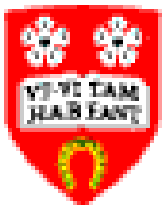

University of Leicester

# Regulation of the procoagulant activity of platelets and platelet-derived microparticles

Thesis submitted for the degree of
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at
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# Regulation of the procoagulant activity of platelets and platelet-derived microparticles

## Abstract

Procoagulant microparticles (MPs) in the circulation are increasingly recognized as playing a role in haemostasis and inflammation and may prove useful biomarkers for clinical studies. Platelets are known to generate MPs in response to stimulation, and platelet-derived MPs (PDMPs) form the majority of the MPs found in the normal circulation and can be elevated in a number of disease states. The current study has focused on the procoagulant activity of platelets and PDMPs following stimulation with the collagen-mimetic peptide CRP-XL. Their activity in accelerating thrombin generation can be accurately measured by the Calibrated Automated Thrombogram (CAT) assay using 1pM TF reagent to initiate the reaction, with the finding that plasma from patients with chronic renal disease has significant thrombin generation due to increased procoagulant activity of MPs. Conversely premature MI patients in a stable condition have thrombin generation comparable to matched healthy control. In all subjects, removal of MPs from plasma by filtration (>0.2 μm) eliminates this procoagulant activity, indicating the importance of MP size in driving the procoagulant response. The procoagulant activity of activated platelets and PDMPs showed a strong correlation with annexin-V binding measured using flow cytometry. Comparison of the regulatory mechanism of the procoagulant activity of platelet and PDMPs upon activation with platelets undergoing apoptosis showed that although both activation and apoptosis resulted in exposure of the procoagulant surface, apoptotic platelets did not release procoagulant MPs or show any markers of activation such as P-selectin expression, fibrinogen binding or aggregation. Reactive oxygen species (ROS) are generated during platelet activation or apoptosis mainly through the NADP(P)H oxidase pathway. The procoagulant activity of platelets and PDMPs was significantly attenuated (as measured by thrombin generation and annexin-V binding) by inhibition of the NAD(P)H oxidase pathway by apocynin, which had similar inhibition on other platelet responses including on 12-HETE generation, $TxB_2$ production and aggregation. However antioxidants only inhibited apoptosis-induced platelet procoagulant activity. These data demonstrate significant involvement of ROS in platelet procoagulant activity induced by both activation and apoptosis and suggest the involvement of lipid peroxidation during apoptosis.

Hassan A. A. Hamali
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# Statement by the candidate

This thesis is the result of work carried out wholly 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.

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Hassan A A Hamali

August 2011

# Publications

**(A). Papers related to this thesis that have been published or submitted**

- Thomas CP, Morgan LT, Maskery BH, Murphy RC, Kuhn H, Hazen SL, Goodall AH, **Hamali HA**, Collins PW, O'Donnell VB. (2009). *Phospholipid-esterified Eicosanoids are generated in agonist-activated human platelets and enhance tissue factor dependent thrombin generation.* The Journal of Biological Chemistry; **285**(10):6891-6903. [Impact factor 5.8].

- Vogler M, **Hamali HA**, Sun X, Bampton ETW, Dinsdale D, Snowden RT, Dyer MJS, Goodall AH, Cohen GM. (2011). *Pathways involved in platelet apoptosis and activation following BCL2-inhibition*. Blood. [Impact factor 10.5].

- Burton JO, **Hamali HA**, Stansfield R, Patel A, Goodall AH, and Brunskill NJ. (2011). *Elevated levels of procoagulant plasma microparticles in dialysis patients*. Submitted to Kidney international. [Impact factor 5.6]

**(B). Published abstracts have been presented at national and international meetings/conferences**

- **Hamali HA**, Appleby JA, O'Donnell VB, Goodall AH. *The role of 12-lipoxygenase and NAD(P)H in the generation of procoagulant phospholipids on platelets activated through GPVI.* Presented as a talk at the First French-UK platelet meeting, Toulouse, France. October 2009.

- **Hamali HA**, Goodall AH. *Analyzing the effect of platelet-derived microparticles on the calibrated automated thrombography: assay and pre-analytical variables*. Presented as a talk at the International Society for Laboratory Haematology, Brighton, UK. May 2010. Published in International Journal of Laboratory Haematology; 2010;32(s1):1-176.

- **Hamali HA,** Appleby JA, Goodall AH. *Role of ROS production in the collagen-mediated procoagulant response in platelets*. Presented as a talk at the Nottingham Platelet Conference, Nottingham, UK. July 2010. Published in Platelets; 2010; 21(5):393-419.

- Burton JO, **Hamali HA**, Stansfield R, Patel A, Goodall AH, and Brunskill NJ. *Elevated Levels of Plasma Microparticles in Dialysis Patients are Associated with a Pro-coagulant State.* Presented as a poster at the American Society of Nephrology, USA. October 2010.

- Vogler M, **Hamali HA**, Bampton ETW, Dinsdale D, Dyer MJS, Goodall AH, Cohen GM. *Platelet Toxicity Induced By the BCL2-Inhibitor ABT-263, A Novel Anti-Cancer Agent 2011.*

- **Hamali HA**, Vogler M, Cohen GM, Goodall AH. *Differences between platelet activation and apoptosis: the role of ROS production in the procoagulant response.* Presented as a poster at the Saudi international Conference V, Warwick University, Warwick, UK. June 2011.

- **Hamali HA**, Vogler M, Cohen GM, Goodall AH. *Differences between platelet activation and apoptosis: the role of ROS production in the procoagulant response.* BSTH annual meeting, Brighton, UK. October 2011.

**(C). Manuscripts planned**

- **Hamali HA,** Alsahli MA, Goodall AH. The effect of platelet-derived microparticles by the calibrated automated thrombography: assay and pre-analytical variables**.**

- **Hamali HA,** Appleby JA, Gardiner S, Harrison P, Goodall AH. *Factors regulating the prothrombotic potential of platelets and platelet-derived microparticles.*

- **Hamali HA,** O'Donnell A**,** Goodall AH. *Role of ROS in the regulation of the collagen-mediated procoagulant response in human platelets.*

- **Hamali HA,** Krishnan U, Burton JO, Samani, NJ, Brunskill NJ, Goodall AH. *Role of Elevated Levels of procoagulant microparticles in the circulation of patients with cardiovascular disease Patients are Associated with a Pro-coagulant State.*

- **Hamali HA**, Vogler M, Cohen GM, Goodall AH. *Differential roles of ROS in the regulation of thrombin generation by platelets undergoing apoptosis and activation.*

