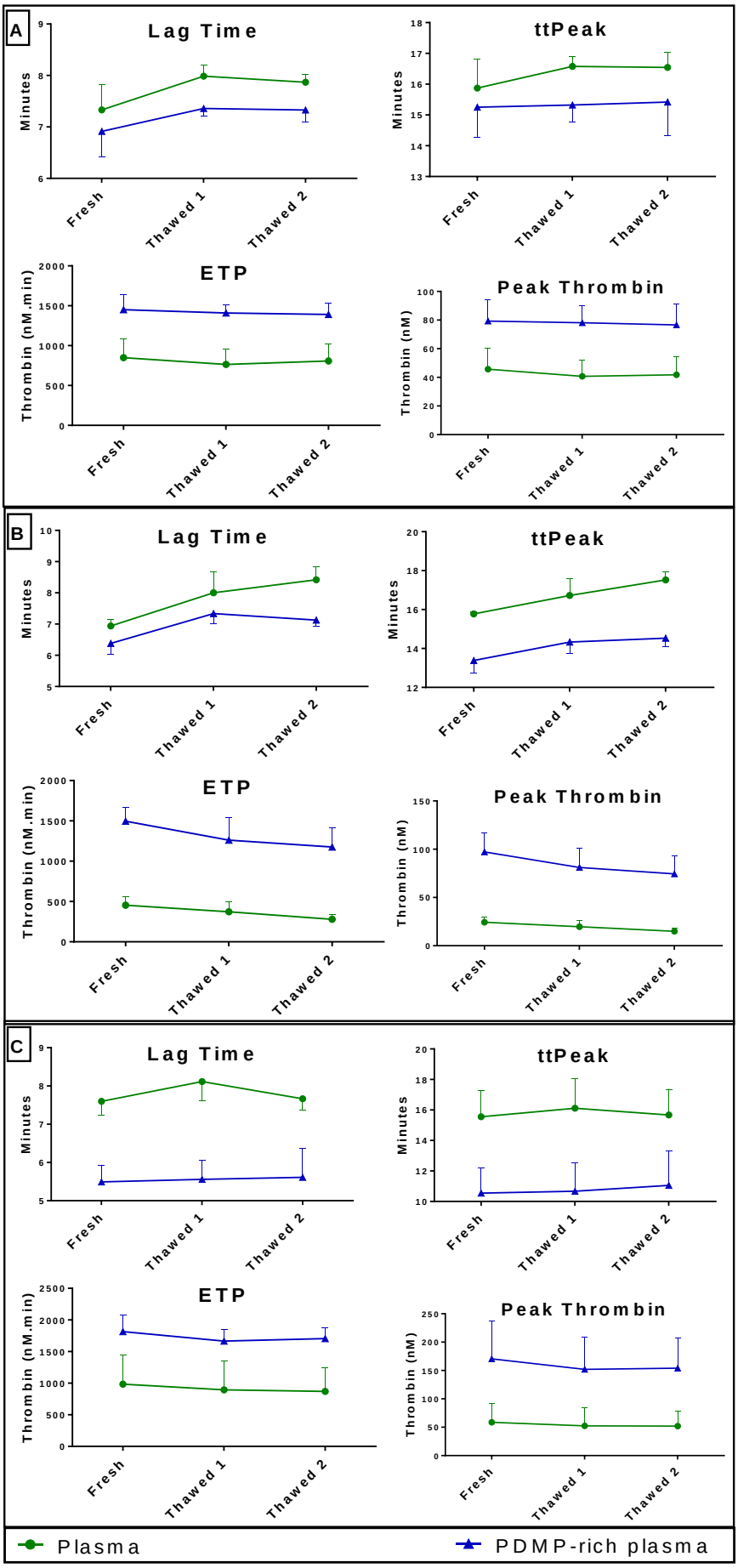
Having established that freezing and thawing of samples has significant effect on the procoagulant activity of coagulation factors but not PDMPs, it was sensible to examine whether the procoagulant activity of MPs form different parent cells are also unaffected by repeat freezing and thawing.

Consistent with previous finding of PDMPs generated from PRP samples, freezing and thawing had no significant effect on the PPL activity of MPs derived from washed platelets using the 1 pM TF reagent assay (Figure 3.26 A). Additionally, repeat freezing and thawing had similar effect on both ECDMP-rich and MDMP-rich plasma as well as on normal plasma (Figure 3.26 B and C). This finding is consistent with the early finding that freezing and thawing causes moderate coagulation factor deterioration but no significant impact on the PPL activity of MPs. Interestingly, there was a more pronounced effect of freezing and thawing on the lag time and ttPeak values of both normal and PDMP-rich plasma samples

compared to those seen in Figure 3.13 A. However, it should be noted that these samples were prepared using pooled plasma that had been frozen and thawed once.

On the other hand, repeat freezing and thawing showed a potential to cause an increase in the TF activity of the ECDMPs and MDMPs. Using the MP reagent assay, lag time and ttPeak values of both ECDMPs and MDMPs were slightly reduced after each of the freezing cycles (Figure 3.27 A and B). Additionally, similar trends of reduction in the lag time and ttPeak values of MDMPs were seen when analysed with the no reagent assay (Figure 3.27 C). Although freezing and thawing caused an increase in the TF activity of MPs, PDMPs generated from washed platelets failed to support thrombin generation in the PM and no reagent assays even with repeated freezing and thawing (data not shown), further confirming the previous finding that PDMPs are deficient in TF activity.

Collectively, results from this study clearly show that repeat freezing and thawing of plasma samples consistently show reduction trends in the thrombotic capacity, suggesting that it may cause coagulation factor degradation. On the other hand, freezing and thawing has no significant impact on the PPL activity of MPs, whereas it increases TF activity. It is unclear; however, whether this is due to MP fragmentation or to changes in the TF conformation leading to decryption.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Thrombin (nM.min)
Peak Thrombin
Thrombin (nM)
Fresh
Thawed 1
Thawed 2
Plasma
PDMP-rich plasma

Figure 3.26. Effect of freezing and thawing on the PPL activity of MPs derived from different cell types. Panel A: PDMPs were generated from washed platelets by stimulation with 2 µg/mL CRP-XL for 10 minutes at 37°C (n=4). Panel B: EA.hy926 cells were stimulated with 10 ng/mL TNF-α for 24 hours (n=3). ECDMPs were isolated from media suspension and purified from cell debris by centrifugation at 160g for 20 minutes followed by 1500g for 15 minutes. ECDMPs were washed two times in HBS. Panel C: THP-1 cells were transformed into macrophages using 100 ng/mL PMA for 72 hours (n=3). Macrophages were stimulated with 10 ng/mL LPS for 6 hours at tissue culture incubator. MDMPs were purified from cell debris by sequential centrifugation steps: 160g for 20 minutes, 1500g for 15 minutes and 13000g for 2 minutes. MDMPs were washed two times in HBS. MP preparations were resuspended into CTI-inhibited pre-filtered pooled plasma. The PPL activity of MP was analysed in CAT assay using Stago PRP reagent (1 pM TF). Samples were analysed as fresh and after subjecting to two freeze/thaw cycles. Data are presented as mean±SD.

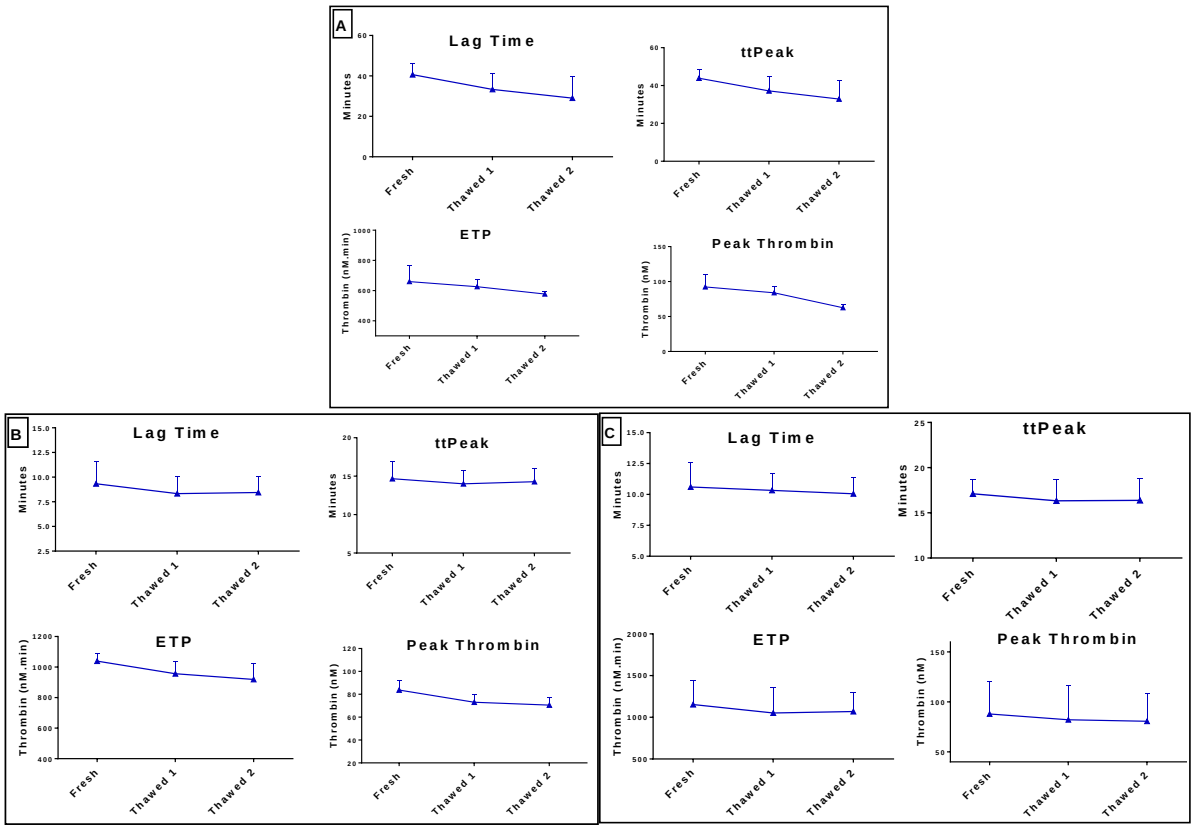


Figure 3.27. Effect of freezing and thawing on the TF activity of ECDMPs and MDMPs. Panel A: shows thrombin generation of ECDMPs using the MP reagent assay (4 µM PL). Panel B and C: show thrombin generation of MDMPs using the MP reagent and no reagent assays respectively. Samples were analysed as fresh and after subjecting to two freeze/thaw cycles. Data are from three separate experiments as in Figure 3.26 and presented as mean±SD.

3.3 **Discussion**

3.3.1 Impact of pre-analytical and analytical variables on the CAT assay

The first aim of the present study was to evaluate the effects of the pre-analytical and analytical variables on thrombin generation of normal and MP-rich plasma samples. Currently, the lack of uniform consensus regarding the pre-analytical and analytical conditions has hindered MP analysis (Freyssinet and Toti 2010, Jy, *et al* 2004, Piccin, *et al* 2007, Yuana, *et al* 2011). The most critical and discrepant pre-analytical steps are (1) plasma preparation, (2) centrifugation protocols, (3) freezing and thawing temperature and (4) storing conditions. Several studies have been recently conducted to investigate and evaluate the impacts of pre-analytical variables on MP analysis (Ayers, *et al* 2011, Dey-Hazra, *et al* 2010, Lacroix, *et al* 2012, Lee, *et al* 2012a, Robert, *et al* 2009, Shah, *et al* 2008, Trummer, *et al* 2008, Trummer, *et al* 2009). In addition, the SSC of the ISTH organised a first collaborative working party aimed to address issues related to MP detection, quantification and phenotyping and since then important progress has been made in the field of MP analysis (Jy, *et al* 2004). However, despite the ongoing efforts of the SSC and other researchers, there is still a need for guidelines on MP analysis, and standardisation of pre-analytical conditions. Additionally, the majority of the studies that addressed the pre-analytical variables of MP analysis focused mainly on MP count, whereas only a few studies have explored the impact on the procoagulant activity of MPs (Keuren, *et al* 2006, Lacroix, *et al* 2012).

The initial experimentation for the CAT assay precision reveals that the assay used according to our laboratory protocol has good inter-assay reproducibility with %CV below 10% for the measured thrombin generation parameters using different reagents. On the other hand, thrombin generation showed an important inter-individual variability, which is consistent

with previous reports (Gerotziafas, *et al* 2005, Vanschoonbeek, *et al* 2004). Since thrombin generation is a global assay that measures the endpoint of the coagulation process there are many factors that might have an impact on the inter-individual variability such as variations in procoagulant and anticoagulant factors in the blood (Butenas, *et al* 1999, Hemker and Beguin 1995). The %CV values of the inter-individual variability in the present study were high with all assay reagents used. These have been designed to be sensitive to different components (see Table 2.3), reflecting the significant inter-individual variability in the activity of clotting factors, natural inhibitors of coagulation and endogenous levels of MPs.

One focus of the current study was to evaluate all of the commercially available thrombin generation reagents for their sensitivity and reliability to detect and characterise the procoagulant properties of MPs in terms of the PPL and TF activities. Despite the fact that the CAT assay is widely used to detect and evaluate the functional properties of MPs, it appears that there is no general agreement on which is the most appropriate reagent for MP analysis. Most studies used the 1 pM TF reagent assay (Stago PRP reagent) to detect and evaluate the functional properties of MPs (Bidot, *et al* 2008, Keuren, *et al* 2006, Pereira, *et al* 2006), whereas others used the 5 pM TF/4 µM PL reagent assay (Stago PPP reagent) (Macey, *et al* 2010, Macey, *et al* 2011). Additionally, some studies investigated the TF activity of MPs using the 4 µM PL reagent assay (Stago MP reagent) (Van Der Meijden, *et al* 2012) and other used the no reagent assay (Ollivier, *et al* 2010).

The present study demonstrated that the PPP (5 pM TF/4 µM PL), PPP low (1 pM TF/4 µM PL) and MP (4 µM PL) reagents show very low, if any, sensitivity to the plasma levels of PDMPs as they failed to discriminate between normal and PDMP-rich plasma samples. However, it is noteworthy that PDMPs have been shown in the present study and other studies to exert high PPL activity while they are deficient to TF activity. It appears that the addition of 4 µM exogenous PL to the thrombin generation in the PPP, PPP low and MP

assays leads to normalisation of the PPL activity of the MPs present in the plasma, therefore the assays lose their sensitivity to the levels of MPs in plasma. In contrast, when the exogenous PL was omitted from the assay, as with the PRP reagent (1 pM TF), the assay showed great sensitivity to the PPL activity MPs in the samples; this was particularly the case for the ETP and peak thrombin values. The addition of the exogenous 1 pM TF is enough to trigger the coagulation process and remains dependent on the endogenous PPL activity of MPs to propagate thrombin generation, which in turn reflects the amount of PPL activity in the samples. On the other hand, the MP reagent assay showed good sensitivity to the levels of TF-bearing MPs (ECDMPs and MDMPs) but not TF-negative MPs (PDMPs). This assay is dependent on the endogenous TF activity of MPs to trigger thrombin generation. Using serial dilutions of moderately and strongly TF-positive MPs, ECDMPs and MDMPs respectively, showed that the MP reagent enhances the sensitivity of the CAT assay to the plasma levels of TF-bearing MPs.

Overall, it appears that the PRP reagent assay is the most sensitive assay to detect MPs derived from different cell types and can be used to evaluate the PPL properties of MPs, whereas the MP reagent assay is sensitive to TF-bearing MPs and can be used to evaluate the TF activity of MPs. This finding supports that rationale that omitting one of the procoagulant factors, either PPL or TF, makes the assay dependent and sensitive to the endogenous levels of the missing factor.

MP isolation is considered to be one of the most critical pre-analytical steps that causes variability between different studies. Several centrifugation protocols have been proposed and evaluated for MP isolation. The present study demonstrated that single-step centrifugation protocols, either at 2000g for 20 minutes or at 1800g for 30 minutes, are not recommended for MP isolation from blood samples due to the presence of platelet contamination in the MP suspension. This finding is in agreement with several studies that

showed an increase in the MP concentration after samples freezing using single-step centrifugation protocols at 1500-2000 for 15-30 minutes (Ayers, *et al* 2011, Lechner and Weltermann 2008, Shah, *et al* 2008). As shown in the present study, and by Mobarrez *et el* (Mobarrez, *et al* 2010), platelet contamination in sample preparations leads to false increase in MP activity and count due to platelet fragmentation during freezing and thawing. On the other hand, the two-step protocols, the 1500g for 15 minutes followed by 13000g for 2 minutes and more preferably the double spin at 2500g for 15 minutes each, appear to be the most appropriate currently available centrifugation protocols for MP isolation as they showed the least platelet contamination, whilst retain MP activity. This is in the line with previous findings that recommended these centrifugation protocols for MP isolation (Jy, *et al* 2004, Lacroix, *et al* 2012), but here it has been extended to investigate the effect of these centrifugation protocols on the procoagulant activity of MPs and specifically measures platelet contamination.

The present study also investigated the impact of sample storage conditions on the procoagulant activity of plasma and MP samples. It appears that plasma stored at -80°C should ideally be used within two months and no longer than six months. Interestingly, according to the results in Figure 3.3, the procoagulant activity of MPs seems to be less affected by the period of storage as shown by the PRP reagent assay compared to the activity of clotting factors as shown by the PPP and PPP low reagent assays.

Additionally, because in most cases, samples are stored before analysis, and in some cases samples are exposed to repeated freezing and thawing, the present study investigated the effect of freezing and thawing on the procoagulant activity of the coagulation factors and MPs. The present study consistently showed that freezing and thawing had a significant impact on the procoagulant activity of normal and MP-rich plasma samples as measure with

all thrombin generation reagents, indicating that repeated freezing and thawing causes decay of the clotting factors. Conversely, freezing and thawing showed a modest increase in the TF activity of the ECDMPs and MDMPs. It could be proposed that the increase in the TF activity in frozen MPs was due to TF decryption by freezing and thawing process (Bach 2006, Chen and Hogg 2013, Rao, *et al* 2012). This finding, however, contradicts findings in the literature that showed no significant difference in TF activity of fresh MPs versus frozen MPs (Lee, *et al* 2012a, Tesselaar, *et al* 2007). The most likely explanation for this contradiction is related to the sensitivity of the used assays and the type of MPs that may possess different TF properties. Although Lee *et al* (2012a) found no significant increase in TF activity in frozen samples, there was donor to donor variability where 3 of 5 donors used in that study showed a modest and statically significant increase in the TF activity after sample freezing, suggesting that TF activity of MPs may be affected differently between individuals. Furthermore, the present study observed that the effect of freezing and thawing was more pronounced in the TF activity of the ECDMPs compared to the MDMPs.

On the other hand, the present study demonstrated that there was no major impact of repeated freezing and thawing on the PPL activity of the all MP types used, providing the plasma is free of platelets and cell fragments. The effect of freezing and thawing on the MP analysis remains highly controversial. Some studies reported a significant increase in the MP count in frozen samples compared to fresh samples (Ayers, *et al* 2011, Dey-Hazra, *et al* 2010), whereas others showed no increase in the count when samples were appropriately prepared using two-step centrifugation protocols (Lacroix, *et al* 2012, Shet, *et al* 2003, Trummer, *et al* 2009). The most likely explanation for the contradicting findings about the increased MP activity in frozen samples is related to platelet and cell fragment contamination in MP preparation as the most of the studies that showed an increase in the MP count after freezing used single-step centrifugation protocol in MP isolation.

In conclusion, MPs are receiving a great deal of attention in recent years, as diagnostic and investigative tools as well as prognostic biomarkers. However, it is essential to validate, optimise and standardise the pre-analytical and analytical conditions of MP isolation and storage to enable comparison findings between different studies. The present study focused mainly on investigation of the impact of pre-analytical and analytical variables on the procoagulant activity of MPs using the CAT assay. The major pre-analytical and analytical points in the present study can be highlighted as following: 1) the double-spin, but not the single-step, centrifugation protocols should be used for MP isolation from blood samples, 2) platelet contamination should be sufficiently removed from MP preparation, 3) freezing and thawing of pure MP preparations has no major impact on the PPL activity of MPs but enhances their TF activity and causes a small decay of clotting factors, 4) frozen samples should be analysed within six months, 5) the PRP reagent assay is the most sensitive assay for MP detection and PPL activity evaluation, whereas the MP reagent assay is appropriate for TF-bearing MPs.

3.3.2 Procoagulant activity of PDMPs, ECDMPs and MDMPs

The present study specifically compares the relative procoagulant activity of MPs derived from platelets, endothelial cells and monocyte-derived macrophages in terms of their PPL and TF activities. There has, to my knowledge, been no previous study that compared the procoagulant activity of PDMPs, ECDMPs and MDMPs in a single set of experiments that would enable directly comparison of their relative PPL and TF activities, thus help to define their potential contributions in haemostasis and/or thrombosis.

The present study showed that the all three types of MPs have relatively high PPL activities. In thrombin generation assay, after the initiation of thrombin generation with low

concentration of TF (the 1 pM TF reagent assay), the PDMPs, MDMPs and to a lesser extent the ECDMPs increased the amount of thrombin generation in plasma. This finding is in agreement with the well-established role of MPs in promoting thrombin generation through the influence of PPLs (Berckmans, *et al* 2001, Bidot, *et al* 2008, Keuren, *et al* 2006). However, there was a discrepant result in the PS concentration of the PDMPs determined by the MP activity ELISA and their PPL activity in the CAT assay, which has been discussed in more details in section 3.2.7.2.1.

On the other hand, MPs derived from the three different cell types showed a significant variation in their ability to exert TF activity, reflecting they heterogeneously contribute to thrombus initiation. MPs generated from washed platelets neither supported thrombin generation in TF-dependent manner (i.e. with using the 4 µM PL reagent in the CAT assay in the presence of contact pathway inhibitor, CTI) nor showed a measurable amount TF in the MP-TF ELISA, indicating that they lack functional TF.

The debate around TF expression in platelets and their MPs remains highly controversial. On one hand, Zillman and co-authors showed that whole blood stimulation with collagen for five minutes results in TF expression in platelets (Zillmann, *et al* 2001). However, this study did not explore whether platelet-associated TF activity was actually of platelet origin or was taken up from TF presented in the whole blood. It has been shown that platelets have the capacity to take up TF from monocytes or TF-bearing MPs in the circulation, a process that is mainly due to fusion with MPs from other cells (in particular monocytes) mediated via the interaction of P-selectin glycoprotein ligand-1 (PSGL-1) and P-selectin on the surface of platelets (del Conde, *et al* 2005, Falati, *et al* 2003, Lopez-Vilchez, *et al* 2009). Interestingly, studies by independent groups demonstrated that quiescent platelets express physiologically functional TF as well as TF mRNA, which increase in response to activation (Camera, *et al* 2003, Panes, *et al* 2007, Schwertz, *et al* 2006, Siddiqui, *et al* 2002). Additionally, one study

revealed that resting platelets contain very low concentrations of TF stored within α-granules and the open canalicular system, which is readily to be exposed on the platelet surface after stimulation (Muller, *et al* 2003). On the other hand, other studies have failed to detect the presence or synthesis of physiologically active TF in both un-stimulated and stimulated platelets (Aleman, *et al* 2011, Bouchard, *et al* 2012, Bouchard, *et al* 2010, Osterud and Olsen 2013, Osterud, *et al* 2009, Van Der Meijden, *et al* 2012). These findings are in agreement with early work of Butenas *et al* demonstrating that whole human blood containing CTI did not show functional TF activity in the thrombin generation assay (Butenas, *et al* 2005). It was initially thought that the wide variability between different reports was due to the transient and dynamic process of platelet TF expression (Camera, *et al* 2010); however, others failed to show TF activity on platelets using similar experimental conditions (Osterud and Olsen 2013).

These contradicting results in the literature indicate that the ongoing controversy on the platelet TF expression has not been resolved. It appears the most likely explanation for the contradicting findings about the TF activity on platelets is related to technical issues in the measurement of TF such as (1) different methods of sample preparations (e.g. whole blood, PRP or washed platelets), (2) different platelet stimuli, (3) different durations of platelet stimulation, (4) differences in assay sensitivity, (5) TF detection antibody specificity, (6) lack of TF inhibitory step and (7) presence of monocytes and TF-positive MPs contamination in sample preparation. Further investigations are required with taking into the account the technical issues to resolve this ongoing controversy.

Despite the contracting findings in the literature about the TF activity on platelets and PDMPs, the present study demonstrated that platelet stimulation with CRP-XL for 10 minutes in washed platelet preparation (i.e. in the absence of other cell contamination) results in the release of MPs that promote thrombin generation only after the addition of exogenous

TF, suggesting that PDMPs are TF-negative. PDMPs have been shown to induce thrombin generation independently from TF and through the intrinsic pathway activation (Van Der Meijden, *et al* 2012). Therefore, it could be speculated that the main thrombotic role of PDMPs is to propagate thrombus formation only after it is induced by TF exposure or contact pathway activation. This is in agreement with the rationale that more than 70% of all circulating MPs in the peripheral blood of healthy individuals are derived from platelets or megakaryocytes, which do not spontaneously induce thrombosis (Flaumenhaft, *et al* 2009, Shantsila, *et al* 2010).

The level of circulating ECDMPs in healthy conditions appears to be very low (Berckmans, *et al* 2001, Leroyer, *et al* 2007). ECDMPs plasma levels have been shown to be elevated in many physiological and pathophysiological conditions (Burton, *et al* 2013, Gonzalez-Quintero, *et al* 2003, Mallat, *et al* 2000). Despite the fact they represent a relatively small fraction of circulating MPs, ECDMPS have been shown to play critical roles in haemostasis and thrombosis (Chironi, *et al* 2009, Dignat-George and Boulanger 2011, Markiewicz, *et al* 2013). In the present study, ECDMPs showed moderate PPL and TF activities that enable them to independently initiate and promote thrombin generation in TF- and PL-dependent manners. These data support previous reports (Abid Hussein, *et al* 2008, Banfi, *et al* 2005, Combes, *et al* 1999, Kushak, *et al* 2005), but extend to specifically evaluate their relative PPL and TF procoagulant activity and compare it with MPs derived from different cell origins. However, it has to be emphasised that ECDMP characteristics are highly dependent on the nature of the stimulation (Peterson, *et al* 2008), therefore, extra caution should be considered when interpreting these data. In the present study, TNF-α stimulation of endothelial cells was used to generate ECDMPs *in vitro*. Unfortunately, the precise mechanisms of *in vivo* ECDMP generation are poorly understood and may have distinct properties. Nevertheless, it has been demonstrated that ECDMPs isolated from patients with

different pathological conditions also have PPL and TF activity (Dignat-George and Boulanger 2011, Markiewicz, *et al* 2013).

Similar to ECDMPs, MDMPs were able to independently initiate and enhance thrombin generation in TF- and PL-dependent manners, but with the observation that MDMPs had significantly higher TF and PL activity and concentration compared to ECDMPs as shown by the CAT and ELISA assays. Interestingly, data also revealed that MDMPs had more PPL and activity compared to PDMPs as shown by peak thrombin of thrombin generation. This finding is in the line with a previous report of Tracy *el al* (1983) demonstrating monocytes induce enhancement in prothrombinase activity more than platelets (Tracy, *et al* 1983), which was recently supported by Aleman and colleagues (Aleman, *et al* 2011). MPs derived from monocytes have been shown to express high TF activity and thought to be the predominant circulating TF-bearing MPs in health and disease (Owens and Mackman 2011). It is noteworthy that MDMPs used here were generated from monocyte-derived macrophages, which might have different properties than MPs derived from monocytes. However, MPs derived from monocytes have been shown to possess high TF and PPL activities (Aleman, *et al* 2011, Van Der Meijden, *et al* 2012), which is consistent with the present findings.

It has to be stressed that it is necessary to inhibit the intrinsic pathway activation when evaluating TF activity. It has been demonstrated that MPs generated from platelets and red blood cells can initiate thrombin generation in a FXII-dependent manner, independently from TF activity (Van Der Meijden, *et al* 2012). This may lead to miss-interpretation of the measurement of TF activity of MPs in thrombin generation assays. Therefore, in the present study CTI was used in all samples to ascertain that thrombin generation is induced via TF-dependent manner and not via intrinsic pathway activation.

Several limitations of the present study need to be highlighted. First, the procoagulant activity was investigated in MPs but not in their parent cells. For instant, the fact that PDMPs lack TF activity, it is unclear at this stage whether TF is also not expressed in the CRP-XL stimulated platelets but not packaged into their PDMPs remains to be elucidated. However, as discussed above, several studies have failed to detect TF activity and antigen in either platelets or their MPs. Second, the present study used CRP-XL as the main platelet agonist to induce platelet procoagulant response and MP formation; other stimuli may result in the release of MPs that express TF activity. Nevertheless, data in Chapter 5 of this thesis showed that rhodocytin platelet stimulation through CLEC-2 also results in the generation of PDMPs that lack TF activity. Additionally, other studies which used different platelet agonists than CRP-XL or rhodocytin to generate MPs have showed similar procoagulant properties of PDMPs that support the idea that they lack TF activity (Aleman, *et al* 2011, Bouchard, *et al* 2010, Osterud and Olsen 2013, Van Der Meijden, *et al* 2012). Third, the present study did not exclude the effects of thrombin generation negative regulator factors that may present on MPs such as TFPI and protein C. This may have led to underestimates of the procoagulant activity of the MPs. Furthermore, TF encryption was not explored, which may have also inhibited the TF activity. However, despite the anti-coagulant factors or inactivity of TF, the main aim of the present study was to evaluate the natural contribution of MPs in plasma procoagulant activity including their pro- and anti-coagulant activities. Fourth, these MPs were generated *in vitro* from isolated parent cells and from cell lines in the case of ECDMPs and MDMPs, which may have different characteristics to those generated *in vivo*. However, as discussed above, data in the present study are consistent with several studies that reported similar procoagulant properties of MPs generated from given cell types. Finally, MP count was performed using the NanoSight NS500 NTA system in MP samples that had been suspended into pooled plasma. Although NTA has been favourably used to measure the size and concentration of

MP population, it is unable, with the current protocol settings, to discriminate between MPs and other small particles present in the plasma such as exosomes, protein aggregates and lipoproteins. Therefore, the number of MPs may have been overestimated by the presence of the other small particles in the plasma. However, since all used MPs were suspended into the same pooled plasma at the same volumes, this should have enabled consistent measurement of their relative concentrations across all the samples.

In conclusion, the present study provides a new insight into the different contributions of MPs derived from platelets, endothelial cells and monocyte-derived macrophages in the procoagulant activity of plasma. All given MPs have relatively potent PPL activity, whereas PDMPs lack TF and ECDMPs and MDMPs possess moderate and potent TF activity respectively. These findings clearly indicate that that MPs generated from different cell types have different roles in haemostasis and thrombosis. However, it is noteworthy that their roles are not limited by their PPL or TF activities. For instance, ECDMPs have been shown to express ultra-large vWF multimeres that promote platelet aggregation and increase clot stability (Jy, *et al* 2005). On the other hand, MPs have been shown to express negative regulators of thrombin generation such as TFPI and protein C (Perez-Casal, *et al* 2005, Steppich, *et al* 2005), indicating that their role in haemostasis and thrombosis is dependent on both their pro- and anti-coagulant activities. It would be valuable to investigate the other pro- and anti-coagulant activities of MPs derived from different cellular origins.

CHAPTER 4: **Mechanisms of generating platelet procoagulant response and MP formation**

4.1 Introduction

The contribution of procoagulant platelets in haemostasis and thrombosis is well studied using wide range of approaches such as PS exposure, FVa expression (Alberio, *et al* 2000), thrombin generation (Cosemans, *et al* 2005) and prothrombinase assays (Fager, *et al* 2010). It is well established that the mechanism of generating platelet procoagulant response and MP release requires a prolonged and potent increase in the cytosolic Ca^{2+} concentration to activate phospholipid scramblase and protease calpain, which ultimately leads to breakdown of the cytoskeleton and consequent PS exposure and MP formation (reviewed by (Heemskerk, *et al* 2005). The ability of platelets to express a procoagulant surface is dependent on the nature of stimulation and the type of agonist used. For years it has been known that platelet stimulation through the GPVI collagen receptor is a potent inducer, by its sole action, of platelet PS exposure and MPs generation (Nieswandt and Watson 2003). It appears that the procoagulant response induced via GPVI is restricted to a subpopulation of platelets (Alberio, *et al* 2000, Dacharyprigent, *et al* 1993). In addition, another platelet ITAM-containing receptor, FcγRIIA, has been shown to exclusively generate platelet procoagulant surfaces and MPs, which contribute in the pathophysiology of HIT (Warkentin, *et al* 1994).

On the other hand, soluble agonists such as thrombin, ADP and TxA_2, which activate platelets through GPCRs, are considered potent agonists that induce platelet shape change, aggregation, degranulation and secretion (Offermanns 2006, Stegner and Nieswandt 2011). Additionally, is well established that platelet co-stimulation with collagen and thrombin provides the most potent physiological stimulation in generating procoagulant platelet

surfaces, known as coated-platelets (Alberio, *et al* 2000, Dale 2005, Keuren, *et al* 2005). Despite their potency in inducing platelet aggregation, the ability of GPCR agonists to induce platelet procoagulant response and MP formation remains controversial. Nevertheless, ADP and TxA_2 have been shown to play a key role in the positive feedback mechanism for platelet procoagulant response induced via GPVI. Therefore, it has been suggested that the main role of GPCRs is to promote and amplify platelet procoagulant response rather than exclusively inducing it (Stegner and Nieswandt 2011).

The first aim of this study was to comprehensively evaluate the capability of each of the platelet GPCRs and ITAM-containing receptors in generating a procoagulant surface on human platelets, and to generate release of procoagulant MPs. Additionally, the synergistic effect of GPCRs with ITAM-containing receptors in this regard was also examined. Finally, since GPVI and FcγRIIA are the primary platelet receptors capable of inducing a platelet procoagulant response and since the third platelet ITAM-containing receptor, CLEC-2, shares the same downstream signalling pathway of these receptors, we hypothesised that CLEC-2 is also capable of generating a procoagulant response and MP formation in platelets. Therefore, the second aim of this project was to investigate the role of CLEC-2 in this aspect, characterise some of its mechanisms underlying this process and evaluate its functional properties.

4.2 Results

4.2.1 ITAM-containing receptors but not GPCRs are capable to potently induce platelet procoagulant response and MP formation

To compare the relative activity of each of platelet GPCRs and ITAM-containing receptors in their capability of inducing platelet procoagulant response, Annexin V binding was used to measure platelet PS exposure and MP formation by flow cytometry. A two-colour protocol was employed, using a platelet-specific marker CD42b-PE and Annexin V-FITC. Additionally, platelet degranulation was measured, using P-selectin-FITC. Unless otherwise stated, the experiments in this chapter were performed using washed platelets suspended into HBS pH 7.4 containing 2mM $CaCl_2$ at a platelet density of 1.5 x 10^8/mL. Additionally, platelet-platelet interaction was further minimised by inhibiting GPIIb/IIIa using RFGP56 antibody. RFGP56 is a mouse MAb generated in Professor Goodall's laboratory in 1986 that blocks fibrinogen binding. Figure 4.1 demonstrates that platelet aggregation was inhibited by approximately 80% by RFGP56 at a final concentration of 10 µg/mL. This concentration was used in the subsequent experiments.

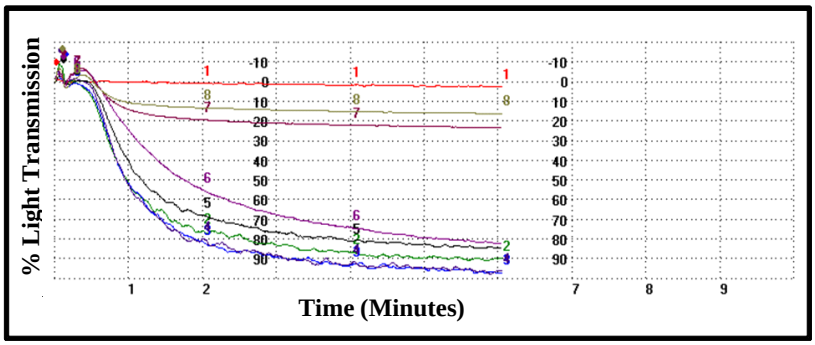
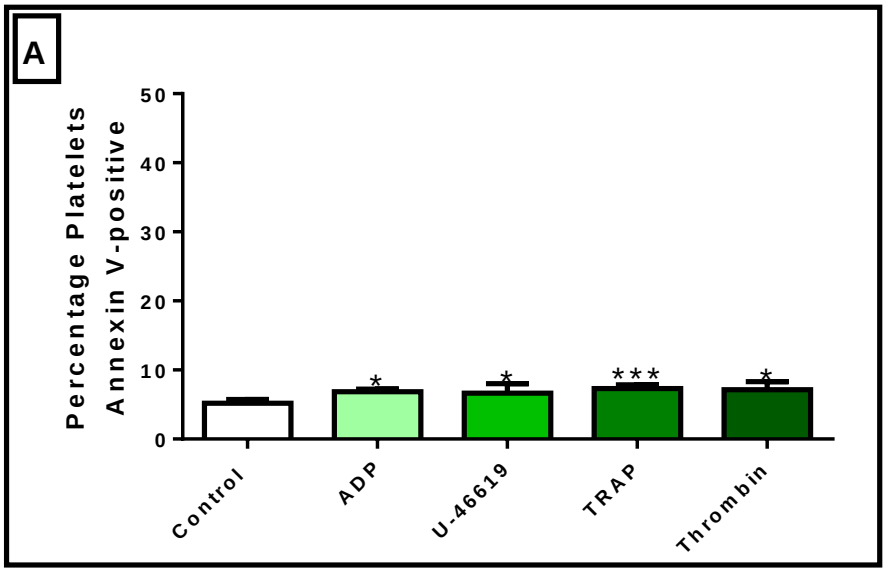


Figure 4.1. RFGP56 antibody dose titration. PRP was incubated with 0, 0.1, 0.3, 1, 3, 10 and 30 µg/mL RFGP56 antibody in lines 2 to 8 respectively. Line 1 was control PRP. 2ug/mL CRP-XL was added to samples in lines 2 to 8 and platelet aggregation was measured. 10 and 30 µg/mL doses (lines 7 and 8) showed the maximum inhibition of platelet aggregation.

Maximum concentrations of ADP, TxA_2 (TP) receptor agonist U-46619, TRAP and thrombin, which were more than required to induce full platelet degranulation, were used to examine their capability of generating platelet procoagulant surfaces. Platelet activation by these agonists resulted in slight but statistically significant increase in Annexin V binding (Figure 4.2 A), however, very little MP release could be detected in flow cytometry (Figure 4.2 B). This finding suggests that platelet activation through GPCRs alone does not play a major role in initiating the platelet procoagulant response. In sharp contrast, platelet activation through GPVI using serial doses of CRP-XL showed a significant increase in PS exposure in a dose-dependent manner (Figure 4.3A). This was accompanied by the release of strongly Annexin V^{+ve} MPs, which formed the majority of the Annexin V^{+ve} particles (Figure 4.3 B). Platelets showed a maximum response to CRP-XL at 3 µg/mL where 24.5±7.0 % of platelets bound Annexin V. Higher concentrations of CRP-XL did not generate a further increase in Annexin V binding, which supports the previous findings that procoagulant activity in collagen-stimulated platelets is restricted to a subpopulation of platelets (Alberio, *et al* 2000, Dacharyprigent, *et al* 1993). Platelet degranulation, unlike PS exposure, was induced in the majority of collagen-activated platelets, and maximum activation was achieved with lower concentrations of CRP-XL than needed to induce PS exposure. As can be seen from the dose-response curve of CRP-XL in Figure 4.3, nearly 90% of platelets were positive for P-selectin with only 0.3 µg/mL CRP-XL, which supports the view that platelet degranulation and procoagulant response occur through different mechanisms.



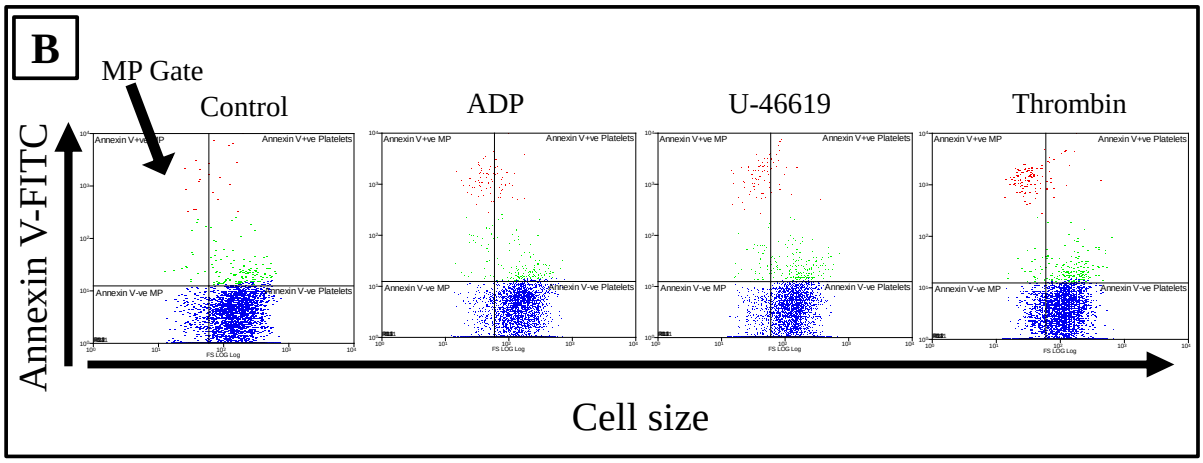


Figure 4.2. GPCRs are not capable to induce platelet PS exposure. Panel A: Washed platelets were activated for 10 minutes at 37°C with high doses of ADP (3 x10^{-5} M), U-46619 (3 x10^{-6} M), TRAP (1 x10^{-4} M) and thrombin (0.32 U/mL). Panel A, none of the used GPCRs agonist was able to exclusively induce a marked increase in Annexin V binding as measured by flow cytometry. Data are from six separate experiments performed using samples from different donors. Data are shown as mean±SD. P-values are versus resting control platelets; *$p<0.05$; ***$p<0.0001$. Panel B: Representative flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter of platelets stimulated with indicated agonists. In the dot-plot graphs; the upper left-hand quadrant shows Annexin V^{+ve} events in MP-size gate, the lower left-hand quadrant shows Annexin V^{-ve} events in MP-size gate, the upper right-hand quadrant shows Annexin V^{+ve} events in platelet-size gate, the lower right-hand quadrant shows Annexin V^{-ve} events in platelet-size gate; blue dots are Annexin V^{-}; green dots are weak Annexin V^{+}; red dots are strong Annexin V^{+++}.