# List of Abbreviations

**aa:** amino acid
**AA:** arachidonic acid
**Ab(s):** antibody/antibodies
**$\alpha_2\beta_1$:** Integrin alpha 2 beta 1 [Glycoprotein (GP) Ia-IIa]
**$\alpha IIb\beta_3$:** Integrin alpha IIb beta 3 [Glycoprotein (GP) IIb-IIIa]
**α2M:** α2-macroglobuin
**ACD:** acid citrate dextrose
**ACS:** acute coronary syndrome
**ADP:** adenosine diphosphate
**ANOVA:** analysis of variance
**AxV -ve:** annexin-V negative events
**AxV +ve:** annexin-V positive events
**AxV ++ve:** intermediate
**AxV +++ve:** strong annexin-V positive events
**APAF-1:** apoptotic protease-activating factor-1
**APC:** activated protein C
**aPTT:** Activated Partial Thromboplastin Time
**ASA:** aspirin or acetylsalicylic acid
**AT:** antithrombin
**ATP:** adenosine diphosphate
**AU:** arbitrary units
**AUC:** area under the curve
**BH4:** tetrahydrobiopterin
**BHT:** butylated hydroxytoluene
**BMI:** body mass index
**BSA:** Bovine serum albumin
**$Ca^{2+}$:** calcium ion
**$[Ca^{2+}]i$:** intracellular calcium
**CAD:** coronary artery disease
**cAMP:** cyclic adenosine monophosphate
**CAT:** calibrated automated thrombography
**CD**: cluster of differentiation
**CKD**: chronic kidney disease
**CM-$H_2$DCFDA**: 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate acetyl ester
**COX-1:** cyclooxygenase-1
**CRP-XL:** cross-linked collagen-related peptide
**CTI:** corn trypsin inhibitor
**CV:** coefficient of variation ((SD/mean)x100%)
**CVDs:** cardiovascular diseases
**Cys A:** Cyclosporine A
**Cys/CySS:** cysteine/cystine.
**DAG:** Diacylglycerol

**DIC:** disseminated intravascular coagulation
**DMSO:** dimethyl sulphoxide
**DPI**: Diphenyliodonium
**DTS:** Dense tubular system
**DVT:** deep venous thrombosis
**EC(s):** endothelial cell(s)
**EDTA:** ethylenediaminetetra-acetic acid
**EGTA:** *ethylene glycol tetra-acetic acid*
**$E_h$:** redox state
**ELISA:** enzyme-linked immunosorbent assay
**EM:** electron microscope
**EDMPs:** endothelial cell-derived microparticles
**EPCR:** endothelial cell protein C receptors
**EPR-1:** effector protease recptor-1
**ESRD:** end stage renal disease
**ETP:** endogenous thrombin potential
**Fc:** crystalline fragment of antibody
**FcRγ:** Fc receptor gamma-chain
**FII:** prothrombin; coagulation factor II
**FIIa:** thrombin activated factor II
**FITC:** fluorescein isothiocyanate
**FS:** forward scatter
**FII:** prothrombin
**FII(a):** thrombin
**FIX(a):** coagulation factor IX (activated)
**FV(a):** coagulation factor V (activated)
**FVII(a):** coagulation factor VII (activated)
**FVIII(a):** coagulation factor VIII (activated)
**FX(a):** coagulation factor X (activated)
**FXI(a):** coagulation factor XI (activated)
**FXII(a):** coagulation factor XII (activated)
**FXIII(a):** coagulation factor XIII (activated)
**GSH/GSSG**: glutathione/oxidised glutathione
**Gla:** γ-carboxygluatmic acid
**GP(s):** Glycoprotein(s)
**GPIb-IX-V:** Glycoprotein Ib-IX-V receptor complex
**GPVI:** Glycoprotein VI
**GPCR(s):** G-protein coupled receptor(s)
**GPx:** glutathione peroxidase
**HDL:** high density lipoprotein
**HBS:** HEPES buffer saline
**HBS/$Ca^{2+}$:** HEPES buffer saline + 2mM $CaCl_2$
**$^{\cdot}OH$ :** hydroxyl radical
**HD:** haemodialysis (HD)
**HEPES:** 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
**$H_2O$:** water

**$H_2O_2$:** hydrogen peroxide
**HDL:** high density lipoprotein
**HMWK:** high molecular weight kininigen
**HSA:** human serum albumin
**IHD:** ischaemic heart disease
**Ig:** immunoglobin
**IP3:** 1,4,5-triphosphate
**ICAM-1:** intracellular adhesion molecule-1
**ISTH:** International society of thrombosis and haemostasis
**ITAM:** Immunereceptor tyrosine-based activation motif
**LDL:** low density lipoprotein
**L-NAME:** L(G)-nitro-L- arginine methyl ester
**LT:** lag time
**Mab:** monoclonal antibody
**MDA:** malondialdehyde
**MFI:** median fluorescence intensity
**MI:** myocardial infraction
**Milli.Q.d$H_2O$:** ultra-pure water
**MINAP:** Myocardial Infarction National Audi Project
**MP(s):** microparticle(s)
**MPTP**: mitochondrial permeability transition pore
**NAC:** N-Acetyl-Cysteine
**NAD(P)H:** Nicotinamide adenine dinucleotide (phosphate) oxidase
**NIBSC:** National Institute for Biological Standards and Control
**NS:** not statistically significant
**NO:** nitric oxide
**NOS:** nitric oxide synthase
**NTA:** nanoparticles tracking analysis
**$O^{\cdot}_2^-$ :** superoxide
**$ONOO^-$:** peroxynitrite
**OCS:** Open canalicular system
**PA:** phosphatidic acid
**PAF:** platelet-activating factor
**PAR-1/-4:** protease activated receptor -1/-4
**PBS:** phosphate buffered saline
**PC:** phosphatidylcholine
**PD**: peritoneal dialysis
**PDI:** Protein disulphide isomerase
**PDMPs:** platelets-derived microparticles
**PE:** phosphatidylethanolamine
**$PGI_2$:** prostaglandin $I_2$ sodium salt
**PI:** phosphatidylinositol
**PIP:** phosphatidylinositol-4-bisphosphate
**$PIP_2$:** phosphatidylinositol 4,5-bisphosphate
**$PIP_3$:** phosphatidyl-inositol-3,4,5 triphosphate
**PI3-K:** phosphatidylinositol 3 kinase

**PK:** pre-kallikrein
**PKC:** protein kinase C
**PL(s):** phospholipid(s)
**PLA2:** phospholipase A2
**PLCβ2:** phospholipase C beta2
**PLCγ2:** phospholipase C gamma2
**Plts:** Platelets
**PMA:** phorbol myristate acetate
**PP:** pooled plasma
**PP±CTI:** pooled plasma with/without CTI
**PPP:** platelet-poor plasma
**PRP:** platelet-rich plasma
**PS:** phosphotidylserine
**$PS^{+ve}$:** phosphotidylserine positive
**PSGL-1:** P-selectin glycoprotein ligand-1
**PT:** peak thrombin
**PUFA:** polyunsaturated fatty acid
**PVD:** peripheral vascular disease.
**$R^2$:** the square of the coefficient of correlation
**RE:** reticuloendothelial
**RGD:** arg-gly-asp
**ROCK-1:** Rho-associated kinase
**ROS:** reactive oxygen species
**RPE:** R-Phycoerythrin
**RPM:** revolutions per minute
**RT:** room temperature
**SD:** Standard deviation
**SEM:** standard error
**SH3:** Src Homology 3 domain
**SM:** sphingomyelin
**SMC:** smooth muscle cells
**SOD:** superoxide dismutase
**SS:** side scatter
**TBA:** thiobarbituric acid
**TBARS:** thiobarbituric acid reacting substances
**TF:** tissue factor
**TFPI:** tissue factor pathway inhibitor
**TG:** Thrombin generation
**TriG:** triglyceride
**TM:** thrombomodulin
**TNF:** tumor necrosis factor
**TRAP:** thrombin receptor activating peptide
**TRAF-4:** TNF-receptor associated receptor 4
**ttP:** time to peak
**$TxA_2$:** thromboxane $A_2$
**$TxB_2$:** thromboxane $B_2$

**WBC:** whole blood count
**vWF:** von Willebrand factor
**5-HT:** serotonin
**12-HETE:** 12-hydroxyeicosatetraenoic acid
**12-LOX:** 12-lipoxygenase
**ΔΨm**: mitochondrial membrane potential

# Chapter 1: Introduction

## 1.1 Haemostasis

Haemostasis is a normal physiological process that retains the integrity of the vascular system by stemming blood loss following vascular injury. It is a highly regulated mechanism that prevents bleeding while also limiting excessive clot formation. This involves platelets and coagulation factors leading to fibrin formation and also involves circulatory microparticles (MPs). Once wound healing has commenced, fibrinolysis works to dissolve any unwanted clots. In pathological conditions, there is impairment or disruption to the regulatory mechanism, which leads to excessive thrombin generation and therefore to uncontrolled thrombus formation, which in turn leads to thrombosis. Thrombosis can lead to arterial diseases, including myocardial infarction (MI) and stroke, and to venous thrombosis, which all have life-threatening consequences. Arterial thrombosis are platelet-rich whereas venous thrombosis are fibrin-rich (Ruggeri 2002).

Haemostasis comprises four overlapping, well-coordinated events that are initiated by vascular constriction at the site of vascular injury to limit blood flow, followed by primary haemostasis involving platelet adhesion to von Willebrand factor (vWF) and collagen exposed on the subendothelium. Platelets subsequently become activated, change their shape and degranulate, releasing a second wave of agonists, in particular adenosine diphosphate (ADP), thromboxane $A_2$ ($TxA_2$) and other metabolites, which then aggregate to each other via activated glycoprotein (GP) IIb/IIIa bridged by fibrinogen. At this stage, the plug formed by platelets is loose and is subsequently

stabilized by the coagulation system, which generates thrombin that cleaves fibrinogen to form a fibrin mesh. Once a clot is formed, and bleeding has stopped, the final process is fibrinolysis, which is the process that dissolves the clot after the damaged vessel is healed or repaired, mainly mediated by the action of plasmin. This thesis comprises a study of the contribution of the procoagulant activity of platelets and MPs in normal individuals, and of MPs in patients with cardiovascular risk (including those with coronary artery disease, CAD, and end stage renal disease - ESRD) in order to understand their contributions to normal haemostasis and pathological conditions.

## 1.2. Coagulation

### 1.2.1 Coagulation cascade

Coagulation involves a series of cumulative activations of serine protease enzymes in the plasma, which results in thrombin generation and, subsequently, fibrin clot formation. These coagulation factors normally circulate in their inactive form as 'zymogens'. The classical model of coagulation is known as the "waterfall model", as described in 1964 by two independent groups (Davie and Ratnoff 1964, Macfarlane 1964) (Fig 1.1). In the cascade model, coagulation is initiated by one of two independent pathways, intrinsic or extrinsic, which leads to the activation of factor X (FX) in the common pathway. The extrinsic pathway is triggered by tissue factor (TF), which binds to activated factor VII (FVIIa). A low level of FVIIa is present in the circulation (Eichinger*, et al* 1995, Morrissey*, et al* 1993). The TF-FVIIa complex activates FX in the common pathway. The intrinsic pathway is initiated by contact activation factors including FXII, high molecular weight kininogen (HMWK) and pre-

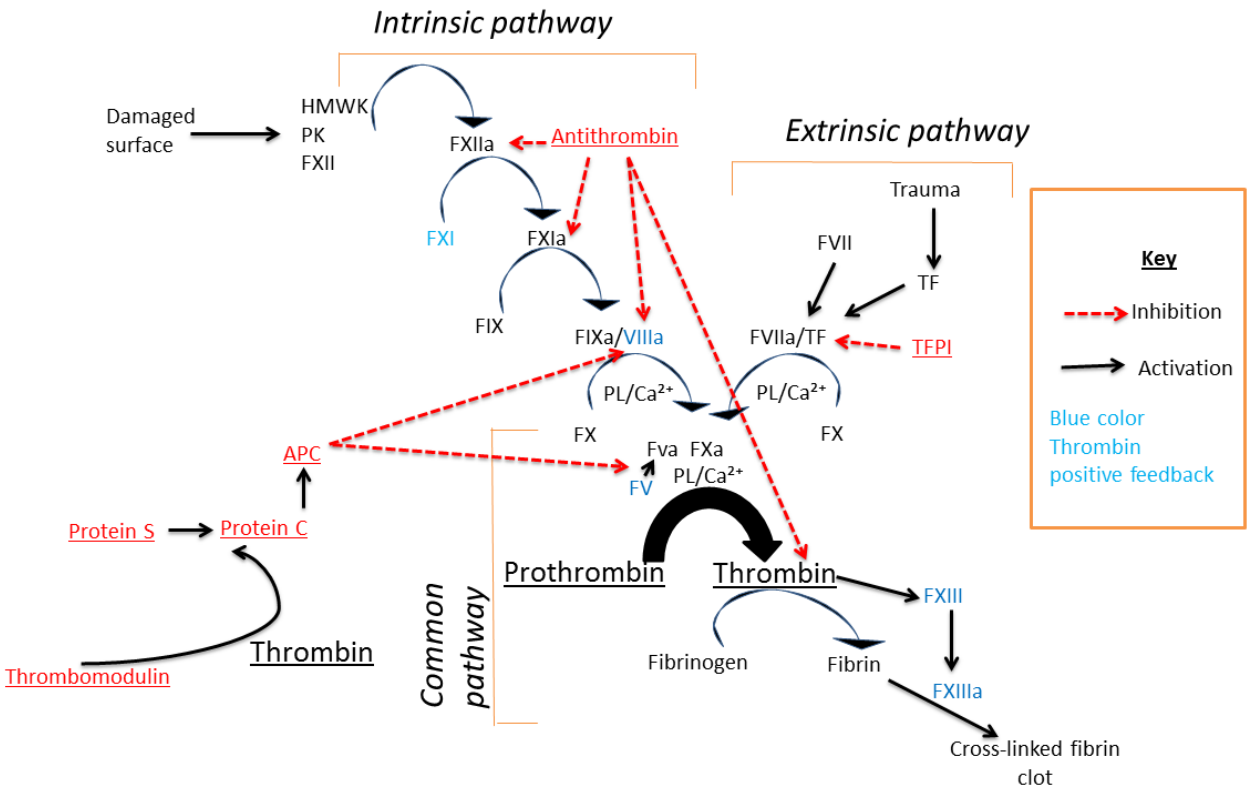



**Fig 1.1. The model of coagulation cascade.** The coagulation cascade is represented by two independent pathways, intrinsic and extrinsic, which emerge in the activation of FX in the common pathway. The positive feedback loop of thrombin is shown in blue. The inhibitory pathways, including antithrombin, tissue factor pathway inhibitor (TFPI), protein C and its cofactor protein S, activated protein (APC) and thrombomodulin, are illustrated in the cascade in red. High molecular weight kininogen (HMWK) and pre-kallikrein (PK)Adapted from Monroe, *et al* (2002).

kallikrein (PK), resulting in activation of FXI. FXIa activates FIX and its cofactor (FVIII), leading to activation of FX to at least 50-fold higher levels than by the TF-VIIa complex (Lawson and Mann 1991). The binding of FXIa to FVIIIa forms the tenase complex, and the binding of cofactor FVa to FXa forms the prothrombinase complex, which both take place on a negatively charged membrane surface, and this enhances the conversion of prothrombin (FII) to thrombin (FIIa). FXa converts prothrombin to thrombin, which acts on fibrinogen to produce a fibrin, which polymerises to form a fibrin clot. FXIII is activated by the feedback loop of thrombin to produce a stable clot by cross linking the fibrin polymers. The coagulation mechanism (Fig 1.1) is crucially dependent on calcium ($Ca^{2+}$) and anionic negatively-charged phospholipids (PLs) which provide "the procoagulant surface", mainly phosphotidylserine (PS) (Lawson and Mann 1991), for the binding and assembly of the coagulation complexes and coagulation factors including FXa (Jesty and Nemerson 1976, Lawson and Mann 1991) in order to enhance their procoagulant activity. It had been thought that the negatively charged PLs provides a passive surface that only exists for the cumulative reaction of the coagulation proteins to take place upon, but an understanding of the involvement of cells in coagulation has changed the idea of it from a passive surface to a regulatory surface of coagulation process (discussed in section 1.1.3.1).