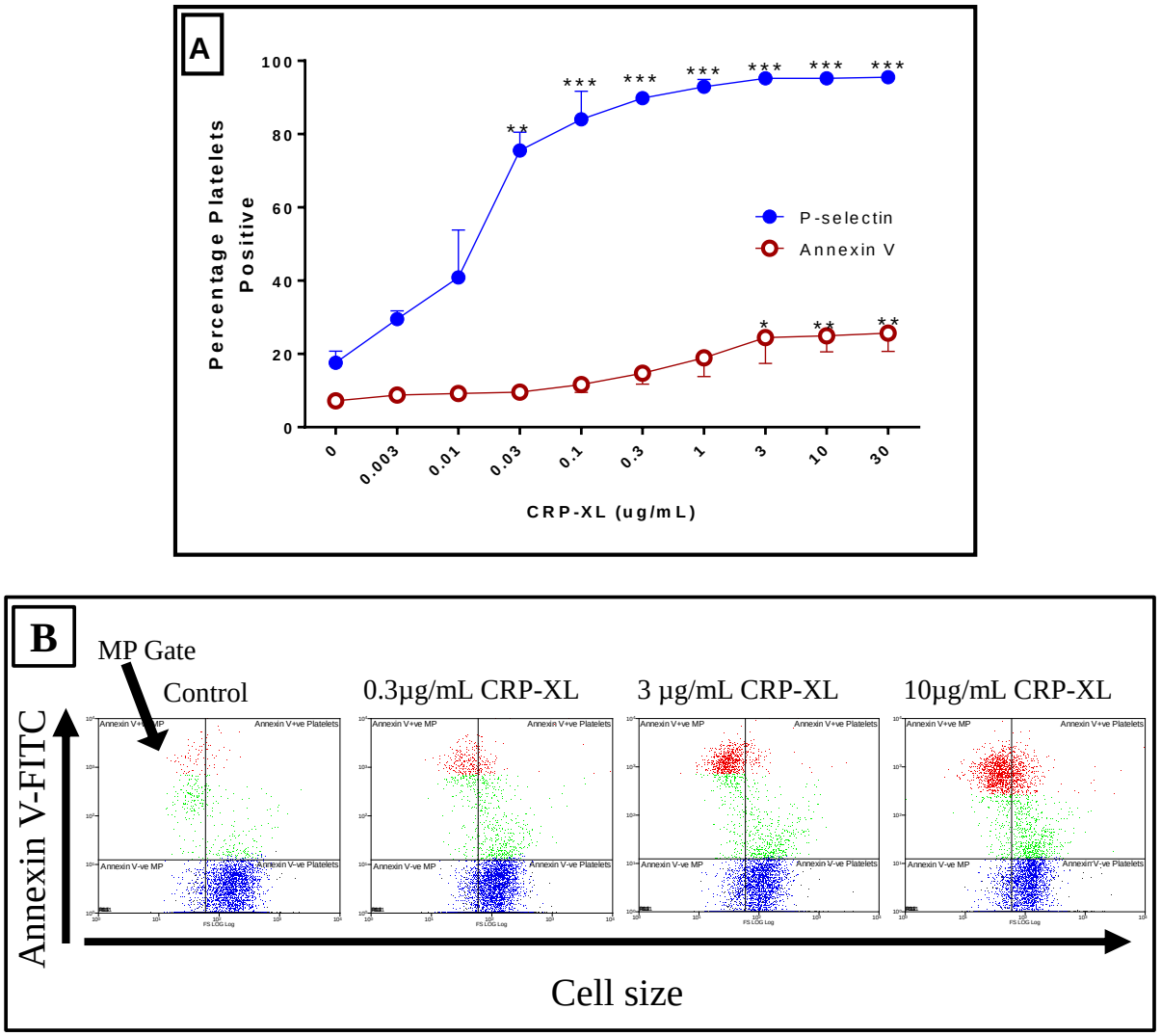


Figure 4.3. Platelet surface expression of P-selectin and PS exposure induced by serial doses of CRP-XL. Panel A: Washed platelets were stimulated with indicated concentrations of CRP-XL for 10 minutes at 37°C. Annexin V binding and P-selectin expression were measured by flow cytometry. Data are from three different donors and are shown as mean±SD. P-values are compared to resting platelets; *p<0.05; **p<0.001; ***p<0.0001. Panel B: Representative flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter of platelets stimulated with indicated does of CRP-XL. In the dot-plot graphs; the upper left-hand quadrant shows Annexin V^{+ve} events in MP-size gate, the lower left-hand quadrant shows Annexin V^{-ve} events in MP-size gate, the upper right-hand quadrant shows Annexin V^{+ve} events in platelet-size gate, the lower right-hand quadrant shows Annexin V^{-ve} events in platelet-size gate; blue dots are Annexin V^{-}; green dots are weak Annexin V^{+}; red dots are strong Annexin V^{+++}.

The potential of the other platelet ITAM-containing receptor, FcγRIIA, to generate a platelet procoagulant response and MP formation was also evaluated. Comparably to GPVI, FcγRIIA consists of two extracellular immunoglobulin domains; however, the ITAM motifs of FcγRIIA are present in the cytoplasmic tail of the receptor, whereas in GPVI are in the FcRγ-chain dimers associated with GPVI receptor (Hibbs, *et al* 1988, Huang, *et al* 1992). Despite the distinct structural difference, the signalling pathway events are similar. Platelet activation through FcγRIIA was achieved by allowing IV.3 Fab fragments to bind to FcγRIIA for 3 minutes then cross-linking these with a secondary antibody, $F(ab')_2$ fragment goat anti-mouse IgG, for 10 minutes at 37°C. The concentration of the secondary antibody used was 7.5 µg/mL for each 1 µg/mL IV.3 (Table 4.1; personal communication with Monica Arman Albert, School of Clinical and Experimental Medicine, University of Birmingham). Activation of platelets through FcγRIIA elicited very powerful procoagulant activity. It induced Annexin V binding in 26.4±8.4 % positive of platelets at 10 µg/mL IV.3 Fab fragment (Figure 4.4 A). The strongly Annexin V^{+ve} particles were induced through FcγRIIA were a mixed of larger, platelet-sized and smaller, MP-sized particles (Figure 4.4 B). Interestingly, unlike GPVI, PS exposure in FcγRIIA-stimulated platelets increased proportionally with increasing concentrations of IV.3 and did not plateau. At a concentration of 50 µg/mL, the highest concentration used in this study, IV.3 Fab fragment was capable of inducing Annexin V binding in 40.6±11.1 % positive of platelets. Higher concentrations of IV.3 Fab fragment were not used due to the limitation of the concentration of the stock solution. This finding may suggest that there is a distinct difference in signalling through GPVI and FcγRIIA in respect of their action in generating platelet procoagulant activities, which needs further investigation. Activation through FcγRIIA also induced platelet degranulation in the majority of platelets. However, unlike the effects of activation through GPVI, regardless of the magnitude of P-selectin expression and PS exposure in FcγRIIA-

stimulated platelets, both P-selectin expression and PS exposure reached the maximum nearly at equivalent doses of IV.3 Fab fragment. From the IV.3 Fab fragment dose-titration curve in Figure 4.4 A, an intermediate dose, 15 µg/mL, was selected for use in subsequent experiments. Neither IV.3 Fab fragments nor its secondary antibodies induced a platelet procoagulant response when they interacted with platelets, confirming that the receptor dimerization/clustering is critical for FcγRIIA platelet procoagulant activity (Figure 4.4 C).

IV.3 Fab fragment	1 µg/mL	2 µg/mL	5 µg/mL	10 µg/mL	20 µg/mL	50 µg/mL
Goat anti-mouse IgG	7.5µg/mL	15 µg/mL	37 µg/mL	75 µg/mL	150µg/mL	370µg/mL

Table 4.1. Antibody concentrations used to activate platelets via FcγRIIA. Washed platelets were incubated with indicated doses of IV.3 fab fragments for 3 minutes then with the corresponding dose of the secondary antibodies, $F(ab')_2$ fragment goat anti-mouse IgG for10 minutes at 37°C.

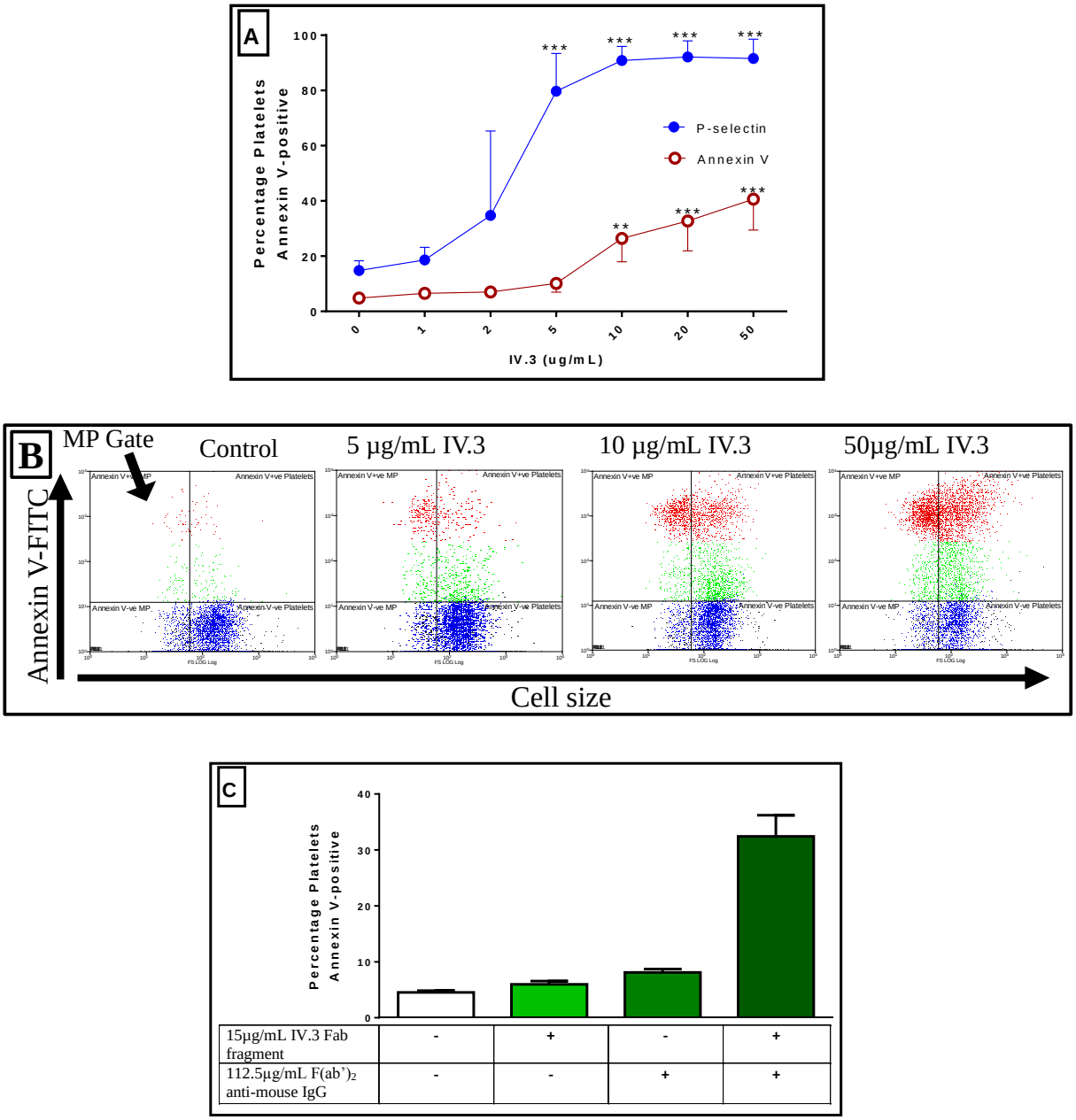


Figure 4.4. FcγRIIA Exclusively induces platelet procoagulant response. Panel A: Washed platelets were incubated with different doses of IV.3 fab fragments for 3 minutes then with the corresponding dose of the secondary antibodies, $F(ab')_2$ fragment goat anti-mouse IgG for10 minutes at 37°C. Annexin V binding and P-selectin expression were measured by flow cytometry. Data are from four different donors and are shown as mean±SD. P-values are compared to resting platelets; **p<0.001; ***p<0.0001. Panel B: Representative flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter of platelets stimulated with indicated does of IV.3 fab. Panel C: Annexin V binding to washed platelets incubated with either 15µg/mL IV.3 fab fragment, 112.5µg/mL $F(ab')_2$ fragment goat anti-mouse IgG or combination of both for10 minutes at 37°C (n=4).

Platelets contain a third ITAM-containing receptor, CLEC-2, which has not previously been studied in relation to its role in inducing the platelet procoagulant response. Unlike the typical ITAM-containing receptors that contain two YxxL motifs, CLEC-2 contains only a single YxxL motif (classed as a hemITAM motif) in its cytoplasmic tail. In addition, there is a distinct difference in the proximal phosphorylation mechanisms between CLEC-2 and GPVI. Signalling through CLEC-2 is believed to be mediated by both Src and Syk tyrosine kinases, whereas GPVI signalling is mediated exclusively by Src kinases (Spalton, *et al* 2009). However, despite the distinct differences in the proximal phosphorylation mechanisms between CLEC-2 and GPVI, the downstream signalling events are similar. Therefore, the question addressed here was whether CLEC-2 is also capable generating platelet procoagulant surfaces and MPs, similar to the other two platelet ITAM-containing receptors, GPVI and FcγRIIA. To address this question, washed platelets were activated through CLEC-2 using increasing doses of the snake venom, rhodocytin. Platelet activation through CLEC-2 induced Annexin V binding in relatively similar manner to activation through GPVI and FcγRIIA. Interestingly, rhodocytin, like CRP-XL, induced platelet PS exposure in a dose-dependent manner and reached a maximum at 30 nM where 24.2±10.9 % positive of platelet population bound to Annexin V, after which there was no further increase with higher doses of rhodocytin (Figure 4.5 A). The platelet procoagulant response induced by rhodocytin was also accompanied by the release of strongly Annexin V^{+ve} MPs, which formed the majority of the Annexin V^{+ve} events (Figure 4.5 C). CLEC-2 also induced P-selectin surface expression in the majority of rhodocytin-activated platelets. However, similar to FcγRIIA but not GPVI, platelet P-selectin expression in rhodocytin-stimulated platelets increased simultaneously with PS exposure, regardless to the magnitude of the expression of both markers. Because there was a sharp increase in both markers of activation between 10 and 30nM rhodocytin, a narrower range of doses was used between 1 and 300 nM rhodocytin to

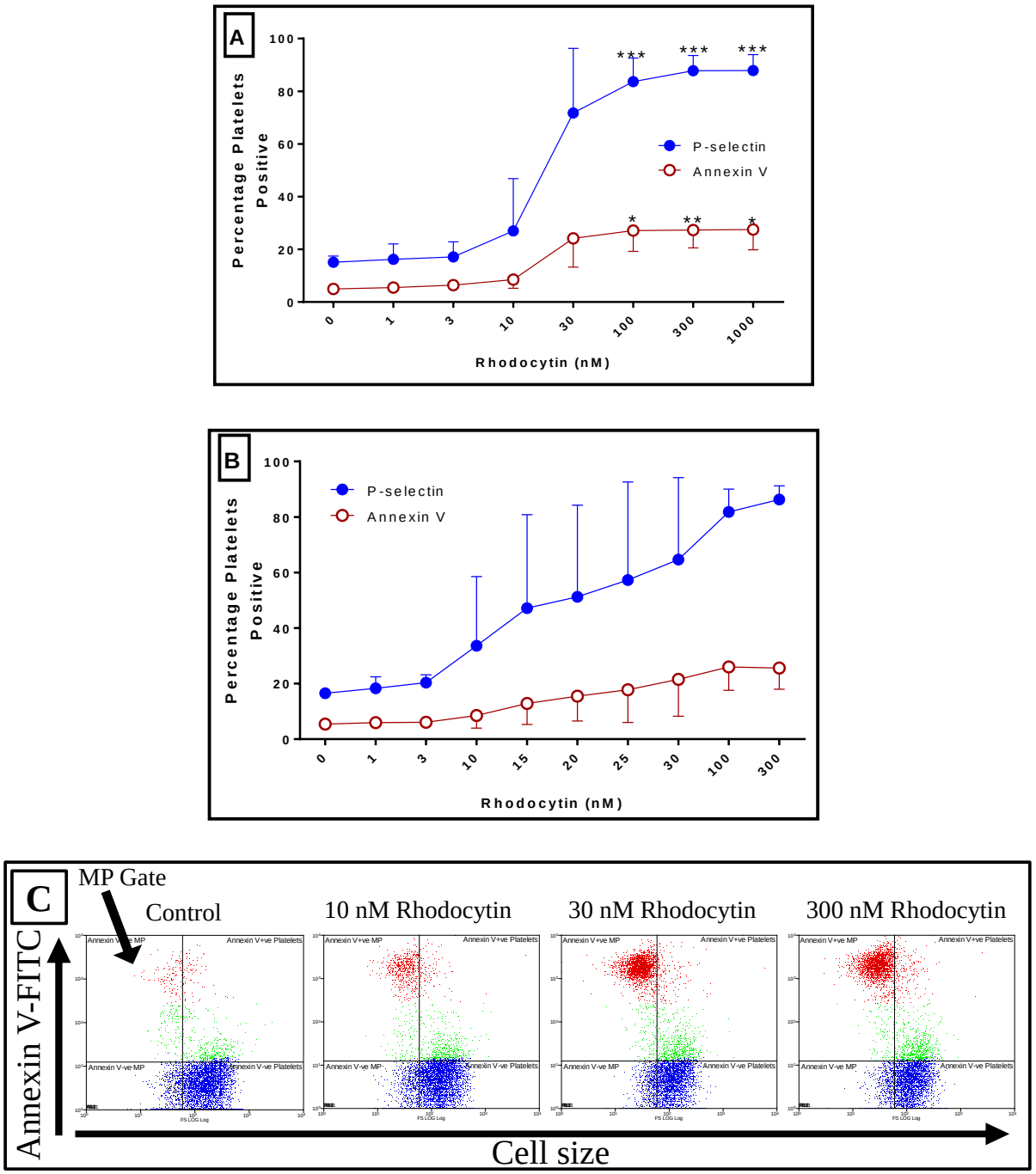


Figure 4.5. Platelet activation through CLEC-2 is procoagulant. Washed platelets were analysed for P-selectin expression and Annexin V binding by flow cytometry after stimulation by indicated concentrations of rhodocytin for10 minutes at 37°C. Data are from five different donors (Panel A), of which three included 10, 15 20 and 25 nM rhodocytin doses (Panel B). Data are presented as mean±SD and P-values are versus resting control platelets; *p<0.05; **p<0.001; ***p<0.0001. Panel C: Representative flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter of platelets stimulated with indicated does of rhodocytin.

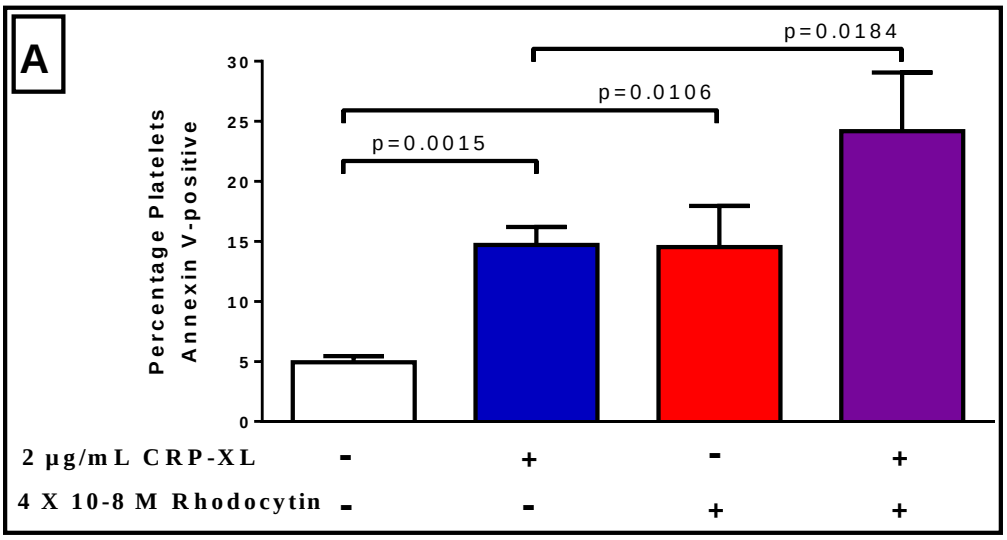
confirm whether the increase in P-selectin and Annexin V binding occurred simultaneously. Figure 4.5 B shows that over this dose range P-selectin expression and PS exposure increased in parallel. 40 nM rhodocytin was chosen as the concentration for subsequent experiments investigating the role of CLEC-2 platelet procoagulant activity.

Overall these date indicate that signalling through ITAM-containing receptors, in the absence of other agonists, or of outside-in signalling through integrins, is sufficient to induce a procoagulant response. However, there were differences in the relative potencies of the agonists for the three ITAM receptors agonists in inducing degranulation and PS exposure. Notably, signalling through CLEC2 or GPVI supports the view that the procoagulant response is restricted to a subpopulation of platelets, whereas signalling through the FcγRIIA receptor induced PS exposure on a higher percentage (~40%) of platelets. It is not possible to say whether the FcγRIIA-mediated effect would result in more than 40% of platelets, as higher concentrations of the IV.3 antibody could not be achieved.

4.2.2 Additive effect of ITAM-containing receptors on other ITAM-containing receptors

The possibility whether ITAM-containing receptors synergise with each other in their platelet procoagulant activity was examined. For this purpose, platelets were simultaneously co-stimulated with CRP-XL, rhodocytin or IV.3 or combination of these agonists. Strikingly, each of the ITAM-containing receptor showed a significant additive effect on the procoagulant activity of the others despite the fact they share similar downstream signalling events (Figure 4.6). Additionally, platelet MP release was enhanced when they were activated using more than one ITAM-containing receptor (Figure 4.7). Interestingly, platelet Annexin V binding was further enhanced when platelets were activated via all ITAM-containing

receptors (i.e. with IV.3, CRP-XL and rhodocytin together), suggesting that each ITAM-containing receptor may have its independent downstream signalling pathway. However, this needs further investigations to be established. It is noteworthy that the magnitude of the enhancement in the platelet procoagulant response is more pronounced with the combination of the hemITAM (CLEC-2) with typical ITAM (GPVI or FcγRIIA) receptors than the combination of two typical ITAM-containing receptors (i.e. GPVI and FcγRIIA). Furthermore, despite the enhancement in platelet Annexin V binding by co-stimulation with different combinations of agonists, the procoagulant response remained restricted to less than 60% of platelets. Notably, as observed above that platelet activation with IV.3 alone resulted in a generation of strongly Annexin V^{+ve} particles that was a mixture of larger, platelet-sized and smaller, MP-sized populations (Figure 4.4 B), co-stimulation with IV.3 and CRP-XL and/or rhodocytin showed similar two size-distinct strongly Annexin V^{+ve} populations. Interestingly, the larger, platelet-sized strongly Annexin V^{+ve} population was not observed in platelet stimulation with CRP-XL or rhodocytin or even with the combination of both agonists.



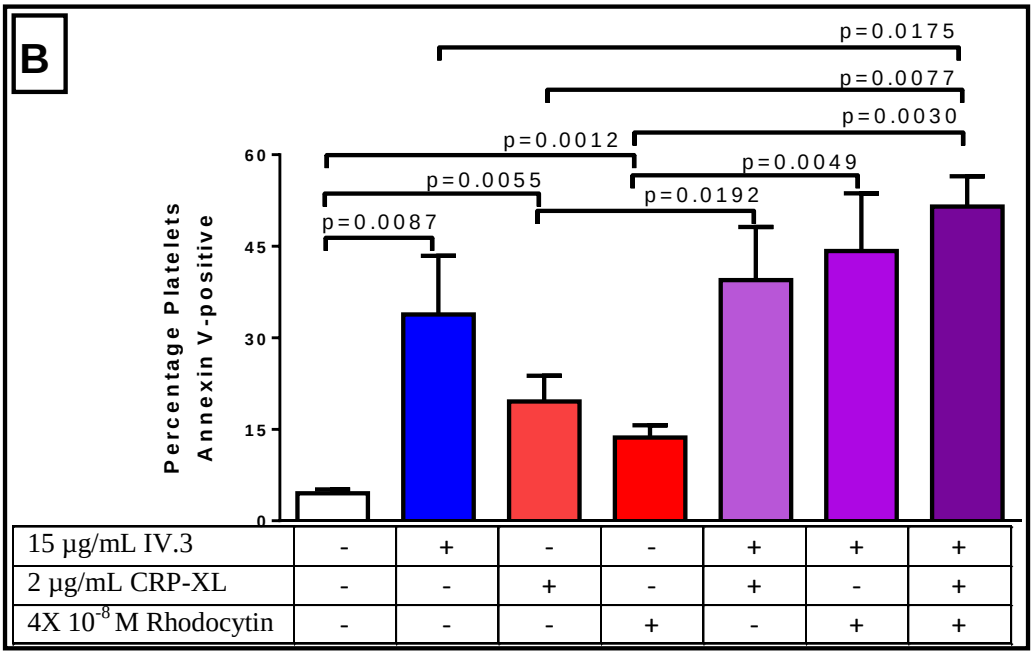


Figure 4.6. Additive effect of ITAM-containing receptors on other ITAM-containing receptors. Washed platelets were simultaneously co-stimulated with indicated agonists and analysed on flow cytometry for Annexin V binding. Panel A: additive effect of GPVI with CLEC-2 (n=4); Panel B: additive effect of FcγRIIA, GPVI and/or CLEC-2 (n=4). Data are from separate experiments performed using blood from different donors and presented as mean±SD.

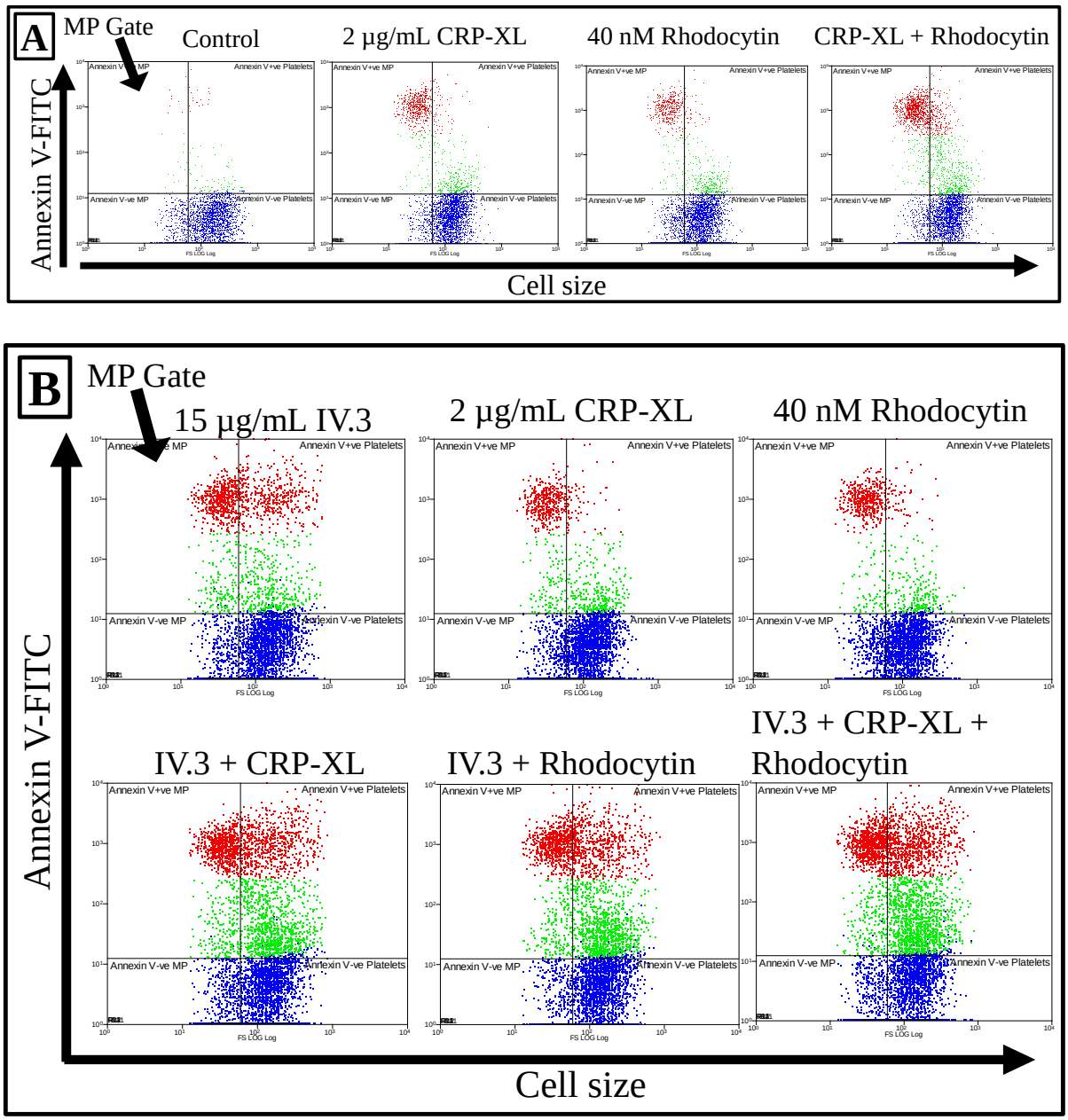
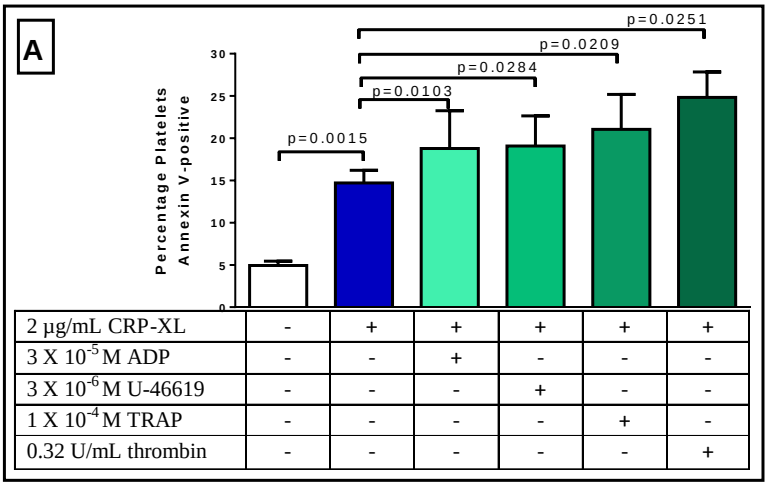


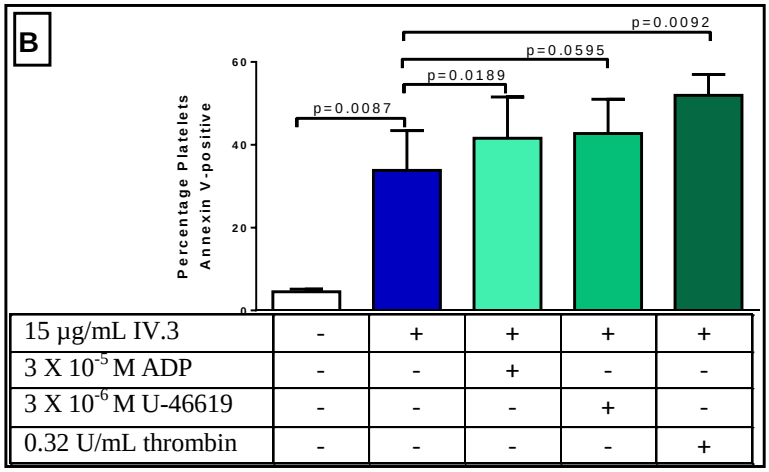
Figure 4.7. ITAM-containing receptors enhance each other in inducing platelet MP generation. Washed platelets were simultaneously co-stimulated with indicated agonists and analysed on flow cytometry for Annexin V binding. Panel A: shows representative examples of the additive effect of GPVI with CLEC-2; Panel B: shows representative examples of the additive effect of FcγRIIA, GPVI and/or CLEC-2. Flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter of platelets stimulated with indicated agonists. In the dot-plot graphs; the upper left-hand quadrant shows Annexin V^{+ve} events in MP-size gate, the lower left-hand quadrant shows Annexin V^{-ve} events in MP-size gate, the upper right-hand quadrant shows Annexin V^{+ve} events in platelet-size gate, the lower right-hand quadrant shows Annexin V^{-ve} events in platelet-size gate; blue dots are Annexin V^{-}; green dots are weak Annexin V^{+}; red dots are strong Annexin V^{+++}.

4.2.3 Synergistic effect of GPCRs on ITAM-containing receptors

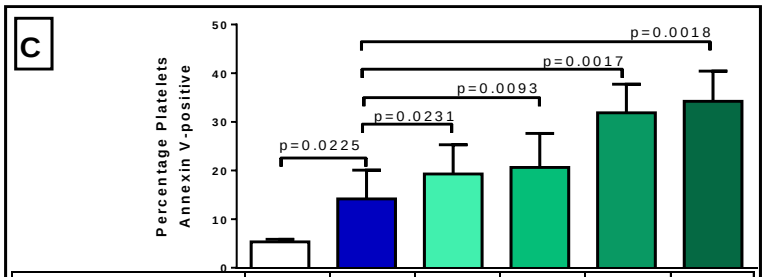
To define the relative contribution of GPCRs to the role of each ITAM-containing receptor in inducing the platelet procoagulant response, experiments were designed to evaluate the effect of each GPCR agonist on platelet PS exposure in ITAM-containing receptor activated platelets. For this purpose, platelets were simultaneously co-stimulated with one of each of the ITAM-containing receptor agonists alongside ADP, U-46619, TRAP or thrombin at concentrations previously determined to induce maximal platelet activation, as defined by P-selectin expression. ADP, U46619, TRAP and thrombin all showed a potent synergistic effect on the platelet procoagulant activity of GPVI, CLEC-2 and FcγRIIA inducing a significant enhancement in platelet PS exposure (Figure 4.8) and MP generation (Figure 4.9). It can be clearly seen that thrombin is more potent than ADP or TxA_2 in enhancing the procoagulant activity of any of the ITAM-containing receptors. This finding is in agreement with previous reports that showed that thrombin plus collagen is the most potent platelet procoagulant inducer (Alberio, *et al* 2000, Bevers, *et al* 1983). TRAP also showed comparable potency to thrombin in terms of the synergistic effect to platelet procoagulant activity induced by CRP-XL and rhodocytin. This finding suggests that PAR1 is the main mediator for thrombin aaditive effect, which is in the line with previous reports (Andersen, *et al* 1999, Keuren, *et al* 2005).



2 µg/mL CRP-XL	-	+	+	+	+	+
3 X 10^{-5} M ADP	-	-	+	-	-	-
3 X 10^{-6} M U-46619	-	-	-	+	-	-
1 X 10^{-4} M TRAP	-	-	-	-	+	-
0.32 U/mL thrombin	-	-	-	-	-	+



15 µg/mL IV.3	-	+	+	+	+
3 X 10^{-5} M ADP	-	-	+	-	-
3 X 10^{-6} M U-46619	-	-	-	+	-
0.32 U/mL thrombin	-	-	-	-	+



4 X10^8 M Rhodocytin	-	+	+	+	+	+
3 X 10^{-5} M ADP	-	-	+	-	-	-
3 X 10^{-6} M U-46619	-	-	-	+	-	-
1 X 10^{-4} M TRAP	-	-	-	-	+	-
0.32 U/mL thrombin	-	-	-	-	-	+

Figure 4.8. Synergistic effect of GPCRs on ITAM-containing receptors procoagulant activity. Washed platelets were simultaneously co-stimulated with indicated agonists and analysed on flow cytometry for Annexin V binding. Panel A: synergistic effect of GPCRs on GPVI (n=4); Panel B: synergistic effect of GPCRs on FcγRIIA (n=4); Panel C: synergistic effect of GPCRs on CLEC-2. Data are from five separate experiments performed using blood from different donors and shown as mean±SD.

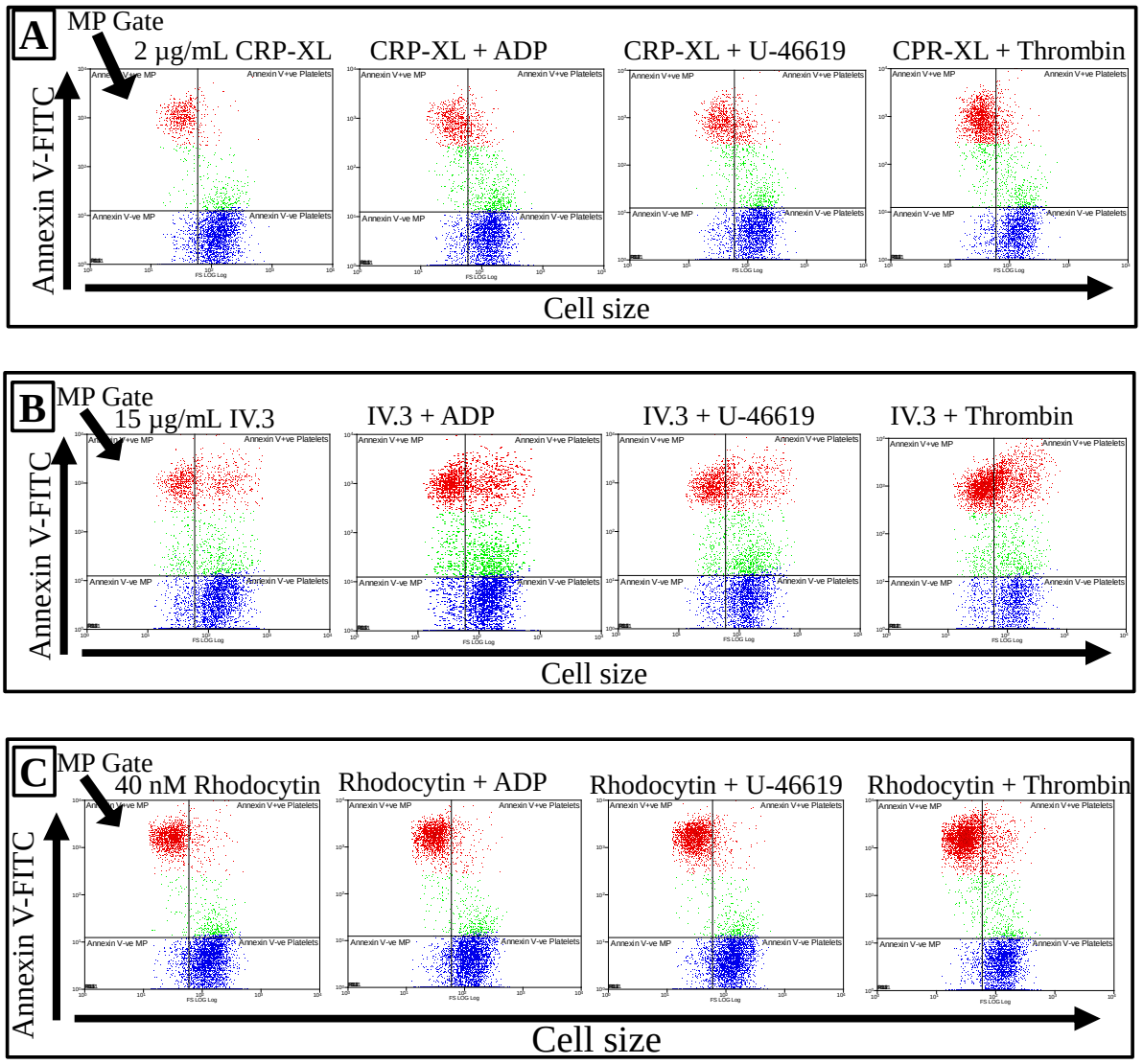


Figure 4.9. GPCRs show strong synergistic effect on platelet MP release induced by ITAM-containing receptors. Washed platelets were simultaneously co-stimulated with indicated agonists and analysed on flow cytometry for Annexin V binding. Panels A, B and C show representative examples of the synergistic effect of GPCRs on the MP generation induced by GPVI, FcγRIIA and CLEC-2 respectively. Flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter of platelets stimulated with indicated agonists. In the dot-plot graphs; the upper left-hand quadrant shows Annexin V^{+ve} events in MP-size gate, the lower left-hand quadrant shows Annexin V^{-ve} events in MP-size gate, the upper right-hand quadrant shows Annexin V^{+ve} events in platelet-size gate, the lower right-hand quadrant shows Annexin V^{-ve} events in platelet-size gate; blue dots are Annexin V^{-}; green dots are weak Annexin V^{+}; red dots are strong Annexin V^{+++}.

4.2.4 Mechanisms underlying platelet procoagulant response induced through CLEC-2

Having established that CLEC-2 induces platelet procoagulant response, further characterisation of some of the mechanisms underlying this novel function for CLEC-2 were investigated. Since the role of GPVI in inducing the platelet procoagulant response is well studied, it was logical to compare CLEC-2 with GPVI in these experiments.

4.2.4.1 **Role of released secondary mediators**

As shown in section 4.2.3 exogenous ADP and TxA_2 showed strong synergistic effect on the platelet procoagulant activity of all platelet ITAM-containing receptors. ADP and TxA_2 are known to play a critical role in positive feedback mechanism required for sustained platelet activation, therefore, it was reasonable to conduct further investigation into the role of the released endogenous levels of these agonists in platelet procoagulant response induced by CLEC-2 and GPVI.

4.2.4.1.1 **ADP**

Activation through ADP receptors was inhibited using specific antagonist of $P2Y_1$ (10 µM MRS 2179), or the $P2Y_{12}$ (3×10^{-7} M AR-C69931MX), or both. In order to ensure that all ADP effects had been abolished, a further sample was stimulated in the presence of ATP/ADP scavenger apyrase (at a final concentration of 80 µg/mL). These experimental conditions had been previously established by Jackie A. Appleby (PhD thesis, 2006, Department of Cardiovascular Sciences, University of Leicester) to evaluate the role of endogenous ADP in platelet procoagulant activity induced by CRP-XL, but using 1:10 diluted whole blood rather than washed platelets. Therefore, it was decided to evaluate the

role of ADP receptors in CLEC-2 platelet procoagulant activity using the same above conditions but in washed platelet preparation. Strikingly, and in contrast to the previous findings in diluted whole blood, inhibiting $P2Y_1$ with MRS 2179 produced a significant enhancement in Annexin V binding in rhodocytin-stimulated platelets (from 11.9±4.7 to 17.7±7.0 % positive, p=0.0387) (Figure 4.10). However, AR-C69931MX and apyrase caused a dramatic reduction in Annexin V binding to rhodocytin-stimulated platelets (from 11.9±4.7 to 6.5±1.9 and 7.00±2.2 % positive respectively) although it did not completely abolish the effect, which is consistent with the previous findings for CRP-XL-stimulated platelets.