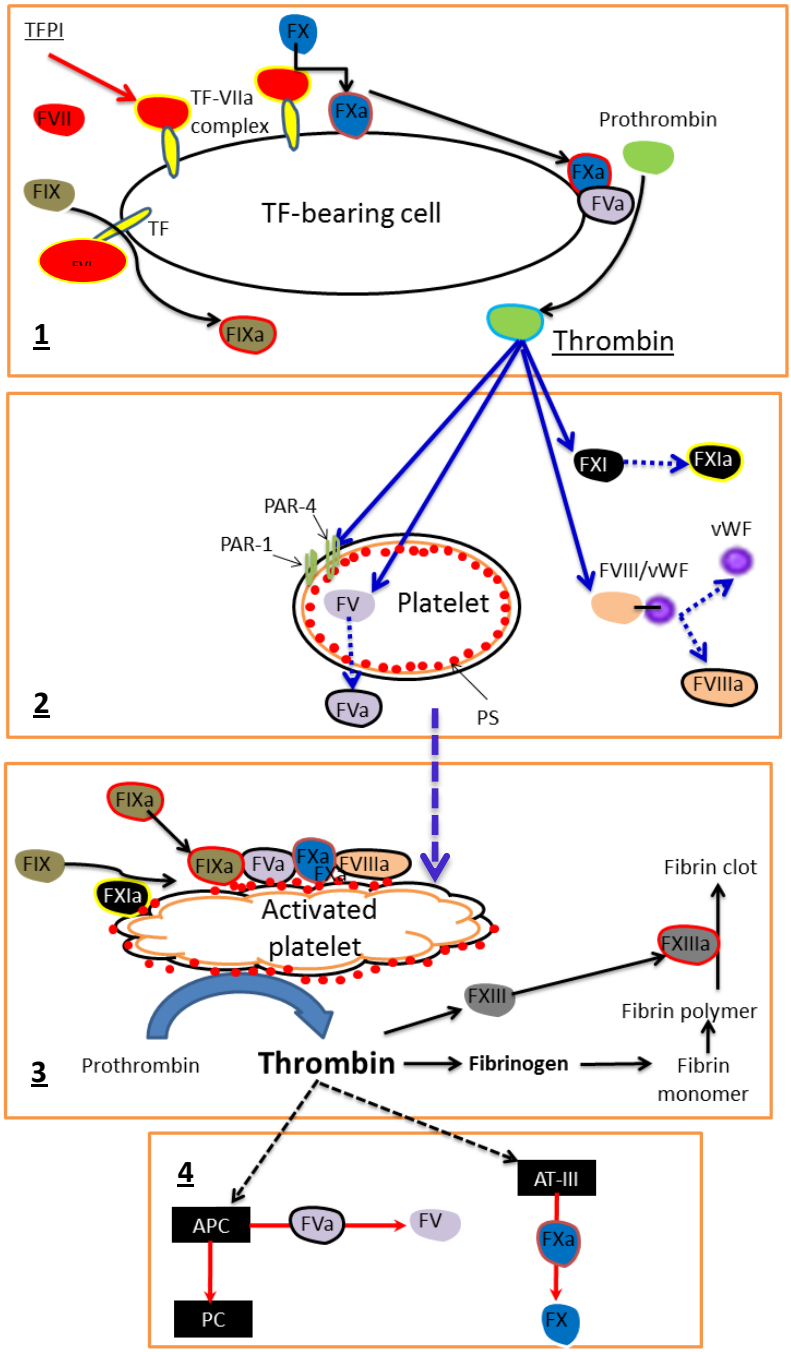
For many years, the cascade model was thought to be consistent with the physiological processes observed *in vivo*. This model is examined by two conventional

laboratory tests: the prothrombin time and the activated partial thromboplastin time (aPTT), which measure the extrinsic and intrinsic pathways, respectively. However, this model focuses only on the chemical interactions among the coagulation factors while ignoring the important role of cells, and specifically platelets. In addition, it is inconsistent with certain clinical observations; for example, the lack of contact factors, including FXII, HMWK or PK, is not associated with a bleeding diathesis. In contrast, the question should actually be: "Why do haemophilic patients bleed?" These observations have prompted investigators to wonder whether the model can be initiated by either the extrinsic or the intrinsic pathways, and whether the extrinsic pathway can compensate for the lack of the intrinsic pathway factors. In addition, it has been established that FIX can be activated by the FVIIa/TF complex (Lawson and Mann 1991) and/or by thrombin on the procoagulant surface provided by activated platelets (Gailani and Broze 1991). All of these observations, in combination with other reports (Bom and Bertina 1990, Mertens and Bertina 1984), indicate that the interaction of the coagulation factors is not separate from platelets. This prompted the revision of the cascade model, as a more complicated overlapping process, rather than a simple cascade, and comprised of the interactions among coagulation factors, platelets and other cells.

### 1.2.2. Cell-based model of haemostasis

The cell-based model of haemostasis was a concept proposed a decade ago describing the interaction of coagulation factors with blood cells, including platelets, for providing the procoagulant PLs, in addition monocytes or fibroblast as a source of TF (Kjalke*, et al* 1998), as reviewed by Hoffman and Monroe (2001), Roberts*, et al* (2006) and others

(Smith 2009) (Fig 1.2). The process involves four overlapping phases: initiation, amplification or "priming", propagation and shut-down. The overlap reflects the fact that each stage generates different levels of thrombin, because thrombin is the central enzyme of coagulation, featuring positive and negative feedback loops on platelets, coagulation factors and other soluble factors. At the site of vascular injury, exposed TF cells within the intima (e.g. fibroblasts) initiate the coagulation process, leading to FX and FIX activation by the TF/VIIIa complex. FXa activates its cofactor FV (prothrombinase complex) (Monkovic and Tracy 1990), which generates thrombin from prothrombin (Monroe*, et al* 1996, Nesheim*, et al* 1979). At this stage FXa is labile, and if it leaves TF-bearing cell environment it can be easily and rapidly inactivated by tissue factor pathway inhibitor (TFPI) or antithrombin (AT). The low quantity of thrombin generated does not has the ability to cleave fibrinogen to fibrin, but it does prime the process (positive feedback loop) by activating platelets, FXI, FVIII, FV and FIX. For instance, thrombin cleaves the vWF/FVIII complex to free vWF and FVIIIa leading to an amplification of its own generation (Butenas*, et al* 1997, Gailani and Broze 1991, Hamer*, et al* 1987, Hultin 1985, Kumar*, et al* 1994, Oliver*, et al* 1999, Pieters*, et al* 1989, Suzuki*, et al* 1982). Then, the reaction moves onto the surface of activated platelets. On activated platelets the process is amplified not only due to the activation of coagulation factors but also due to the increase in the procoagulant surface provided by platelets that adhere to the site of vascular injury (Diaz-Ricart*, et al* 2000). The binding of tenase and prothrombinase complexes on the activated platelet via the negatively charged PLs, lead to a burst in the generation of thrombin in the propagation phase. The amount of thrombin generated in this phase is sufficient




**(Panel 1 "Initiation phase")** TF initiates the coagulation process to produce a small amount of thrombin through the action of FXa/FVa, which is activated by TF/FVIIa/FX. TFPI inhibits this activation at early stage. The small amount of thrombin generated is able to activate platelets via PAR-1 and -4 receptors and coagulation factors (**panel 2 "amplification phase").** This leads to a massive production of thrombin (**panel 3 "Propagation phase").** The "shut-down phase" **(panel 4)** ensures fibrin clot formation stops after bleeding has stopped.

**Fig 1.2: Cell-based model of haemostasis.** This mode involves four overlapping phases, initiation, amplification, propagation and shut-down. Activated protein C (APC), protein C, antithrombin (AT), coagulation factor (F), phosphotidylserine (PS), tissue factor (TF), tissue factor pathway inhibitor (TFPI), von Willebrand factor (vWF), and protease activated receptors 1 and 4 (PAR-1 and -4). Adapted from Monroe, *et al* (2002).

to cleave fibrinogen, leading to fibrin polymerization. FXIIIa cross-links fibrin to stabilize the clot (Mosesson 1992). The final phase is to ensure the "shut down" of the process by the inactivation of coagulation factors; this involves thrombomodulin (TM), AT and activated protein C (APC) to inactivate the clotting process (discussed in section 1.1.3.2).

## 1.3. Regulation and control mechanisms of coagulation

### 1.3.1 Membrane PLs and receptors

It is well established that the procoagulant, negatively-charged PLs surface, mainly PS, plays a crucial role in the binding of coagulation factors (Lawson and Mann 1991, Sims*, et al* 1989, Thiagarajan and Tait 1990), but other reports have suggested the presence of protein and other binding receptors for coagulation factors as well, as reviewed by (Heemskerk*, et al* 2002) and the observation on those, non-lipid binding sites (non-negatively charged) have been postulated from the observation that annexin-V does not completely inhibit FXa generation (London*, et al* 1996), with evidence of the involvement of FV as a receptor for FX. In addition, effector protease receptor-1 (EPR-1) has been proposed as a potential receptor for FX (Larson*, et al* 1998). However, the main focus is on the binding of coagulant and anticoagulant factors to the negatively-charged PLs, which regulate the coagulation process by enhancing the assembly and the catalytic activity of the tenase and prothrombinase complexes. The importance of exposure of the negatively-charged PLs, mainly PS, is demonstrated by haemorrhage episodes in humans (Sims*, et al* 1989) and animals (Brooks*, et al* 2002), with Scott syndrome, who lack PS expression and MP formation (Weiss*, et al* 1979). Coagulation factors, mainly the vitamin K-dependent proteases (II, FVII, FIX and FX), bind to the

negatively-charged PLs via the γ-carboxyglutamic acid (Gla) domain at the N-terminal, which is a positively charged site in the presence of $Ca^{2+}$ (Davie*, et al* 1991, Mann*, et al* 1990, Thompson 1986). Coagulation factors that lack the Gla domain, known as non-Gla-domain-containing factors, bind to the platelet's surface via, other non-PS, membrane sites, or via membrane GPs. For example, FIX binds to platelets via the dimeric structure of FXI; as one chain of dimeric FXIa binds platelets, while the other chain binds to FIX (Gailani*, et al* 2001). In addition, those factors that lack the Gla-domain residue, including thrombin (De Marco*, et al* 1994), FIX (Baglia*, et al* 2002), FXII (Bradford*, et al* 2000), and HMWK (Joseph*, et al* 1999) have been shown to bind to the GPIbα component of the GPIb-V-IX complex. Other platelet GPs, including GPIIb-IIIa, also play a key role in the binding of coagulation factors such as prothrombin (Byzova and Plow 1997), FV and FXI (Monkovic and Tracy 1990). In addition, the negatively charged PL is a binding site for the anticoagulant factors. The binding of the anticoagulant factors to the negatively-charged PLs regulates the inactivation of FVa by APC (Ravanat*, et al* 1992). According to Sumner, *et al* (1996), the negatively charged PLs is not only required for coagulation but also for other proteins and receptor to perform their function adequately.

### 1.3.2 Control mechanisms

There are several control mechanisms of coagulation which exist to ensure the localization of the fibrin clot at the site of vascular injury, the inhibition of thrombin formation and fibrin clot formation after bleeding has stopped, and the removal of the thrombus once the wound is healed. These anticoagulant systems are TFPI, TM, APC and AT. The initial step is inactivation by TFPI, which participates in the inhibition of

coagulation in the priming phase. TFPI is the main inhibitor of TF and mainly acts on TF-FVIIa-FX to inactivate coagulation (Fig 1.2) (Mann 1999); this is achieved through the three kuntiz inhibitory domains of TFPI (Carson and Konigsberg 1981). TFPI is found on the endothelium, in the plasma and, to a lesser extent, in platelets (Bajaj*, et al* 2001) and monocytes (Ott*, et al* 2001) and can be synthesised by endothelium and megakaryocytes. TM is found as an integral membrane protein on the surface of vascular endothelial cells (ECs) and peripheral blood cells. It is a cofactor for thrombin. Thrombin binds to TM on ECs around the site of damage. This switches thrombin from a procoagulant entity to an anticoagulant by changing the substrate specificity of thrombin (Esmon 2000a). Then the thrombin-TM complex binds to protein C with high affinity, leading to the activation of protein C. Through this process, APC acts to inactivate and degrade FVa and FVIIIa and to separate them from the procoagulant surface. The activity of APC is enhanced by the presence of protein S (its cofactor), because protein S enhances the binding of APC to the platelet on ECs surface. In addition, APC can also bind to endothelial cell protein C receptor (EPCR), which switches the substrate specificity of APC giving a similar effect to that of TM when it binds with thrombin (Esmon 2000a, Esmon 2000b). Freeing FXa from its cofactor makes it vulnerable to inactivation by AT. AT is a serpine inhibitor that inhibits serine protease factors, mainly thrombin and other coagulation factors, including FXa, FIXa FXIa and FXIIa. AT circulates in the blood in the α-isoform and is found as the β-isoform in the vessel wall (Swedenborg 1998). AT alone inactivates thrombin and FXa slowly, this effect is enhanced 10,000-fold in the presence of heparin, the therapeutic anticoagulant (Rau*, et al* 2007).

### 1.3.3 Thrombin

Thrombin is a trypsin-like serine protease, which is generated through the cleavage of its precursor II by the prothrombinase complex, leading first to the intermediate form of thrombin with a 49-residue A chain and a 259-residue B chain and then to the α-thrombin. The A and B chains of thrombin are covalently linked by a disulphide bridge. The B chain has similar trypsin and catalytic active site domains, which recognizes its own A chain (as a substrate) and cleaves it at residue 13. This occurs in the intermediate form of thrombin to generate α-thrombin consisting of a 36-residue A chain and a 259-residue B chain. Thrombin is the most potent soluble agonist of platelets and coagulation factors. It causes full platelet response, including a transient, elevation of intracellular $Ca^{2+}$ ($[Ca^{2+}]i$), protein phosphorylation, $TxA_2$ synthesis, shape change and aggregation. In addition, it has anticoagulant properties (as discussed previously). Thrombin not only plays a major role in orchestrating haemostasis (Rickles*, et al* 2003), but also thrombosis, inflammation and angiogenesis.