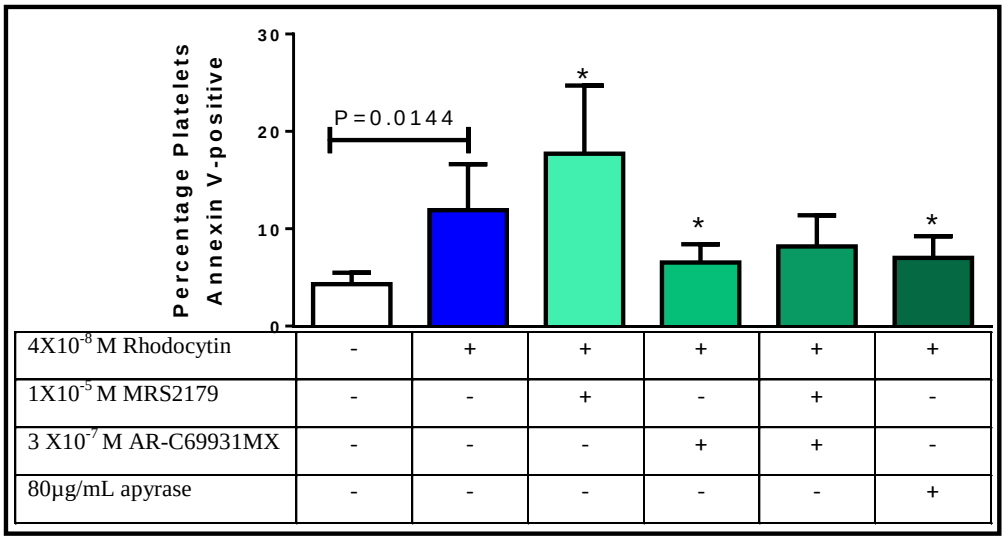


Figure 4.10. Contribution of $P2Y_1$ and $P2Y_{12}$ receptors to PS exposure induced by rhodocytin in washed platelets. Annexin V binding in rhodocytin-activated washed platelets in the presence or absence of the ADP receptor inhibitors MRS 2179 and AR-C69931MX or apyrase as indicated. Data are from five different donors and are shown as mean±SD. P-values are compared to rhodocytin-activated platelets unless indicated; *p<0.05.

Since blocking the $P2Y_1$ receptor in washed platelets showed an increase with MRS 2179 in response to rhodocytin stimulation, in contrast to the previous findings with CRP-XL-stimulated platelets in whole blood, it was sensible to conduct further investigations to determine whether this was due to an artefact caused during platelet sample preparation. Washed platelets were therefore stimulated with CRP-XL in the presence or absence of MRS 2179 and analysed for Annexin V binding. Interestingly, the presence of MRS 2179 also markedly enhanced Annexin V binding in CRP-XL-stimulated platelets (from 20.6±2.9 to 31.3±2.6 % positive) (Figure 4.11). This finding suggests that the washing procedure may de-sensitise the P2Y1 receptor.

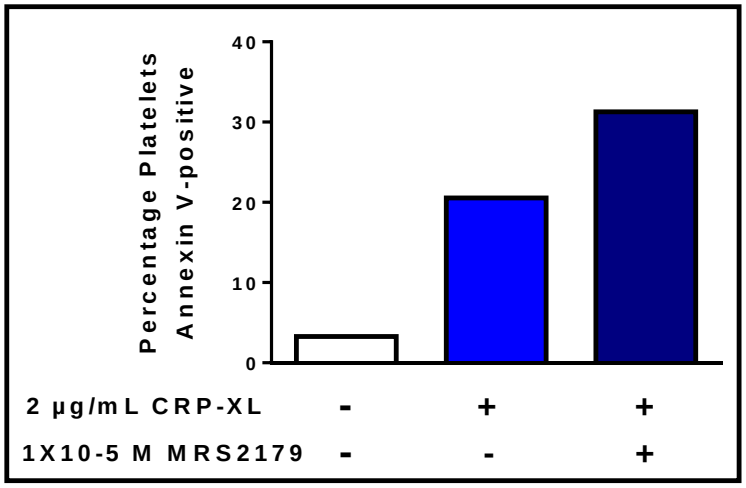
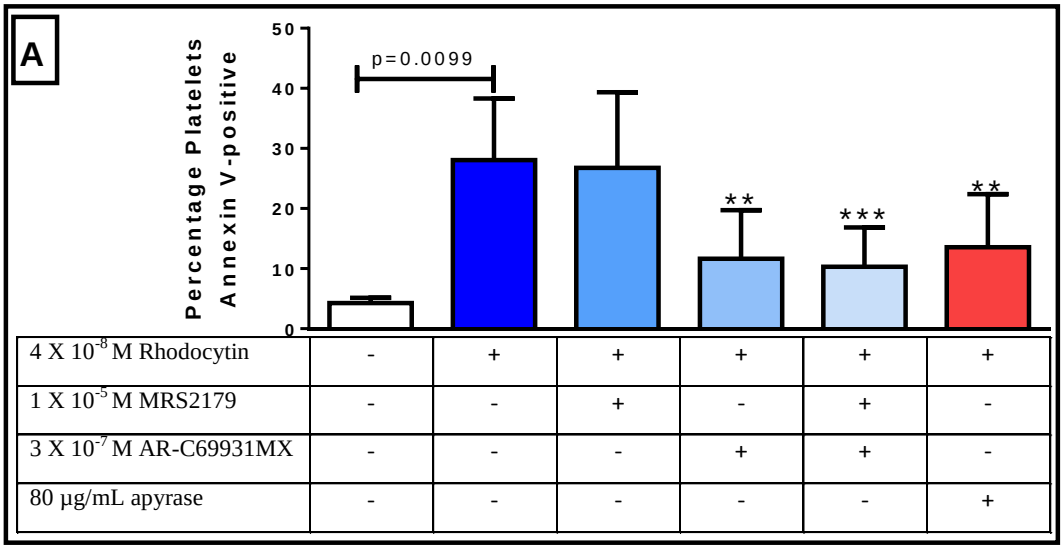


Figure 4.11. Blocking $P2Y_1$ by MRS 2179 enhances platelet procoagulant response induced by CRP-XL. Washed platelets were stimulated with 2 µg/mL CRP-XL for 10 minutes at 37°C in the presence or absence of the $P2Y_1$ antagonist MRS 2179 and analysed on flow cytometry for Annexin V binding. Data are shown as mean (n=2).

The effects of ADP receptors in the CLEC-2 platelet procoagulant activity was therefore retested in PRP samples, adding GPRP peptide at 0.5 mg/ml to prevent fibrin polymerisation. Figure 4.12 A shows that Annexin V binding in rhodocytin-stimulated platelets was slightly inhibited in the presence of MRS 2179, in line with the previous findings of CRP-XL-stimulated platelets in whole blood sample preparation, whereas AR-C69931MX markedly inhibited the response. Blocking both of ADP receptors in the PRP samples showed an even greater reduction in platelet Annexin V binding. These data suggest that stimulation via $P2Y_{12}$ plays the major role in enhancing platelet procoagulant activity induced through CLEC-2. In addition, $P2Y_{12}$ seems to play the major role in MP generation as it is shown in Figure 4.12 B, where AR-C69931MX showed a significant decrease in the number of MPs, but not in the number of PS-exposing platelets. However, blocking $P2Y_1$ with MRS 2179 showed negligible amount of reduction in the number of both Annexin V positive platelets and MPs. Overall, the inhibitory effect of blocking ADP as well as the enhancement induced by the exogenous ADP that was seen in section 4.2.3, all indicate that ADP plays a critical role in CLEC-2 platelet procoagulant activity and MP formation.



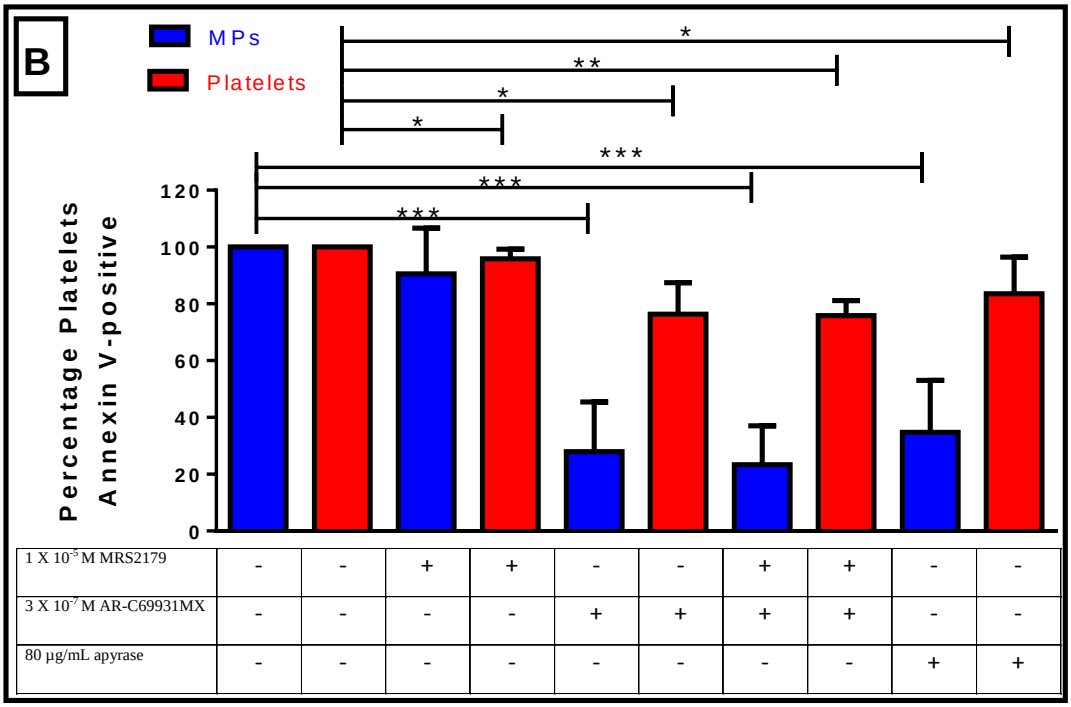
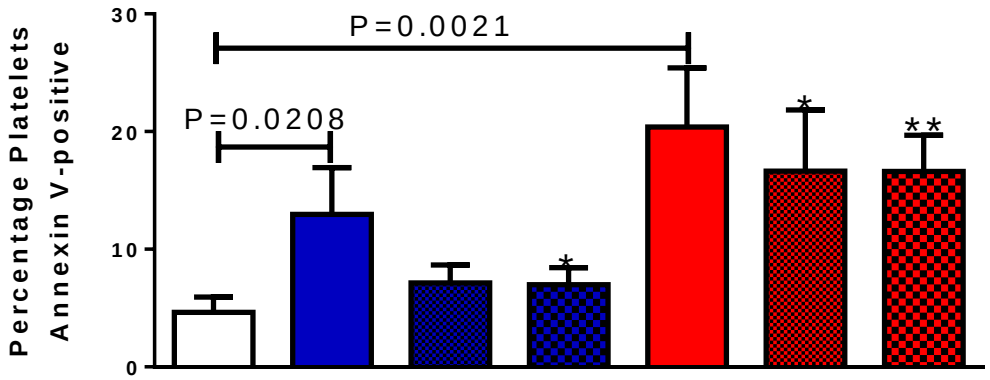


Figure 4.12. Different impact of ADP receptors on platelet PS exposure induced by rhodocytin in PRP samples. (A) Annexin V binding in rhodocytin-activated platelets in PRP samples the presence or absence of the ADP receptor inhibitors MRS 2179 and AR-C69931MX or apyrase as indicated. (B) Percentage reduction in procoagulant platelets and MPs in rhodocytin-activated PRP samples after inhibition by apyrase, MRS 2179 and/or AR-C69931MX. MPs were discriminated from platelets according to their size. Data are from four separate experiments performed using blood from different donors and shown as mean±SD. P-values are compared to rhodocytin-activated platelets unless indicated; *p<0.05; **p<0.001; ***p<0.0001.

4.2.4.1.2 **TxA_2**

To investigate the role of endogenously released TxA_2 in the platelet procoagulant response induced through CLEC-2 and GPVI, TxA_2 synthesis was blocked by aspirin. Aspirin (acetylsalicylic acid: ASA) is one of the most widely used anti-platelet drugs which irreversibly blocks COX-1, thus blocks TxA_2 synthesis. Washed platelets were pre-incubated for 30 minutes with two doses of aspirin (low dose; 8.3×10^{-5} M and high dose; 5×10^{-4} M which are roughly equivalent to therapeutic doses of 75 and 450 mg respectively) followed by activation with CRP-XL or rhodocytin. Interestingly, platelet Annexin V binding induced by rhodocytin was significantly inhibited by both doses of aspirin (from 13.0±4.0 to 7.1±1.5 and 7.0±1.5 % positive for low and high doses respectively). On the other hand, aspirin had only a modest effect, at either dose on Annexin V binding in CRP-XL-stimulated platelets (from 20.4±5.0 to 16.7±5.2 and 16.6±3.1 % positive for low and high doses respectively) compared to rhodocytin-stimulated platelets (Figure 4.13). However, it could be argued that since the magnitude of Annexin V binding in CRP-XL-stimulated platelets was higher than with rhodocytin-stimulated platelets, the effect of aspirin would be less. The inhibitory effect of aspirin further supports previous finding of the contribution of exogenous TxA_2 (TP) receptor agonist U-46619 in enhancing the platelet procoagulant activity of CLEC-2 and GPVI (section 4.2.3).

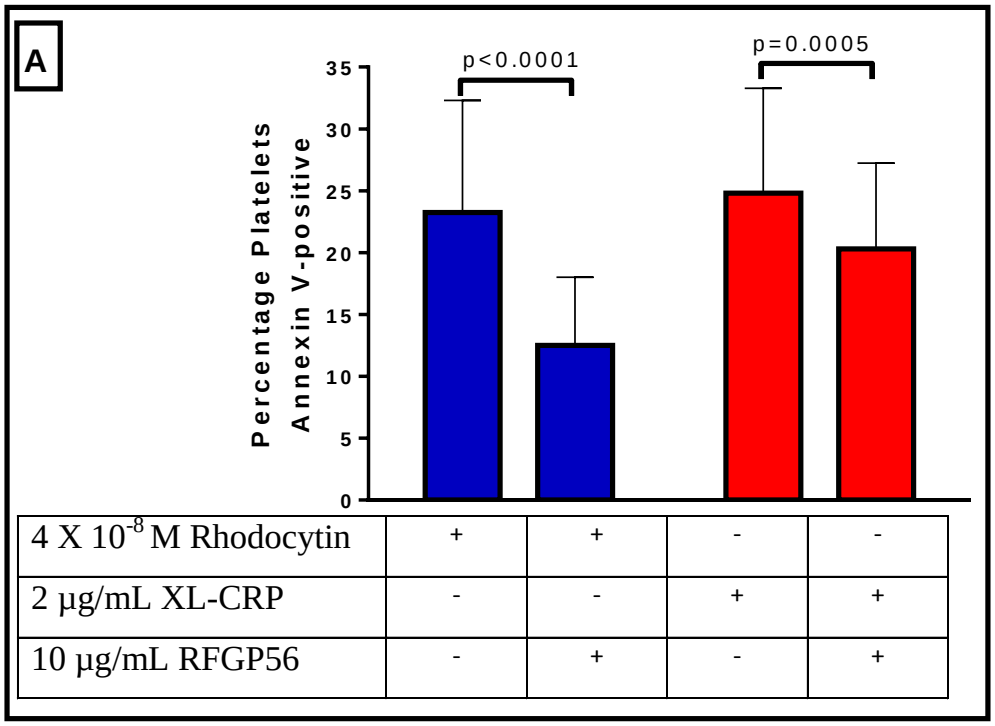


4 X 10^{-8} M Rhodocytin	-	+	+	+	-	-	-
2 µg/mL CRP-XL	-	-	-	-	+	+	+
8.3 X 10^{-5} M Aspirin (n=3)	-	-	+	-	-	+	-
5 X 10^{-4} M Aspirin (n=5)	-	-	-	+	-	-	+

Figure 4.13. Annexin V binding in aspirinated platelets. Washed platelets were analysed for PS exposure by flow cytometry after activation with rhodocytin or CRP-XL in the presence or absence of two doses of aspirin as indicated. Data are from five separate experiments performed using blood from different donors, of which three included 8.3 X 10^{-5} M aspirin dose. P-values are versus activated platelets in the absence of aspirin unless indicated; *p<0.05; **p<0.001; ***p<0.0001.

4.2.4.2 **Role of outside-in signalling through GPIIb/IIIa**

Outside-in signalling through GPIIb/IIIa plays a crucial role in platelet activation. Ligand binding to GPIIb/IIIa induces the phosphorylation of the two NxxY motifs in the cytosolic tail of GPIIIa through SFK-dependent signalling events, which ultimately activates PLCγ2 (reviewed by (Li, *et al* 2010)). Since GPIIb/IIIa shares, to some extent, similar downstream signalling pathways of GPVI and CLEC-2 where all activate PLCγ2, it was decided to block GPIIb/IIIa in all the previous experiments to make platelet activation more dependent on GPVI and CLEC-2 signalling pathways. To assess the contribution of inhibiting GPIIb/IIIa in platelet PS exposure induced through CELC-2, GPVI and FcγRIIA, washed platelets were stimulated with rhodocytin, CRP-XL and IV.3 respectively in the presence or absence of RFGP56 antibody. The presence of the anti-GPIIb/IIIa antibody produced a significant decrease in the percentage of PS-exposing platelets in response to rhodocytin (from 23.3±9.0 to 12.5±5.5 % positive) (Figure 4.14 A). This was greater than the inhibition seen when GPIIb/IIIa was blocked for CRP-XL-stimulated platelets (from 24.8±8.5 to 20.3±6.9 % positive). These findings are in agreement with previous reports that GPIIb/IIIa inhibition results in a decrease of platelet procoagulant activity including prothrombinase activity and PS exposure using various agonists (Dormann, *et al* 2000, Furman, *et al* 2000, Lages and Weiss 2001, Ramstrom, *et al* 2003). It is unclear, however, whether this reduction in platelet procoagulant activity through CLEC-2 and GPVI is due to the inhibition of the GPIIb/IIIa signalling pathway or of platelet-platelet interaction. It is less likely to be platelet-platelet interaction because the experiments were carried out in in the absence of fibrinogen, although it should be acknowledged that activated platelets release fibrinogen from their α-granules. In sharp contrast, RFGP56 antibody showed no significant impact on the procoagulant activity of platelets mediated through FcγRIIA (from 30.2±10.1 to 32.4±7.6 % positive; p=0.1742) (Figure 4.14 B).



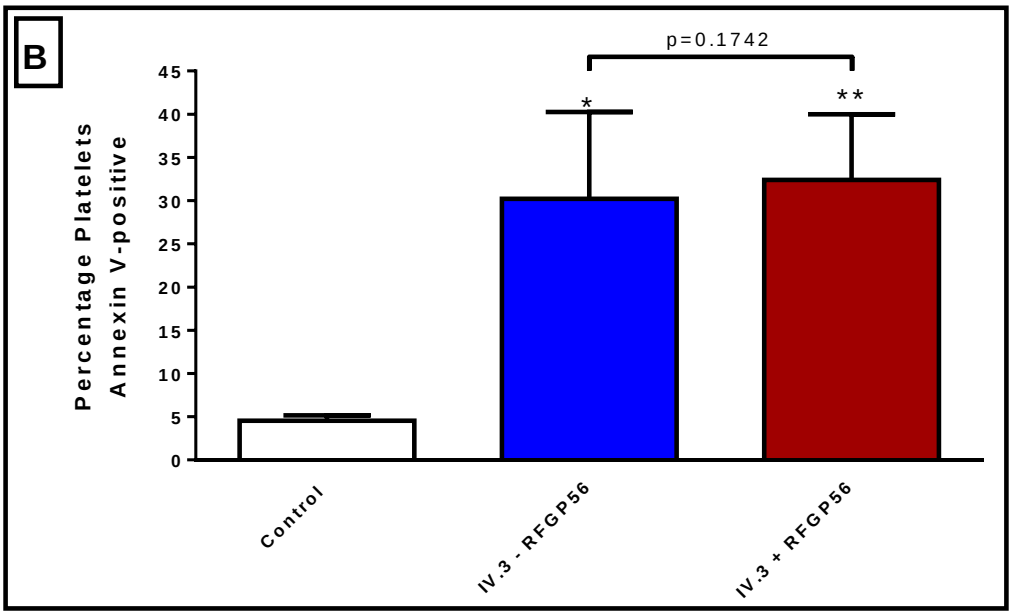


Figure 4.14. Blocking GPIIb/IIIa inhibits CLEC-2 and GPVI but not FcγRIIA mediated platelet procoagulant response. Washed platelets were activated with rhodocytin, CRP-XL (A; n=11) and IV.3 (B; n=4) in the presence or absence of the GPIIb/IIIa blocking antibody, 10µg/mL RFGP56. Platelets PS exposure was measured using Annexin V binding on flow cytometry. Data are presented mean±SD. Unless indicated, P-values are versus control platelets; *p<0.05; **p<0.001.

4.2.4.3 **Role of Src and Syk kinases**

An interesting difference between CLEC-2 and GPVI signalling pathways is that the phosphorylation of the ITAM motif of the GPVI/FcRy-chain is mainly dependent on the Src family kinases phosphorylation which subsequently leads to the activation of Syk kinases, whereas for CLEC-2 this is mediated by both Src and Syk tyrosine kinases (Spalton, *et al* 2009). Therefore, it was decided to evaluate the relative contribution of Src and Syk kinases in CLEC-2 and GPVI platelet procoagulant activity. Dasatinib and PP2, Src family tyrosine kinase (SFK) inhibitors, were used to inhibit the Src signalling pathway. Additionally, Syk kinase activity inhibitors, PRT-318 and R405, were used to investigate the role of Syk kinases. Serial dilutions of each inhibitor were used to determine the optimal doses (data not shown), which were based on those used previously in the literature (Hantschel, *et al* 2007, Reilly, *et al* 2011, Spalton, *et al* 2009). However, due to the limitations of the stock concentration, lower concentration of PP2 than those used in the literature were used (5 µM instead of 20 µM). Figure 4.15 A shows that platelet PS exposure induced by rhodocytin or CRP-XL was completely abolished when Src was inhibited by 1 µM dasatinib. Similarly, 5 µM PP2 showed significant, although not complete, inhibition of CLEC-2 and GPVI platelet procoagulant activity (Figure 4.15 B). However, the partial inhibition may due to the relatively low concentration of PP2 used. Inhibition of Syk kinase activity by 1 µM PRT-318 completely abrogated platelet Annexin V binding induced through CLEC-2 and GPVI (Figure 4.15 C). The other Syk kinase activity inhibitor, R406, at 1 µM also gave complete inhibition of PS exposure in CLEC-2-stimulated platelets; however, Annexin V binding to CRP-XL-stimulated platelets was also partially inhibited (Figure 4.15 D). Despite the variations between the inhibitors used, which probably stem from different levels of specificity of these agents, these data confirm that both Src and Syk kinases are critical for platelet procoagulant response induced though CLEC-2 and GPVI.

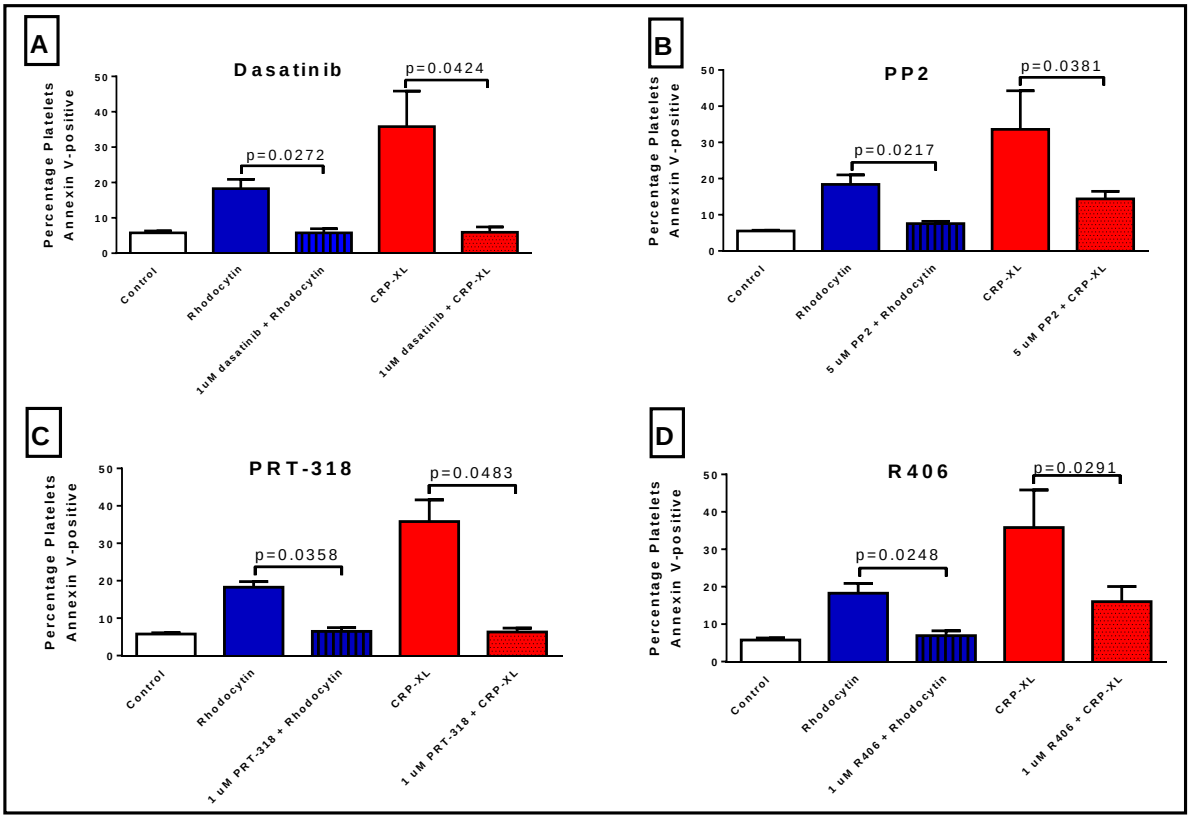
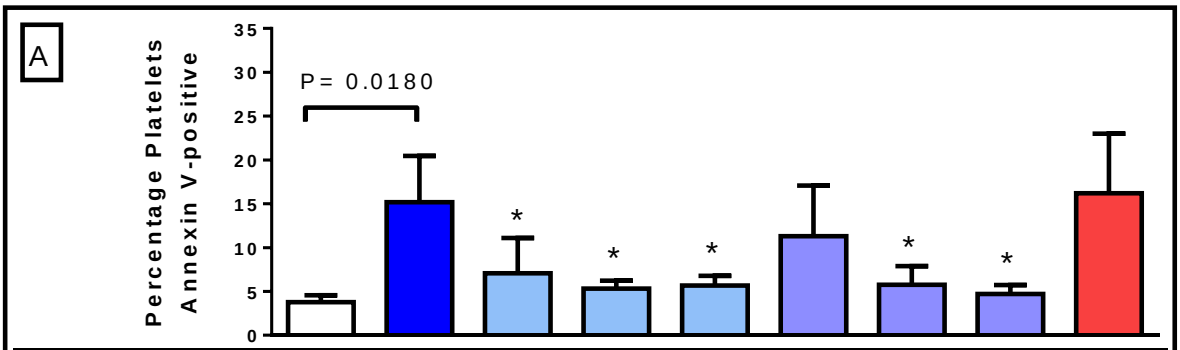


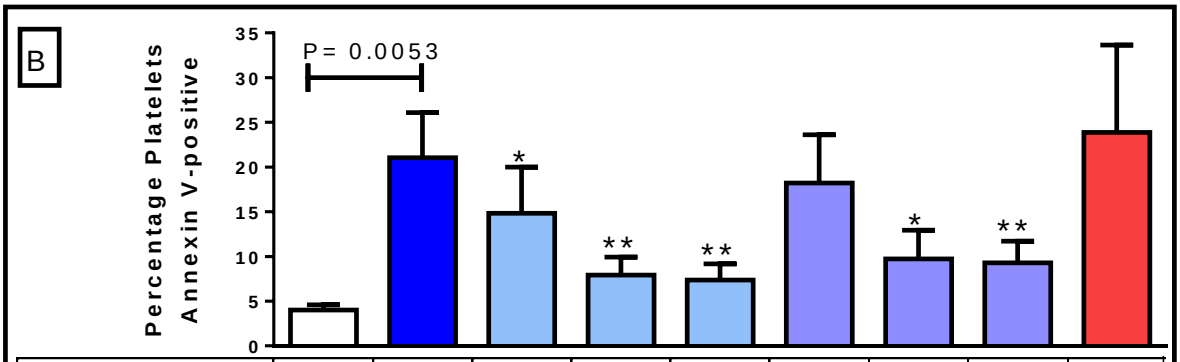
Figure 4.15. CLEC-2 and GPVI platelet procoagulant activity is dependent on Src and Syk kinases. Washed platelets were pre-treated with 1 µM dasatinib (Panel A), 5 µM PP2 (Panel B), 1 µM PRT-318 (Panel C) or 1 µM R405 (Panel D) prior to platelet stimulation. Platelets were activated by 40 nM rhodocytin or 2 µg/mL CRP-XL and analysed for PS exposure using Annexin V binding assay. Data are from four separate experiments performed using blood from different donors and shown as mean±SD.

4.2.4.4 **Role of PI3K**

PI3K plays a central role in mediating the activation of PLCγ2, which is essential for platelet activation through ITAM-containing receptors. To confirm the role of PI3K in inducing platelet PS exposure induced through CELC-2 and GPVI, platelets were pre-incubated with two different PI3K inhibitors, LY294002 and Wortmannin, at a range of concentrations. As shown on Figure 4.16 A, both LY294002 and Wortmannin significantly inhibited, although not completely abrogated, PS exposure in rhodocytin-stimulated platelets in dose-dependent manner. Additionally, in a similar manner to rhodocytin, CRP-XL-stimulated platelets showed a significant reduction in Annexin V binding in the presence of both PI3K inhibitors (Figure 4.16 B). The vehicle, DMSO, when diluted 1:40, the same dilution as the highest concentration of LY294002, showed a slight increase in the percentage of PS-exposing platelets with both rhodocytin and CRP-XL. This finding suggests that the presence of DMSO may have resulted in an underestimation of the effect of both inhibitors.



4 X10^{-8} M Rhodocytin	-	+	+	+	+	+	+	+	+
2.5 uM LY294002	-	-	+	-	-	-	-	-	-
12.5 uM LY294002	-	-	-	+	-	-	-	-	-
25 uM LY294002	-	-	-	-	+	-	-	-	-
10 nM Wortmannin	-	-	-	-	-	+	-	-	-
50 nM Wortmannin	-	-	-	-	-	-	+	-	-
100 nM Wortmannin	-	-	-	-	-	-	-	+	-
Vehicle	-	-	-	-	-	-	-	-	+



2 µg/mL XL-CRP	-	+	+	+	+	+	+	+	+
2.5 uM LY294002	-	-	+	-	-	-	-	-	-
12.5 uM LY294002	-	-	-	+	-	-	-	-	-
25 uM LY294002	-	-	-	-	+	-	-	-	-
10 nM Wortmannin	-	-	-	-	-	+	-	-	-
50 nM Wortmannin	-	-	-	-	-	-	+	-	-
100 nM Wortmannin	-	-	-	-	-	-	-	+	-
Vehicle	-	-	-	-	-	-	-	-	+

Figure 4.16. PS exposure in rhodocytin- and CRP-XL-stimulated platelets is mediated through PI3K. Washed platelets were incubated with PI3K inhibitors or vehicle (DMSO) as indicated for 15 minutes at 37°C prior to activation with rhodocytin (figure A) or CRP-XL (figure B). Subsamples were then analysed for PS exposure by flow cytometry using Annexin V binding assay. Data are from four separate experiments performed using blood from different donors and shown as mean±SD. P-values are versus activated platelets unless indicated; *p<0.05; **p<0.001.

4.2.4.5 **Inter- and intra-individual variability in procoagulant response**

There is known to be a marked inter-individual variation in the platelet response to CRP-XL, due largely to polymorphisms in the GPVI receptor (Jones, *et al* 2009). To see whether signalling through CLEC2 was also variable between individuals the platelet procoagulant response to maximum doses of rhodocytin and CRP-XL was evaluated in fifty normal healthy donors. There was considerable variation in the response to both agonists but interestingly, there was more inter-individual variability in response to rhodocytin than to CRP-XL. There was more than 9-fold difference between the percentage of PS-exposing platelets in the lowest and highest responders to rhodocytin in tested donors (SD; 13.3±7.5 range: 3.9-36.4 % positive), compared to less than 4-fold in CRP-XL in this aspect (SD; 23.1±7.6 range: 11.2-42.5 % positive) (Figure 4.17 A). In addition, and in line with previous findings donors showed significant higher platelet Annexin V binding in response to CRP-XL than rhodocytin. Interestingly, donors showed a strong correlation in their platelet procoagulant responses to these two stimuli (Figure 4.17 B). To demonstrate that this variability was not simply due to inter-assay variation, the procoagulant responses to rhodocytin and CRP-XL were measured on up to four separate occasions in a number of the donors. Figure 4.18 shows that donors maintained their relative response to rhodocytin and CRP-XL throughout the different visits, reasonably well. Donors' relative procoagulant responses were also consistent over the range of doses of rhodocytin, suggesting that the inter-individual variability is not simply due to the sensitivity to rhodocytin (Figure 4.19). All these finding together confirm that the variability between donors in their platelets ability to respond to rhodocytin and CRP-XL is mainly dependent on the relative procoagulant activity of CLEC-2 and GPVI.

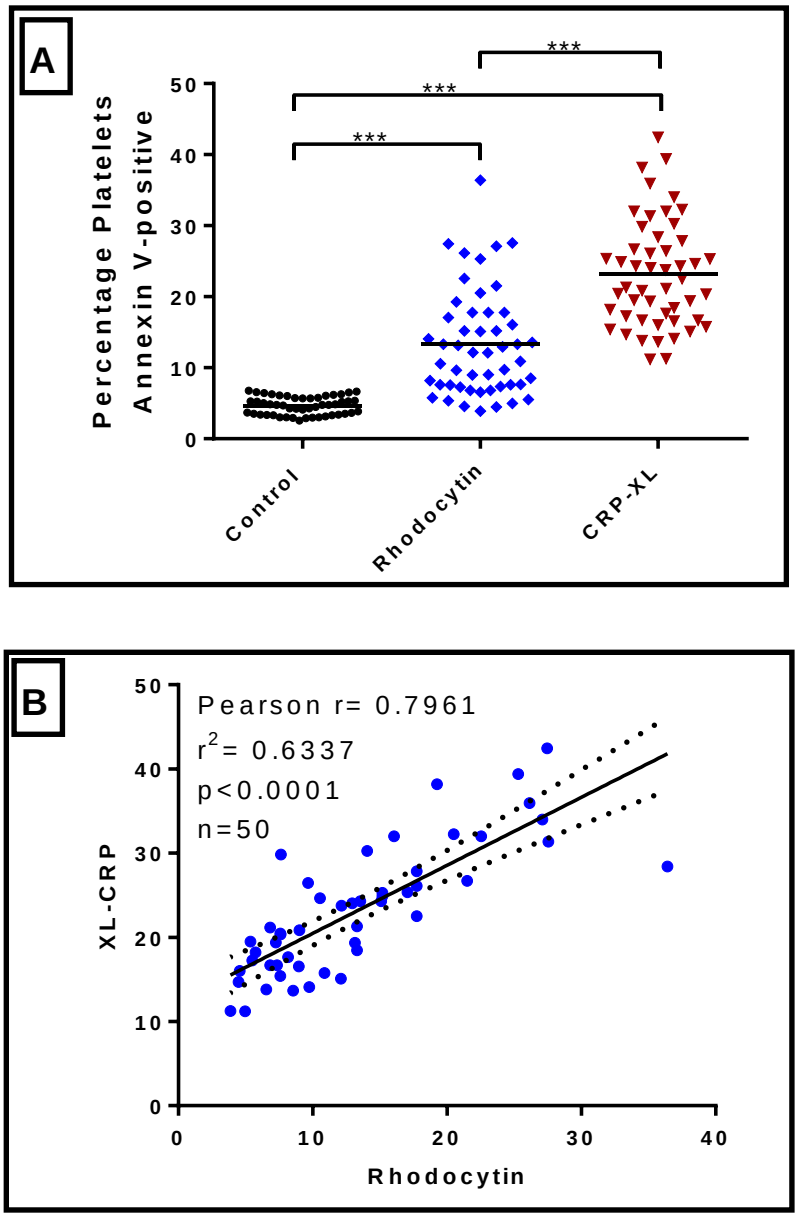


Figure 4.17. Variability in individual responses to rhodocytin and CRP-XL. Washed platelets from normal, healthy donors were stimulated with 40 nM rhodocytin or 2 µg/mL CRP-XL for 10 minutes at 37°C (n=50). (A) shows Annexin V binding, whereas (B) shows the correlation between individuals in their platelet procoagulant responses to rhodocytin vs. CRP-XL. P-values; ***p<0.0001.

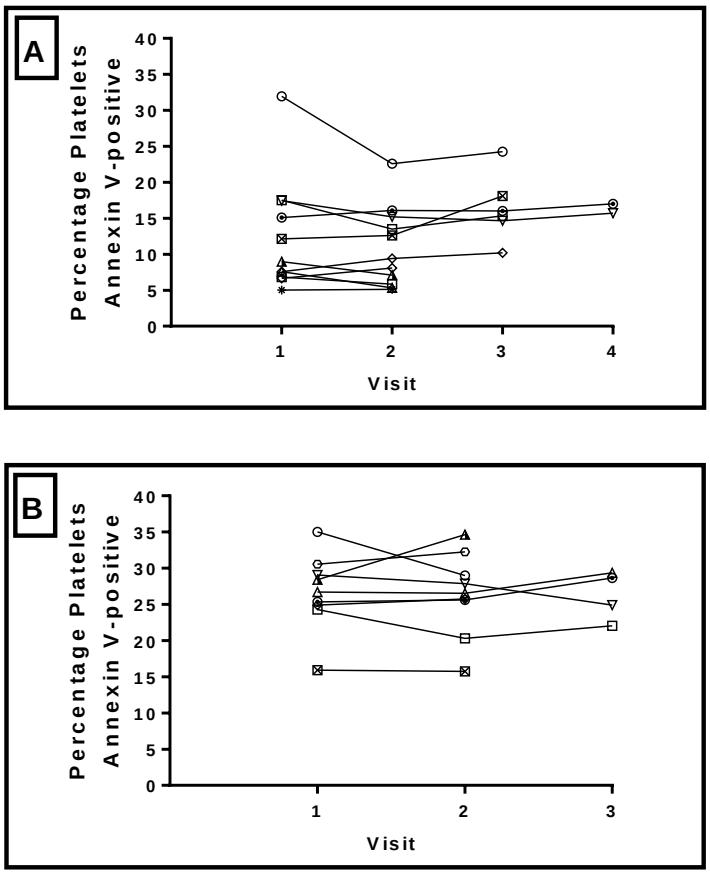


Figure 4.18. Intra-individual variation in platelet PS exposure induced by rhodocytin and CRP-XL. Washed platelets from different donors were activated with 40 nM rhodocytin (Panel A; n=11) or 2 µg/mL CRP-XL (Panel B; n=9) in 2-4 separate visits and measured for Annexin V binding using flow cytometry. Plotted data are from different experiments performed in this project and each symbol represents an individual platelet response.

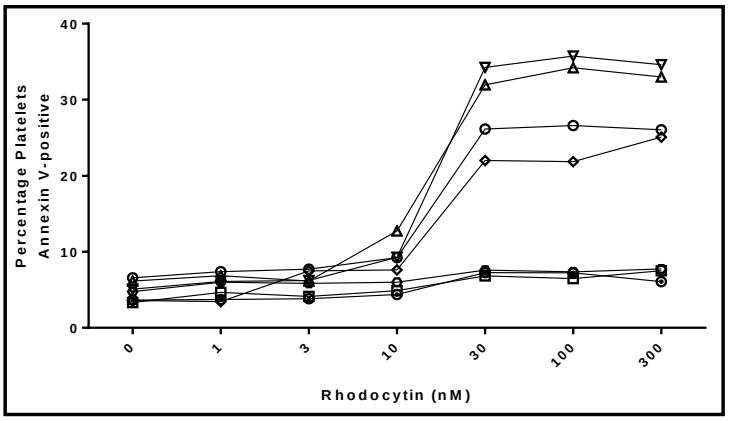


Figure 4.19. Inter-individual variability in procoagulant response to rhodocytin over a range of concentration. Washed platelets from seven donors were activated with 0-300 nM rhodocytin for 10 minutes at 37°C and PS exposure was measured using Annexin V binding assay. Data are from seven different donors, of which three used in Figure 4.5. Each symbol represents an individual platelet response.

4.2.4.6 **Rhodocytin platelet activation time course**

Since some individuals showed only a slight increase in platelet Annexin V binding after 10 minutes activation even with the maximum dose of rhodocytin, platelets were incubated for a longer time to ensure that a maximum response had been reached. Platelets from six individuals who showed weak response to rhodocytin were activated with 40 nM rhodocytin and subsamples were analysed for Annexin V binding at 10 and 30 minutes time points. Figure 4.20 shows that although there was a slight but statically significant increase in the percentage of PS-exposing platelets after rhodocytin stimulation for both time points (from 3.5±1.1 to 6.6±1.3 and form 5.0±0.5 to 7.1±1.7 % positive for the 10 and 30 minute time points respectively), there was no further increase between 10 and 30 minutes activation (6.6±1.3 vs. 7.1±1.7 % positive; p=0.2462).

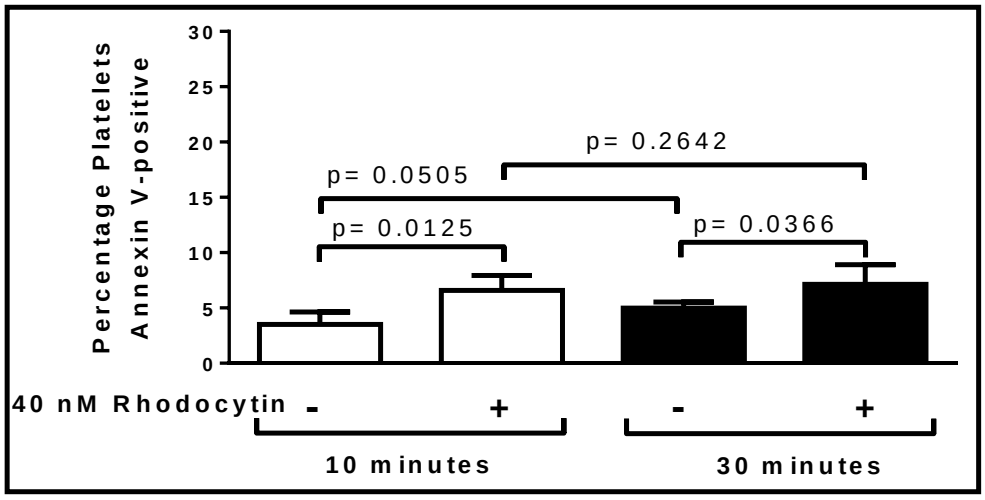


Figure 4.20. Rhodocytin platelet activation time course. Washed platelets from six donors who had shown weak procoagulant response to rhodocytin were activated with 40nM rhodocytin for 10 (white bars on the left of the graph) and 30 (black bars on the right of the graph) minutes at 37°C. PS exposure was measured using Annexin V binding assay. Data are presented as mean±SD.