### 1.3.4 Tissue factor (TF)

TF is the principle physiological initiator of coagulation. It is a 47-kD type 1 transmembrane protein mainly expressed in non-vascular cells, including adventitia, brain, kidney, lung and placenta cells (Fleck*, et al* 1990, Wilcox*, et al* 1989). It has three domains: an extracellular domain, a transmembrane domain and an intracellular domain. Normally, TF is sequestered from the circulation, in the extravascular cells surrounding the blood vessels. Once there is an injury, exposed TF binds to FVIIa in the circulation to initiate coagulation. In the circulation, leukocytes (mainly monocytes) have also been confirmed to express TF (including mRNA, TF antigen and TF functional

protein) (Eilertsen and Osterud 2004, Rauch and Nemerson 2000) upon various stimuli, mainly LPS (Gregory*, et al* 1989), but in an encrypted form (inactive, dormant). The encryption of TF is suggested to be a control mechanism of the procoagulant activity *in vivo*. Indeed, increased levels of TF have been confirmed to increase the procoagulant activity of the plasma in pathological conditions such as acute coronary syndrome (ACS), disseminated intravascular coagulation (DIC) and thrombosis. In addition, platelets enhance TF expression in monocytes (Scholz*, et al* 2002). Moreover, it was recently suggested that platelets release TF upon collagen stimulation (Panes*, et al* 2007), which can be transferred to monocytes (Scholz*, et al* 2002) and from monocytes to platelets (Rauch*, et al* 2000). It has also become increasingly clear that there is a pool of TF in the circulation (Giesen*, et al* 1999). However, it is unclear whether this "blood-borne TF" is a soluble protein, or is in form of MPs. The precise mechanism by which this blood-borne TF takes part in the haemostatic response remains to be elucidated. TF-bearing MPs have been shown to be involved in the development of thrombi. Blood-borne TF is thought to be found in the circulation in an encrypted form. The circulating encrypted form of TF is suggested to be playing a role in clot propagation, which becomes active in the growing thrombus, but not initiation of coagulation (Giesen*, et al* 1999). Recent data has demonstrated that the de-encryption of TF involves a step for oxidation of the disulphide bonds in TF by protein disulphide isomerase (PDI) (Ahamed*, et al* 2006, Chen*, et al* 2006, Cho*, et al* 2008).

## 1.4 Platelets

Platelets play a major role in haemostasis, thrombosis, inflammation and wound healing, and are also implicated in the development and progression of pathological

disease status (Gawaz*, et al* 2005, Ruggeri 2002, Weyrich*, et al* 2003). Platelets are small anucleated cells that are released into the circulation from the cytoplasm of megakaryocytes in the bone marrow through a highly regulated process (Thon and Italiano 2010). The lifespan of platelets in the circulation is ~10 days (at a count of 150-400x$10^9$/L), after which they are removed by macrophages in the spleen and liver. In their resting state platelets are discoid in shape, range from 2-4µm in size, are ~0.5µm thick, and never undergo firm adhesion with the vascular wall. Platelets are featureless from the outside with membrane invaginations that are connected to the internal membrane system, known as the open canalicular system (OCS), which provide platelets with more surface area to enhance the activation process. The OCS is close to the platelet storage granules; upon activation, platelet granules fuse with OCS to be connected to the membrane surface. Platelets also contain a full complement of organelles, including mitochondria, a dense tubular system, endoplasmic reticulum, lysosomes, peroxisomes and glycogen (White 2007).

In general, platelets are composed of three main components: a cytoplasmic membrane, a cytoskeleton (microtubules and microfilaments) and specialized granules (Michelson 2007). The cell membrane of all blood cells, including platelets, consists of three types of lipid: PLs, glycolipids and cholesterol. Of these three types, the PLs play the main role in coagulation. In the resting state, the distribution of the PLs across the cell membrane lipid bilayer is asymmetrical (Bretscher 1972). The outer layer is rich in neutral choline-PLs, including phosphatidylcholine (PC) and sphingomyelin (SM), and the inner layer at the cytoplasmic membrane is rich in negatively-charged anionic-PLs such as PS and phosphatidylethanolamine (PE), with some less common types of PL

such as phosphatidic acid (PA), phosphatidylinositol (PI), phosphatidylinositol-4-monophosphate (PIP) and phosphatidylinositol 4, 5-bisphosphate ($PIP_2$). The PLs content of cell membranes in platelets has been thoroughly investigated, and it has been found that PC and PE each account for approximately 35%, SM ~20%, PS <13%, and PI <13% of the total membrane PLs (Boesze-Battaglia and Schimmel 1997, Owen*, et al* 1981) (Fig 1.3).

The platelet's discoid shape is supported by the cytoskeleton located (actin filament) beneath the cytoplasmic membrane, which has a role in platelet morphological changes during activation. Actin represents a fifth of total protein in platelets (Hartwig 1992). Upon platelet activation, the shape changes, because actin, which is cross-linked by filamin and linked it to the cytoplasmic tail of GPIbα via the actin-binding domain, assemble onto the fast growing ends of actin filaments.

Platelets contain three types of granule: the α-granules (50-80 per platelet) are the most abundant (Harrison*, et al* 1989) and contain high molecular weight compounds including soluble proteins, such as vWF, FV, fibrinogen, fibronectin, platelet-derived growth factor, CD40 ligand, cytokines, plasminogen activator inhibitor-1, β-thromboglobulin and thrombospondin-1, and membrane-bound proteins, such as P-selectin, GPIIb/IIIa and PECAM; the dense granules, of which there are 3-9 per platelet, and which contain low molecular weight compounds such as $Ca^{2+}$, serotonin (5-HT), histamine, polyphosphate, adenosine triphosphate (ATP) and ADP (Holmsen and Weiss 1979, White 1969); and the lysosomal granules, which store hydrolase enzymes, cathespins D and E, and CD63 (Ciferri*, et al* 2000).

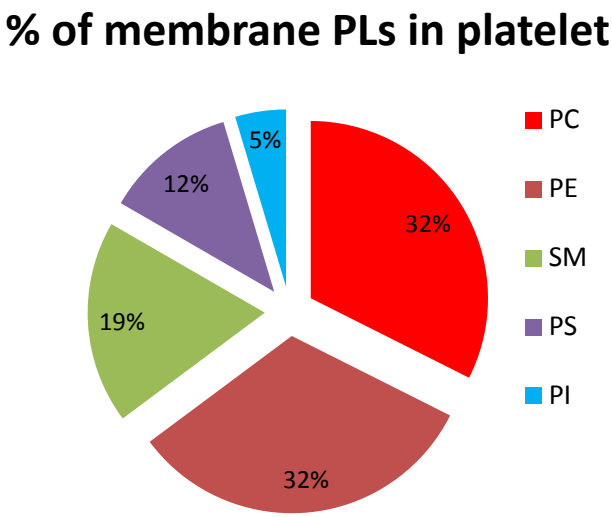



**Fig 1.3. Percentage of membrane phospholipids (PLs) in platelets.** Phosphotidylcholine (PC), Phosphatidylethanolamine (PE), Sphingomylein (SM) and Phosphotidylserine (PS) Adapted from Boesze-Battaglia and Schimmel (1997).