4.2.4.7 **Relationship between platelet degranulation and procoagulant response**

It has been suggested that platelet degranulation and procoagulant responses could be regulated through different mechanisms. This was supported by the fact that there is a marked variation in platelet responses to different agonists in terms of degranulation and procoagulant responses. This can be clearly seen in the early data shown in section 4.2.1 that whilst platelet activation using different agonists induced surface expression of P-selectin in the majority of the platelets, only a subset of platelets showed PS exposure even at the highest concentrations of the agonists or with combinations of more than one agonist. This contrasts with the effect of the calcium ionophore A23187, which results in more than 98% of platelets exposing PS and generating MPs (data not shown). Nevertheless, it could be argued that calcium ionophore A23187 is not a physiological agonist and the above findings support the view that platelet procoagulant response, unlike degranulation, is restricted to a subpopulation of platelets.

In addition, maximal platelet P-selectin expression was induced by lower doses of CRP-XL compared to Annexin V binding (Figure 4.3), which also further supports the hypothesis that platelet degranulation and procoagulant responses are mediated through different signalling mechanisms. However, one of the key differences between CRP-XL, rhodocytin and IV.3 platelet activation that has been revealed in this current study is that in rhodocytin and IV.3, unlike CRP-XL, generate a concurrent increase in both P-selectin expression and Annexin V binding (Figure 4.4 and Figure 4.5). This finding suggests that CRP-XL platelet stimulation is distinct from the other ITAM-containing receptors agonists in this regard. Therefore, it was decided to compare the relationship between platelet degranulation and procoagulant response to CRP-XL and rhodocytin in more detail.

Platelet P-selectin expression and PS exposure induced by 2 µg/mL CRP-XL showed no correlation (Figure 4.21 A), whereas platelet stimulation with 40 nM rhodocytin showed a significant correlation between these markers (Figure 4.21 B). Additionally, the effect of blocking GPIIb/IIIa using RFGP56 antibody also showed a distinct difference between CRP-XL and rhodocytin platelet stimulation. Whilst blocking GPIIb/IIIa showed a slight reduction in Annexin V binding in CRP-XL-stimulated platelets (from 24.4±9.6 to 20.4±6.7 % positive; p=0.0640), P-selectin expression was not affected (from 90.8±2.5 to 90.1±5.5 % positive; p=0.7728) (Figure 4.22 A). In sharp contrast, both Annexin V binding and P-selectin expression were significantly inhibited in rhodocytin-stimulated platelets after blocking GPIIb/IIIa (from 23.4±13.2 to 12.8±7.0 % positive; p=0.0042 and from 81.3±12.1 to 62.1±24.5 % positive; p=0.0125 respectively) (Figure 4.22 B). Furthermore, inhibiting of PI3K with either LY294002 or Wortmannin, at a single (high) concentration, resulted in a dramatic reduction in Annexin V binding induced by CRP-XL or rhodocytin, whereas P-selectin expression was only reduced in the rhodocytin-stimulated platelets but not CRP-XL-stimulated platelets (Figure 4.23). Although these data are from a single experiment, they are in agreement with the above findings.

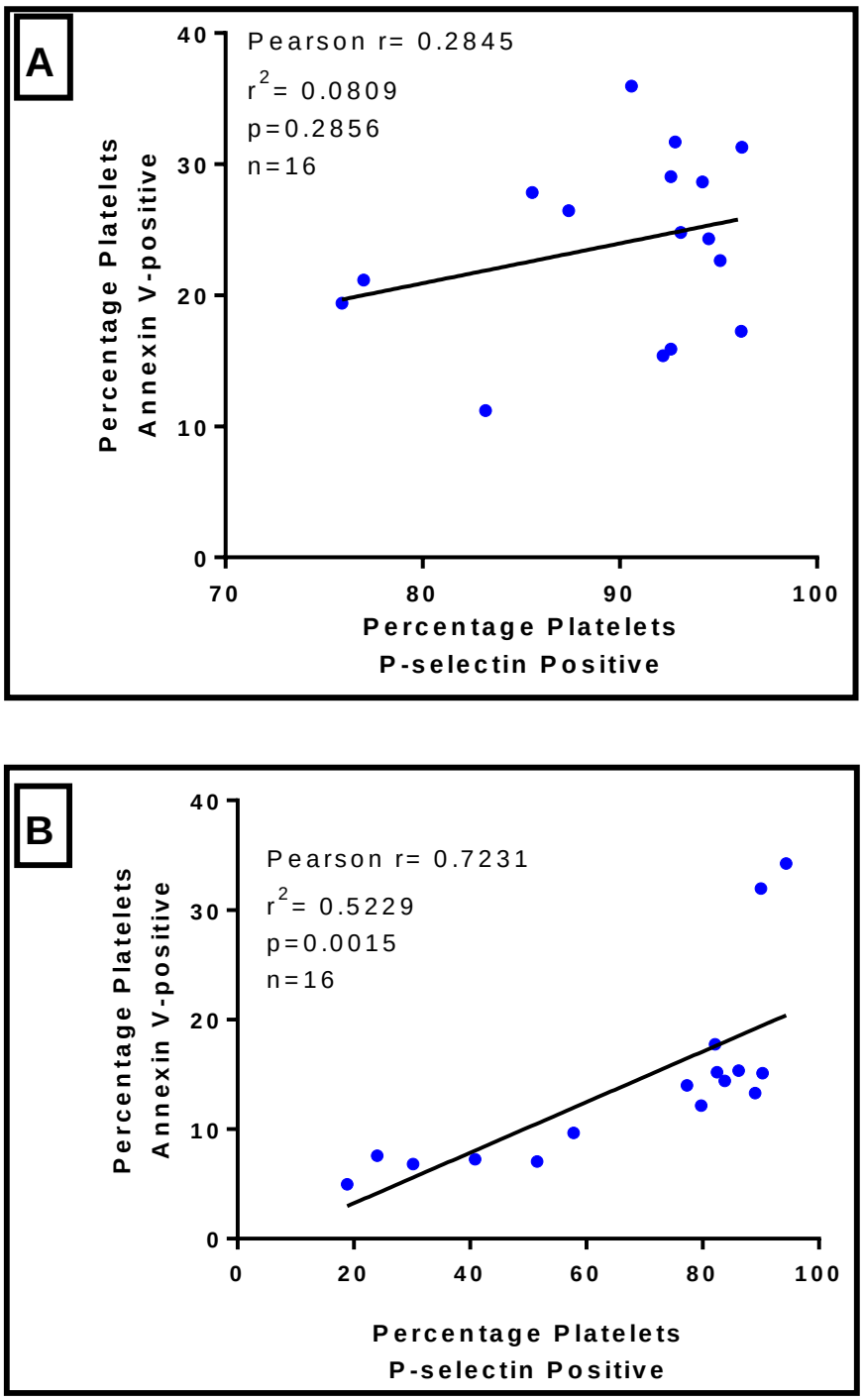


Figure 4.21. Correlation between P-selectin expression and Annexin binding induced by CRP-XL and rhodocytin. Washed platelets were activated by 2 µg/mL CRP-XL (Panel A; n=16) or 40 nM rhodocytin (Panel B; n=16) for 10 minutes at 37°C. PS exposure and surface P-selectin expression were measured by flow cytometry.

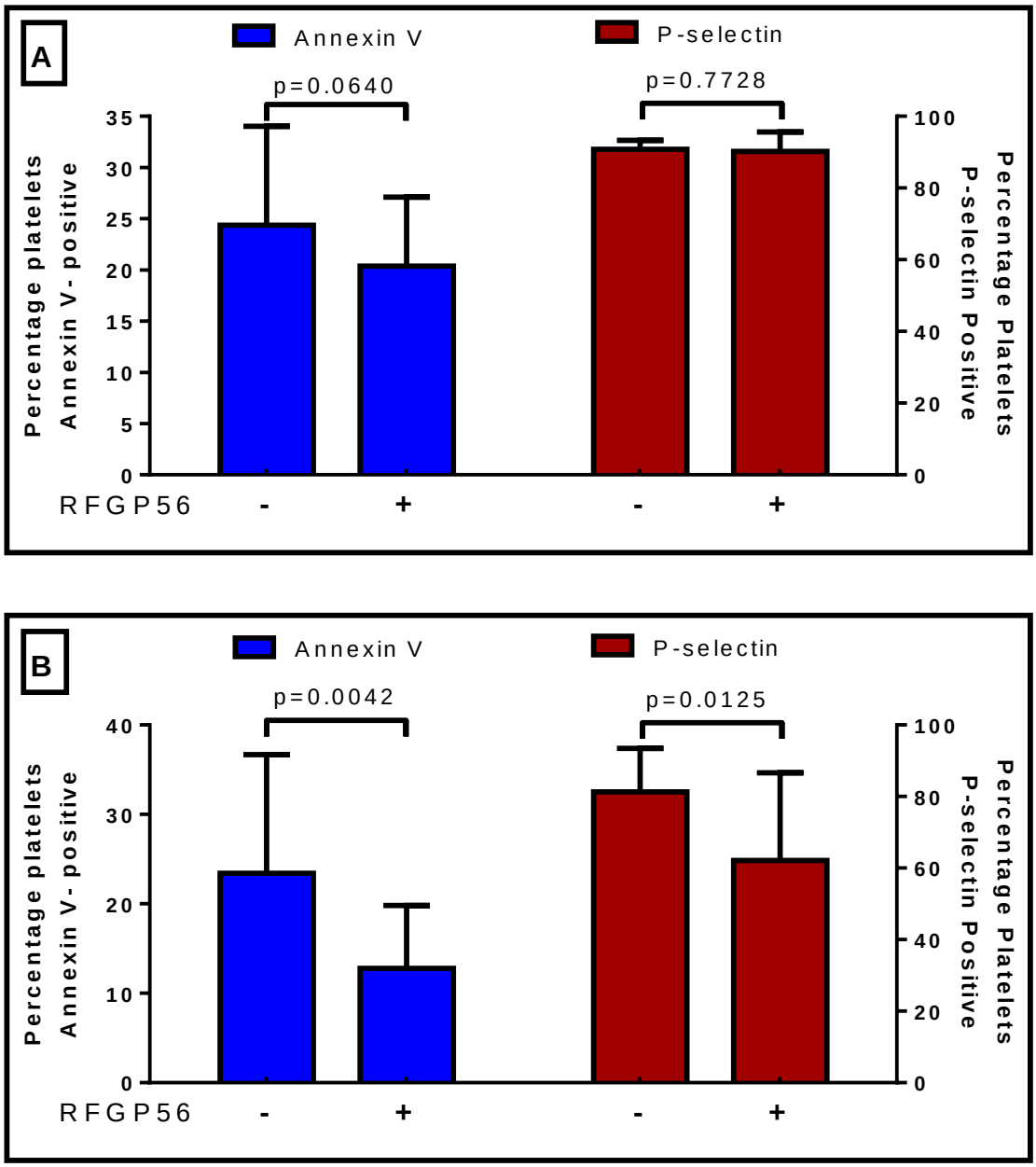


Figure 4.22. Effect of blocking GPIIb/IIIa on Annexin V binding and P-selectin expression induced by CRP-XL and rhodocytin. Washed platelets were activated by 2 µg/mL CRP-XL (Panel A; n=5) or 40 nM rhodocytin (Panel B; n=8) for 10 minutes at 37°C in the presence or absence of the GPIIb/IIIa blocking antibody at final concentration of 10 µg/mL RFGP56. PS exposure and surface P-selectin expression were measured by flow cytometry. Data are presented as mean±SD.

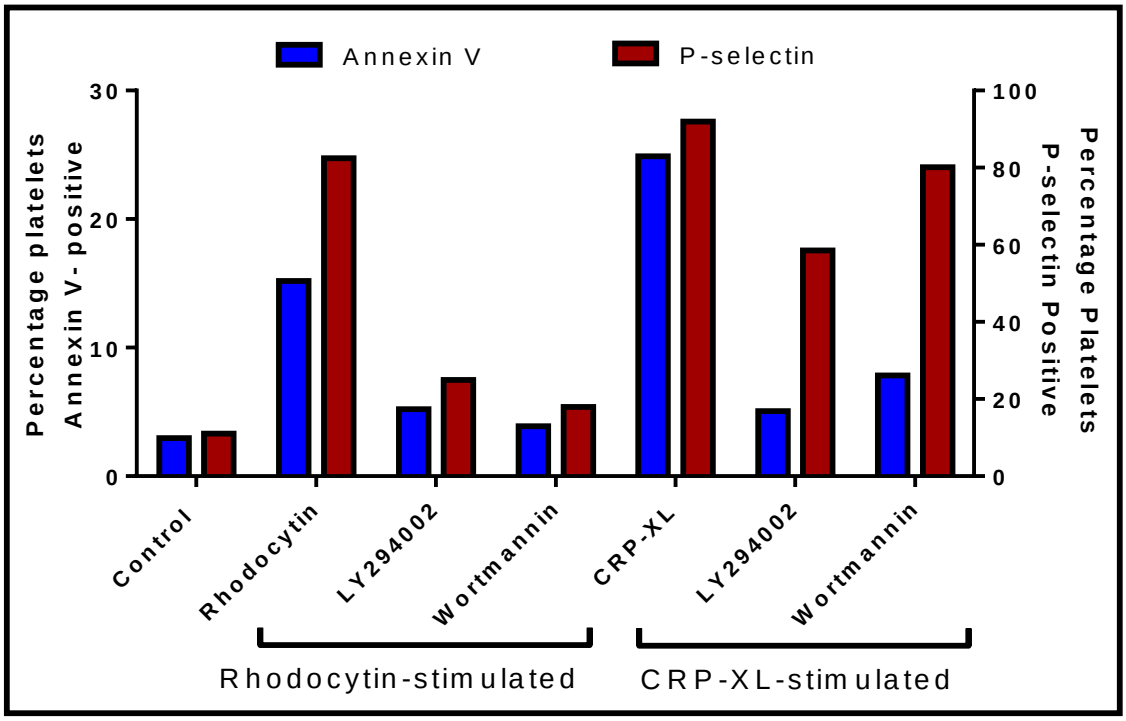


Figure 4.23. Role of PI3K in Annexin V binding and P-selectin expression induced by CRP-XL and rhodocytin. Washed platelets were incubated with 25 µM LY294002 or 100 nM Wortmannin for 15 minutes at 37°C prior to activation with 40 nM rhodocytin or 2 µg/mL CRP-XL. Annexin V binding P-selectin expression were measured by flow cytometry (n=1).

The effect of released endogenous ADP on platelet degranulation induced by rhodocytin was also investigated. As it has been shown, inhibition of $P2Y_{12}$ with AR-C69931MX resulted in a marked reduction of Annexin V binding in washed platelets stimulated with 40 nM rhodocytin (Figure 4.10 A). Similar to the reduction in Annexin V binding, inhibition of $P2Y_{12}$ also resulted in a dramatic decrease in P-selectin expression in rhodocytin-stimulated platelets (Figure 4.24). The ADP scavenger, apyrase, showed a similar inhibitory effect in platelet degranulation. Furthermore, when analysed in PRP inhibiting ADP receptors using MRS 2179 and AR-C69931MX results in a reduction of P-selectin expression in rhodocytin-stimulated in similar pattern to Annexin V binding shown in Figure 4.12 (data not shown).

The above data reinforce the notion that platelet degranulation induced by GPVI is distinct from the procoagulant response, while with CLEC2 stimulation the two responses are regulated in a more similar manner.

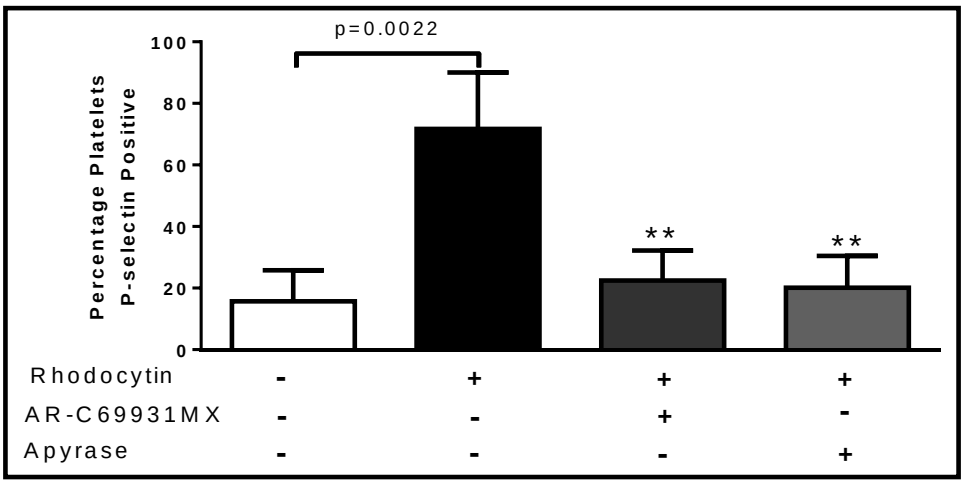


Figure 4.24. Impact of $P2Y_{12}$ on platelet degranulation induced by rhodocytin. Washed platelets were activated with 40 nM rhodocytin in the presence or absence of $3X10^{-7}$M AR-C69931MX or 80 µg/mL apyrase. Annexin V binding was measured by flow cytometry (n=5). P-values are versus activated platelets unless indicated; **p<0.001.

4.2.5 Platelet activation by podoplanin induces procoagulant response and MP formation

Having established that the CLEC2 ligand rhodocytin induces the platelet procoagulant response it was decided to investigate the role of the only known endogenous ligand for CLEC-2, podoplanin, in platelet procoagulant activity. Podoplanin, also called Aggrus, is a type I transmembrane sialomucin-like glycoprotein which is expressed on the surfaces of several cells such as lymphatic endothelial cells, kidney podocytes, fibroblastic reticular cells, lung type I alveolar cells, and some tumour cells, but not on vascular endothelial cells (Navarro-Nunez, *et al* 2013, Nieswandt, *et al* 2011, Suzuki-Inoue, *et al* 2011, Watson, *et al* 2010).

4.2.5.1 **Platelet activation by recombinant podoplanin**

In order to investigate the role of podoplanin in generating platelet procoagulant activity, recombinant mouse-podoplanin fused with human Fc was used to bind to CLEC-2 then cross-linked using anti-human Fc antibodies (the recombinant mouse-podoplanin and anti-human Fc antibodies were generously provided by Alice Y. Pollitt and Steve Watson, School of Clinical and Experimental Medicine, University of Birmingham). Increasing doses of recombinant mouse-podoplanin induced a potent, dose-dependent platelet procoagulant response, accompanied by the release of strongly Annexin V^{+ve} MPs (Figure 4.25 A and B). Maximum platelet Annexin V binding was seen at 20 µg/mL recombinant mouse-podoplanin (27.8±8.5 % positive; p=0.0391), which then was used for the subsequent experiments. Recombinant mouse-podoplanin cross-linked with anti-human Fc also induced surface P-selectin expression in the majority of platelets (Figure 4.25 C). It is noteworthy that the recombinant mouse-podoplanin required cross-linking by the anti-human Fc antibodies to

induce platelet activation, confirming that dimerization is critical for CLEC-2 to induce platelet activation.

Podoplanin-induced platelet procoagulant activity was confirmed in six healthy donors, which also showed a significant increase in Annexin V binding in podoplanin-stimulated platelets (from 4.1±0.6 to 26.2±7.6 % positive, p=0.0009) (Figure 4.26 A). However, because recombinant mouse-podoplanin used in this study was fused with human Fc, it was important to block FcγRIIA. Blocking FcγRIIA was achieved with the use of 20 µg/mL IV.3 Fab fragments, which completely inhibited platelet response to HIT immune-complexes from known HIT-positive patient's plasma (data not shown). Podoplanin induced a potent and significant platelet procoagulant activity even after blocking FcγRIIA (Annexin V binding increased from 6.4±2.0 to 23.5±7.2 % positive, p=0.0019) (Figure 4.26 B), suggesting that podoplanin is capable of inducing a potent platelet procoagulant activity through CLEC-2 independently from FcγRIIA.

It can be clearly seen from the previous data that the recombinant mouse-podoplanin induced more platelet Annexin V binding compared to rhodocytin, even after blocking FcγRIIA. In order to ascertain that the higher percentage of PS-exposing is dependent on the level of individual response through CLEC-2, podoplanin and rhodocytin platelet responses from fourteen normal donors were compared. As might be expected, the donors' responses to recombinant mouse-podoplanin showed a strong correlation with that induced by rhodocytin.

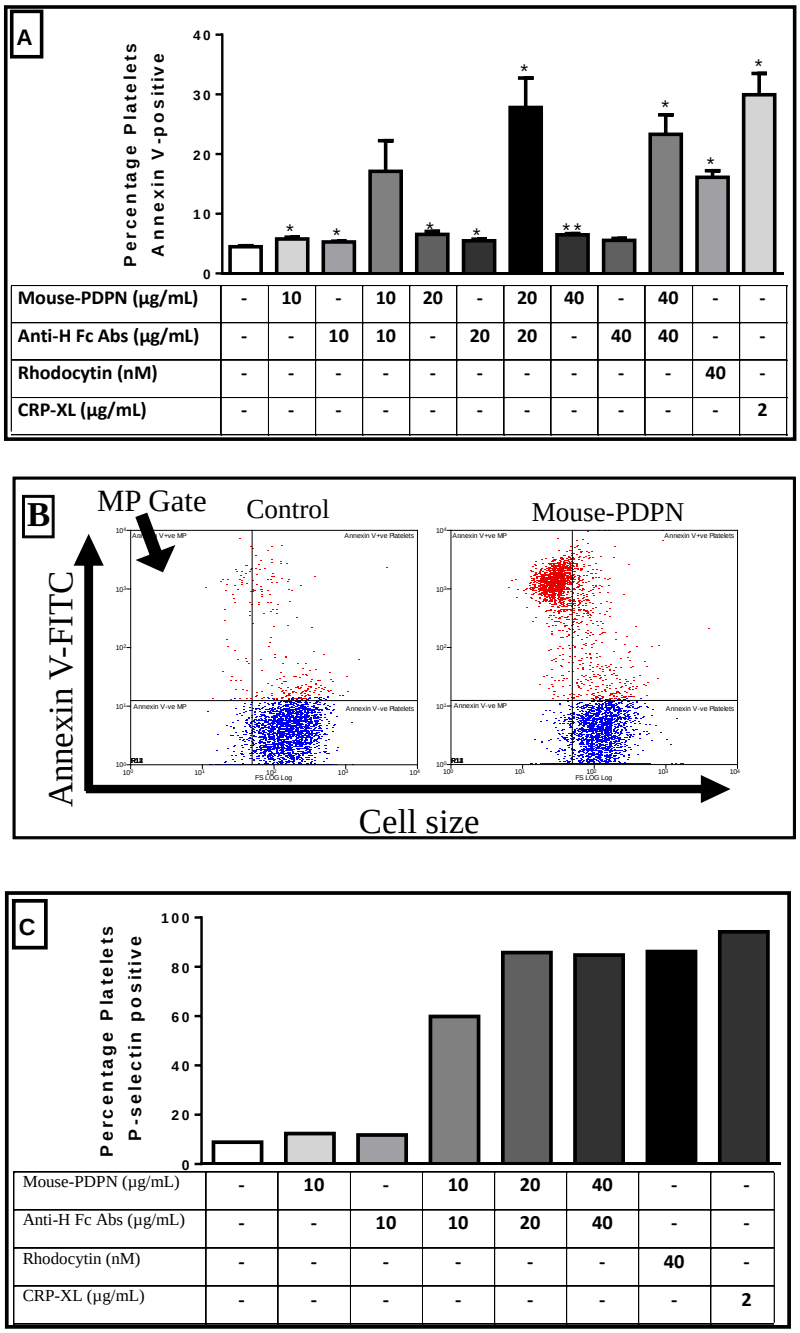


Figure 4.25. Platelet Annexin V binding and P-selectin expression induced by serial doses of recombinant mouse podoplanin. Washed platelets were incubated with serial doses of recombinant mouse-podoplanin (Mouse-PDPN) fused with human Fc then cross-linked using anti-human Fc antibodies (Anti-H Fc Abs) and incubated for 10 minutes at 37°C. Rhodocytin and CRP-XL platelet activation were also included. Annexin V binding (Panel A; n=3) and P-selectin expression (Panel C; n=1) were measured on flow cytometry. P-values are versus control platelets; *$p<0.05$; **$p<0.001$. Panel B: Representative flow cytometry dot-plot graphs of Annexin V-FITC plotted against forward scatter; the upper left-hand quadrant shows Annexin V^{+ve} events in MP-size gate, the lower left-hand quadrant shows Annexin V^{-ve} events in MP-size gate, the upper right-hand quadrant shows Annexin V^{+ve} events in platelet-size gate, the lower right-hand quadrant shows Annexin V^{-ve} events in platelet-size gate; blue dots are Annexin V^{-}; red dots are Annexin V^{+}.

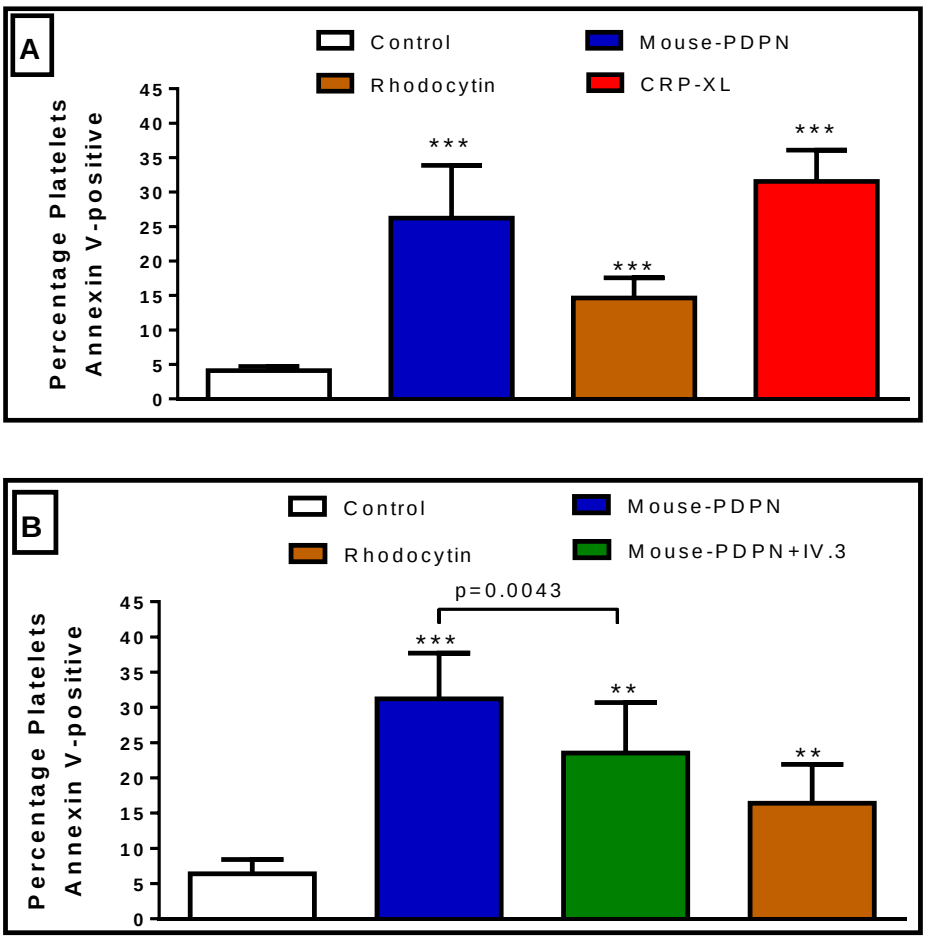


Figure 4.26. Platelet Annexin V binding induced by recombinant mouse podoplanin. Panel A: Annexin V binding from washed platelets stimulated with 20 µg/mL recombinant mouse podoplanin (Mouse-PDPN), 40 nM rhodocytin or 2 µg/mL CRP-XL for 10 minutes at 37°C (n=6). Panel B: Annexin V binding from washed platelets stimulated with 20 µg/mL Mouse-PDPN with and without blocking FcγRIIA using 20 µg/mL IV.3 Fab fragments (+IV.3) (n=5). Platelet stimulation with 40 nM rhodocytin was also included. Data are presented as mean±SD. P-values are versus control platelets unless indicated; **p<0.001; ***p<0.0001.

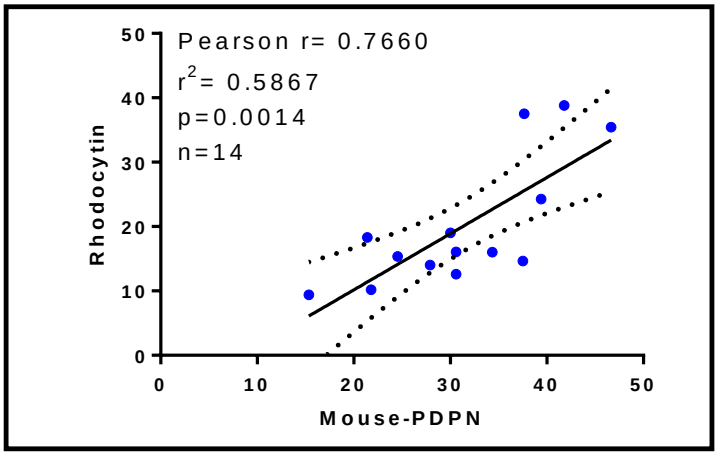


Figure 4.27. Correlation between Annexin V binding induced by podoplanin and rhodocytin. Washed platelets were stimulated with 20 µg/mL Mouse-PDPN or 40 nM rhodocytin for 10 minutes at 37°C. Platelet PS exposure was measured using Annexin V binding assay. Data are from fourteen different individuals measured on separate occasions.

4.2.5.2 **Role of podoplanin-expressing cells**

Interaction between platelets and podoplanin-expressing cells has been shown to play several physiological and pathological roles (Nieswandt, *et al* 2011, Suzuki-Inoue, *et al* 2011, Watson, *et al* 2010). This interaction has been well studied, but most of the studies examined platelet aggregation only. Therefore, the role of podoplanin-expressing cells in generating platelet procoagulant activity was studied. This was achieved with the use of Chinese Hamster Ovary (CHO) cells transfected with full-length human podoplanin (termed PDPN-CHO cells hereafter). Mock transfected (control-CHO) cells were used as a negative control. PDPN-CHO cells had some morphological differences from control-CHO cells such as multiple membrane protrusions, a tendency to grow in clusters (Figure 4.28 A), and were less adherent. Surface expression of human podoplanin by the PDPN-CHO cells was assessed periodically by flow cytometry (Figure 4.28 B).

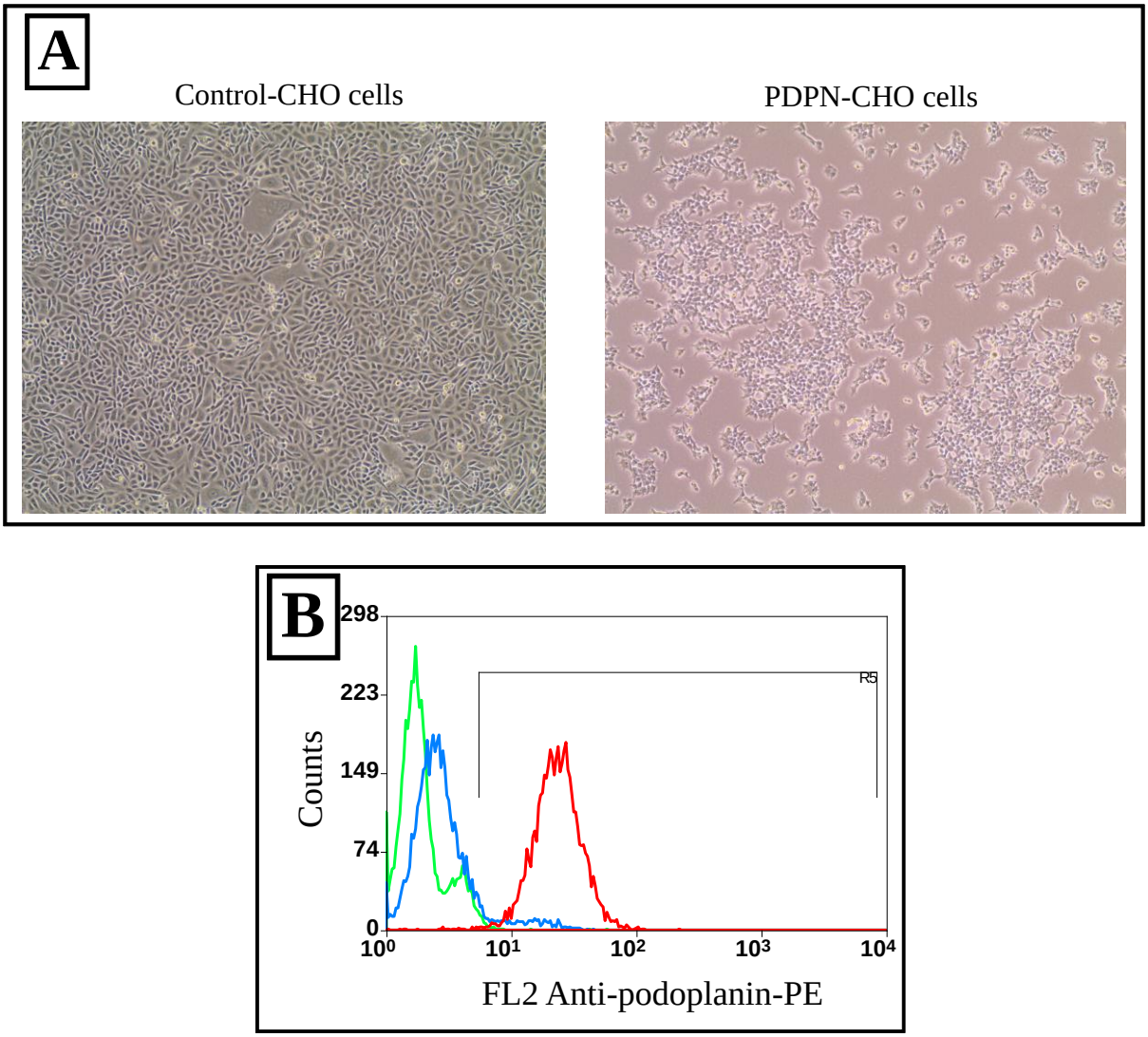


Figure 4.28. Podoplanin expression of CHO cells. Panel A: Microscopic pictures of control-CHO (left) and PDPN-CHO (right) cells. Panel B: Level of human podoplanin surface expression of CHO cells using PE-conjugated anti-podoplanin antibody NZ-1; green: PE isotype; blue: control-CHO cells; red: PDPN-CHO cells.

Washed platelets from normal donors were incubated with harvested control-CHO or PDPN-CHO cells in suspension, isolated by centrifugation (200g for 20 minutes) and analysed for Annexin V binding and MP formation. Figure 4.29 shows PDPN-CHO cells induced potent and significant platelet procoagulant response accompanied by the release of strongly Annexin V^{+ve} MPs, whereas control-CHO cells failed to induce a significant increase in platelet Annexin V binding. The nature of the interaction between platelets and PDPN-CHO cells was also examined by fluorescent microscopy. CD42b-Cy5 was used as platelet-specific marker and P-selectin-FITC or Annexin V-FITC were used to detect platelet degranulation and PS exposure respectively. The nuclei of CHO cells were counterstained with Hoescht stain. Platelets showed adhesion, aggregation, degranulation and PS exposure when they interacted with PDPN-CHO but not with the control-CHO cells (Figure 4.30). Many platelets showed P-selectin expression and Annexin V binding while they were adhered to the surfaces PDPN-CHO cells. However, there were large numbers of unattached platelets to PDPN-CHO cells that also showed exposure of both markers. It has to be noted that this experiment was performed under static conditions; however, platelets have been shown to adhere and aggregate on podoplanin-expressing cells under flow conditions (Uhrin, *et al* 2010).

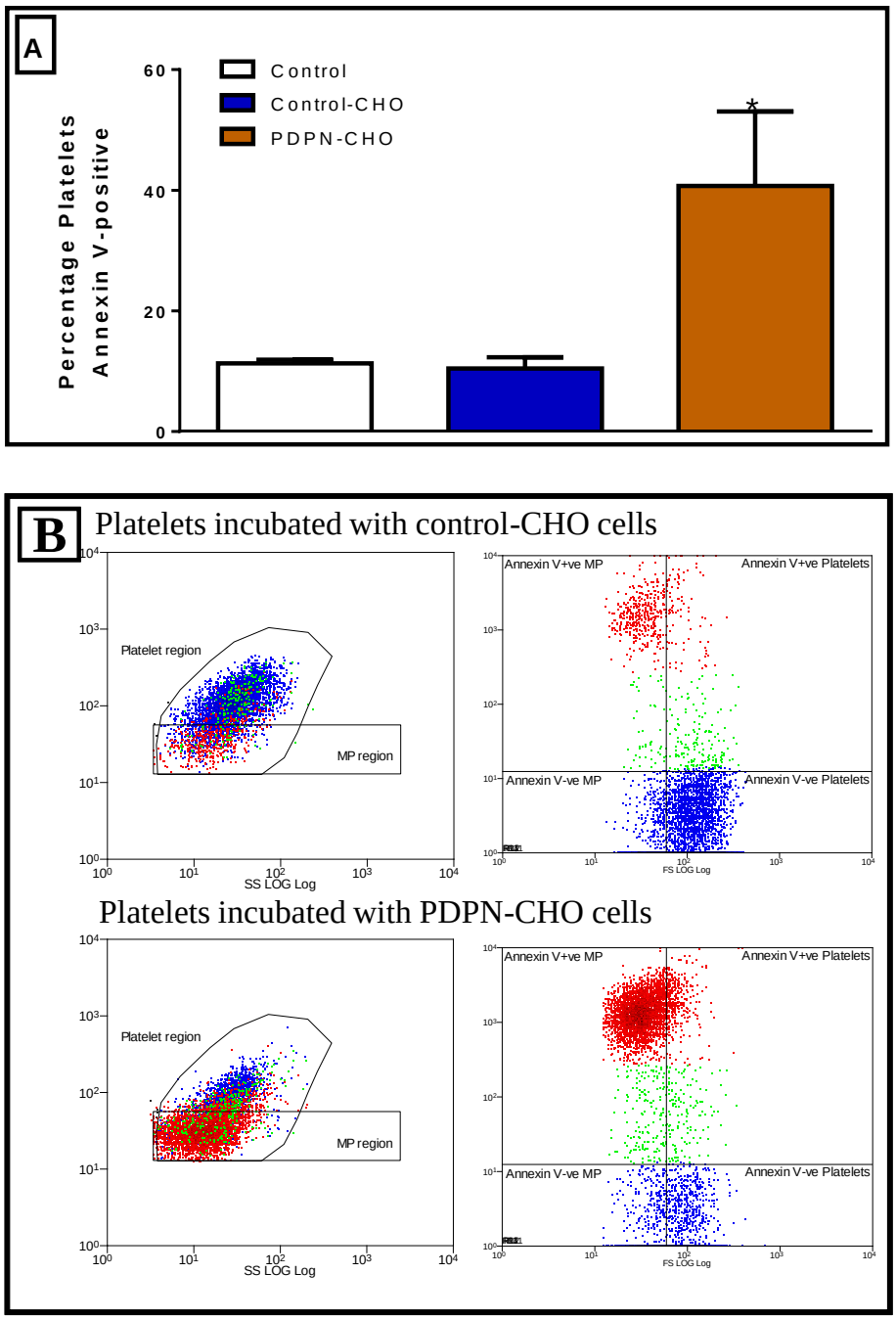
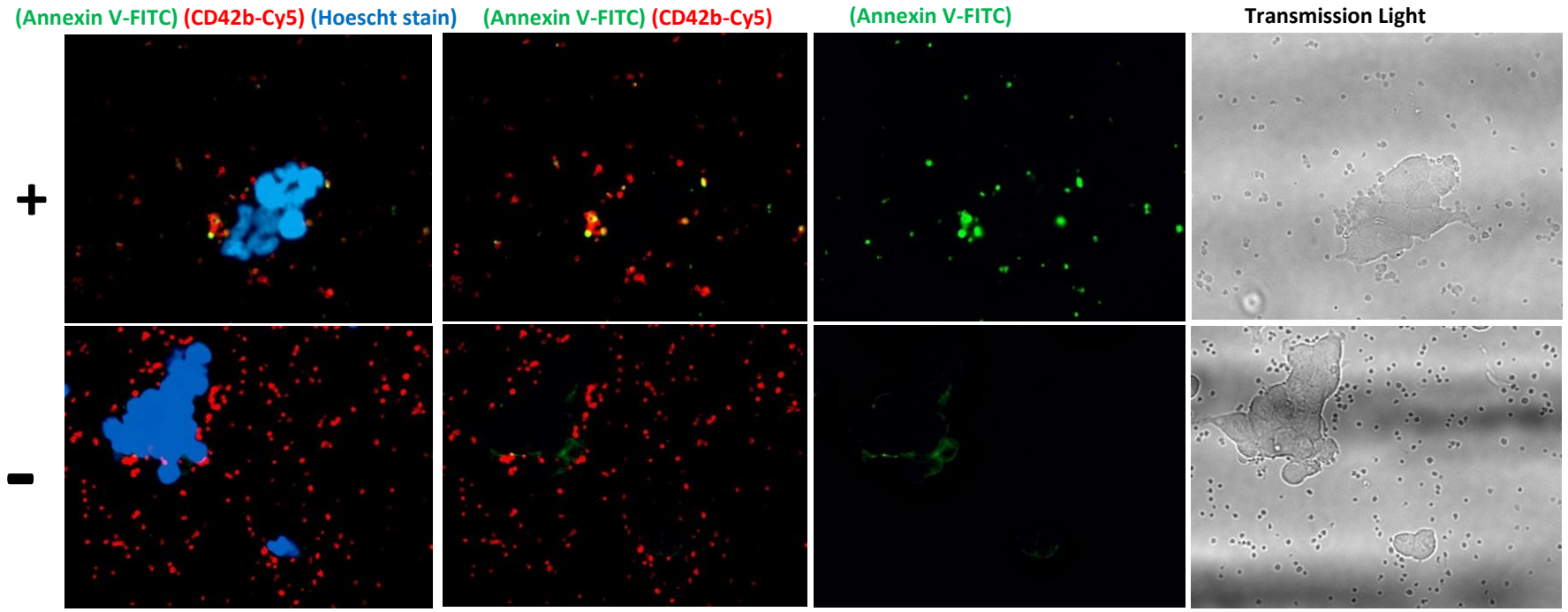


Figure 4.29. PDPN-CHO cells induce platelet procoagulant response and MP generation. Washed platelets were incubated with harvested control-CHO or PDPN-CHO cells in suspension for 10 minutes at 37°C. Platelets were isolated from the cells by centrifugation at 200g for 20 minutes. Panel A: Numerical data of Annexin V binding from four separate experiments using blood from four different donors and shown as mean±SD. P-value is versus control platelets; *$p<0.05$. Panel B: Representative flow cytometry histograms; left panels, scatter-plot of forward scatter plotted against side scatter; right panels, dot-plot of Annexin V-FITC plotted against forward scatter; blue dots are Annexin V^-; red dots are Annexin V^+. From the flow cytometry histograms it can be clearly seen that there was no CHO cell contamination in the platelet preparation.


(Annexin V-FITC) (CD42b-Cy5) (Hoescht stain)
(Annexin V-FITC) (CD42b-Cy5)
(Annexin V-FITC)
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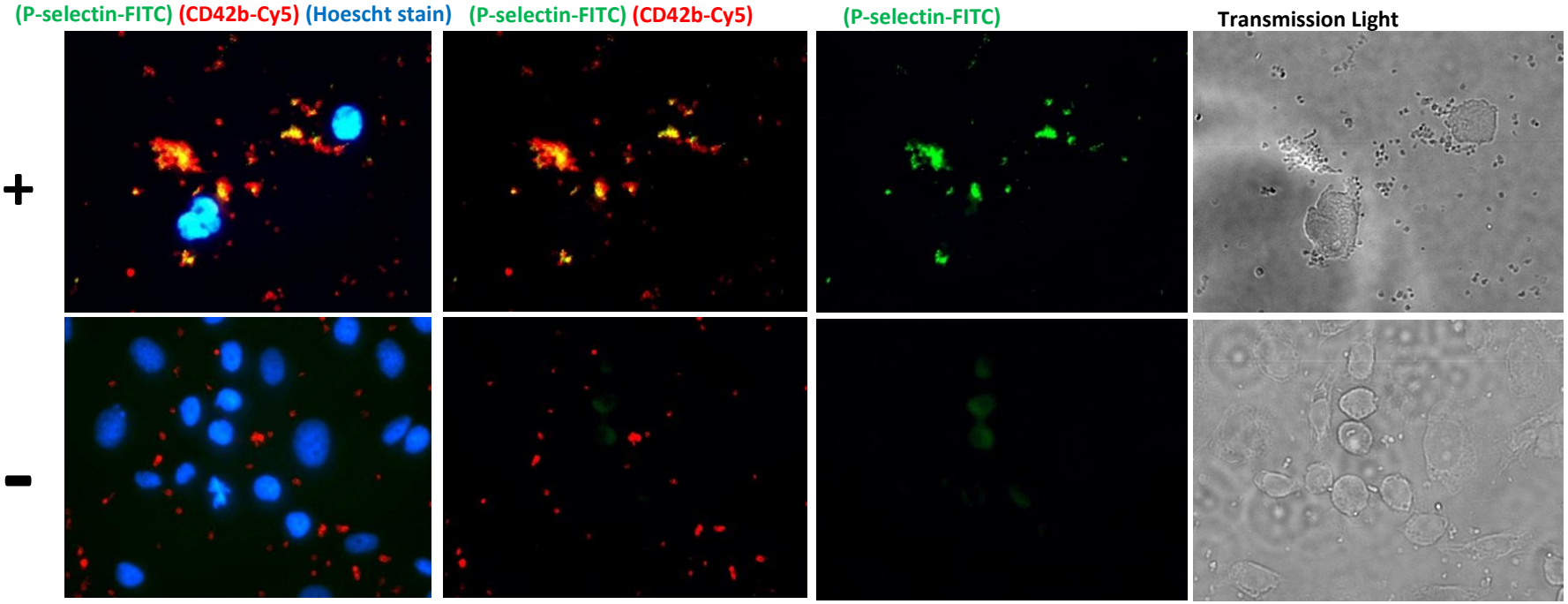
(P-selectin-FITC) (CD42b-Cy5) (Hoescht stain)
(P-selectin-FITC) (CD42b-Cy5)
(P-selectin-FITC)
Transmission Light
+
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Figure 4.30. Platelets adhere, aggregate and express procoagulant surfaces when exposed to podoplanin-expressing cells. Washed platelets were incubated with PDPN-CHO (+) or control-CHO (-) cells on chamber slides for 10 minutes at 37°C. Platelets then were labelled for CD42b-Cy5 (red) and Annexin V-FITC (green) or P-selectin-FITC (green). The nuclei were counterstained with Hoescht stain (blue). Slides were examined using a EVOS AMG fl LED fluorescent microscope with the use of X40 power magnification. Platelets showed aggregations on the PDPN-CHO cells surface as shown by the co-localization of platelet marker and PDPN-CHO cell surfaces. In contrast, platelets did not show surface expression of PS or P-selectin or cell attachment when they were incubated with control-CHO cells.

4.2.6 CLEC-2 activated platelets and their MPs promote thrombin generation

The functional properties of procoagulant platelets and their MPs generated by CLEC-2 stimulation were assessed by measuring their ability to support thrombin generation. Washed platelets from healthy donors were stimulated with CRP-XL and rhodocytin. Samples were centrifuged at 600g for 15 minutes to pellet platelets and MP-rich supernatants were then centrifuged at 1500g for 15 minutes followed by 13000g for 2 minutes to remove residual platelets and cell debris from MP suspensions. MPs were pelleted by centrifugation at 20000g for 30 minutes. Platelet and MP pellets were resuspended into CTI-inhibited pre-filtered pooled plasma and analysed for thrombin generation on the CAT assay using the 1pM TF reagent assay, which is sensitive to the levels of PPL activity. CRP-XL- and rhodocytin-stimulated platelets and their MPs supported thrombin generation at a faster rate and higher levels than untreated platelets (Figure 4.31). Specifically, CRP-XL- and rhodocytin-stimulated platelets gave a significantly shorter lag time and higher peak thrombin. Similarly, MPs derived from CRP-XL- and rhodocytin-stimulated platelets showed a significant enhancement of thrombin generation. In order to ascertain that the extra centrifugation step

(600g for 15 minutes) to pellet platelets did not induce non-specific platelet activation, subsamples were analysed for surface P-selectin expression and Annexin V binding using flow cytometry (Figure 4.32). Additionally, separate control experiments were performed in order to ascertain that rhodocytin did not interfere with thrombin generation (Figure 4.33).

The procoagulant activity of MPs generated from platelets after exposing to podoplanin-expressing cells was also evaluated in the CAT assay. Washed platelets from normal donors were incubated with control-CHO or PDPN-CHO cells in suspension for 10 minutes at 37°C. MPs were isolated MPs after first removing platelet and cell debris by serial centrifugation steps; 600g for 15 minutes, 1500g for 15 minutes and 13000g for 2 minutes. Isolated MPs were then pelleted by centrifugation at 20000g for 30 minute, resuspended into CTI-inhibited pre-filtered pooled plasma and analysed for thrombin generation on the CAT assay using the 1pM TF reagent assay. MPs isolated from platelets incubated with PDPN-CHO cells showed a significant enhancement of thrombin generation (lag time; from 10.3±0.6 to 7.7±0.3 and peak thrombin; from 33.2±8.9 to 121±6.4) (Figure 4.34). However, the supernatant of platelets incubated with control-CHO cells failed to show a significant increase in the thrombin generation, indicating that control-CHO cells do not induce platelet procoagulant MPs.

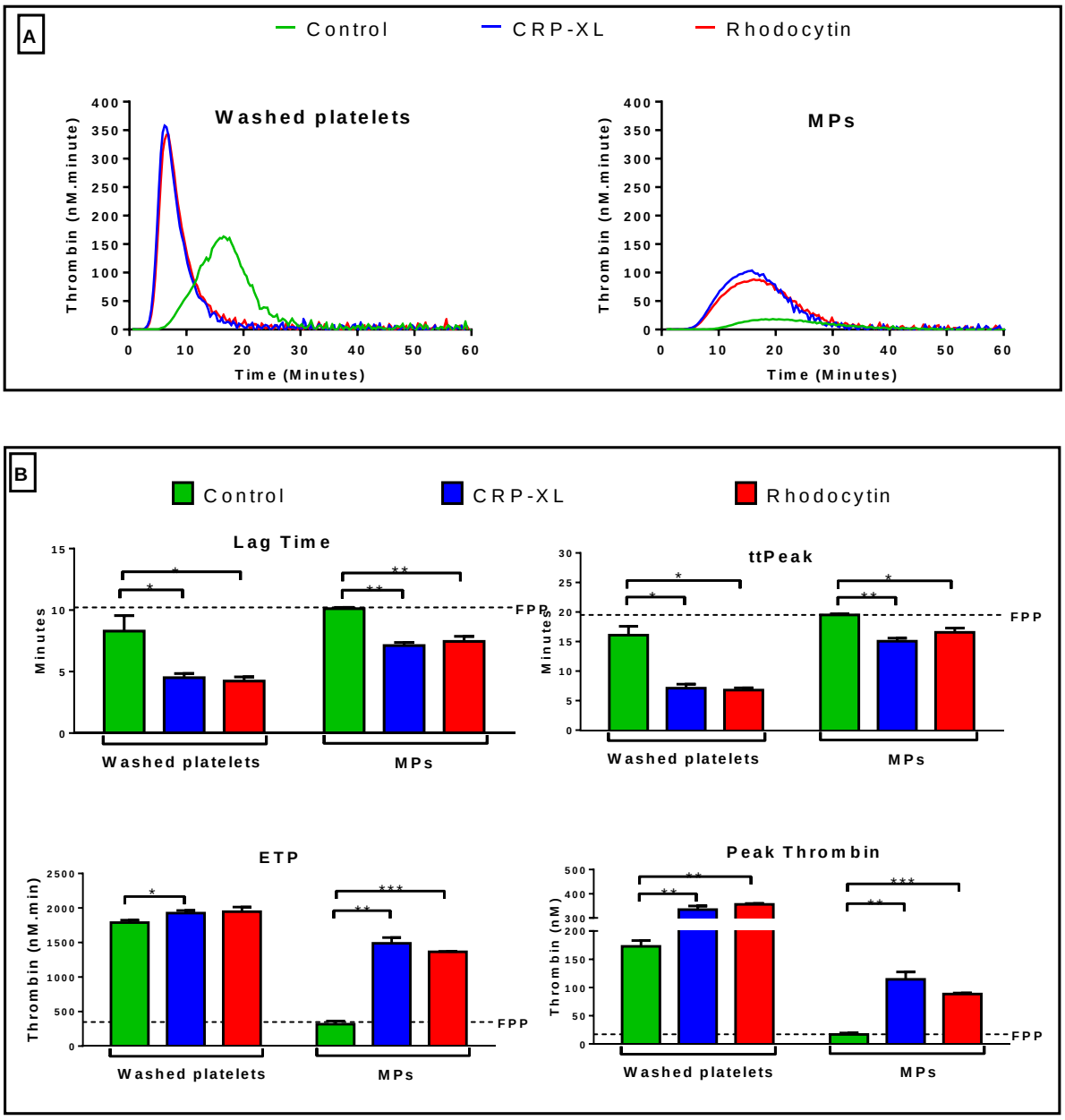

Figure 4.31. Thrombin generation from platelets and platelet-derived MPs induced through CLEC-2. Washed platelets were stimulated with 2 µg/mL CRP-XL or 40 nM rhodocytin for 10 minutes at 37°C. Washed platelets were pelleted from their MPs by centrifugation at 600g for 15 minutes. MPs suspension was purified by centrifugation at 1500g for 15 minutes followed by 13000g for 2 minutes then pelleted at 20000g for 30 minutes. Pellets of washed platelets and MPs were suspended into CTI-treated, pre-filtered normal-pooled plasma and run on the CAT assay using the 1 pM TF reagent assay, which is sensitive to PPL activity. Panel A: Thrombin generation curves of washed platelets (left) and their MPs (right). Panel B: Numerical data for thrombin generation parameters. Dashed lines represent filtered pooled plasma (FPP). Data are from three separate experiments using blood from different donors presented as mean±SD. P-values; *p<0.05; **p<0.001; ***p<0.0001.

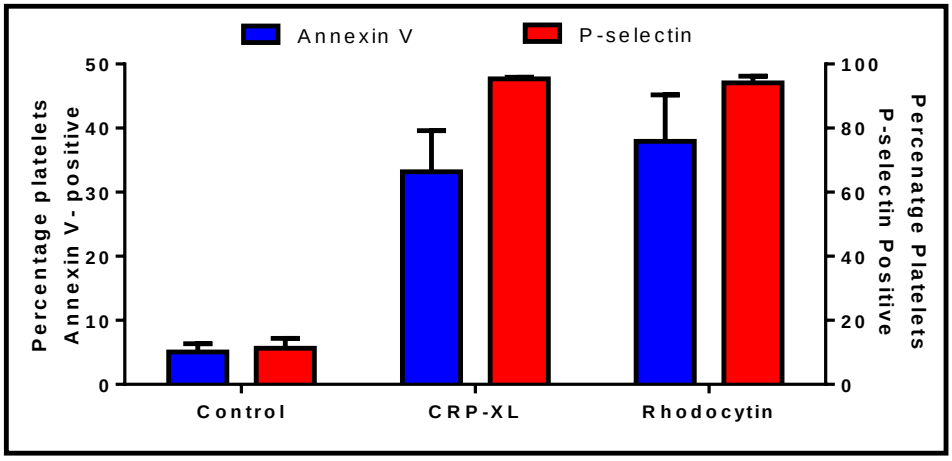

Figure 4.32. Washed platelet preparation for thrombin generation does not induce non-specific platelet activation. Subsamples used in thrombin generation as shown in Figure 4.31 were analysed on flow cytometry for Annexin V binding (blue bars) and P-selectin expression (red bars) before they were used on the CAT assay. The extra centrifugation step (600g for 15 minutes) to pellet platelets for thrombin generation assay did not induce non-specific platelet activation as control platelets showed low Annexin V binding and P-selectin expression. Data are shown as mean±SD.

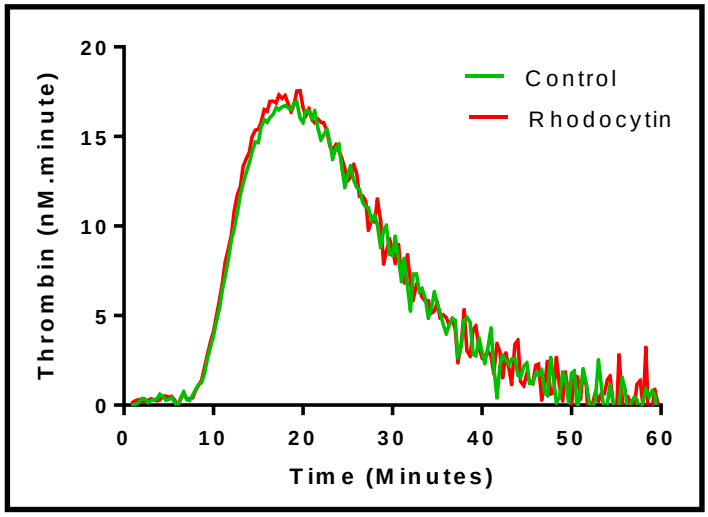

Figure 4.33. Effect of rhodocytin on thrombin generation. Thrombin generation curves using the 1 pM TF reagent assay of filtered pooled plasma in the presence (red) or absence (green) of the maximum used concentration of rhodocytin, 40 nM Rhodocytin does not have an effect on thrombin generation measurement. Data are presented as the mean of three separate experiments.

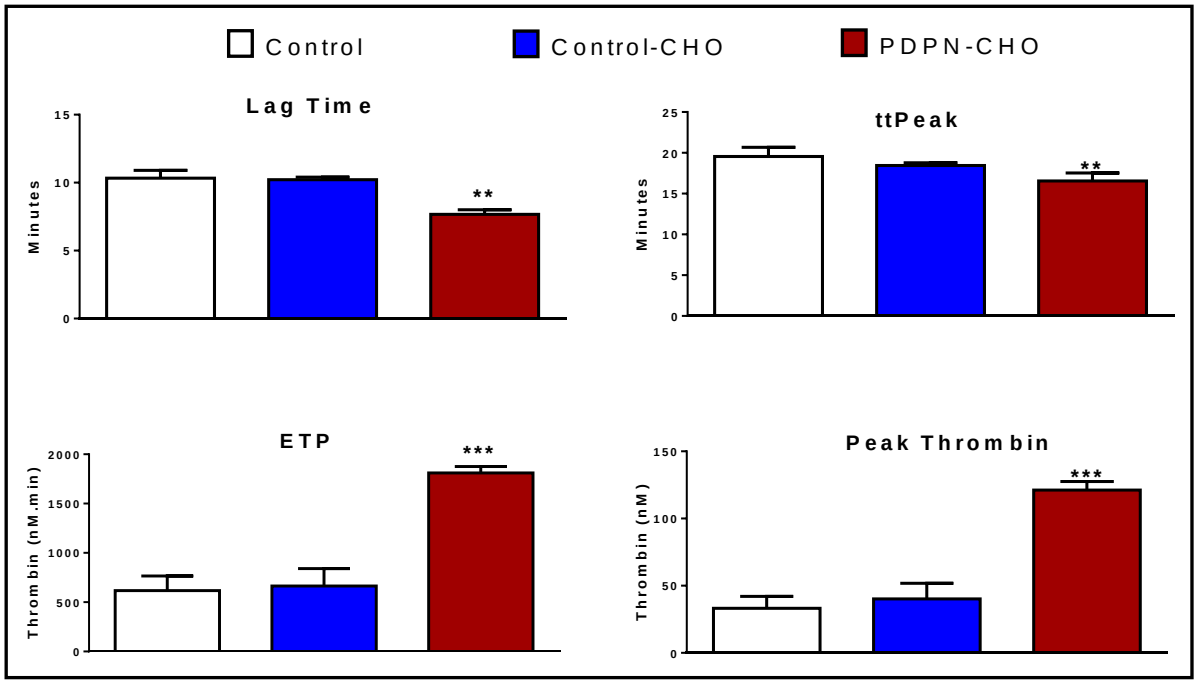

Figure 4.34. CAT assay results of MPs derived from platelets after exposing to podoplanin-expression cells. Washed platelets were incubated with control-CHO or PDPN-CHO cells in suspension for 10 minutes at 37°C. MPs were isolated from platelets and other cells and cell debris by serial centrifugation steps (600g for 15 minutes, 1500g for 15 minutes followed by 13,000g for 2 minutes) then pelleted at 20,000g for 30 minutes. MP pellets were resuspended into CTI-treated, pre-filtered normal-pooled plasma and analysed on CAT assay using the 1 pM TF reagent assay. Data are from three separate experiments using blood from different donors and are shown as mean±SD. P-values are versus control platelets; **p<0.001; ***p<0.0001.

4.2.7 Up-regulation of podoplanin mRNA

Since podoplanin is the only known endogenous ligand for CLEC-2 and there is no evidence that it is present in normal circulation, it was decided to investigate whether the podoplanin gene could be up-regulated in the circulation. In particular, the question was asked whether monocytes could be induced to express podoplanin. A qRT-PCR method was used to measure podoplanin gene (*PDPN*) expression using cDNA samples prepared by Robert E. Turnbull (Department of Cardiovascular Sciences, University of Leicester).

4.2.7.1 **Podoplanin gene up-regulation in monocytes following platelet activation**

Citrated whole blood from healthy donors was incubated for up to 8 hours at 37°C in the presence of an intermediate dose of CRP-XL, 0.5 µg/mL, which induces platelet activation and consequent platelet-monocyte aggregation. Monocytes were extracted at 0, 2, 4, 6 and 8 hours using CD14 Dynabeads and *PDPN* expression was measured. Interestingly, CRP-XL-activated samples showed a strong and steady increase in the relative expression of *PDPN* over time (mean fold change 42.7±8.9, 426±92.5, 894±269 and 4824±1699, at 2, 4, 6 and 8 hours respectively) (Figure 4.35 A). These levels were significantly higher than the *PDPN* gene expression in whole blood incubated for 8 hours without the addition of CRP-XL; mean fold change 97.9±22.8 (Figure 4.35 B). This slight increase in *PDPN* in the control cells probably reflects a low level of platelet activation occurring during the 8 hour incubation since the platelets were not inhibited in this experiment.

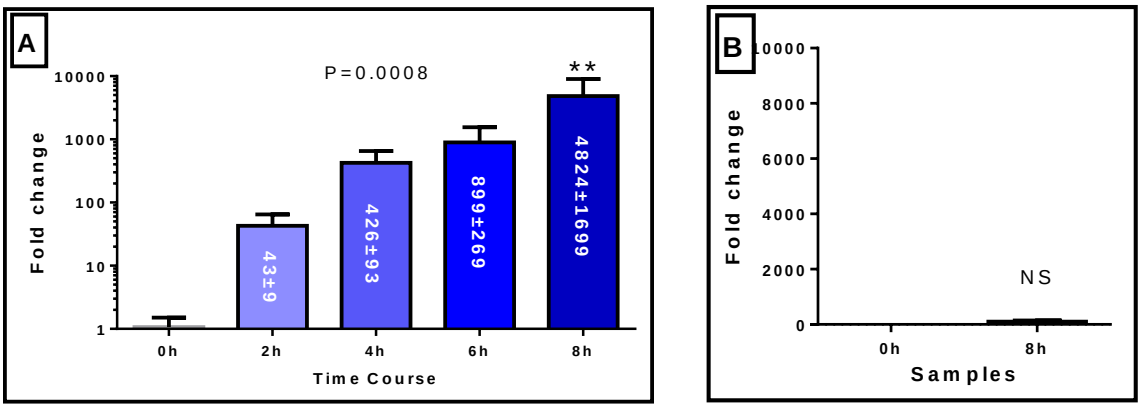

Figure 4.35. Induction of monocyte PDPN gene expression following platelet stimulation with CRP-XL. Monocytes were isolated from whole blood using CD14 Dynabeads, mRNA extracted, reverse transcribed and analysed for *PDPN* expression using qRT-PCR. Data were normalised against *B2M* and shown as relative expression (Fold change). (A) shows monocyte *PDPN* expression isolated at 0, 2, 4, 6 and 8 hours after platelet stimulation with 0.5 µg/mL CRP-XL and incubated at 37°C (n=6). (B) shows monocyte *PDPN* expression isolated from whole blood after incubation at 37°C for 8 hours without the addition of CRP-XL (n=3). Data are shown as mean±SD. P-values are versus 0 hour time point; **p<0.001; NS, not significant.

4.2.7.2 **Podoplanin gene expression in a thrombus**

The relative contribution of monocytes to *PDPN* expression in monocytes incorporated into a thrombus was also examined. For this purpose, thrombi were prepared for 6 hours at 37°C, in a Chandler loop (Jones and Goodall 2003), using whole blood with and without monocyte-depletion using CD14 Dynabeads. Thrombi collected at 6 hours were analysed for *PDPN* expression. Whilst all thrombi with the presence of monocytes showed *PDPN* amplification at cycle threshold (C_T) around 31, there were only two of the six monocyte-depleted thrombi showed *PDPN* amplification at C_T around 36, indicating that monocytes play the major role in *PDPN* up-regulation in thrombus. Because there was no *PDPN* amplification C_T values for four of the six monocyte-depleted thrombi, the measurement of the relative contribution of monocytes to *PDPN* upregulation was impossible. Therefore, *PDPN* amplification products from thrombus samples were visualised on agarose gel (Figure 4.36). It can be clearly seen from the *PDPN* amplification product density that podoplanin gene up-regulation in thrombus is mainly driven by monocytes, confirming the C_T value finding.

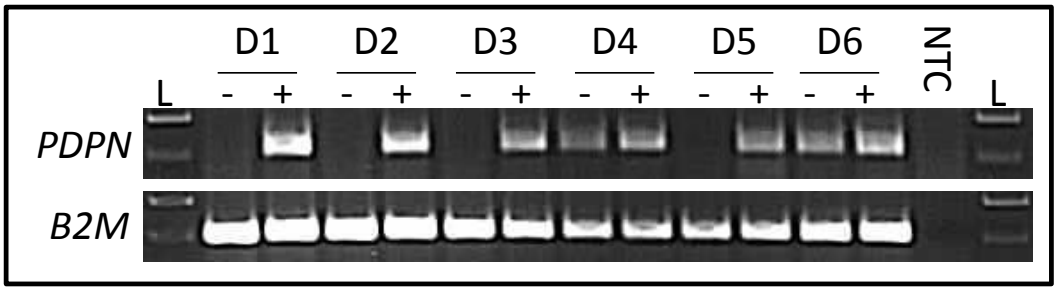

Figure 4.36. Podoplanin gene upregulation in thrombus is mainly from monocytes. Thrombi were formed in Chandler loop from six different donors (D1 to D6) with (+) and without (-) monocyte depletion using CD14 Dynabeads. Thrombi at 6 hours were collected, mRNA extracted, reverse transcribed and analysed for *PDPN* expression using RT-PCR. PCR products, 100 bP ladder (L) and no template control (NTC) PCR products were run alongside with the samples and visualised on 2% agarose gel stained with GelRed.

4.3 Discussion

4.3.1 Different roles GPCRs and ITAM-containing receptors in mediating platelet secretion and procoagulant response

The present study examined the role of individual platelet GPCRs and ITAM-containing receptors in inducing platelet procoagulant surfaces and MP generation using washed platelet preparation and measuring surface P-selectin expression and PS exposure as markers for platelet degranulation and procoagulant response respectively. The washed platelet preparation protocol has been validated to confirm that it does not cause non-specific platelet activation. It was decided to use washed platelet preparation for several reasons. First, it was thought that the snake venom, rhodocytin, might activate the coagulation system in whole blood or PRP sample preparations. Preliminary experiments showed that rhodocytin at high concentrations (1 µM) caused clot formation in PRP samples; despite the fact that the blood was anti-coagulated using sodium citrate and GPRP. Second, washed platelet preparation allows the study of the platelet response without the interference of other blood components such as leukocytes. Third, a diluted washed platelet suspension minimises platelet-platelet interaction. However, although platelet-platelet interaction is minimised and no fibrin fibres should be present in the washed platelet suspension, platelets release fibrinogen from their α-granules upon activation. Therefore, platelet-platelet interaction was further minimised by blocking GPIIb/IIIa, making the procoagulant activity more dependent on the signalling pathways through the individual receptors.

Platelet PS exposure and MP generation were measured by flow cytometry using Annexin V binding. Although flow cytometry is the most common used method to measure and characterise MPs, it is limited by the small size of MPs (1-0.1 µm in diameter). Despite this,

the flow cytometer used in this study (Beckman Coulter MCL-XL) showed a clear discrimination between platelets and their MPs on the basis of their size. However, it is likely that the smaller MPs remained undetected below the size threshold of the machine (0.5 µm diameter according to the manufacturer's specification). In addition, it has been reported that Annexin V is less sensitive in detecting PS exposure than lactadherin (Dasgupta, *et al* 2006), which may have underestimated the levels of platelet procoagulant responses.

4.3.1.1 **GPCRs**

GPCRs signalling pathways are distinct from ITAM-containing receptor signalling pathways. The data presented in this chapter confirm the notion that the platelet activation through individual GPCRs, with minimal platelet-platelet interaction, does not play a major role in mediating the procoagulant response. It has been reported that ADP-treated platelets are unable to express procoagulant surfaces (Ahmad, *et al* 2000). However, there have been conflicting data about the potency of thrombin in this respect. This may be due to variation in experimental conditions such as platelet preparation, platelet concentration, activation conditions and the endpoint measurement, which make comparing these studies difficult. There is accumulating evidence that thrombin-induced platelet activation results in only a small, or even no, increase in Annexin V binding (Alberio, *et al* 2000, Goodwin, *et al* 1994, London, *et al* 2004, Pedicord, *et al* 1998, Ramstrom, *et al* 2003). In sharp contrast, it has been reported that 60-80% of the platelet population expose PS when stimulated with 0.2-0.5 U/mL thrombin (Dormann, *et al* 2000). However, the latter study was performed using gel-filtered platelets activated with thrombin for 25 minutes at RT with gentle shaking, which may have resulted in greater stimulation of the platelets, for example through platelet-released ADP, or through platelet-platelet interaction. Subsequent experiments using relatively similar experimental conditions to Dormann *et al* study have failed to show a

marked increase in Annexin V in thrombin-stimulated platelets (Ramstrom, *et al* 2003). Overall, it appears that the transient increase in the levels of the cytosolic calcium after platelet activation through GPCRs is only sufficient to induce a small degree of PS exposure. However, the ADP and TxA_2 provide critical feedback signalling for platelet procoagulant activity induced via ITAM-containing receptors (discussed below).

4.3.1.2 **ITAM-containing receptors**

4.3.1.2.1 **GPVI**

It has been well established that platelet activation solely through GPVI mediates platelet procoagulant response and MP formation (Munnix, *et al* 2005, Ramstrom, *et al* 2003). This finding has been confirmed in the present study and extended with an interesting observation that GPVI is distinct from other platelet ITAM-containing receptor in terms of inducing platelet secretion (discussed below). However, despite the fact that GPVI is the primary receptor eliciting platelet procoagulant responses and has been used as a standard protocol in many laboratories to induce platelet procoagulant response, there is some inconsistency in literature regarding its relative potency in this aspect. Some studies showed only a small subpopulation of GPVI-stimulated platelets expressed PS (Alberio, *et al* 2000, Keuren, *et al* 2005), whereas others showed higher percentage of platelets bound to Annexin V (Ramstrom, *et al* 2003). However, the previous discussion regarding experimental condition differences applies here. What is agreed is that less than 100% of platelets activated through GPVI can be induced to become procoagulant.

4.3.1.2.2 **FcγRIIA**

Platelet activation through FcγRIIA has also been shown, by itself, to generate platelet procoagulant surfaces and MPs. Warkentin and coworkers showed that platelet activation by immune complexes in HIT patient sera act via FcγRIIA leading to platelet activation, PS

exposure, MP generation, thrombus formation and consequent thrombocytopenia (Warkentin, *et al* 1994). FcγRIIA can be also activated by soluble immune complexes and certain anti-platelet antibodies. It has been suggested that immune complexes that activate platelet through FcγRIIA are able to directly cross-link the receptor (Dereys, *et al* 1993), whereas anti-platelet antibodies make a bridge between their antigens and the receptor (Anderson, *et al* 1991, Rubinstein, *et al* 1995, Rubinstein, *et al* 1991). Platelet FcγRIIA signalling mechanism is not well understood. However, it is believed that FcγRIIA shares the same signalling pathway of GPVI. Early studies have shown that cross-linking of FcγRIIA leads to Syk tyrosine kinase phosphorylation followed by PLCγ2 activation (Maxwell, *et al* 1999, Ozaki, *et al* 2000, Qi, *et al* 1999), which resembles the signalling mechanism of GPVI.

The platelet procoagulant response induced through FcγRIIA was confirmed in this study, which showed relatively higher potential in inducing platelet procoagulant surfaces and MPs than activation through GPVI. This seems to contradict a previous finding that showed that platelet co-stimulation with the GPVI agonist convulxin and thrombin resulted in more COAT (convulxin/collagen and thrombin-induced) platelet production than co-stimulation with IV.3 and thrombin (Batar and Dale 2001). However in that study, a very low concentration of IV.3 was used (1 µg/mL compared to 15 µg/mL used in the present study) and the end points measured were FV, fibrinogen and vWF binding, but not Annexin V binding. In the present study, increasing concentrations of IV.3 showed a steady increase in Annexin V binding, which did not reach a plateau. This finding contradicts the notion that platelet procoagulant activity is restricted to a subpopulation of platelets, as seen in platelet stimulation with collagen or combination of collagen and thrombin. However, it is unclear at this stage whether these high percentages of platelet procoagulant response were due to individual variations, or difference in signalling mechanism of FcγRIIA. Further work is required to confirm this difference between FcγRIIA and other platelet ITAM-containing

receptors in this regards. Nevertheless, data in chapter 5 of this thesis also showed that platelet activation through FcγRIIA by HIT patient sera induces similar, or even higher, levels of PS-exposing platelets.

4.3.1.2.3 **CLEC-2**

In recent years, the role CLEC-2 in platelet activation has attracted considerable interest; however, its patho/physiological role still poorly understood. Since the downstream signalling mechanism of CLEC-2 resembles the signalling of other platelet ITAM-containing receptors, the question arises whether CLEC-2 is also capable of generating a procoagulant platelet surface and MPs. This question was specifically addressed in the present study. Platelet activation via CLEC-2 was induced using the snake venom, rhodocytin. Rhodocytin was initially thought to induce platelet activation via integrin α2β1 (Suzuki-Inoue, *et al* 2001). Another independent study also reported that rhodocytin mediates platelet activation by binding to integrin α2β1and GPIbα, but not to GPVI (Navdaev, *et al* 2001). However, these findings were shortly later disproven by the finding that rhodocytin is able to stimulate platelets lacking integrin α2β1, GPVI or the extracellular domain of GPIbα (Bergmeier, *et al* 2001). Five years later, CLEC-2 was identified as the rhodocytin receptor (Suzuki-Inoue, *et al* 2006). The same study showed that platelet activation induced by anti-CLEC-2 is independent from FcγRIIA. CLEC-2 induces platelet activation through Src kinases family, but independently from the FcγRIIA and GPVI/FcRγ-chain complexes.

Data in the present study clearly show, for the first time, that CLEC-2 induces platelet procoagulant response and MP generation in a similar manner to GPVI. However, during the writing of this thesis, a paper (from Bernhard Nieswandt group in collaboration with Steve P. Watson who is also involved in the present study) has appeared showing that CLEC-2 induces Annexin V binding in more than 90% of mouse platelets (Dutting, *et al* 2014). There were some differences between the study of Dutting *et al* and our own in addition to the

difference in species; they used mouse platelets compared to ours in humans. While it is well established in literature that platelet procoagulant activity is restricted to a subpopulation of platelets even with co-stimulation with more than one agonist, the report of Dutting *et al* that the majority, if not all, of the platelets exposed PS. They collected blood into 20 U/mL heparin, which may itself induce some platelet activation. In addition, washed platelets were activated at high platelet density and the activation was stopped by adding only 500 µL buffer, which increases the chances of platelet-platelet interaction.

The role of the only known natural ligand of CLEC-2, podoplanin, in inducing platelet procoagulant response and MP formation was also investigated in the present study. Platelet activation by recombinant mouse podoplanin through CLEC-2 showed potent platelet degranulation, procoagulant response and MP generation. Additionally, data in the present study revealed that platelets interact with podoplanin expressing cells and they become degranulated, expose PS and release procoagulant MPs.

Podoplanin is the only known endogenous ligand for CLEC-2. It is a highly O-glycosylated type I transmembrane sialomucin-like glycoprotein that is expressed in wide variety of different cell types including lymphatic endothelial cells, kidney podocytes, fibroblastic reticular cells, lung type I alveolar cells, and some tumour cells, but it is absent in the normal vasculature (Navarro-Nunez, *et al* 2013, Watson, *et al* 2010). The question arises whether podoplanin could be upregulated in the circulation. This question was specifically addressed in the present study using two models: (1) platelet-induced podoplanin up-regulation in monocytes and (2) thrombus-induced podoplanin up-regulation. Both models showed that monocytes have a high potential to express podoplanin when they exposed to activated platelets or when they incorporated into thrombus. However, the time scale for podoplanin gene up-regulation in both models suggests that podoplanin monocyte up-regulation does not play a major role in thrombosis, as it requires very quick response. Interestingly, a recent

report has revealed that podoplanin is expressed on inflammatory macrophages and is markedly upregulated by LPS. These macrophages directly activate platelets via CLEC-2, which suggests that CLEC-2 may play a role in atherosclerosis (Kerrigan, *et al* 2012). This suggestion was further supported by the finding that podoplanin was found to be expressed in smooth muscle and macrophages in atherosclerotic lesions in abdominal aortas (Hatakeyama, *et al* 2012). Podoplanin expression increased in advances lesions compared to early lesions. These findings suggest that podoplanin/CLEC-2 platelet activation plays an important role in inflammation rather than thrombosis.

The novel finding in the present study of the role of CLEC-2 in inducing platelet procoagulant response and MP formation adds a key piece of information to the current understanding of the patho/physiological role of CLEC-2. It has been shown that CLEC-2 plays a crucial role in lymph/blood vessel separation in the embryonic development (Bertozzi, *et al* 2010, Carramolino, *et al* 2010, Finney, *et al* 2012, Herzog, *et al* 2013). Additionally, it has been recently shown that CLEC-2, similar to GPVI, plays a critical role in maintaining vascular integrity at sites of inflammation (Boulaftali, *et al* 2013). Since CLEC-2 has been shown to play a critical role in formation of lymphatic vessel and maintaining vascular integrity and since it induces platelet degranulation and procoagulant response, it can be speculated that these functions involve platelet degranulation and procoagulant response rather than aggregation. This speculation can be supported by the finding that mice with aggregation-incompetent platelets do not exhibit the lymphatic vascular defects (Hodivala-Dilke, *et al* 1999), whereas mice with CLEC-2 function impairments develop embryonic/neonatal lethality due to blood/lymphatic mixing (Bertozzi, *et al* 2010, Suzuki-Inoue, *et al* 2010). However, this hypothesis requires further work to be confirmed.

Furthermore, there is accumulating evidence that podoplanin is upregulated in a variety of cancer cell types, which induces platelet aggregation surrounding tumour cells. It has been

suggested that platelet aggregates contribute in metastasis and/or progression of cancer by protecting circulating tumour cells from shear stress and immune cells, facilitating their extravasation from the circulation (reviewed in (Navarro-Nunez, *et al* 2013).Cancer patients are known to have higher thrombotic risk due to increased levels of circulating procoagulant MPs (Kleinjan, *et al* 2012). Since certain tumour cells express podoplanin and since the data in the present study shows that CLEC-2 induces platelet procoagulant response and MP generation, this leads to speculation that CLEC-2 may play a role in increasing the risk of thrombosis in tumour patients not only by inducing platelet aggregation but also by MP production. However, further work is required to establish this assumption.

On the other hand, the role of CLEC-2 in haemostasis and thrombosis is poorly understood, with some contradictory findings. On one hand, it was reported that CLEC-2 depletion results in a significant increase in the bleeding time in mice (May, *et al* 2009), while on the other hand other reports showed only a mild increase in the bleeding time in CLEC-2-defecient mice (Hughes, *et al* 2010a, Suzuki-Inoue, *et al* 2010). However, since the endogenous ligand of CLEC-2 that directly plays a role in haemostasis and thrombosis is still unknown, it is difficult to determine the exact functional role of CLEC-2 in haemostasis and thrombosis. This has led to a focus on identifying an additional ligand of CLEC-2 within the vasculature or membrane proteins expressed upon activation.

4.3.1.2.4 **Clustering of ITAM-containing receptors is critical for their activity**

Data in the present study support the concept that ITAM-containing receptor clustering is critical for their functional platelet activation. FcγRIIA platelet activation using IV.3 antibody requires the secondary antibody to cross-link the receptor, which is in agreement with previous reports (Ozaki, *et al* 2000, Qi, *et al* 1999). In terms of CLEC-2, platelet activation using recombinant mouse podoplanin fused with human Fc also clearly shows that CLEC-2 activates platelets only after the cross-linking by the secondary antibody. This finding is

supported by several studies using antibodies to activate CLEC-2 (May, *et al* 2009, Spalton, *et al* 2009, Suzuki-Inoue, *et al* 2006). Additionally, crystal structures of rhodocytin show that it assembles a tetramer, which is thought to induce activation of CLEC-2 through clustering (Hooley, *et al* 2008, Watson, *et al* 2008). Furthermore, it has been demonstrated for mouse platelets that GPVI cross-linking using GPVI-specific antibody JAQ1 is required for signal transduction (Nieuwland, *et al* 2000).

4.3.2 Additive effect of GPCRs and ITAM-containing receptors

It is well established that platelet co-stimulation by thrombin and collagen is the most potent platelet procoagulant inducer (Alberio, *et al* 2000, Bevers, *et al* 1983). Additionally, thrombin has been shown to enhance the procoagulant activity of FcγRIIA (Batar and Dale 2001). In the present study, combined activation of thrombin with each of platelet ITAM-containing receptors shows more enhancement than ADP and TxA_2 in generating procoagulant platelets and MPs. Data also shows that TRAP has a comparable levels, although slightly less, to thrombin in the potential of enhancing the platelet procoagulant activity of CRP-XL and rhodocytin. This finding suggests that PAR1 is the main mediator for thrombin synergistic effect, which is in the line with previous reports (Andersen, *et al* 1999, Keuren, *et al* 2005). This finding also suggests that PAR4 and GPIbα, the third thrombin receptor, play a minimal role in the synergistic effect of thrombin on the platelet procoagulant activity of CRP-XL and rhodocytin. Furthermore, a recent report showed that platelet activation through FcγRIIA increases platelet sensitivity to thrombin (Berlacher, *et al* 2013). In that study, platelets were stimulated through FcγRIIA using heat-aggregated IgG prior to the activation with thrombin. This finding is in line with the findings in the present study,

although different approaches were used. The authors used the term “hypersensitivity” to describe the synergistic effect.

Data in this study clearly show that platelet activation via more than one ITAM-containing receptor enhances the procoagulant activity. The question arises as how ITAM-containing receptor enhances other ITAM-containing receptor procoagulant activity since they share the same downstream signalling events. It is not clear whether each of the ITAM-containing receptors has any independent signalling pathways to account for this additive effect. Interestingly, although platelet activation through CRP-XL or IV.3 individually showed higher potential than rhodocytin in inducing platelet Annexin V binding by their sole actions, the co-activation with IV.3 and rhodocytin showed more procoagulant potential than co-stimulation with IV.3 and CRP-XL. This could be due to the metalloproteinase-dependent shedding of the GPVI ectodomain after ITAM-dependent platelet activation (Gardiner, *et al* 2004).

The concept of coated-platelets, formerly known as COAT-platelets, has attracted considerable interest since they were first described in 2000 (Alberio, *et al* 2000). Platelet co-activation with collagen and thrombin results in a subset of platelets that bind high levels of FV, fibronectin, fibrinogen, vWF and thrombospondin (Dale, *et al* 2002). In addition, the same research group demonstrated that the dual stimulation via FcγRIIA agonists and thrombin results a subpopulation that resembles coated-platelets, named FcRT-platelets (Fc receptor and thrombin-activated platelets) (Batar and Dale 2001). Whether the procoagulant platelets induced by co-stimulation with rhodocytin and thrombin resemble coated- or RcRT-platelets was not addressed in the present study. However, since platelet co-activation with rhodocytin exhibits similar pattern of procoagulant activity to those in GPVI and FcγRIIA when combined with thrombin, this leads us to assume this co-activation may also result in a similar platelet subpopulation of coated- or RcRT-platelets.