Although de-novo protein synthesis in platelets was identified more than 40 years ago (Warshaw*, et al* 1967), it has recently been discovered that platelets contain unprocessed RNA and can synthesise protein (Denis*, et al* 2005, Kieffer*, et al* 1987, Lindemann*, et al* 2001, Panes*, et al* 2007). Platelets lack nuclei, so cannot transcribe DNA to mRNA; however, some evidence has emerged to show that platelets do contain some mRNA that can be translated into new proteins in a highly-regulated manner (Weyrich*, et al* 1998), but the majority of platelet components are acquired from and synthesized by megakaryocytes, and some granule proteins, such as fibrinogen and albumin, are taken up by platelets in the circulation (Handagama*, et al* 1993, Harrison*, et al* 1989).

### 1.4.1 Platelet receptors

Platelets have different receptors such as the collagen receptor (GPVI), integrin receptors ($\alpha_{IIb}\beta_3$, $\alpha_2\beta_1$) and leucine-rich repeated receptors (GPIbα). Platelets are also rich in G-protein coupled receptors (GPCRs) which are used mainly for soluble agonists such as thrombin (protease-activated receptors 1 and 4; PAR-1 and PAR-4), ADP ($P2Y_1$ and $P2Y_{12}$ receptors), $TxA_2$ (TPα and TPβ receptors) for reviews see (Rivera, *et al* 2009, Stegner and Nieswandt 2010). In the next paragraphs these receptors will be explained and their main signalling pathways, followed by a description of the platelet responses induced by the binding of platelet receptors to their ligands (Fig 1.4).

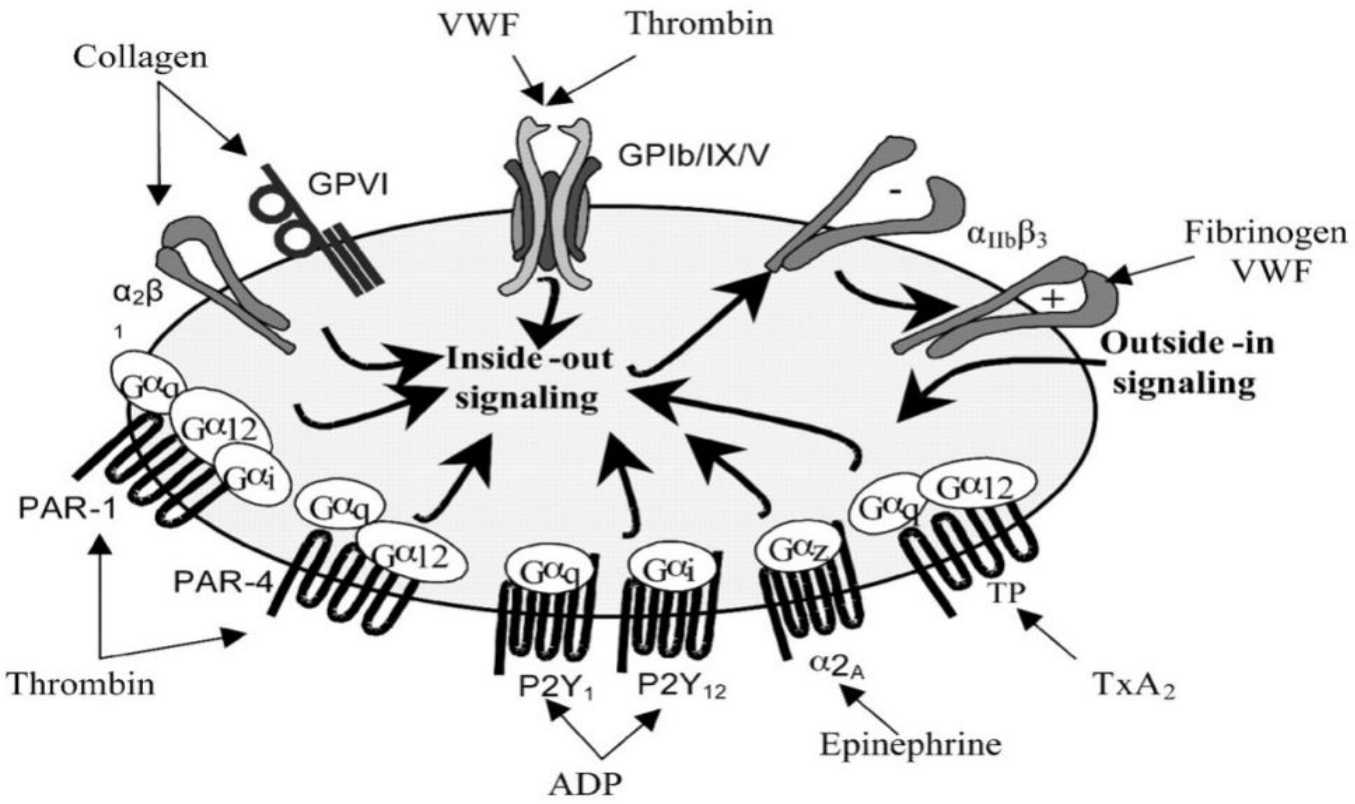



**Fig 1.4: Major platelet receptors and their agonists.** Adapted from Rivera, et al (2009).

### 1.4.1.1 Integrin receptors

Integrins are heterodimers comprised of α- and β-subunits; the β-subunits in platelets are of the β1 or β3 types subunits. Platelets have three β1 integrins: α2bβ1 (<1000 per platelet, a collagen receptor), α5β1 (<1000 per platelet, a fibronectin receptor) and α6β1 (<1000 per platelet, a laminin receptor); and two β3 integrins: $\alpha IIb\beta_3$, (GPIIb-IIIa; ~80,000 copies per platelet with additional copies in the α-granules and OCS) (Wagner*, et al* 1996) and αVβ3 (~1000 copies per platelet, a vitronectin receptor). β1 and β3 subunits in resting platelets normally have low affinity for their receptor; once the platelets become activated they switch to a high affinity state that allow them to bind their ligands (Emsley*, et al* 2000). Platelets also express GPIbα (25,000 per platelet) a member of the leucine-rich repeated receptor which binds vWF (Coller*, et al* 1983) (discussed in section 1.4.2.1).

### 1.4.1.2 Glycoprotein VI (GPVI) "the Collagen receptor"

Collagen is a potent platelet agonist that induces a full range of platelet responses, because it induces sustained elevation in $[Ca^{2+}]i$ in platelets (Heemskerk*, et al* 1999, Siljander*, et al* 2001). The GPVI receptor (~1,000 per platelet) is a 63-KDa-type transmembrane GP expressed uniquely in megakaryocytes and platelets (Fig 1.5). It is a member of the immune receptor family, and is the major signalling receptor for collagen on platelets (Clemetson*, et al* 1999, Watson*, et al* 2001). GPVI is composed of 319 amino acid (aa) residues with two extracellular domains, a transmembrane domain and a cytoplasmic domain. The two extracellular domains are mucin-like domains; both consisting of a total of 249 aa, and are attached to a glycosylated pericellaular stem by two immunoglobin (Ig) domains. The transmembrane region

comprises 19 aa; it binds Src kinases (Fyn and Lyn). It is non-covalently coupled with disulphide-linked Fc receptor-γ (FcR-γ) dimer by a salt bridge. The co-assembly of the two proteins is necessary for stabilizing the receptor. Each FcR-γ contains an immune receptor tyrosine based activation motif (ITAM), which works as a signal transduction unit (Gibbins*, et al* 1997, Poole*, et al* 1997). The cytoplasmic domain is made of 51 aa, and possesses binding sites for calmodulin and Src homology (SH3)-binding proteins of Src family tyrosine kinases (Andrews*, et al* 2002, Suzuki-Inoue*, et al* 2002).