4.3.3 Mechanisms underlying the platelet procoagulant response

4.3.3.1 **Platelet procoagulant response heterogeneity**

Data in the present study support the concept that not all platelets are capable of expressing the same procoagulant activity, a notion that is supported by several studies (Alberio, *et al* 2000, Batar and Dale 2001, Dacharyprigent, *et al* 1993, Heemskerk, *et al* 1997, Kempton, *et al* 2005, Ramstrom, *et al* 2003). The present study clearly shows that only a fraction of platelets is capable of exposing PS after activation through ITAM-containing receptors, despite the enhancement of Annexin V binding in combined stimulation by more than one agonist. On the other hand, platelet activation through these receptors results in essentially all platelets expressing P-selectin. This suggests that all platelets are able to release their α-granule contents when they are exposed to ITAM-containing receptor agonists, whilst only a fraction is able to expose PS. It has been demonstrated that young (reticulated) platelets are more likely to exhibit a procoagulant response than older platelets (Alberio, *et al* 2000).

4.3.3.2 **Role of the secondary mediators**

It well established that platelets release the secondary mediators ADP and TxA_2 upon activation, which play an important positive feedback role in mediating platelet activation of GPVI (Atkinson, *et al* 2003, Nieswandt, *et al* 2001). In addition, a recent report showed CLEC-2-mediated platelet aggregation is highly dependent on ADP and TxA_2 as the aggregation was abolished with combined inhibition of ADP, using apyrase, and TxA_2, using indomethacin (Pollitt, *et al* 2010). Data in this project further confirm the role of the secondary mediators to CLEC-2 and GPVI but in the aspect of platelet procoagulant activity rather than aggregation. Both exogenous concentrations and endogenously released ADP and TxA_2 showed a significant enhancement to the platelet procoagulant activity of these

receptors, although they varied in their degrees of contribution (see below). In addition, this study also investigated the contribution of the different ADP receptors to CLEC-2-mediated procoagulant activity. Consistent with the study of Pollitt *et al*, $P2Y_{12}$, but not $P2Y_1$, plays the main role of ADP contribution to CLEC-2 platelet activation. Data in the present study also shows that $P2Y_{12}$ plays a main role in MP production, but not in platelet PS exposure, as its inhibition showed a dramatic reduction in MP formation but platelet Annexin V binding was not significantly affected. This may suggest that ADP activation through $P2Y_{12}$ may be more important in regulating mechanisms involved in MP budding such as activation of calpain. The role of ADP receptors in GPVI-mediated platelet procoagulant activity was not investigated in this project; however, a previous study in this group (Jackie Appleby PhD thesis) showed a similar role for the ADP receptors in respect of augmenting GPVI signalling compared to their effects on the CLEC-2 response as shown in the present project.

The contribution of the endogenously released TxA_2 to CLEC-2 and GPVI platelet procoagulant activity was also investigated with the use of COX-1 inhibitor, aspirin. Data in the present study clearly showed that CLEC-2 platelet activation is highly dependent on TxA_2, which is supported by a previous finding (Pollitt, *et al* 2010). This finding could be linked to the well-established protective effect of aspirin in certain cancer diseases taking into the account that some tumour cells express podoplanin, which in turn activates platelets via CLEC-2 and increases the mortality through facilitating tumour metastasis as well as increases the risk of thrombosis (Burn, *et al* 2011, Jacobs, *et al* 2012, Rothwell, *et al* 2012). This hypothesis could be supported by the finding that experimental tumour metastasis was abrogated by blocking podoplanin-platelet interactions using NZ-1 antibody, which neutralises the interaction between podoplanin and CLEC-2 (Kato, *et al* 2008). However, this hypothesis requires further work to be confirmed. Aspirin also showed a significant inhibition of GPVI-mediated procoagulant activity, which is in line with previous reports (Atkinson, *et*

al 2003, Nieswandt, *et al* 2001). Interestingly, our data showed that CRP-XL platelet procoagulant activity is less dependent on TxA_2 than rhodocytin. This finding is in agreement with Pollitt *et al* who found that CLEC-2 phosphorylation is distinct from GPVI as CLEC-2, but not GPVI/FcRy-chain, is highly dependent on actin polymerisation, release of ADP and TxA_2 and the small G protein Rac1 (Pollitt, *et al* 2010).

Despite the fact that the positive feedback role of the secondary mediators in platelet activation is well established, the exact mechanism by which the secondary mediators contribute to CLEC-2 and GPVI platelet procoagulant activities still poorly understood. Pollitt and co-workers found that CLEC-2, but not GPVI/FcRy-chain, tyrosine phosphorylation was dramatically reduced in the presence of apyrase and indomethacin, whereas the presence of ADP and U46619 had no significant effect on CLEC-2 phosphorylation (Pollitt, *et al* 2010). This finding indicates that the role of secondary mediators in regulating CLEC-2 tyrosine phosphorylation is indirect. In that study, the authors suggested that platelet activation through CLEC-2 occurs in the following steps: (1) CLEC-2 translocates to lipid rafts; (2) initiates CLEC-2 tyrosine phosphorylation which is dependent of actin polymerization; (3) CLEC-2 phosphorylation and downstream signalling events are reinforced by the release of the secondary mediators; and (4) Rac1 activation plays a role in this feedback mechanism of CLEC-2 activation.

4.3.3.3 **Role of GPIIb/IIIa**

It is well established that ligand binding to GPIIb/IIIa not only mediates platelet aggregation, but also initiates a series of intracellular signalling events, involving the phosphorylation of the two NxxY motifs in the cytosolic tail of GPIIb/IIIa through SFK-dependent signalling events and activation of PLCγ2 (reviewed by (Li, *et al* 2010, Watson, *et al* 2005)). GPIIb/IIIa clustering is also critical for its role in platelet activation as only the multimeric

macromolecular ligands such as fibrinogen, but not the monomeric peptide induce signalling events (Leisner, *et al* 1999). Data in the present study clearly show that GPIIb/IIIa plays a role in mediating platelet procoagulant activity induced through GPVI and CLEC-2 with an observation that CLEC-2, unlike GPVI, is more dependent on outside-in signalling through GPIIb/IIIa. These findings are in agreement with previous reports that GPIIb/IIIa inhibition results in a decrease of platelet procoagulant activity including prothrombinase activity and PS exposure using various agonists (Dormann, *et al* 2000, Furman, *et al* 2000, Gemmell, *et al* 1993, Lages and Weiss 2001, Ramstrom, *et al* 2003). In contrast, platelet procoagulant activity mediated through FcγRIIA was not affected by GPIIb/IIIa inhibition. This finding is in disagreement with previous finding of Boylan *et al*, which showed that outside-in signalling though GPIIb/IIIa increases FcγRIIA phosphorylation (Boylan, *et al* 2008). In that study; however, a different approach was used. The authors investigated the contribution of GPIIb/IIIa to FcγRIIA-mediated platelet activation by inducing outside-in signalling with fibrinogen, whereas in the present study GPIIb/IIIa was inhibited with RFPG56 antibody.

Data in the present study do not give a clear indication as to how GPIIb/IIIa contributes to the procoagulant activity of CLEC-2 and GPVI. However, because the experiments were carried out in diluted washed platelet suspension, which minimises the chance for platelets to aggregate, this may suggest that the inhibition is not simply because of inhibiting platelet-platelet interaction. This could be supported by the fact that fibrinogen was absent in the washed platelet suspension, although activated platelets do release some fibrinogen from their α-granules. This leads to speculation that GPIIb/IIIa signalling pathway mechanism is required for CLEC-2 and GPVI procoagulant activity. Nevertheless, the exact mechanism of how GPIIb/IIIa contributes to platelet procoagulant activity is still poorly understood. One route may be via calpain activation (Fox, *et al* 1993). This is further supported by the current

finding that GPIIb/IIIa plays a role platelet PS exposure, which requires calpain activation, but not in degranulation (Ilveskero and Lassila 2003).

On the other hand, data from the present study clearly showed that that 10 µg/mL RFGP56 antibody markedly inhibits platelet aggregation, but does not, by itself cause platelet activation. In sharp contrast, several reports showed that certain GPIIb/IIIa-specific IgG antibodies can cause robust platelet activation (Anderson, *et al* 1991, Horsewood, *et al* 1991, Rubinstein, *et al* 1991, Tomiyama, *et al* 1992). However, these studies also showed that it was the Fc portions of these antibodies that activated platelets as blocking FcγRIIA with IV.3 antibody completely abolished their activity. Several potential mechanisms have been suggested of how Fc region of anti-GPIIb/IIIa IgG activates FcγRIIA, but the most likely is that the Fab portion of the antibody binds to its antigen target on platelet surface while the Fc region interacts with FcγRIIA either on the same platelet (intra-platelet) or on adjacent platelets (inter-platelet). This variation between anti-GPIIb/IIIa antibodies in inducing platelet activation may due to the differences in their epitope specificities.

4.3.3.4 **Signalling mechanisms of CLEC-2 and GPVI**

As discussed, one of the differences between in CLEC-2 and GPVI signalling mechanisms is that CLEC-2 phosphorylation is more dependent on actin polymerisation, release of ADP and TxA_2 and Rac1. Another interesting differences is that the phosphorylation of the ITAM motif of the GPVI/FcRy-chain is mainly dependent on the Src family kinases phosphorylation which subsequently leads to the activation of Syk kinases, whereas in CLEC-2 is mediated by both Src and Syk tyrosine kinases (Spalton, *et al* 2009). This finding was achieved with the use of the selective inhibitor of Syk, R406, which completely inhibits CLEC-2 phosphorylation, but not GPVI/FcRy-chain. In the present study, the role of Src and Syk tyrosine kinases signalling pathway mechanisms of CLEC-2 and GPVI were investigated

at the functional assay rather than phosphorylation levels. Both CLEC-2 and GPVI platelet procoagulant activities were completely dependent on Src kinases as the Annexin V binding in rhodocytin and CRP-XL stimulated platelets was completely abolished with the use of the SFK inhibitor, dasatinib. The other SFK inhibitor, PP2, at the concentration used, further supported this finding. However, the concentration of PP2 used may have been insufficient to completely inhibit the procoagulant activity of GPVI. Nevertheless, these findings are consistent with the well-established role of Src family kinases in mediating platelet activity through CLEC-2 and GPVI (Ezumi, *et al* 1998, Quek, *et al* 2000, Severin, *et al* 2011, Suzuki-Inoue, *et al* 2006). On the other hand, the use of the Syk kinase inhibitors PRT-318 and R405 showed that the procoagulant activity of CLEC-2 and GPVI is also completely dependent on Syk kinases, despite the fact that there is a distinct difference in the proximal events in their signalling mechanisms. This finding suggests that Syk kinases play a critical role further downstream in CLEC-2 and GPVI signalling cascades. Of note, 1 µM R405 showed complete inhibition of rhodocytin platelet procoagulant activity, whereas marked but not complete inhibition of CRP-XL. This finding could be supported by the fact that R405 has little effect of the GPVI/FcRy-chain phosphorylation.

It is well established that PI3K plays a critical role in mediating platelet activation induced through GPVI (Gilio, *et al* 2009, Kim, *et al* 2009). Data in the present study confirm the key role of PI3K in mediating GPVI platelet procoagulant activity. Whilst largely known about the key role of PI3K in mediating GPVI platelet activation, the data presented here clearly showed that PI3K also plays a similar role in CLEC-2 platelet procoagulant activity.

4.3.4 Individual variability in platelet procoagulant activity

Data in the present study clearly show that there was a higher degree of individual variability in the platelet procoagulant response to rhodocytin compared to CRP-XL. Analysis of the platelet response in certain individuals at different time points showed that individuals maintain their relative platelet procoagulant response to these agonists over time, indicating that the variability is inherent to the individual. Additionally, low responders to rhodocytin showed a negligible amount of increase in platelet procoagulant activity when incubated with rhodocytin for 30 minutes compared to 10 minutes, confirming that there is no lag in the effect of this agonist. These findings together support the notion that individuals vary considerably in their procoagulant response mediated through CLEC-2.

Another striking finding in the variation between individuals in their platelet procoagulant responses to rhodocytin and CRP-XL is that individuals showed a strong correlation in their responses to these agonists, despite the magnitude of the response. Since CLEC-2 and GPVI share similar downstream signalling mechanisms, it could be speculated that the response variation through GPVI and CLEC-2 is largely regulated by the variation in the downstream signalling pathway mechanisms. Interestingly, it has been reported that up to 35% of individual response variation to CRP-XL is associated with genetic polymorphisms in the *GP6* gene (Jones, *et al* 2009). However, further work is required to investigate other factors such as levels of receptors expression, genetic variation and platelet hyperactivity.

4.3.5 Relationship between degranulation and procoagulant response

It has been suggested that platelet degranulation and procoagulant response are regulated through different mechanisms (Ilveskero and Lassila 2003, Thiagarajan and Tait 1990). This notion was based on platelet activation by collagen, and is supported by the data in the present study that showed variation between platelet degranulation and procoagulant response only seen in GPVI platelet stimulation. In contrast, CLEC-2 platelet stimulation showed degranulation and procoagulant response seem to be regulated through the same mechanism. This evidence is derived from the following observations: (1) whilst the CRP-XL dose titration showed P-selectin expression is induced by low dose of CRP-XL compared to Annexin V binding, the rhodocytin and IV.3 dose titrations showed P-selectin expression and Annexin V binding occur concurrently; (2) the lack of correlation between P-selectin expression and Annexin V binding in individuals' responses to CRP-XL, whereas there was a strong correlation in response to rhodocytin; and (3) GPIIb/IIIa and PI3K inhibition resulted in a significant inhibition in PS exposure but not in P-selectin expression in CRP-XL-stimulated platelet, whereas P-selectin expression and PS exposure were both significantly inhibited in rhodocytin-stimulated platelets. These findings together show that platelet α-granules release and PL rearrangement are regulated in a similar manner in CLEC-2, but not in GPVI platelet activation.

4.3.6 CLEC-2 activated platelets and their MPs promote thrombin generation

Since the present study confirmed that CLEC-2 mediates the platelet procoagulant response and MP generation, it was important to evaluate the functional properties of this response.

Platelet negatively charged PL surfaces have been long known to enhance the thrombin generation by assembling and activating the two major coagulation factor complexes, tenase and prothrombinase. However, although platelet membrane PL rearrangement plays a key role in the procoagulant activity of platelets, it is not the only factor that regulates this response. Platelet glycoproteins for instance have been shown to provide binding sites for prothrombin, factor XI and FV (Heemskerk, *et al* 2005).

The thrombin generation assay is a well-established method of measuring the overall haemostatic response of plasma and the contribution of procoagulant cells and MPs. As described earlier in this thesis, the CAT assay has been optimised to provide a sensitive measurement of the contribution of the negatively charged PLs of platelets and MPs to thrombin generation. As reported in this thesis and in the literature (Aleman, *et al* 2011), PDMPs from washed platelets fail to support thrombin generation in the absence of TF and contact activation pathway. Therefore, 1 pM exogenous TF was used to trigger thrombin generation in this study. CLEC-2-activated platelets and their generated MPs clearly support thrombin generation in PL-dependent manner, indicating the functional properties of CLEC-2 platelet procoagulant activity. This was analysed alongside CRP-XL-activated platelets and their MPs, which also showed similar findings. Additionally, MPs isolated from platelets exposed to podoplanin-expressing cells (CHO cells) also showed a significant enhancement of thrombin generation.

Taken together the data in this chapter show that activation of human platelets through any of the three ITAM receptors can induce a procoagulant surface on platelets and release of procoagulant MPs through mechanisms that are largely similar, but with some important differences that probably relate to differences in their down-stream signalling pathways. It is likely that these receptors play important roles in causing platelets to generate thrombin in different physiological situations; GPVI in haemostasis, CLEC2 in development, and

FcγRIIA in the innate immune response to certain bacteria. All three receptors may play a role in thrombin generation in pathological situations; GPVI, and possibly CLEC2 in thrombosis; CLEC2 in cancer and FcγRIIA in HIT.

CHAPTER 5: **Evaluation of the procoagulant activity of platelet-derived microparticles in heparin-induced thrombocytopenia**

5.1 Introduction

5.1.1 Definition

HIT is a transient, life-threating, prothrombotic disorder initiated by heparin, typically after five to fifteen days of treatment (Arepally and Ortel 2006, Greinacher, *et al* 2010, Warkentin 2003). It was first described more than five decades ago (Weismann and Tobin 1958), and since then more insights into the pathogenesis of HIT have revealed that it is mediated by antibodies against PF4 and heparin complexes (PF4/heparin complexes) (Amiral, *et al* 1992). PF4 is a positively-charged, tetrameric, platelet-specific chemokine that is stored in α-granules (Li, *et al* 2002).

Unfractionated heparin (UFH) is one of the most commonly used anticoagulants. It is a glycosaminoglycan (GAG) that binds to antithrombin through a pentasaccharide sequence leading to changing antithrombin activity from a slow to very rapid thrombin inhibitor by altering the affinity of antithrombin for thrombin. Low-molecular weight heparin (LMWH) has been developed using synthesised pentasaccharide sequence and is less able to cause HIT compared to UFH (less than 1% compared to up to 5% respectively) (Greinacher, *et al* 1995). Despite the low platelet count in HIT, the major clinical complication is venous and arterial thrombosis due to the presence of high levels of procoagulant MPs in the circulation (Greinacher, *et al* 2010).

5.1.2 Pathogenesis

Under normal conditions, PF4 is found in low levels in human plasma; however, its level increases dramatically after heparin infusion, by displacement from the surface of endothelial cells (Arepally and Ortel 2010). The binding of PF4 to heparin forms linear multi-molecular clusters with multiple neoepitopes on PF4, termed PF4/heparin complexes. These complexes circulate in a soluble form or bind to platelet surfaces through the negative charge of heparin (Figure 5.1) (Warkentin 2003). The neoepitopes on PF4 can trigger an immune response leading to the production of IgM, IgA and/or IgG antibodies. These antibodies are not exclusively specific for PF4/heparin complex, but also recognise PF4 complexes with other anionic GAGs (Goad, *et al* 1994, Greinacher, *et al* 1992). The antibodies bind to PF4 forming large immune complexes known as HIT-immune complexes. There is accumulating evidence that the main pathogenic isotype of HIT antibodies is IgG (Amiral, *et al* 1992, Amiral, *et al* 1996, Suh, *et al* 1997). When several IgG antibodies bind to PF4/heparin complexes on platelet surfaces, the Fc region of the IgG molecules cross-link FcγRIIA either on the same platelet or on adjacent platelets, leading to platelet activation, aggregation, degranulation, procoagulant response and MP generation (Hughes, *et al* 2000, Reilly, *et al* 2001, Warkentin, *et al* 1994). The procoagulant platelets and their released MPs induce thrombin generation resulting in arterial or venous thrombosis. Additionally, activated platelets release more PF4 from platelet α-granules, which in turn leads to formation of additional HIT-immune complexes through a positive-feedback mechanism (Newman and Chong 2000).

Other cells may contribute in the thrombotic nature of HIT such as endothelial cells and monocytes. For example, HIT antibodies bind to endothelial cells and induce TF expression, which is enhanced in the presence of platelets (Cines, *et al* 1987). Monocytes also have been shown to synthesis TF when PF4/heparin complexes bind to the monocyte surface GAGs

(Arepally and Mayer 2001, Pouplard, *et al* 2001). More recently, HIT-immune complexes have been shown to activate monocytes through FcγRI receptor and induce the production of TF-positive monocyte-derived MPs (Kasthuri, *et al* 2012).

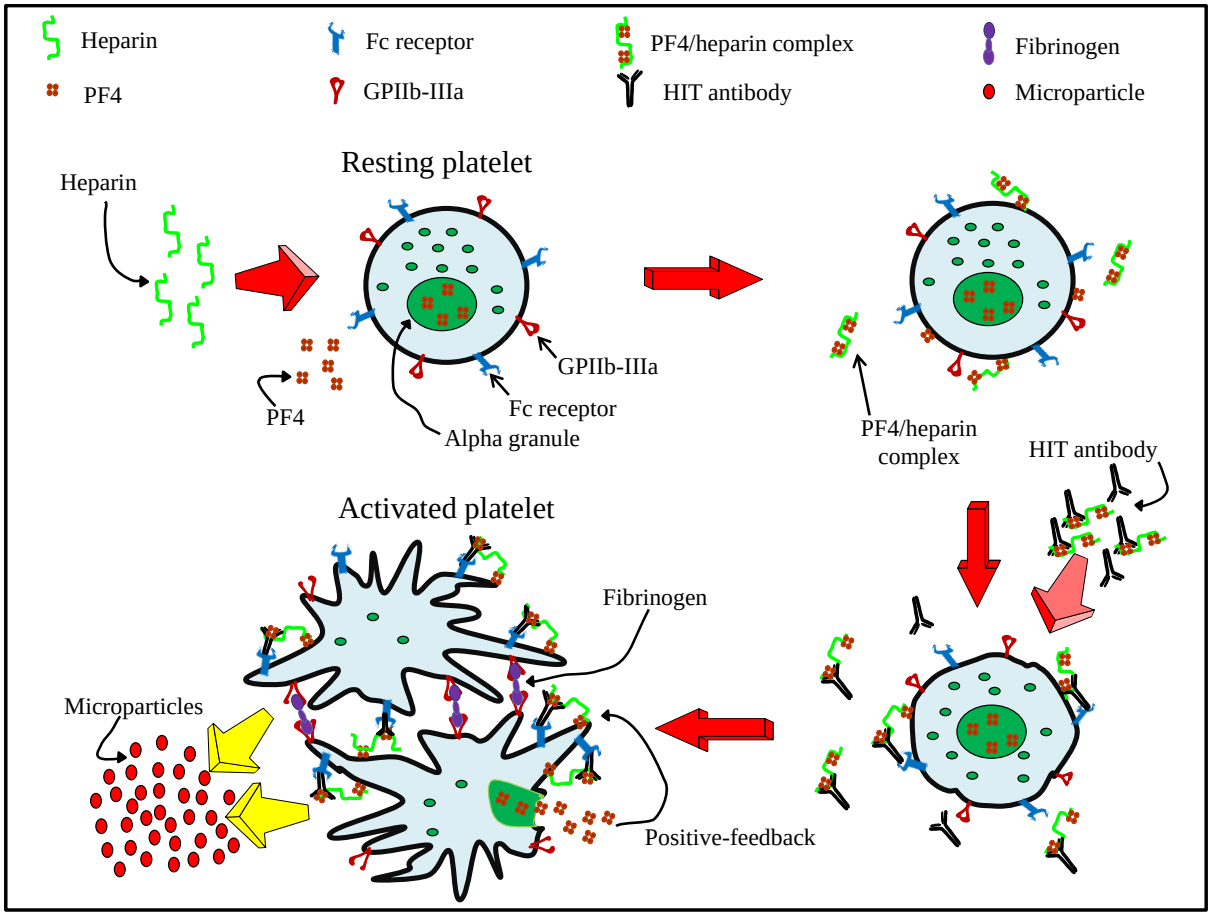

Figure 5.1. Schematic presentation of platelet activation in HIT. Heparin binding to PF4 results in the formation of PF4/heparin complexes that bind to platelet surfaces or circulate in free form. PF4/heparin complexes expose new epitopes, leading to formation of anti-PF4/heparin complex antibodies. The Fc part of IgG antibody in HIT-immune complexes binds and cross-links the platelet FcγRIIA, resulting in platelet activation, aggregation and MP shedding. Adapted from Warkentin (2003).

5.1.3 Diagnosis

The diagnosis of HIT can be challenging, given the fact that there is no gold standard laboratory test for the diagnosis. Therefore, it is commonly accepted that laboratory investigations should be interpreted in the context of the clinical presentation of the patient (Arepally and Ortel 2010).

5.1.3.1 **Clinical diagnosis**

Two main features are essential for establishing a clinical diagnosis: (1) presence of clinical complications (thrombocytopenia and/or thrombosis) and (2) absence of other causes of thrombocytopenia. Other causes of platelet fall such as bacterial infection, bone marrow disease and drugs other than heparin should be excluded. The timing of HIT presentation is also critical in the clinical diagnosis. Typically, the clinical complications of HIT occur within 5-14 days after heparin exposure for heparin-naïve patients, once the immune response has developed. However, HIT complications can occur within 24 hours of heparin infusion in patients who had heparin exposure in last three months (Arepally and Ortel 2010, Warkentin 2003), indicative of a pre-existing antibody response.

Various algorithms have been proposed to be used as a guideline for the clinical diagnostic criteria of HIT (Greinacher, *et al* 1994, Sheridan, *et al* 1986, Warkentin 2003). These algorithms are based on (1) severity of thrombocytopenia, (2) timing of thrombocytopenia, (3) onset of thrombosis and (4) exclusion of other causes of thrombocytopenia. The most commonly used algorithm is the 4T Score, which is a scoring system (0, 1 and 2) based on the four clinical features (Table 5.1). Cumulative scores are used to determine the probability of HIT, scores of high (6-8 points), intermediate (4-5 points) or low (≤3 points) (Lo, *et al* 2006, Warkentin 2003).

	2	1	0
Thrombocytopenia	Platelet count fall >50% and platelet nadir ≥20 x 10^9 L^{-1}	Platelet count fall 30%-50% or platelet nadir 10-19 x 10^9 L^{-1}	Platelet count fall <30% or platelet nadir <10 x 10^9 L^{-1}
Timing of platelet count fall	Clear onset between days 5-10; or ≤1 day (prior heparin exposure within 30 days)	Consistent with days 5-10 fall, but not clear (e.g. missing platelet counts); onset of after day 10 or fall ≤1 day (prior heparin exposure 30-100 days ago)	Platelet count fall <4 days without recent exposure
Thrombosis and other sequel	New thrombosis (confirmed); skin necrosis; acute systemic reaction post-intravenous UFH bolus	Progressive or recurrent thrombosis ; non-necrotising (erythematous) skin lesion; suspected thrombosis (not proven)	None
Other causes for thrombocytopenia	None apparent	Possible	Definite

Table 5.1. 4T clinical scoring system. Adapted from (Lo, *et al* 2006, Warkentin 2003).

5.1.3.2 Laboratory diagnosis

Laboratory investigations for HIT are based on either the immunological detection of anti-PF4/heparin complex antibodies, or the functional ability of these immune complexes to cause platelet activation.

Several immunological assays have been developed to detect antibodies that bind to either PF4/heparin or PF4/polyvinylsulphate complexes bound to a solid phase ELISA plate. They are designed to detect either polytypic antibodies of IgG/IgM/IgA classes, or IgG only, depending on the secondary antibodies used. Several immunological ELISA assay kits are commercially available. These kits give slightly different results but show overall good correlation with each other, particularly with high titre of PF4/heparin antibodies (Arepally and Ortel 2010, Cuker 2011, Greinacher, *et al* 2010, Leo and Winteroll 2003). Recently, fully automated semi-quantitative chemiluminescent immunological assays (HemosIL AcuStar)

for either IgG or polytypic immunoglobulin classes have been introduced as rapid methods for HIT diagnosis (Legnani, *et al* 2010, Minet, *et al* 2013, Van Hoecke and Devreese 2012).

Immunological assays are widely used in clinical centres because of their high sensitivity (more than 99%), relatively rapid turnaround time and ease of performance. However, HIT immunological assays are associated with low positive predictive values due to their limited capacity to discriminate between platelet-activating from clinically insignificant antibodies (Arepally and Ortel 2010).

The second approach of laboratory diagnosis of HIT, the functional assays, are all essentially dependent on the ability of HIT-immune complexes in patient samples to activate fresh platelets from healthy donors *in vitro*, in the presence of a clinically-relevant dose of heparin. Several functional assays have been developed measuring different platelet activation parameters. The most two widely used functional assays are (1) the serotonin release assay (SRA), which measures the release of radioactive-labelled serotonin from donor platelets (Sheridan, *et al* 1986) and (2) the heparin-induced platelet aggregation assay (HIPA), which measures aggregation of donor platelets (Greinacher, *et al* 1991). Flow cytometry is also applied in the measurement of platelet P-selectin expression (Jy, *et al* 1999), Annexin V binding (Poley and Mempel 2001) and MPs release (Lee, *et al* 1996). Thrombin generation has also been used to measure the platelet procoagulant response, but not MP generation (Tardy-Poncet, *et al* 2009). MP generation assays have been shown to be more relevant biomarker than activated platelet assays (Denys, *et al* 2008, Tomer, *et al* 1999).

The big advantage of the functional assays is that they reflect the clinically significant antibodies of HIT, which is directly relevant to the pathogenesis of HIT. Therefore, functional assays are generally more specific and give higher predictive values compared to the immunological assays (Greinacher, *et al* 2010, Leo and Winteroll 2003). However, due to

the technical demand of these assays and the high variability in responsiveness among platelet donors, the sensitivity and specificity of these assays can vary widely between laboratories.

5.1.4 Aim and hypothesis

The primary aim of this project was to develop and validate a novel functional assay using thrombin generation to detect and measure the procoagulant activity of MPs generated by HIT-immune complexes, called HIT-induced microparticle assay (HIMA) hereafter. The performance of this novel assay was compared with patients' clinical outcomes and other assays for HIT diagnosis. The secondary aim was to characterise the procoagulant activity of PDMPs generated by platelet activation through the ITAM-containing receptor, FcγRIIA. This was achieved using the ability of HIT-immune complexes in HIT-positive patient samples, which are known to cross-link donor platelet FcγRIIA receptors and induce platelet procoagulant response and MP formation.

5.2 Study design

5.2.1 Patient sample preparation and clinical diagnosis

In this study, a total of 75 patients with suspected HIT were referred to three centres: (1) Leicester Royal Infirmary, (2) Royal Hallamshire Hospital and (3) Royal Brompton Hospital. Blood was collected from each patient into serum gel tubes and left to completely clot over 1 hour then centrifuged at 1800g for 30 minutes at RT. Serum supernatants were collected and stored at -80°C until they were used. On the day of the experiment frozen patient samples

were thawed at 37°C for 15 minutes. To remove endogenous MPs, patient sera were centrifuged at 17000g for 30 minutes before they were used in the assays. Clinical diagnosis was performed in each centre based on the 4T Scores algorithm system. Two functional assays were performed in our laboratory and the other immunological assays had been used in other centres (section 5.2.3).

5.2.2 Functional assays

In our laboratory, two functional assays; CAT assay and flow cytometric detection of platelet Annexin V binding, were performed in parallel as shown in Figure 5.2.

5.2.2.1 **Donor platelet preparation**

Whole blood was collected from "O" type blood group healthy donors as described in section 2.2.1.1. The first tube was collected into a serum gel tube and serum was prepared as described in 5.2.1. All subsequent blood was collected into 4.5 mL sodium citrate tubes and PPP and PRP samples were prepared as described in sections 2.2.1.2 and 2.2.1.4. Platelet count was adjusted at 450 x 10^3/µL using autologous PPP. The PRP was used within 1 hour of collection.

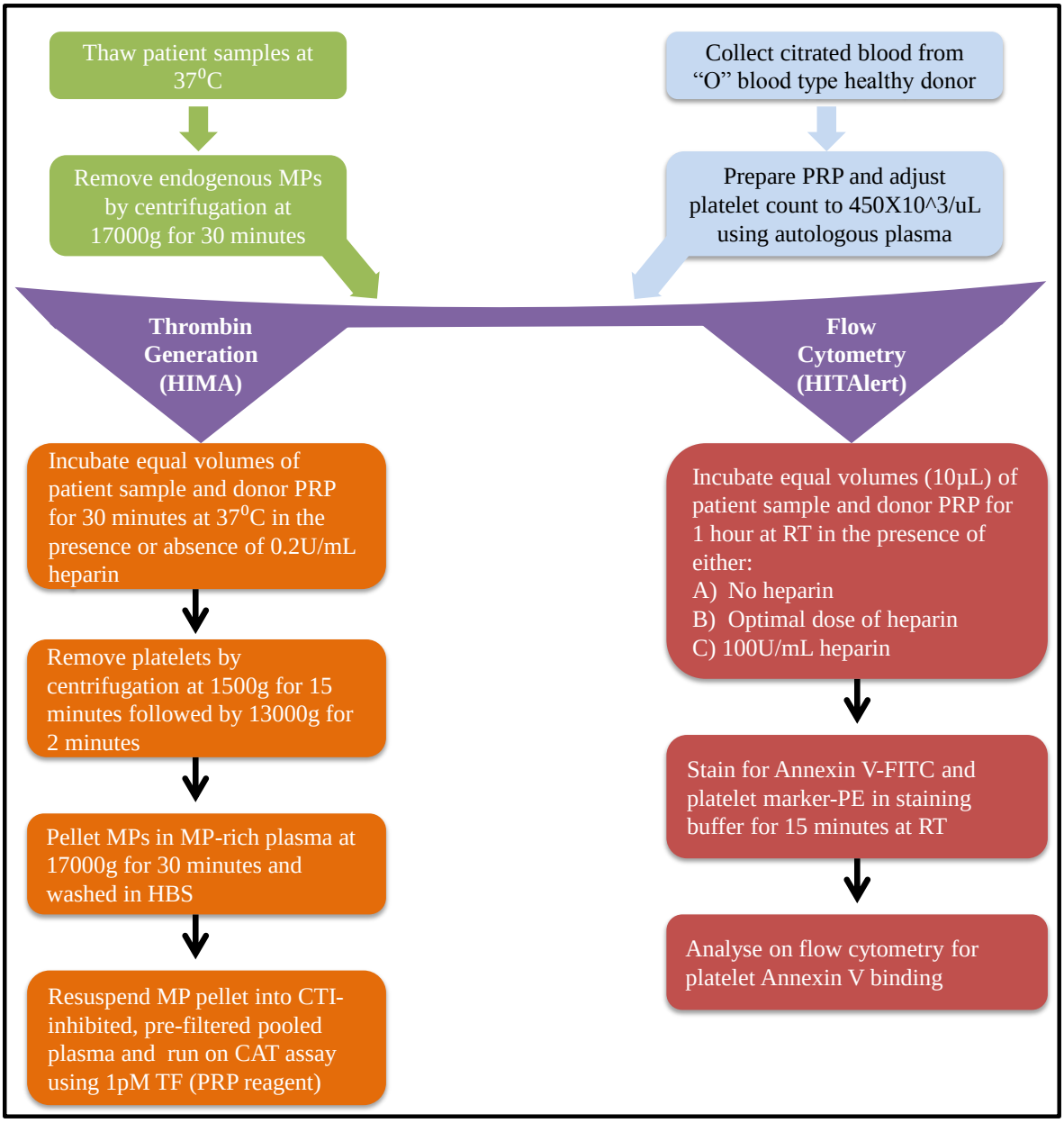

Figure 5.2. Schematic diagram of overview of assays for thrombin generation and flow cytometry for HIT diagnosis.

5.2.2.2 **CAT assay (HIMA)**

The CAT assay was used to measure the procoagulant activity of MPs generated from donor platelets when incubated with patient serum containing HIT immune complexes. 200 µL of patient samples was mixed with equal volumes of freshly prepared donor PRP in the presence or either UFH at final concentration of 0.2 U/mL or HBS buffer, incubated at 37°C for 30 minutes, shaking gently on a horizontal orbital shaker at 75rpm. At the end of this incubation, the MP-rich supernatant samples were collected by centrifugation at 1500g for 15 minutes followed by 13000g for 2 minutes. To remove residual UFH, MPs were washed in HBS buffer by centrifuging the MP-rich supernatant samples at 17000g for 30 minutes. Pellets were resuspended into 1 mL HBS buffer and centrifuged again at 17000g for 30 minutes. Supernatants were discarded and pellets were resuspended into 200 µL CTI-treated, pre-filtered pooled plasma (section 2.2.1.3). The procoagulant activity of the MPs in these suspensions was analysed by CAT assay using the PRP reagent (1 pM TF; Stago) as described in section 2.2.4. For some experiments, samples were analysed in the CAT assay before and after removing MPs by passing the samples through 0.2 µm pore filters using the Ceveron filtration unit. In order to standardise the assay, the serum sample from each donor was incubated with autologous PRP and analysed alongside the patient samples. Patient thrombin generation results were then normalised against the activity in the donor serum.

5.2.2.3 **Flow cytometry (HITAlert)**

The commercially available functional assay, HITAlert, (IQ Products BV, Netherlands) was used to measure the ability of HIT-immune complexes in patient samples to induce Annexin V binding in donor platelets. This was performed by Bhavesh M. Popat, an intercalated BSc student, under my supervision. HITAlert was performed according to the manufacturer's instructions. Briefly, 10 µL of patient serum was mixed with equal volume of freshly

prepared donor PRP. Sample mixtures were immediately added to 30 µL of the supplied assay buffer in the presence (called assay sample IV; according to the manufacturer's instructions) or absence (assay sample III) of a therapeutic dose of UFH (the exact concentration was not provided). In order to determine that platelet activation is dependent on heparin, a parallel sample was incubated in the presence of a high dose of UFH (100 U/mL final concentration; assay sample V). Sample suspensions were incubated for 1 hour at RT on a horizontal orbital shaker. At the end of this incubation, 5 µL of the suspensions were added to 45 µL supplied buffer containing PE-labelled platelet marker and Annexin V-FITC, and incubated for 15 minutes at RT before adding 400 µL of the buffer. The platelets was immediately analysed in Coulter EPICS XL-MCL flow cytometry (Beckman Coulter), gating the platelet population on the PE-labelled platelet marker. Activated platelets were distinguished from resting platelets by the Annexin V-FITC binding.

Patient samples were considered positive only when met two conditions: (1) they showed ≥8% platelet activation (Annexin V binding) in the presence of therapeutic concentration of UFH (assay sample IV) and (2) they showed less than 8% Annexin V binding, or ≥50% reduction of platelet activation in the absence of heparin (assay sample III), or in the presence of high the dose of heparin (assay sample V) (Figure 5.3).

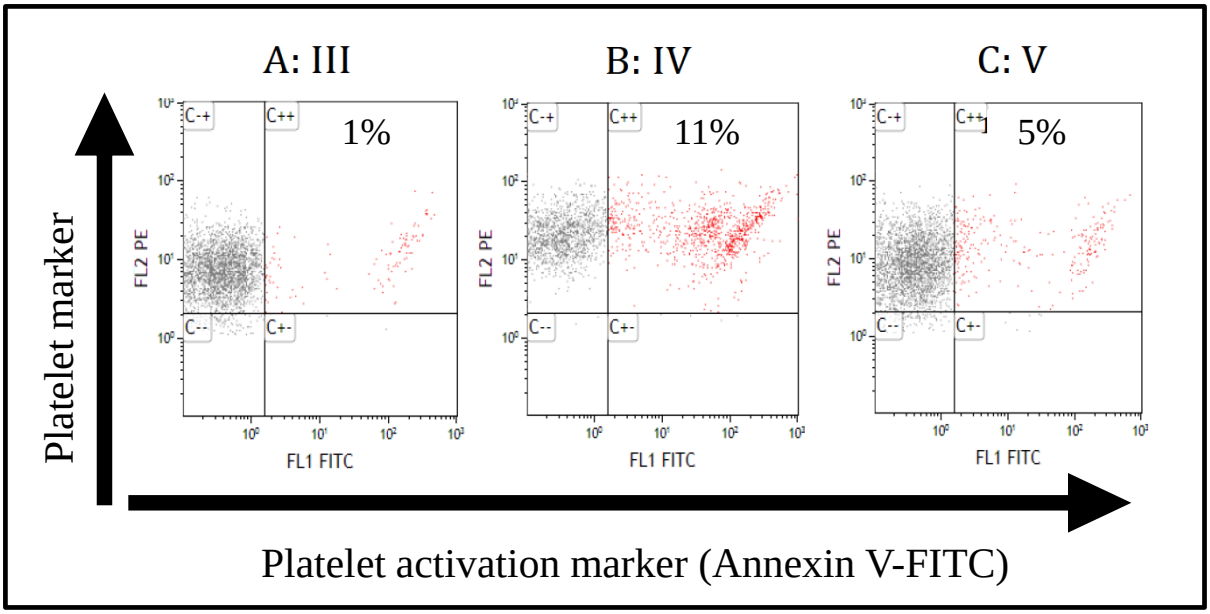

Figure 5.3. Representative HITAlert flow cytometry result of a HIT positive patient. Histograms show platelet marker (PE-labelled) plotted against platelet activation marker (Annexin V-FITC) of donor platelets incubated with HIT suspected patient sera in the presence of different concentrations of heparin. A) shows the result in the absence of heparin (assay sample III), showing 1% platelet Annexin V binding. B) shows the result in the presence of a therapeutic concentration of UFH (assay sample IV), showing 11% platelet Annexin V binding. C) shows the result in presence of super-therapeutic dosage of UFH (assay sample V), showing 5% platelet Annexin V binding.

5.2.3 Immunological assays

The presence of anti-PF4/heparin complex antibodies in patient sera was analysed using several immunological laboratory investigations. These assays were performed according the manufacturer's recommendations and were carried out in the three collaborated centres. A summary of all used assays in the study and number of samples analysed in each assay is provided in Figure 5.4.

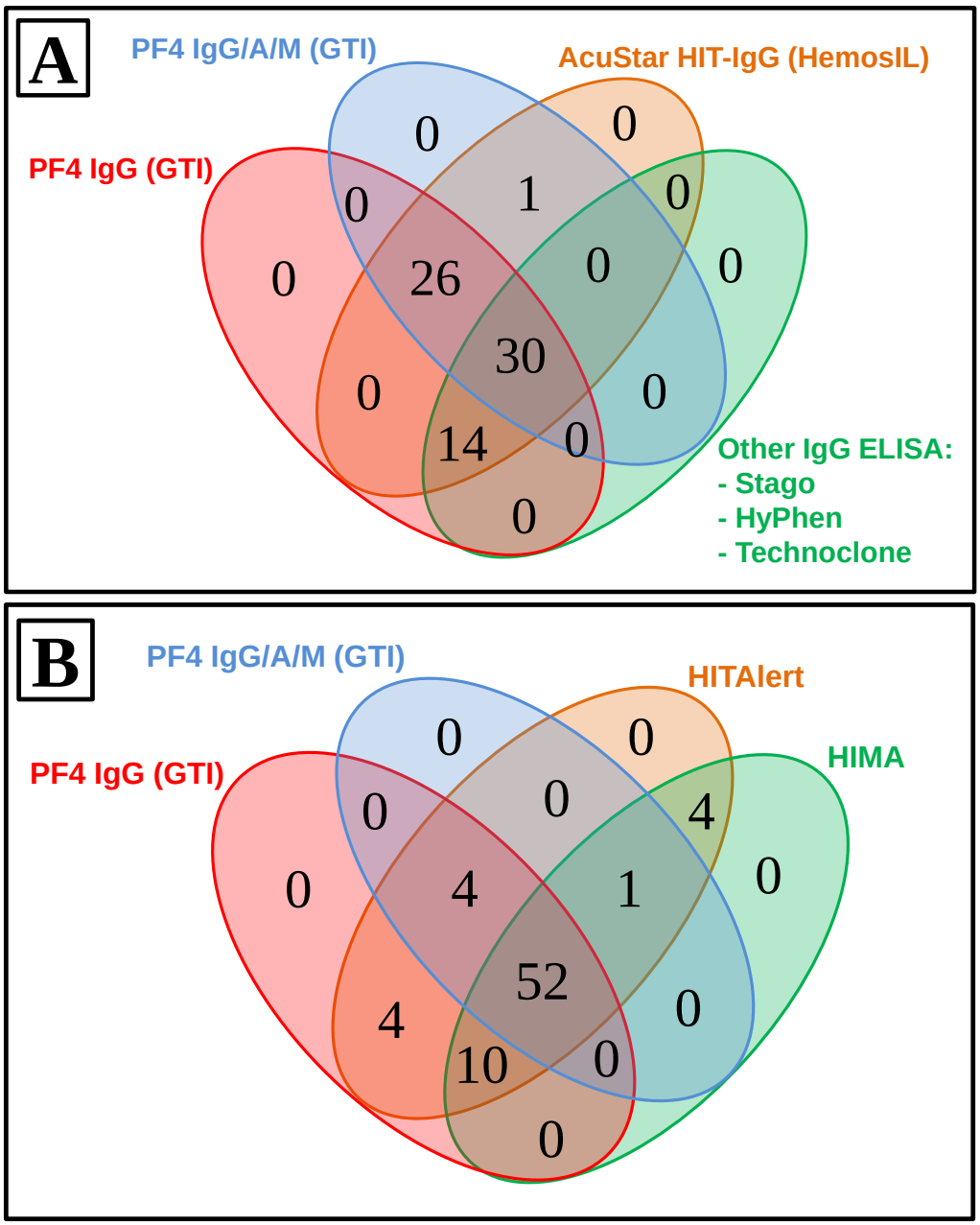

Figure 5.4. Different HIT diagnostic assays used in the study. A) Venn diagram shows the number of samples that were analysed using the four immunological assays, PF4 IgG/A/M (GTI), PF4 IgG (GTI), Asserachrom HPIA-IgG (Stago), Zymutest HIA IgG (Hyphen), Technozym HIT IgG (Technoclone) and AcuStar HIT-IgG (HemosIL). B) Venn diagram shows the number of samples analysed by immunological (represented by the polytypic PF4 IgG/A/M and IgG ELISA) and functional assays, HITAlert and HIMA.

The PF4 IgG/A/M ELISA (GTI Diagnostics) was used to detect and measure the titre of the polytypic HIT antibodies in 57 samples. The titre of HIT IgG antibodies was measured using the following ELISA: (1) PF4 IgG ELISA (GTI Diagnostics) (70 samples), (2) Asserachrom HPIA-IgG (Stago Diagnostic) (44 samples), (3) Zymutest HIA IgG (HyPhen BioMed) (44 samples) and (4) Technozym HIT IgG (Technoclone, Veinna, Austria) (44 samples).

The HemosIL AcuStar HIT-IgG is a two-step fully automated immunoassay that consists of magnetic particles coated with PF4-polyvinyl sulphate and isoluminol-labelled antihuman IgG antibody. A total of 71 samples were analysed using this assay.

5.2.4 Statistical analysis

Receiver operating characteristic (ROC) Curves were used to determine the optimal cut-off values of HIMA compared to the HITAlert, PF4 IgG and AcuStar assays. A Fisher's exact test was used to compare the final diagnosis of HIMA and other diagnoses. The sensitivity and specificity of HIMA were assessed using the PF4 IgG and HITAlert assays as references. The above analyses were performed using Stata/IC 13 (Stata software, version 13, USA).

A Pearson's regression (r) analysis was used to assess the correlation between the HIMA and 4T Scores and other laboratory assays. This was performed using Graph Pad Prism version 6 (GraphPad Software Inc.). A P-value < 0.05 was considered significant.

5.3 Results

5.3.1 Evaluation of donor response to HIT-immune complexes

Since individuals vary widely in their responses to HIT-immune complexes (Warkentin, *et al* 1992), it was sensible to evaluate the response of the platelet donors involved in the study to ensure that their platelets reacted to HIT antibodies. Platelet responses of five healthy volunteers with 'O' blood group were assessed in the HITAlert assay using a known positive HIT sample. Figure 5.5 shows that platelets from donors 1, 2 and 3 gave good reactivity with the HIT positive sample, whereas donors 4 and 5 failed to reach the threshold of 8%. Therefore, it was decided to use platelets from donors 1, 2 and 3 throughout the study.

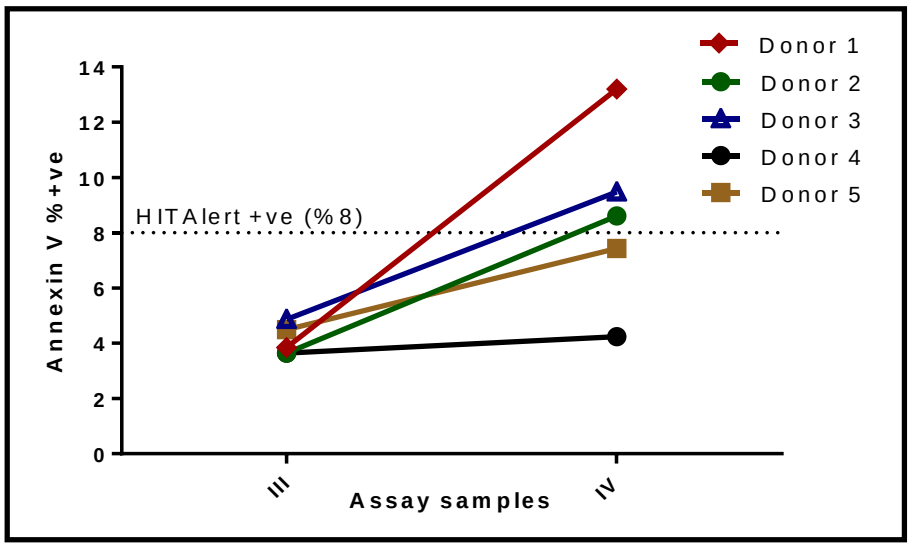

Figure 5.5. Donor platelet reactivity with HIT sample. HITAlert results of PRP samples collected from five health donors with 'O' blood group among the department staff. Donors' PRP samples were incubated with a known HIT positive sample in the absence (assay sample III) or presence (assay sample IV) of therapeutic dose of UFH for 1 hour at RT. Subsamples were analysed for Annexin V binding in flow cytometry.

5.3.2 CAT assay optimisation for HIT diagnosis

The thrombin generation assay for HIT diagnosis was achieved by incubating HIT-suspected patient sera with donor PRP in the presence or absence of 0.2 U/mL final concentration of UFH. The MPs generated were separated from the platelets and their functional procoagulant activity was assessed in the CAT assay using the 1pM TF reagent assay (Stage PRP reagent). However, the presence of heparin in the samples could risk inhibition of thrombin generation; despite the previous report of Trady-Poncet *et al* that HIT positive samples promote thrombin generation in donor PRP samples (Tardy-Poncet, *et al* 2009). Therefore, preliminarily experiments were carried out to determine the effect of heparin on this CAT assay. Samples from eight patients; four of which known to have high HIT probability (patients A, B, C and D) and four with intermediate HIT probability (patients E, F, G and H) according to the clinical diagnosis 4T Scores, were mixed with equal volumes of donor PRP in the presence or absence of UFH at a final concentration of 0.2 U/mL. Sample mixtures were incubated at 37°C for 30 minutes and MP-rich supernatant samples were collected by centrifugation at 1500g for 15 minutes followed by 13000g for 2 minutes. The procoagulant activity of the MP-rich plasma was assessed in the CAT assay using the 1 pM TF reagent assay. Figure 5.6 shows that the thrombin generation was completely abrogated in three of the heparinised samples from patients with high 4T scores, and from all of the samples from the patients with a low 4T score. However, all supported thrombin generation in the absence of heparin. This is an unsurprising finding given the known inhibitory effect of heparin to thrombin generation. Additionally, pooled plasma was incubated with increasing concentrations of serum collected from healthy donor and incubated at 37°C for 30 minutes before measuring thrombin generation using the 1 pM TF reagent. Figure 5.7 shows that thrombin generation was significantly inhibited by the presence of serum in plasma samples in a dose-dependent

manner. These two findings, the effect of heparin and serum on thrombin generation, clearly show that samples that contain heparin and/or serum are not suitable for analysing the procoagulant activity of the generated PDMPs in the CAT assay.

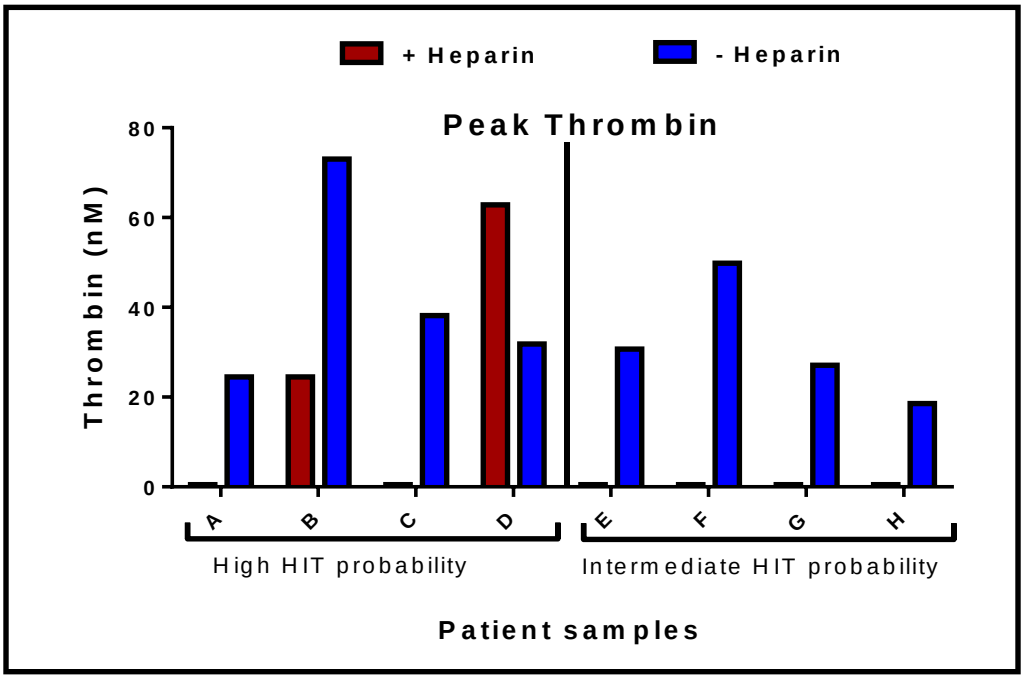

Figure 5.6. Effect of heparin on plasma procoagulant activity. 200 µL of HIT suspected patient sera were mixed with equal volumes of donor PRP samples in the presence (+ Heparin) or absence (-Heparin) of UFH at a final concentration of 0.2 U/mL. Sample mixtures were incubated at 37°C for 30 minutes and MP-rich supernatant samples were collected by centrifugation at 1500g for 15 minutes followed by 13000g for 2 minutes. Thrombin generation was assessed on CAT assay using the 1pM TF reagent assay. Thrombin generation data are represented by PT values.

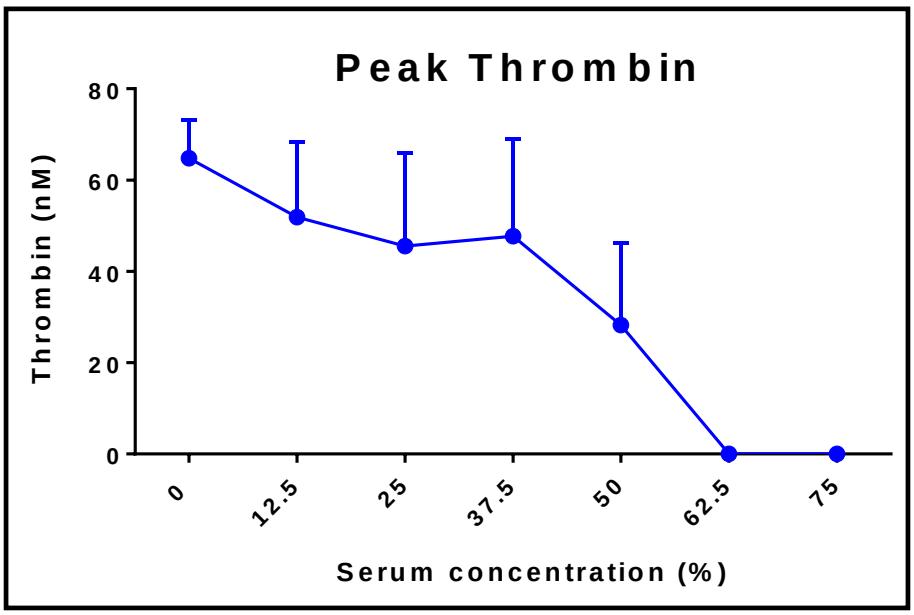

Figure 5.7. Effect of serum on plasma procoagulant activity. Pooled plasma was incubated with increasing concentrations of serum at 37°C for 30 minutes and run in the CAT assay using the 1 pM TF reagent assay. Thrombin generation data are represented by PT values. Data are from six separate experiments performed using sera from different donors.

In order to remove the effect of heparin and serum in the sample mixtures, MPs generated by the patients' serum from healthy PRP were washed, added to CTI-treated, pre-filtered pooled plasma before being analysed in the CAT assay for measuring their procoagulant activity as described in section 5.2.2.2. The use of pooled plasma in the assay eliminates the inter-individual variation in plasma procoagulant activity and makes the assay entirely dependent on the levels of the MPs. In preliminary experiments, samples prepared using donor PRP and autologous serum showed relatively low amount of thrombin generation (Figure 5.8 A). There was no significant difference between samples incubated with and without 0.2 U/mL UFH, confirming that there is no residual heparin in the samples after washing the MPs. The sensitivity of the assay toward HIT diagnosis was investigated using HIT suspected patient

samples. Samples prepared using sera from four patients with a low probability of HIT showed relatively similar thrombin generation curves to those seen with autologous plasma, indicating that there was no MP generation (Figure 5.8 B). In sharp contrast, samples prepared from four patients' sera with high and intermediate probability of HIT showed a marked enhancement in the amount of thrombin generation when incubated in the presence of 0.2 U/mL UFH, but not in the absence of heparin, compared to the basal level curves (Figure 5.8 C). Interestingly, thrombin generation curves remarkably resemble the thrombin generation of PDMPs generated by platelet activation through GPVI and CLEC-2 (see Figure 3.7 and Figure 4.31). The procoagulant activity of the samples was significantly eliminated by filtration, confirming the activity was due to the presence of MPs in the samples. These finding clearly show that the HIT-optimised CAT assay has a potential sensitivity for HIT diagnosis.

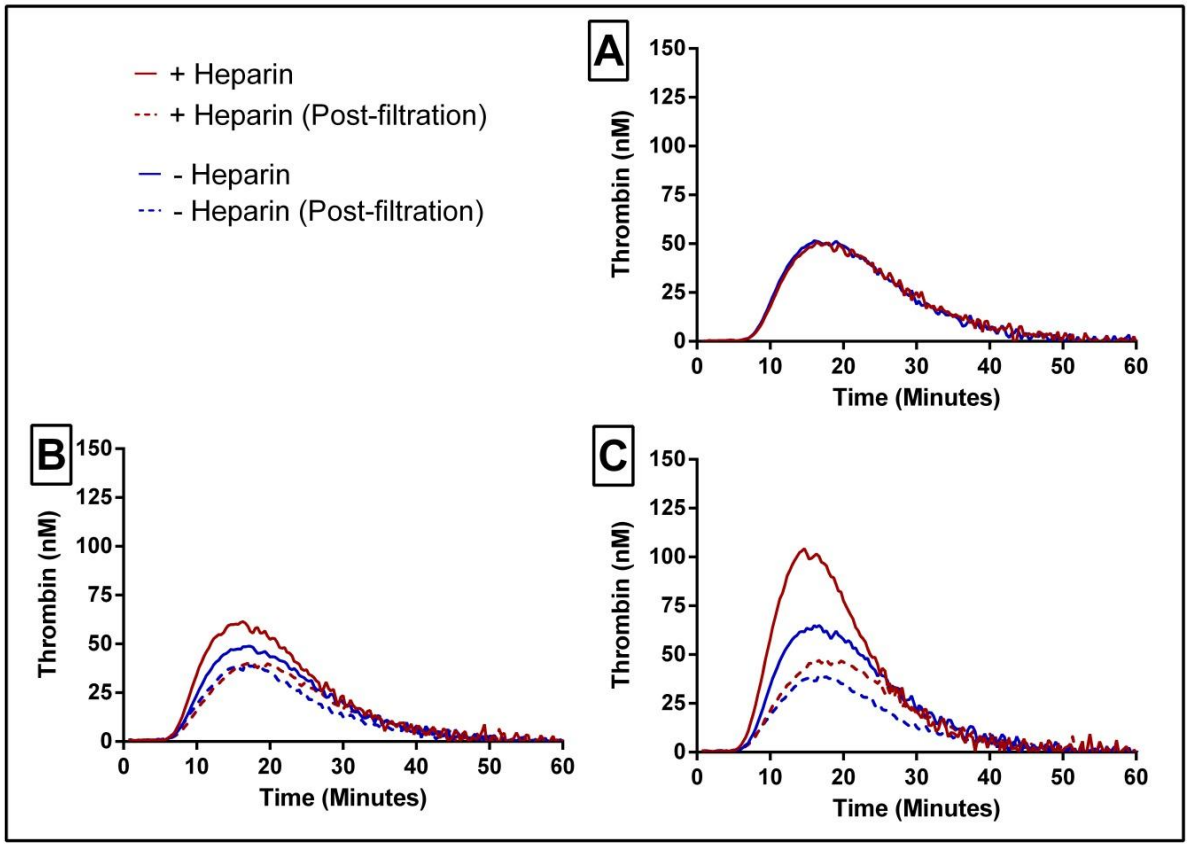

Figure 5.8. CAT assay results of washed MPs generated using HIMA functional assay. Thrombin generation curves represent the mean of four separate experiments using PRP from different donors incubated with (A) autologous sera, (B) low probability HIT suspected patient sera or (C) high and intermediate probability HIT suspected patient sera. 200µL of freshly prepared donor PRP samples was mixed with equal volumes of serum samples in the presence (+ Heparin, red lines) or absence (- Heparin, blue lines) of UFH at a final concentration of 0.2 U/mL. Sample mixtures were incubated at 37°C for 30 minutes and MP-rich supernatant samples were collected by centrifugation at 1500g for 15 minutes followed by 13000g for 2 minutes. MP-rich supernatant samples were centrifuged at 17000g for 30 minutes. Pellets were resuspended into 1mL HBS buffer and centrifuged again at 17000g for 30 minutes. Supernatants were discarded and pellets were resuspended into 200 µL CTI-treated, pre-filtered pooled plasma. Samples were analysed on CAT assay using the 1 pM TF reagent assay. For samples analysed before and after removing the MPs by filtration (Post-filtration, dashed lines), sample volumes were doubled.

5.3.3 Patient HIMA results

Among the 75 patients, 67 patients whom sufficient sample volume was available were analysed for MP generation using the optimised HIT CAT assay. Due to insufficient sample volume eight of the 67 samples were only analysed in the presence of 0.2 U/mL UHF, but not in the absence of heparin. In order to minimise the inter-assay and inter-donor variability, patients' thrombin generation results were normalised against donor's results included in each run. Since peak thrombin shows the highest sensitivity to the procoagulant activity of phospholipids among the thrombin generation parameters, which in turn reflects the levels of MPs in the samples, this parameter was used to describe thrombin generation throughout the study. All patient results, including the clinical diagnosis the 4T Score and laboratory diagnoses, are summarised in Table 5.2.

ROC curve analysis was used to determine the optimal cut-off value for the HIMA, to distinguish between HIT-positive and HIT-negative results. The most optimal separation between HIT-positive and HIT-negative results of HITAlert assay was established at a cut-off value of 135% HIMA, with 100% sensitivity and 96% specificity (Figure 5.9). In addition, ROC curve analysis revealed that this HIMA cut-off value also showed the most appropriate separation between HIT-positive and HIT-negative results of PF4 IgG and AcuStar assays with sensitivity of 67% and 71% and specificity of 84% and 85% respectively. The low sensitivity of the HIMA cut-off value to the PF4 IgG and AcuStar assays is unsurprising given the limited capacity of PF4 IgG and AcuStar assays to distinguish platelet activating from non-activating PF4/heparin complex antibodies.

Patient ID	Clinical Diagnosis	Functional Assays							Immunological Assays											
		HIMA			HITAlert Assay				PF4 IgGMA ELISA		PF4 IgG ELISA		AcuStar HI-IgG		Stago ELISA		Technoclone ELISA		HyPhen ELISA	
	4T Score	UFH U/mL			Assay Samples															
		0	0.2	Inter.	III	IV	V	Inter.	OD	Inter.	OD	Inter.	U/mL	Inter.	OD	Inter.	OD	Inter.	OD	Inter.
1	5			ND	1.26	11.10	1.10	P		ND	2.2	P	12.49	P	3.245	P	2.058	P	3.154	P
2	5			ND	4.39	17.40	7.16	P		ND	1	P	22.58	P	2.516	P	1.25	P	2.855	P
3	5			ND	1.18	1.50	0.93	N		ND	0.36	N	0.41	N	0.144	N	0.076	N	1.343	P
4	8			ND	7.40	39.50	3.27	P		ND	1.7	P	3.62	P	3.29	P	1.171	P	3.415	P
5	6		135.74	P	5.06	8.68	4.54	P		ND	1.4	P	5.14	P	2.98	P	0.318	P	2.669	P
6	ND	103.25	93.50	N	2.19	3.87	3.92	N		ND	1.21	P	0.69	N	2.484	P	0.812	P	2.138	P
7	4	137.49	415.45	P	10.30	20.00	9.09	P		ND	1.67	P	14.15	P	3.288	P	0.924	P	2.962	P
8	ND		258.67	P	8.05	51.30	5.59	P		ND	2.31	P	128	P	3.502	P	1.668	N	3.176	P
9	5		327.58	N	23.20	51.50	27.00	N		ND	0.08	N	0.16	N	0.072	N	0.046	N	0.097	N
10	4		147.23	N	11.30	8.52	8.42	N		ND	0.09	N	0.16	N	0.141	N	0.041	N	0.185	N
11	6	98.48	143.25	P	4.83	64.80	1.44	P		ND	2.09	P	15.26	P	3.353	P	0.607	P	2.995	P
12	6		240.70	P	8.60	18.00	2.42	P		ND	2.24	P	11.12	P	3.266	P	2.12	P	3.09	P
13	6	104.55	163.61	P	3.50	13.30	2.27	P		ND	1.61	P	8.62	P	3.161	P	0.697	P	2.706	P
14	6	106.16	222.72	P	3.99	9.76	4.11	P		ND	1.35	P	9.15	P	3.362	P	1.184	P	2.967	P
15	4	124.53	134.28	N	1.30	1.45	2.18	N	0.069	N	0.02	N	0.11	N	0.018	N	0.053	N	0.07	N
16	4			ND	0.85	1.22	1.37	N	0.114	N	0.05	N	0.12	N	0.053	N	0.046	N	0.097	N
17	4	88.87	97.82	N	3.16	2.84	2.10	N	0.545	P	0.29	N	0.32	N	1.257	N	0.132	N	2.12	P
18	3	101.19	96.46	N	2.68	2.58	2.12	N	0.168	N	0.047	N	0.12	N	0.081	N	0.048	N	0.071	N
19	2	101.75	116.56	N	1.10	0.87	1.03	N	0.183	N	0.03	N	0.12	N	0.074	N	0.027	N	0.073	N
20	6	87.57	94.62	N	3.88	3.11	3.79	N	0.326	N	0.16	N	0.13	N	0.119	N	0.081	N	0.099	N
21	4	120.83	190.61	P	6.94	5.61	5.12	N	0.117	N	0.07	N	0.12	N	0.031	N	0.048	N	0.483	P
22	2	104.87	99.62	N	1.31	1.44	1.43	N	0.191	N	0.11	N	0.16	N	0.052	N	0.062	N	0.188	N
23	ND			ND	1.46	1.02	1.18	N	0.336	N	0.16	N	0.21	N	0.057	N	0.056	N	0.96	P
24	2		99.44	N	2.08	1.06	1.02	N	0.11	N	0.07	N	0.12	N	0.033	N	0.052	N	0.084	N
25	ND		99.30	N	1.71	2.05	2.02	N	0.387	N	0.19	N	0.32	N	0.292	N	0.077	N	0.229	N
26	ND	95.36	98.50	N	3.59	3.72	3.53	N	2.521	P	0.702	P	2.21	P	2.697	P	3.5	P	2.517	P
27	ND	103.35	109.42	N	1.54	1.28	1.36	N	0.142	N	0.06	N	0.14	N	0.179	N	0.032	N	0.221	N
28	3	126.92	127.91	N	2.13	6.23	1.98	N	2.541	P	0.88	P	9.96	P	3.237	P	1.815	P	2.778	P
29	ND	91.19	91.66	N	2.97	5.77	3.93	N	0.379	N	0.21	N	0.31	N	0.205	N	0.171	N	0.211	N
30	2	100.85	101.48	N	4.28	5.01	2.37	N	0.068	N	0.04	N	0.09	N	0.02	N	0.05	N	0.051	N
31	ND	98.48	94.28	N	2.62	5.00	4.17	N	0.276	N	0.14	N	0.12	N	0.09	N	0.051	N	0.309	N
32	4	79.87	89.54	N	0.96	1.06	1.80	N	1.06	P	0.07	N	0.12	N	0.024	N	0.059	N	0.072	N
33	4	105.19	117.54	N	0.81	1.02	0.99	N	0.51	P	0.24	N	0.22	N	0.208	N	0.114	N	0.242	N
34	6	100.21	137.49	P	0.93	15.00	6.82	P	3.056	P	1.68	P	10.91	P	2.983	P	0.347	N	2.9	P
35	2	103.88	91.41	N	2.66	3.07	2.64	N	0.29	N	0.23	N	0.12	N	0.025	N	0.06	N	0.2	N
36	4	108.82	93.97	N	2.73	2.26	4.46	N	1.304	P	0.09	N	0.23	N	0.424	N	0.197	N	0.439	P
37	6	95.66	111.02	N	0.53	1.85	2.38	N	0.207	N	0.07	N	0.12	N	0.063	N	0.045	N	0.097	N
38	4	96.58	97.53	N	1.95	2.41	2.15	N	0.81	P	0.14	N	0.1	N	0.372	N	0.048	N	0.098	N
39	ND	104.22	97.21	N	3.02	1.98	2.25	N	0.589	P	0.078	N	0.18	N	0.038	N	0.064	N	0.111	N
40	ND	110.68	152.19	P	1.87	1.33	1.65	N	0.304	N	0.19	N	0.16	N	0.081	N	0.112	N	0.276	N
41	ND	247.91	418.62	P	42.30	49.00	45.40	P	0.978	P	0.14	N	0.18	N	0.481	N	0.159	N	0.253	N
42	5		98.04	N	2.37	2.11	2.60	N	0.80	P	0.07	N	0.11	N	0.019	N	0.06	N	0.069	N
43	4	104.07	98.34	N	1.80	2.36	2.77	N	0.743	P	0.13	N	0.15	N	0.17	N	0.103	N	0.157	N
44	0	116.19	110.50	N	2.34	3.52	2.84	N	0.122	N	0.06	N	0.2	N	0.023	N	0.073	N	0.154	N
45	ND	120.68	129.11	N	3.62	4.14	4.82	N		ND		ND		ND		ND		ND		ND
46	ND	134.17	325.00	P	8.44	30.60	2.74	P		ND		ND		ND		ND		ND		ND
47	ND	103.54	105.74	N	3.77	3.62	4.32	N		ND		ND		ND		ND		ND		ND
48	ND	123.61	114.28	N	3.25	1.18	1.39	N		ND		ND		ND		ND		ND		ND
49	4	91.44	99.44	N	4.69	4.92	4.63	N	0.19	N		ND	0	N		ND		ND		ND
50	4	120.53	124.94	N	4.5	5.6	5.21	N	0.93	P	0.235	N	0.37	N		ND		ND		ND
51	ND	115.94	99.53	N	4.78	4.7	5.08	N	0.41	P	0.181	N	0.25	N		ND		ND		ND
52	5	80.62	87.03	N	3.73	4.11	3.13	N	2.71	P	1.553	P	2.88	P		ND		ND		ND
53	5	89.18	91.41	N	6.68	6.39	7.17	N	0.5	P	0.126	N	0	N		ND		ND		ND
54	6	104.03	92.88	N	4.78	6.65	3.76	N	0.42	P	0.094	N	0.02	N		ND		ND		ND
55	4	99.13	113.24	N	4.93	4.9	5.74	N	0.39	N	0.127	N	0	N		ND		ND		ND
56	ND	96.19	95.58	N	5.43	7.54	8.99	N	0.29	N	0.063	N	0.02	N		ND		ND		ND
57	ND			ND	5.18	4.66	5.76	N	0.34	N	0.055	N	0.04	N		ND		ND		ND
58	6	124.47	235.35	P	6.59	25.6	5.75	P	2.65	P	1.839	P	24.54	P		ND		ND		ND
59	4	98.83	125.93	N	4.2	3.52	3.68	N	0.1	N	0.044	N	0	N		ND		ND		ND
60	5	90.63	98.66	N	2.89	3.94	3.25	N	1.96	P	0.914	P	7.45	P		ND		ND		ND
61	4	93.18	132.62	N	5.19	5.89	5.5	N	0.37	N	0.07	N	0.02	N		ND		ND		ND
62	5	121.33	164.02	N	4.43	8.23	7.39	N	0.24	N	0.046	N	0	N		ND		ND		ND
63	5	131.83	319.31	P	7.83	20.4	7.46	P	2.47	P	1.909	P	31.98	P		ND		ND		ND
64	3	98.26	90.23	N	9.12	8.48	8.4	N	0.11	N	0.109	N	0	N		ND		ND		ND
65	4			ND	5.28	4.77	5.3	N	0.08	N	0.136	N	0.07	N		ND		ND		ND
66	5	125.95	174.50	P	9.54	11.1	10.1	P	2.15	P	1.687	P	2.29	P		ND		ND		ND
67	5	99.32	116.88	N	4.61	7.41	6.89	N	0.52	P	0.193	N	0.27	N		ND		ND		ND
68	4	104.40	97.36	N	3.49	4.57	3.74	N	0.22	N	0.115	N	0	N		ND		ND		ND
69	5	125.53	127.15	N	8.38	7.52	7.39	N	0.33	N	0.128	N	0	N		ND		ND		ND
70	4		186.74	P	5.68	9.37	7.49	P	0.44	P	0.153	N	0.23	N		ND		ND		ND
71	2	116.41	108.63	N	4.19	4.18	3.77	N	0.07	N	0.12	N	0	N		ND		ND		ND
72	ND	95.74	99.91	N	5.06	5.64	5.25	N	0.36	N	0.169	N	0.23	N		ND		ND		ND
73	5	455.75	430.28	P	61.9	79.4	3.52	P	2.51	P	2.487	P	128	P		ND		ND		ND
74	1	112.09	118.29	N	5.87	5.21	5.19	N	0.15	N	0.094	N	0	N		ND		ND		ND
75	ND	101.60	119.54	N	3.38	5.85	4.65	N	2.58	P	2.572	P	36.13	P		ND		ND		ND

Table 5.2. 4T Score and laboratory assay results for all HIT suspected patients cohort. Table key: P) Positive; N) Negative; ND) Not Determined; OD) Optical Density; Inter.) Interpretation. Colour coding: light green) Positive; red) Negative; yellow) Not Determined.

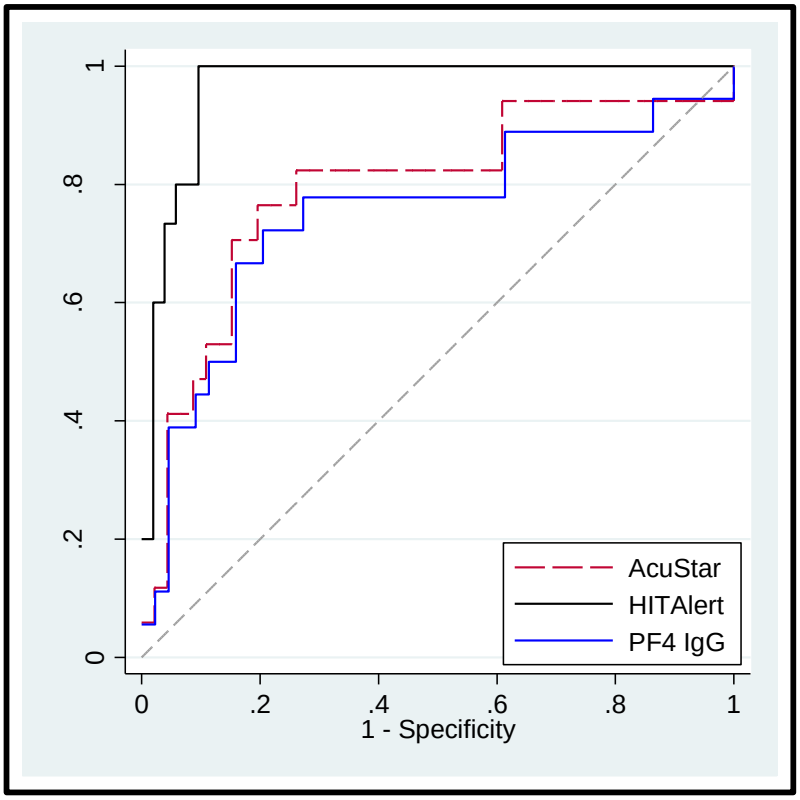

Figure 5.9. ROC curves for HIMA. HITAlert (n=67), PF4 IgG (n=62) and AcuStar (n=63) assays were used as standards for HIMA ROC curve analysis to determine the optimal cut-off value.

Samples were considered positive in HIMA if the result was ≥135% in the presence of 0.2 U/mL UFH and showed a marked reduction in the absence of heparin. As it can be seen from Table 5.2, HIMA showed good correlation with the clinical diagnosis and the other laboratory assays, particularly with HITAlert. However, despite this good agreement, there were some discrepant results between the diagnostic assays. For example, in the case of patient 40, HIMA was positive but all other laboratory investigations were negative. Similarly, patient 21 showed a positive result for the HIMA but negative results with all other laboratory assay apart from the IgG Hyphen ELISA, which showed a weak positive IgG titre. Since the other laboratory investigations of these two patients indicate that they are highly likely to be HIT-negative, the discrepancy of the HIMA results for these patients could be due to a technical artefact.

In the case of patients 7 and 73, their sera induced platelet activation and MP formation in the presence and absence of heparin as shown by HITAlert and HIMA assays. These two patients had an intermediate risk of HIT according to the 4T Score and were positive in the immunological assays, suggesting that they are likely to be HIT-positive. These findings could suggest that the platelet activation induced by anti-PF4/heparin complex antibodies of these two patients is heparin-independent, which has been previously reported on the basis of SRA (Prechel, *et al* 2005) and HIPA assays (Socher, *et al* 2008). This could reflect the strong binding of anti-PF4/heparin complex antibodies to PF4 even in the absence of heparin (Aster 2005). The other possibility of this finding could be the presence of residual amount of heparin in the patient samples, thus activating platelets without the addition of UFH to the assay tubes. The latter possibility is supported by the significant inhibition of platelet activation of these two patients was seen when high dose of UFH was used in HITAlert assay (assay sample IV). The high concentration of heparin causes disassociation of HIT-immune complexes, thus inhibits platelet activation (Greinacher 2009). Since the immunological assays were positive for these two patients, and since the induced platelet activation was inhibited in the inhibitory step in HITAlert assay using high concentration of UFH, the results were considered as positive for the functional assays.

On the other hand, patients 9, 10 and 62 showed relatively strong platelet activation and MP formation in both functional assays; however, the immunological assays failed to detect HIT antibodies in these samples. Interestingly, platelet activation induced by these patients' samples was not markedly inhibited in the presence of high concentration of UFH. This finding suggests that platelet activation may have been induced by other causes rather than anti-PF4/heparin complex antibodies. It has been suggested that false-positive functional assay could be caused by thrombin contamination, or IgG-containing immune complexes present in patient samples (Warkentin 2014). In addition, it has been reported that patients

with lupus or antiphospholipid syndrome (APS) often have anti-PF4 (but not anti-PF4/heparin) antibodies, which may test positive in the functional assays (Pauzner, *et al* 2009, Sikara, *et al* 2010). Therefore, the inhibitory step using high concentration of heparin is critical to rule out the false positive functional assay results in these cases. Since these patients tested negative in the immunological assays, and since they did not fulfil the criteria for HITAlert assay to be positive (no inhibition in the presence of high concentration of UFH in assay sample IV), results for these three patients were considered as negative for the functional assays.

Similarly, patients 41 and 70 showed relatively potent platelet activation and MP formation, which was not inhibited in the presence of high dose of heparin. However, these two patients showed positive results only in the polytypic IgG/A/M ELISA but not in the IgG specific immunological assays. This finding suggest further possibilities. The first possibility is that they are false-positive functional assay results similar to patients 9, 10 and 62 and therefore the same above discussion could be applied here. The second possibility is that the platelet activation is caused by either IgA or IgM present in patient sera. It has been reported that IgA or IgM alone can cause thrombocytopenia (Amiral, *et al* 1996). Therefore, the functional assays for these two patients were considered as positive. However, further investigations are required to confirm the results.

5.3.4 Platelet activation by HIT-immune complexes is induced through FcγRIIA

In order to determine that the platelet activation and MP generation in the assay is dependent on FcγRIIA, donor PRP was pre-incubated with IV.3 Fab fragments prior to the incubation with HIT-positive patient sera. This was carried out in 3 samples where sufficient plasma was available for testing. Figure 5.10 A shows that platelet Annexin V binding induced by two known HIT-positive patient sera (patients 4 and 13) was abolished by 20 µg/mL IV.3 Fab fragments. This was further verified in the CAT assay using three known HIT-positive patient sera (patients 13, 34 and 63). Thrombin generation induced by the HIT-positive patient sera was completely abrogated when FcγRIIA was blocked by 20 µg/mL IV.3 Fab fragments (Figure 5.10 B). These finding clearly show that platelet activation and MP generation in both functional assays is dependent on the cross-linking of FcγRIIA by pathogenic IgG anti-PF4/heparin complex antibodies in HIT patient sera.

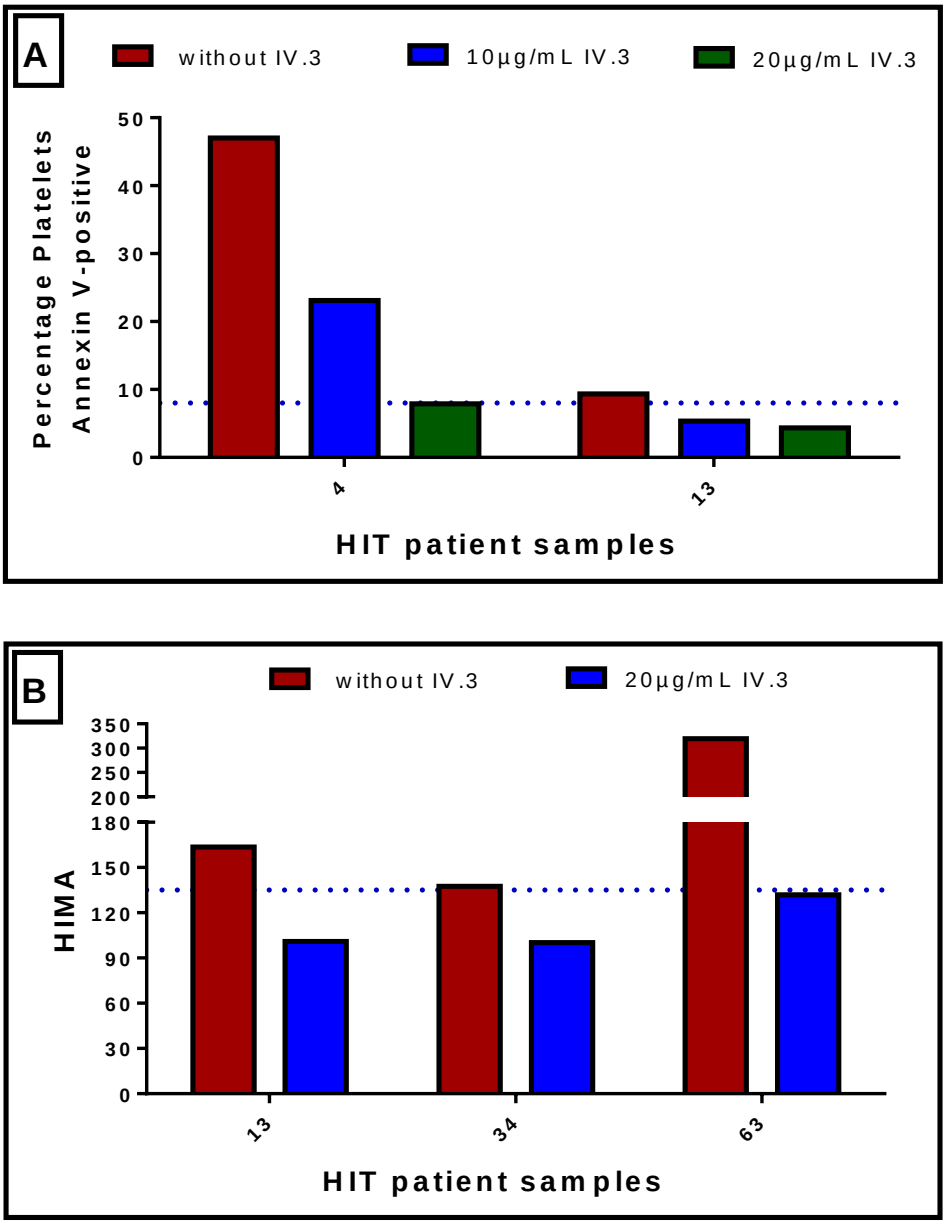

Figure 5.10. Platelet activation and MP generation is induced through FcγRIIA. Donor PRP was pre-incubated with indicated doses of IV.3 Fab fragments for 5 minutes prior to being used in the functional assays. A) shows HITAlert results of patients 4 and 13 sera. Patient sera were incubated with IV.3 pre-treated PRP for 1 hour at RT in the presence of therapeutic dose of UFH (assay samples IV). Subsamples were analysed for platelet Annexin V binding in flow cytometry. B) shows HIMA results of patients 13, 34 and 63. Patient sera were incubated with donor PRP pre-treated with 20µg/mL IV.3 for 30 minutes at 37°C in the presence of 0.2 U/mL UFH. MPs were isolated, washed, mixed into CTI-treated, pre-filtered pooled plasma and analysed in CAT assay using the 1 pM TF reagent assay. Dashed horizontal lines represent the cut-off values of A) HITAlert assay (8%) and B) HIMA (135%).

5.3.5 Comparison of HIMA with other diagnostic tests

5.3.5.1 Comparison of HIMA with 4T Score

4T Score results were available for 49 of 67 patients with suspected HIT. HIMA showed high association with the 4T Score. None of the patients with a low 4T score was positive for HIMA (100% agreement, n=11). Conversely, patients with high HIT risk were associated with HIMA positive results with 70% agreement (n=10). In the group of intermediate HIT probability, a large proportion of patients were found HIMA negative (22/28, n=28) (Table 5.3; Fisher's exact test, P<0.0001, n=49). Additionally, HIMA showed positive correlation with 4T Score (r=0.3018, P=0.0351, n=49) (Figure 5.11).

		Clinical Diagnosis (4T Scores)			
		Low	**Intermediate**	**High**	**P value**
HIMA	**Negative**	11	22	3	Fisher's exact<0.0001
	Positive	0	6	7	
	Total	11	28	10	49

Table 5.3. Comparison of HIMA with 4T Score. HIMA results of HIT suspected patients were divided into two groups, HIMA negative or HIMA positive. Subsequently, these two groups were compared with the clinical diagnosis 4T Score (n=49).

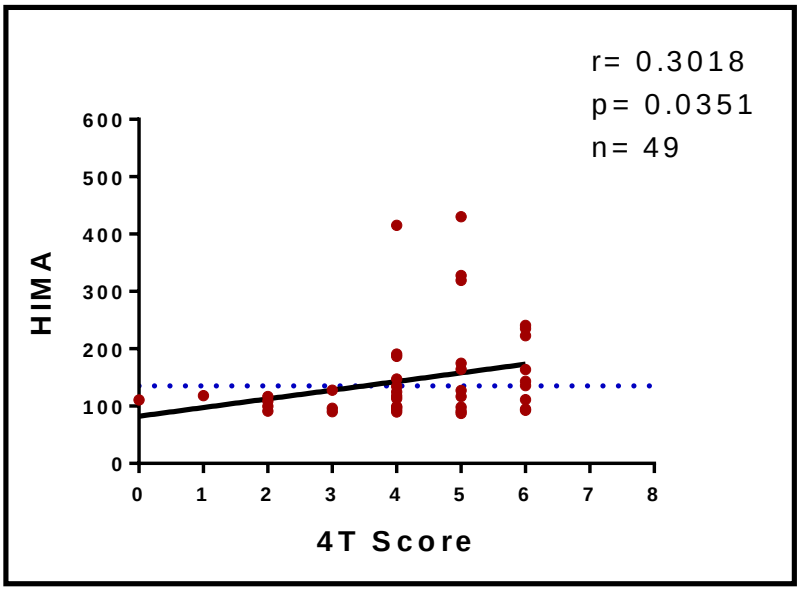

Figure 5.11. Correlation between HIMA and 4T Score. HIMA results of HIT suspected patients were correlated with the 4T Scores (n=49). Dashed horizontal line represent the cut-off value of HIMA, 135%.

5.3.5.2 **Comparison of HIMA with HITAlert assay**

The two functional assays in the study, HIMA and HITAlert, were performed on the same samples of 67 patients with suspected HIT. These assays showed the highest agreement among the other laboratory investigations performed. In the group of HITAlert negative samples, 50 out of 52 were also HIMA negative (96% agreement). On the other hand, HIMA showed 100% agreement with all 15 samples that were positive in the HITAlert assay (Table 5.4; Fisher's exact test, P<0.0001, n=67). In addition, HIMA showed a strong positive correlation with the HITAlert data (r=0.7626, P<0.0001, n=67) (Figure 5.12).

		HITAlert Assay		
		Negative	**Positive**	**P value**
HIMA	**Negative**	50	0	Fisher's exact<0.0001
	Positive	2	15	
	Total	52	15	67

Table 5.4. Comparison of HIMA and HITAlert assay. Agreements of HIMA results with HITAlert results of 67 HIT suspected patients.

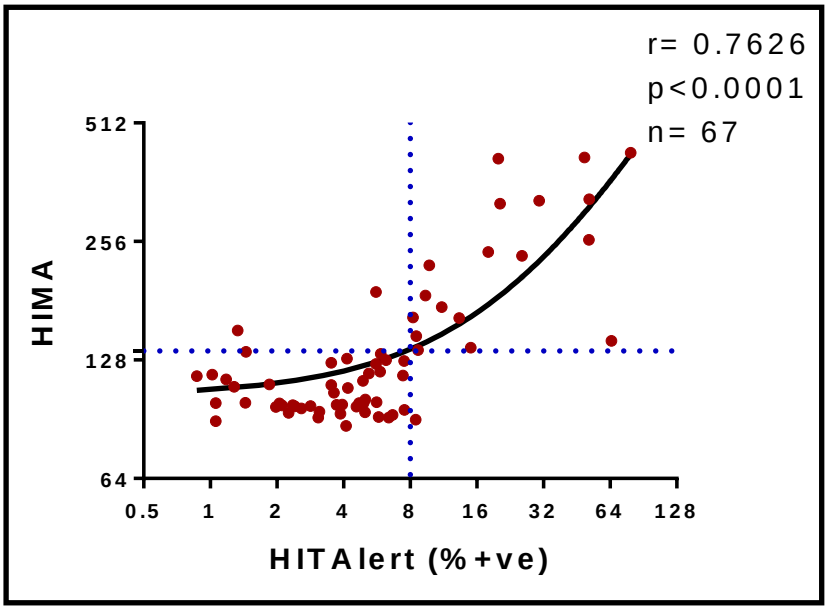

Figure 5.12. Correlation between HIMA and HITAlert assay. HIMA results of HIT suspected patients were correlated with the HITAlert results (n=67). Dashed horizontal line represents the cut-off value of HIMA, 135%. Dashed vertical line represents the cut-off vlaue of HITAlert assay, 8%.

5.3.5.3 **Comparison of HIMA with immunological assays**

The patients' HIMA results were compared with the presence of polytypic IgG/A/M anti-PF4/heparin complex antibodies using the PF4 IgG/A/M ELISA. There was no significant association between the PF4 IgG/A/M ELISA and HIMA results (Table 5.5 A; Fisher's exact test, P=0.123, n=53). A high proportion of PF4 IgG/A/M ELISA positive samples were found to be HIMA negative (18/25, 72% disagreement). However, the majority of HIT-negative samples for the PF4 IgG/A/M ELISA were also HIMA negative (26/28, 93% agreement). These findings indicate the low specificity of the polytypic IgG/A/M ELISA. Nevertheless, HIMA showed significant positive correlation with the IgG/A/M anti-PF4/heparin complex antibody titres (r=0.4336, P=0.0013, n=53) (Figure 5.13 A).

The performance of HIMA was also compared with the IgG-specific immunological assays. The HIMA results were associated with correct classification of 91% (40/44) of PF4 IgG ELISA HIT-negative results. In the group of PF4 IgG-positive patients, HIMA results showed 67% agreement (12/18) (Table 5.5 B; Fisher's exact test, P<0.0001, n=62). Additionally, HIMA results showed a strong positive correlation with IgG anti-PF4/heparin complex antibody titres (r=0.5346, P<0.0001, n=62) (Figure 5.13 B). Similar to PF4 IgG ELISA, HIMA assay showed a relatively similar association and correlation with the AcuStar HIT-IgG assay with 91% (42/46) and 71% (12/17) correct classification of AcuStar-negative and AcuStar-positive results respectively (Table 5.5 C; Fisher's exact test, P<0.0001, n=63) and a strong positive correlation (r=0.4881, P<0.0001, n=63) (Figure 5.13 C). Finally, HIMA performance was also compared with the following IgG-specific ELISA namely; (1) Asserachrom HPIA-IgG, (2) Technozym HIT IgG and (3) Zymutest HIA IgG. HIMA results were available for a total of 38 HIT suspected patients whom were analysed on these three IgG-specific ELISAs. HIMA results were associated with correct classification of 89%

(24/27), 83% (24/29) and 92% (22/24) of HIT-negative results and 73% (8/11), 67% (6/9) and 64% (9/14) of HIT-positive results according to the Asserachrom, Technozym and Zymutest ELISAs respectively (Table 5.5 D-F; Fisher's exact tests, P<0.0001, P=0.009 and P<0.0001 respectively, n=38). Additionally, HIMA results showed positive correlations with the IgG anti-PF4/heparin complex antibody titres measured by the Asserachrom and Zymutest ELISAs (r=0.3558, P=0.0284, and r=0.3336, P=0.0407 respectively) but not with Technoclone ELISA (r=0.2108, P=0.2039) (Figure 5.13 D-F). From the these findings it can be clearly seen that HIMA compares favourably with these IgG-specific ELISAs compared to the polytypic IgG/A/M ELISA. This is an unsurprising finding given it is known that IgA or IgM anti-PF4/heparin complex antibodies are not the main pathogenic HIT antibody types.

		Negative	Positive	P value
(A)		**PF4 IgG/A/M ELISA (GTI)**		
HIMA	**Negative**	26	18	Fisher's exact=0.123
	Positive	2	7	
	Total	28	25	53
(B)		**PF4 IgG ELISA (GTI)**		
HIMA	**Negative**	40	6	Fisher's exact<0.0001
	Positive	4	12	
	Total	44	18	62
(C)		**AcuStar HIT-IgG (HemosIL)**		
HIMA	**Negative**	42	5	Fisher's exact<0.0001
	Positive	4	12	
	Total	46	17	63
(D)		**Asserachrom HPIA-IgG (Stago Diagnostic)**		
HIMA	**Negative**	24	3	Fisher's exact<0.0001
	Positive	3	8	
	Total	27	11	38
(E)		**Technozym HIT IgG (Technoclone)**		
HIMA	**Negative**	24	3	Fisher's exact=0.009
	Positive	5	6	
	Total	29	9	38
(F)		**Zymutest HIA IgG (HyPhen BioMed)**		
HIMA	**Negative**	22	5	Fisher's exact<0.0001
	Positive	2	9	
	Total	24	14	38

Table 5.5. Comparison of HIMA and immunological assays. Agreement of HIMA results with A) PF4 IgG/A/M ELISA (GTI), B) PF4 IgG ELISA (GTI), C) AcuStar HIT-IgG (HemosIL), D) Asserachrom HPIA-IgG (Stago Diagnostic), E) Technozym HIT IgG (Technoclone) and F) Zymutest HIA IgG (HyPhen BioMed).

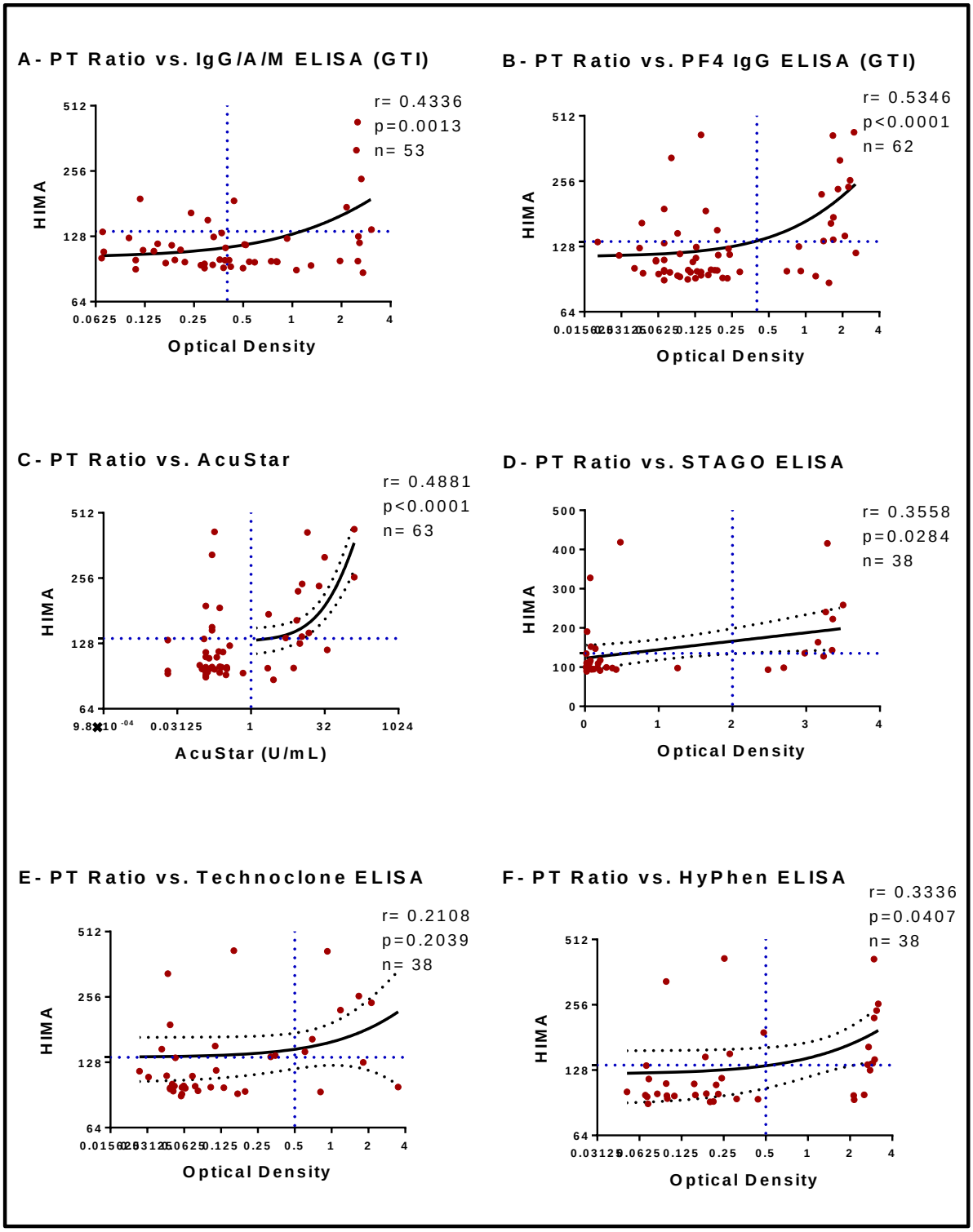

Figure 5.13. Correlation between HIMA Immunological assays. HIMA results of HIT suspected patients were correlated with A) PF4 IgG/A/M ELISA (GTI), B) PF4 IgG ELISA (GTI), C) AcuStar HIT-IgG (HemosIL), D) Asserachrom HPIA-IgG (Stago Diagnostic), E) Technozym HIT IgG (Technoclone) and F) Zymutest HIA IgG (HyPhen BioMed). Dashed horizontal lines represent the cut-off value of HIMA and dashed vertical lines represent the cut-off value of each assay.

5.3.5.4 **HIMA assay sensitivity and specificity**

The sensitivity and specificity of HIMA assay was also evaluated using the HITAlert and PF4 IgG as reference for functional and immunological assays respectively. When compared with HITAlert assay, HIMA assay showed high sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with 100%, 96%, 88% and 100% respectively (Table 5.6). Additionally, HIMA assay showed 67%, 91%, 75% and 87% sensitivity, specificity, PPV and NPV respectively when PF4 IgG ELIAS was used (Table 5.6).

Assay	Sensitivity (%) (95%CI)	Specificity (%) (95%CI)	Positive predictive value (%) (95%CI)	Negative predictive value (%) (95%CI)
HITAlert assay	100 (78-100)	96 (87-100)	88 (64-99)	100 (93-100)
PF4 IgG ELISA	67 (40-87)	91 (87-97)	75 (48-93)	87 (74-95)

Table 5.6. Sensitivity and specificity of HIMA assay. HITAlert (n=67) and PF4 IgG (n=62) assays were used as standards to assess the sensitivity and specificity of HIMA assay.

5.4 **Discussion**

5.4.1 Procoagulant activity of PDMPs in HIT disorder

HIT is a unique prothrombotic disorder that causes both arterial and venous thrombosis. The exact mechanism that initiates thrombosis remains unclear. It has been suggested that this high thrombotic risk of HIT disorder is mediated by the pathogenic effects of HIT antibodies that can cause endothelial cell injury leading to activation of coagulation (Cines, *et al* 1987, Visentin, *et al* 1994), monocyte activation leading to TF expression (Kasthuri, *et al* 2012, Rauova, *et al* 2010) and platelet activation leading to procoagulant MP generation (Arepally

and Ortel 2010, Warkentin 2003). The focus of this project was to investigate the role of HIT immune complexes in generating a platelet procoagulant response and MP generation, and to use this effect to develop a method for detecting functionally-relevant antibodies in patients with suspected HIT.

It is well established that HIT immune complexes induce platelet activation through cross-linking the platelet FcγRIIA via the Fc region of the IgG HIT antibodies. This was further confirmed in this thesis. Data shown in Chapter 4 demonstrates that platelet activation through FcγRIIA is able, by itself, to generate procoagulant platelets and MPs. However, the functional properties of PDMPs generated through FcγRIIA platelet activation are poorly studied. In the present study, the procoagulant activity of PDMPs generated through *in vitro* FcγRIIA platelet activation induced by HIT immune complexes was examined using thrombin generation, which would reflect the *in vivo* pathogenic mechanism of platelet activation in HIT.

In the present study, it was clearly shown that HIT immune complexes, in the presence of optimal dose of heparin, are able to induce a potent platelet procoagulant response (as shown in platelet Annexin V binding by flow cytometry) and the formation of MPs capable of promoting thrombin generation. It was also confirmed that the production of procoagulant platelets and MP generation induced by HIT immune complexes is mediated through FcγRIIA as confirmed by blocking with IV.3 Fab fragments. This finding is in agreement with previous reports that showed that sera from patients suspected of HIT induce the platelet procoagulant response and MP formation through FcγRIIA (Tomer, *et al* 1999, Warkentin, *et al* 1994). Hughes *et al* (2000) showed, by scanning and transmission electron microscopy, that MPs generated from platelet activation by HIT immune complexes results in budding and release of MPs ranges in size from 0.1 to 1μm in diameter. The present study also demonstrates that the PDMPs generated by HIT immune complexes support thrombin

generation in a similar manner to those generated when platelets are activated via the other two ITAM-containing receptor on platelets, GPVI and CLEC-2. PDMPs generated by HIT immune complexes markedly enhance the amount of thrombin generation in a PL-dependent manner, which is reflected by increased amounts of ETP and PT, but they failed to markedly shorten the lag time and ttPeak, suggesting that they lack TF activity.

Unlike the procoagulant response generated through GPVI and CLEC2 (described in Chapter 4) some patients' sera were able to induce Annexin V binding in more than 50% of the platelets; specifically sera from patients 8, 11, and 73 that gave showed 51%, 65% and 79% Annexin V binding respectively. This finding further supports the observation in section 4.2.1 that FcγRIIA has the potential for inducing platelet procoagulant surfaces and MP generation in a greater proportion of platelets than the other platelet ITAM-containing receptors, GPVI and CLEC-2. This substantiates the early observation of Warkentin and Sheppard (1999) who showed that FcγRIIA platelet activation by HIT immune complexes, and other platelet IgG agonists such as heat-aggregated IgG, induce greater numbers of MPs and procoagulant activity than collagen (Warkentin and Sheppard 1999).

5.4.2 Evaluation of HIMA assay as a diagnostic tool for HIT

To prevent thrombotic events in patients suspected of having HIT, rapid and reliable diagnosis is essential. However, HIT laboratory testing is largely agreed to suffer from limitations in specificity, sensitivity and feasibility. In the present study, the CAT assay was optimised and validated to be used as a functional assay for HIT diagnosis by measuring the procoagulant activity of generated MPs from donor platelets.

In general, functional assays for HIT diagnosis are dependent on the ability of the HIT antibodies to activate platelets. They are thus more specific than immunological assays as

they are able to discriminate between the pathogenic and non-pathogenic HIT antibodies. Although several functional assays have been used, the generation of MPs has been suggested to be more relevant diagnostic tool for HIT than other platelet activation endpoint markers as it more closely reflects the effects of HIT antibodies in patients (Denys, *et al* 2008, Tomer, *et al* 1999). Assays for MP generation have been assessed for HIT diagnosis in several studies using flow cytometry (Denys, *et al* 2008, Lee, *et al* 1996, Mullier, *et al* 2010, Mullier, *et al* 2013, Tomer, *et al* 1999), which showed a very good performance toward HIT diagnosis, with high sensitivity and specificity compared with the clinical diagnosis and other laboratory tests. However, MP detection by flow cytometry suffers from several limitations. First, despite the incremental developments in the new generation of flow cytometers to detect the small size particles, the predominant size range of MPs is below the detection threshold of flow cytometers (Lacroix, *et al* 2010). Second, the reported existence of "Annexin-negative MPs" further limits the MPs detection in flow cytometry (Horstman, *et al* 2004, Piccin, *et al* 2007). Finally, it remains uncertain whether the events in the MP gate in the flow cytometry represent single particles or a "swarm" of MPs, which may lead to underestimates of the number of MPs in the samples (Harrison and Gardiner 2012, van der Pol, *et al* 2012). The first attempt to standardise the flow cytometry-based assays for HIT diagnosis was the introduction of a commercially available assay, HITAlert, which is based on platelet Annexin V binding. HITAlert assay was evaluated in the present study and, in accord with other recent studies (Garritsen, *et al* 2014, Solano, *et al* 2013), showed a very good performance toward HIT diagnosis, with high sensitivity and specificity compared with the clinical diagnosis and other laboratory tests. However, the assay, as presented, lacks discrimination between platelets and MPs.

In the present study, to overcome the limitations of flow cytometry in MP detection, the CAT assay was proposed as a method to evaluate the generation of MP by HIT samples. The CAT

assay had already been proposed in 2009 to measure the procoagulant activity of donor platelets in PRP preparation (Tardy-Poncet, *et al* 2009). That study, however, was limited by several issues. First, PRP samples were analysed in the CAT assay in the presence or absence of low concentration of UFH, which may have inhibited thrombin generation as shown in the present study (Figure 5.6). Additionally, some patient samples might contain endogenous heparin, which may further inhibit the ability of the MPs to support thrombin generation. Second, the study did not discriminate between the procoagulant activities of platelets and/or MPs. Third, there was no cut off value to discriminate between HIT-negative and HIT-positive samples due to lack of standardisation. Finally, endogenous MPs were not removed from patient samples before they were used in the assay, leading to possible interference with the assay results.

To overcome these limitations of the CAT assay in measuring the procoagulant activity of MPs generated in HIT sample mixtures, some modifications were introduced in the present study. Endogenous MPs in the patients' serum samples were removed by centrifugation before being used in the assay. MP-free patient samples were mixed with donor platelets and the generated MPs were isolated, washed, mixed into CTI-treated, pre-filtered pooled plasma and analysed in the CAT assay using the 1 pM TF reagent assay (Stago PRP reagent) that is sensitive to the levels of PDMPs in the plasma. The washing step is critical to remove the effect of heparin in the sample mixtures, while the use of pooled plasma eliminates the individual variations in terms of plasma procoagulant activity, making the assay completely dependent on the levels of the generated PDMPs in the samples. To overcome inter-assay and inter-donor variability, it was proposed to use ratios instead of absolute values by normalising the patients' thrombin generation results against donor's results.

As shown in section 5.3.5, the standardised HIMA assay showed very good correlations and associations with other diagnoses, particularly with the other functional assay, HITAlert.

Consequently, HIMA is sufficiently sensitive to detect the clinically pathogenic HIT antibodies, which means it may have a place in HIT diagnosis as a reliable and rapid functional assay.

The strength of the present study are the use of (1) unique set of data including clinical diagnosis, functional (HITAlert) and immunological laboratory assays (PF4 IgG/A/M, PF4 IgG, AcuStar HIT-IgG, Asserachrom HPIA-IgG, Technozym HIT IgG and Zymutest HIA IgG) to assess the performance of HIMA assay, (2) the two functional assays, HITAlert and HIMA, were performed using the same platelet donors, which enables a direct comparison between their performance, (3) multicentre study and (4) standardised CAT assay that is completely dependent on the levels of MPs generated in the assay without the interference of individual variation in the plasma procoagulant activity and heparin. To the best of my knowledge, this is the only study that has removed the endogenous MPs in patient samples before being analysed in HIT functional assays.

On the other hand, the present study has potential limitations. First, the study population was relatively small. Validation in a lager study with including normal healthy controls is required. Second, no high concentration of heparin was used in the HIMA assay. It is recommended to use a high dose of heparin (such as 100U/mL), which causes disassociation of HIT-immune complexes and thus improves the specificity of the assay (Greinacher 2009). However, this was not carried out here due to the small volumes of patients' serum available. Third, the assay was performed in PRP preparation, which may be less sensitive than washed platelet preparation (Chong, *et al* 1993, Warkentin, *et al* 2011). Finally, HIMA, similar to other HIT functional assays, suffers from a series of well-known limitations including: immediate availability of suitable healthy donors, technically demanding, time-consuming, and therefore not easily adaptable to use in routine clinical laboratories.

In rare cases, HIT functional assays also suffer from their limited capacity to discriminate between platelet activation induced by HIT immune complexes or other causes of platelet activation such as in APS. This may lead to false-positive results as was seen in patients 9, 10 and 62 in the present study. Therefore, it has been suggested to use anticardiolipin and anti-β2GPI ELISAs, which test positive in APS patients and negative in HIT-suspected patients (Garritsen, *et al* 2014). In addition, another laboratory strategies that could be suggested is to block the FcγRIIA using IV.3 antibodies to discriminate between platelet activation by genuine HIT-samples and other causes, thus improving the specificity of the HIT functional assays.

In conclusion, this study showed good performance of HIMA toward HIT diagnosis. A follow-up study on a larger cohort of patients suspected of HIT and normal healthy controls would be valuable to confirm the use of HIMA as a new promising HIT diagnostic assay.

CHAPTER 6: General discussion

Originally believed to be just 'platelet dust', MPs are now known to play a critical role in many patho/physiological conditions. Indeed, the field of MP research has developed enormously in a short period of time. However, the MP research field faces a number of challenges with regards to standardisation of MP analysis, which hinder the progress in understanding how MPs achieve their biological effects.

Based on the data in the present study and data from the literature, the following recommendations could be suggested for the pre-analytical and analytical steps in analysing the procoagulant activity of MPs in the CAT assay (Table 6.1).

Step	Recommended	Reference
Blood sampling	Large needle or butterfly (19-21 gauge). Light tourniquet to localise the vein <1 minute. Discard the first millilitres.	(Jy, *et al* 2004, Lacroix, *et al* 2012, van Ierssel, *et al* 2010)
Anti-coagulant	Plastic sodium citrate tubes: (0.105 M sodium citrate with a 1:9 v/v). CTI (18.3 µg/mL), particularly for TF analysis.	(Lacroix, *et al* 2012, Van Der Meijden, *et al* 2012)
Delay before MP isolation	Within one hour and no longer than two hours.	(Lacroix, *et al* 2012, Pearson, *et al* 2009)
Transportation	Avoid agitation, keep tubes vertically.	(Lacroix, *et al* 2012)
Centrifugation	1- Two-step centrifugation at 2500g for 15 minutes (recommended), or 2- 1500g for 15 minutes followed by 13000g for 2 minutes (acceptable). Use light brake, at RT.	Present study and (Jy, *et al* 2004, Lacroix, *et al* 2012)
Storage	-80°C for up to six months. Quick thaw at 37°C or at RT. Repeated freezing and thawing is not recommended but acceptable for MP PPL activity.	Present study and (Ayers, *et al* 2011, Lacroix, *et al* 2012)
CAT assay analysis	1- PRP reagent assay for MP detection and evaluation of PPL activity, and 2- MP reagent assay for evaluation of TF activity	Present study.

Table 6.1. Recommended protocol for the analysis of the procoagulant activity of MPs using CAT assay.

A large needle (19 or 21 gauge) or butterfly with short tubing is recommended for phlebotomy to minimise shear stress during blood collection that can activate platelets. The minimal use of a tourniquet avoids activation of haemostasis through vascular stasis, and discarding the first millilitres of blood avoids contamination of the sample with material from the vessel wall or connective tissue. Collection into vacutainers is preferred to collection via a syringe as this minimises the time without anticoagulant, thereby minimising the chance of activation of platelets and coagulation *in vitro*. Collection into sodium citrate is compatible with assays of coagulation factors, and immediate addition of the FXIIa inhibitor, CTI, is necessary for the analysis of TF and PPL activity to reduce the risk of activation via the contact pathway. Processing the samples within one hour is recommended but no longer than two hours, and transportation without agitation will reduce the risk of activation *in vitro*. A two-step centrifugation procedure, each at 2500g for 15 minutes is recommended as this has been shown to remove cells including platelets and so will prevent the risk of platelets and cell debris being fragmented during freezing and thawing, which can result in an apparent increase in MPs in the sample. Alternatively, centrifugation at 1500g for 15 minutes followed by 13000g for 2 minutes protocol can achieve the same results. Recommendation of storage at -80°C (rather than -20°C) is based on the known greater stability of many proteins at the lower temperature, and the recommendation of storage for a maximum six months is based on the data from this study, which showed a slight deterioration in clotting factor activity, and from others (Ayers, *et al* 2011, Lacroix, *et al* 2012). Rapid thawing of samples at 37°C or RT is a recommendation based on previous guidance for analysis of coagulation factors (Lacroix, *et al* 2012). Running the CAT assay before and after sample filtration allows the contribution of MPs to the assay to be demonstrated. The use of the PRP reagent is recommended to evaluate the PPL activity of MPs and the MP reagent for TF activity.

There have been ongoing efforts to improve MP analysis by improving the sensitivity of the current approaches such as flow cytometry, as well as by developing novel approaches such as NTA, DLS and AFM. However, it is important to optimise and validate these approaches before they become routinely used. Equally important, there is still a need for multi-centre international collaborations to evaluate and standardise the pre-analytical and analytical steps of MP analysis with regards to both numeration and functional characterisation to agree on a well-defined protocol that can be used in research and clinical laboratories. However, it has be taken into the account the MP sampling and analysis performance should be compatible with equipment and logistical issues for clinical laboratory setting. Only then the outcomes from different studies can be directly compared.

MP levels in plasma are dependent on both their generation by activated and apoptotic cells and clearance from the circulation. Therefore, circulating MP levels do not necessary reflect the MP generation, but rather than a dynamic balance between generation and clearance. Phagocytosis is believed to be the main mechanism by which MPs are cleared from the circulation. The phagocytosis of MPs by phagocytic cells appears to be mediated by the PS exposure on MP surfaces and scavenger receptors that bind PS directly (Terrisse, *et al* 2010, Willekens, *et al* 2005), or by opsonisation by IgM that facilitates the binding and uptake of MPs by macrophages (Litvack, *et al* 2011). Defects in the elimination of MPs lead to increased levels in the circulation. It has been shown that mice deficient in the developmental endothelial locus-1 (Del-1), which is thought to mediate endothelial cell uptake of MPs, displayed a higher levels of circulating MPs compared with wild-type mice following endotoxin (LPS) administration (Dasgupta, *et al* 2012). In general, MPs are thought to have a relatively short half-life in the circulation. It has been demonstrated that Annexin V positive MPs circulate only for 5.8 hours following transfusion of platelet concentrates into humans (Rank, *et al* 2011).

The question as whether MPs are simply a consequence of disease processes, or mediators of a pathological process is still highly controversial. The association of high MP levels with many different pathological conditions makes them of particular interest as useful biomarkers not only for diagnostic, but also for the assessment of prognosis and predicting disease outcomes. Beyond their putative role as biomarkers, there are emerging evidences that MPs contribute to pathological processes and exert biological effects.

The best established biological effect of MPs is their ability to promote thrombin generation and cause thrombosis. Their procoagulant properties are largely dependent on the exposure of the negatively-charged PLs and expression of TF. It has been proposed that PDMPs that lack TF may play a role in haemostasis, whereas MDMPs that express TF may contribute to thrombosis, and play only a minor role in haemostasis (Owens and Mackman 2011). Indeed, this notion agrees with the observation that the most abundant MPs in the circulation of healthy subjects are derived from platelets or megakaryocytes and they do not cause spontaneous thrombosis. As discussed before, data in the present study further support this notion, as it showed that PDMPs do not have the ability to initiate thrombin generation, whereas TF-bearing MPs such as those derived from ECDMPs and MDMPs initiate thrombin generation.

MPs are generated in response to pro-inflammatory stimuli such as TNF-α, however, they also contribute to inflammation by stimulating the release of cytokines and chemokines such as IL-6, IL-8 and TNF-α. It has been reported that MPs derived from leucocytes enhance the release of inflammatory cytokines IL-6 and monocyte chemotactic protein in cultured endothelial cells (Mesri and Altieri 1999) and the release of TNF-α and IL-1β from monocytes (Scanu, *et al* 2008). Additionally, administration of ECDMPs to rats resulted in acute lung injury, as shown by neutrophil infiltration and increased pro-inflammatory cytokines, TNF-α and IL-1β (Buesing, *et al* 2011, Densmore, *et al* 2006).

In addition, MPs have been shown to regulate angiogenesis. PDMPs have been shown to enhance the proliferation, survival, migration and tube formation of cultured endothelial cells (Kim, *et al* 2004). Furthermore, MPs isolated from atherosclerotic plaques also promoted endothelial cell proliferation *in vitro* and stimulated angiogenesis *in vivo* after injecting these MPs into mice (Leroyer, *et al* 2008).

Although apoptosis is a potent stimulus for MP generation, there is accumulating evidence that MPs can also induce apoptosis in other cells. MPs isolated from patients with hypertension induce H_2O_2 production, cellular senescence and apoptosis in endothelial progenitor cells (Huang, *et al* 2010). Interestingly, MPs derived from platelets and endothelial cells have been demonstrated to contain caspase 3 (Abid Hussein, *et al* 2005, Boing, *et al* 2008), which is believed to be a protective mechanism by removing pro-apoptotic machinery. It has been also proposed that MPs may transfer caspase 3 to target cells, which in turn may induce apoptosis (Abid Hussein, *et al* 2005). Overall, it appears that MPs serve as both biomarkers of, and effectors in, the patho/physiological processes.

In terms of the mechanisms underlying the generation of the platelet procoagulant response and MP formation, it appears that platelet ITAM-containing receptors are the primary receptors eliciting PS exposure and MP formation, as they were able, by their sole actions, and with minimum platelet-platelet interaction, to induce a potent platelet procoagulant response accompanied with the release of procoagulant MPs. On the other hand, platelet GPCRs play a minor role in inducing platelet procoagulant response on their own. However, they play a critical role in enhancing the ITAM-containing receptor procoagulant activity. This evidence is derived from the following findings: (1) GPCR agonists alone were not able to induce significant platelet procoagulant response and MP generation; (2) exogenous concentrations of GPCR agonists significantly enhanced platelet procoagulant activity mediated through ITAM-containing receptors; and (3) inhibition of endogenous released

ADP and TxA_2 resulted in a significant inhibition of platelet procoagulant activity of ITAM-containing receptors.

Activation of platelets via GPCRs, especially the PAR1 and PAR4 thrombin receptors and the TxA_2 TP receptors, is generally effective in causing platelet degranulation. The question as how these different platelet responses (i.e. platelet degranulation and/or procoagulant response) contribute to the patho/physiological effects of signalling through platelet ITAM-containing receptors, remains to be addressed. It has been recently proposed that GPVI and CLEC-2 play a critical role in maintaining vascular integrity in inflammation, whereas platelet activation through GPCRs is not essential for the maintenance of vascular integrity (Boulaftali, *et al* 2013). In the line with this, CLEC-2 has been shown to play a key role in lymph/blood vessel separation in the embryonic development (Bertozzi, *et al* 2010, Carramolino, *et al* 2010, Finney, *et al* 2012, Herzog, *et al* 2013). However, the mechanism as how GPVI and CLEC-2 contribute to the vessel integrity in inflammation and/or development remains unclear. It can be speculated that a local procoagulant response is required for platelets to achieve their contribution to vessel integrity, since both receptors are potent platelet procoagulant response inducers. This speculation could be supported by the finding that mice with aggregation-incompetent platelets do not exhibit the lymphatic vascular defects (Hodivala-Dilke, *et al* 1999), whereas CLEC-2-defecient mice develop embryonic/neonatal lethality due to blood/lymphatic mixing (Bertozzi, *et al* 2010, Suzuki-Inoue, *et al* 2010). More investigations are required to understand the underlying molecular mechanisms of how platelets contribute to vessel integrity.

The best-studied platelet ITAM-containing receptor in haemostasis and thrombosis at sites of vascular damage and plaque rupture is GPVI. It is the central receptor for collagen, which is present in large amount in ECM and atherosclerotic plaques. Interestingly, deficiency in GPVI in humans and mice is associated with only mild bleeding tendency, suggesting that

this receptor could be a good antithrombotic target (Nieswandt, *et al* 2011, Watson, *et al* 2010). However, it has to be highlighted that GPVI plays a critical role in vascular integrity, which indicates a potential negative side effect of this therapeutic approach.

As discussed above, CLEC-2 has been shown to play a key role in lymph/blood vessel separation in the embryo. One question arises here as whether CLEC-2 plays this role only in early developmental or whether it also has a role in day-to-day physiological functions. On one hand, it has been shown that CLEC-2-defecient mice suffer from only a mild bleeding tendency, suggesting that CLEC-2 may play only a minor role in haemostasis (Hughes, *et al* 2010a, Suzuki-Inoue, *et al* 2010). On the other hand, CLEC-2 has been shown to play a critical role in maintaining vascular integrity at sites of inflammation (Boulaftali, *et al* 2013). However, since the only known endogenous ligand for CLEC-2 podoplanin is not constitutively expressed in ECM or the vessel wall, it is difficult to determine the underlying mechanism as how CLEC-2 contributes to the vascular integrity at the site of inflammation. One possibility is that podoplanin is upregulated in the inflammatory macrophages (Kerrigan, *et al* 2012) or other cells such as smooth muscle (Hatakeyama, *et al* 2012). The data presented in this thesis showed that podoplanin can be upregulated in monocytes within a thrombus suggests that CLEC-2 may also play a role in maintaining thrombus stability. Overall, it appears that CLEC-2 is critical for lymphatic vascular development and vascular integrity at the site of inflammation in adults.

It would be important to study the thrombotic contribution of platelet procoagulant response and MP generation induced through CLEC-2 *in vivo*. This could be achieved by measuring platelet procoagulant activity and MP formation in CLEC-2 deficient compared to wild type mice before and after inducing thrombosis and/or inflammation. Additionally, since podoplanin is upregulated in a variety of cancer cell types (Navarro-Nunez, *et al* 2013), it would be good to evaluate the MP levels in patients with these types of tumours.

Data in the present study demonstrated that the platelet procoagulant activity of GPVI and CLEC-2 are significantly inhibited by $P2Y_{12}$, Src and Syk inhibitors and to less extent aspirin. This makes them of particular of interest as therapeutic strategies especially for CLEC-2 as it is involved in tumour metastasis. More systemic, unbiased studies are required to evaluate these therapeutic approaches.

Since FcγRIIA shares similar signalling mechanisms with GPVI and CLEC-2, and since it evokes similar platelet procoagulant activity, it is not impossible that $P2Y_{12}$, TxA_2, Src and Syk inhibitors have similar inhibitory effect to the platelet procoagulant activity of FcγRIIA, making them promising therapeutic targets for HIT. Unfortunately time constraints, and limitations of HIT serum, did not allow full dissection of the role of these mediators in the present study.

As discussed before, the pathological role of FcγRIIA can be demonstrated in the development of destructive immune response in HIT, leading to a potential life-threatening, high thrombotic risk disorder. On the other hand, the physiological role of FcγRIIA in platelets is still poorly understood. The recent discovery of the FcγRIIA role in the outside-in signalling of GPIIb/IIIa has boosted the understanding of the contribution of FcγRIIA to haemostasis and thrombosis (Boylan, *et al* 2008, Zhi, *et al* 2013). It is proposed that FcγRIIA functions as an adaptor for GPIIb/IIIa signalling. In addition, it is well established that platelets play a major role in the innate immune system (Cox, *et al* 2011, Engelmann and Massberg 2013, Semple, *et al* 2011). One mechanism by which platelets contribute to the innate immune system is that antibody-bound bacteria bind directly to FcγRIIA leading to platelet activation, which in turn speeds their clearance. Indeed, it has been shown that FcγRIIA plays a critical role in bacterial-induced platelet activation (Arman, *et al* 2014, Tilley, *et al* 2013). The question as whether platelet procoagulant response induced by FcγRIIA is required for bacterial clearance remains unclear.

In summary, this thesis focused on the procoagulant properties of MPs derived from platelets and other vascular cells and evaluated the mechanisms whereby platelets generate procoagulant MPs. The pre-analytical and analytical steps of MP procoagulant activity analysis were investigated using the CAT assay, revealing that the MP isolation protocol can be a major source of variation between studies. Proper centrifugation protocols should be used to avoid contamination with platelets and cell fragments. Repeat freezing and thawing has no significant impact on the procoagulant activity of MPs providing the plasma is free of platelets and cell fragments, but does cause a slight decay of clotting factor activity, as does long term storage. Further experiments to validate these findings using different MP analysis approaches such as flow cytometry, NTA, DLS, ELISA, prothrombinase assay and STA Phospholipid Procoag Assay are needed. The present study also showed that MPs derived from platelets, endothelial cells and macrophages are heterogeneous in their procoagulant properties, indicating that they play different roles in haemostasis and thrombosis. It would be important to conduct more investigations to evaluate the procoagulant activities of MPs derived from other cell types and to compare different stimuli. Additionally, it is important to evaluate other aspects of procoagulant characteristics of MPs such as the expression of vWF, FV and fibrinogen. This study has proved that the CAT assay can be very useful functional assay to evaluate the procoagulant properties of MPs. It would be interesting to measure MPs in patient samples for both number and functional characteristics, to gain more of an understanding into the functional properties of these MPs.

It appears that platelet activation through the ITAM-containing, but not through the GPCRs, receptors alone results in the release of MPs that they have similar procoagulant activities. The novel finding of CLEC-2 in provoking platelet PS exposure and MP formation provides a new insight into the patho/physiological role of CLEC-2 that makes it a good antithrombotic target, particularly in certain tumour types where podoplanin-CLEC-2

interaction is involved. There remains more work to be done to further understand the physiological role of CLEC-2, particularly in identifying other possible endogenous ligands for CLEC-2 in haemostasis and thrombosis. The ability of HIT-immune complexes in suspected HIT patients to induce the release of procoagulant MPs through FcγRIIA platelet activation was used to establish a novel diagnostic approach for HIT. Measuring the procoagulant activity of MPs in the CAT assay provided a rapid diagnostic assay for HIT with good correlation and association with other clinical and laboratory investigations. However, this assay needs to be further characterised with a larger number of patients.

MPs are receiving a great deal of attention in recent years, as diagnostic and prognostic biomarkers and mediators for many physiological and pathological conditions. Indeed, the field of MP research has developed enormously in a short period of time and it is expected to expand significantly in the future, especially with the continuous improvements in the detection methods. Although this thesis focused on the thrombotic potential of MPs, they are playing critical roles in so many other patho/physiological processes such as inflammation, vascular reactivity, angiogenesis, immunity, cancer progression, cell communication and signalling. To date, the most studied functions of MPs are pathologic, particularly in thrombosis and cancer. There remains much work to be done to get more insight into their normal biological roles and how they influence cellular processes. Better understanding of the underlying molecular mechanisms of how MPs contribute in different patho/physiological processes will help to develop new therapeutic targets for pathological conditions by modulating MP formation and function.

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