Cross-linking of the GPVI receptor by collagen or cross-linked, collagen related peptide (CRP-XL) is essential to induce Src-kinase dependent intracellular signalling pathways, by phosphorylation of ITAM sequences in FcR-γ and the activation of the Src kinases Fyn and Lyn, which are constitutively linked to the FcR-γ (Ezumi*, et al* 1998). In addition, it has been reported that GPVI signals by an ITAM-independent pathway (Hughan*, et al* 2007, Locke*, et al* 2003, Watson*, et al* 2001, Watson*, et al* 2005). The activation of GPVI is Src family kinase dependent (Ezumi*, et al* 1998, Suzuki-Inoue*, et al* 2002) and leads to the phosphorylation of the SyK kinase, which then activates a complex of downstream cascade that recruits and co-assembles a series of adaptor and effectors proteins, forming a scaffold region known as a signalosome. The main core of this scaffold is composed of adaptors, including LAT, SLP-76 and Gads, and associate with Vav 1/3 and Btk/Tec kinases, which support the activation of phospholipase Cγ2 (PLCγ2), phosphatidylinositol 3-kinase (PI3K) and small G proteins. The adaptor proteins regulate the effectors proteins that lead to the activation of PLCγ2. PLCγ2 catalyses the hydrolysis of $PIP_2$ liberating (i) inositol 1,4,5- triphosphate (IP3), which is required for $Ca^{2+}$ mobilization from the intracellular stores and (ii) 1,2-

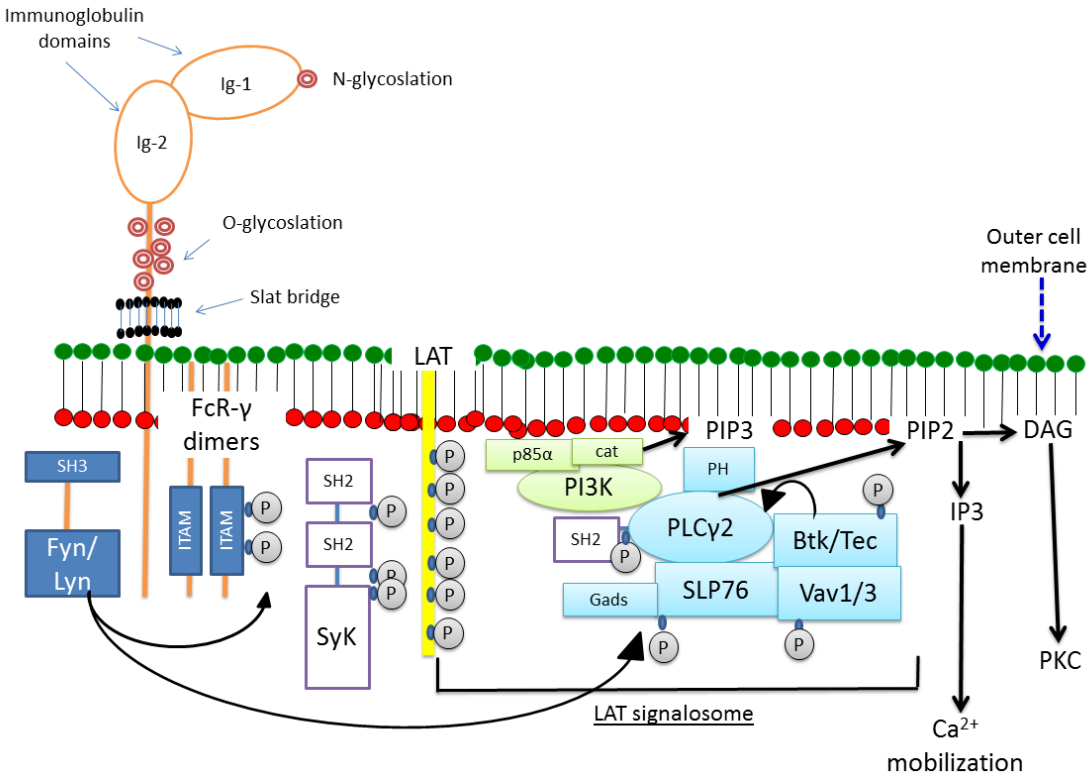



**Fig 1.5. GPVI signalling pathway in platelets.** Adapted from Watson *et al,* (2005).

diacylglycerol (DAG). The PI3K pathway is important for GPVI signalling to attain maximal activation of PLCγ2. GPVI activation leads to a sustained elevation in the $[Ca^{2+}]i$, protein kinase C (PKC) activation, reorganization of the cytoskeleton, mediated by the activation of cytoskeleton proteins, $TxA_2$ synthesis, degranulation, PS exposure, MP formation and activation of integrins including GPIIb/IIIa to facilitate adhesion and aggregation.

The collagen-mimetic peptide, CRP-XL evokes the same signalling pathway as collagen by engaging the GPVI receptor in a similar fashion and is a selective agonist for GPVI (Horii*, et al* 2006). It is composed of triple helical peptides containing a 10-fold glycine-proline-hydroxyproline repeat sequence (Gibbins*, et al* 1997, Kehrel*, et al* 1998, Morton*, et al* 1995).

#### ***1.4.1.2.1. Importance of GPVI***

GPVI is necessary for proper haemostasis and has been implicated in the development of thrombotic events. It is also implicated in the increased risk of MI in patients with sequence dimorphism (Croft*, et al* 2001), although this has not been replicated in other studies. Patients lacking GPVI, due either to gene deletion or who develop auto-antibodies (Abs) for GPVI, that lead to either shed or internalization of the receptor, have been shown to have moderate bleeding diathesis and prolonged bleeding time and their platelets respond weakly to collagen (Moroi*, et al* 1989, Sugiyama*, et al* 1987, Takahashi and Moroi 2001). In addition, deficiency in GPVI in mice has been reported to be associated with bleeding diathesis (Arthur*, et al* 2007). Platelets deficient in GPVI show marked reduction and defective adhesion and aggregation to fibrillar and soluble collagen in mice and humans (Kato*, et al* 2003, Nieswandt*, et al*

2001a), adhesion under flow (Moroi*, et al* 1989, Moroi*, et al* 1996) and thrombus growth (Kato*, et al* 2003). The monoclonal-Ab JAQ1 targeting mouse GPVI has been shown to inhibit adhesion of mouse platelet to collagen under low and high shear flow conditions (Nieswandt*, et al* 2001b). Furthermore, the role of the GPVI receptor in arterial thrombosis is illustrated in the injured carotid artery in mouse models. Using intravital fluorescence microscopy of the mouse carotid artery, Massberg *et al* (2003) found no interaction between platelet and vessel wall after endothelial denudation in GPVI-deficient mice. The platelet response to collagen in mice lacking GPVI or FcR-γ is also impaired in a ferric chloride induced thrombosis model (Dubois*, et al* 2006). In addition, high levels of GPVI expression in platelets have been reported in ACS patients (Bigalke*, et al* 2006). Inhibition of GPVI also reduced platelet adhesion to the atherosclerotic lesion (Penz*, et al* 2005). All these findings illustrate the essential role of GPVI in platelet activation and, therefore, in haemostasis and thrombosis. The expression of GPVI is increased upon platelet activation compared to resting platelets (Cabeza*, et al* 2004, Nieswandt and Watson 2003). Individual responses to CRP-XL varies among the population, and this can mainly be ascribed to genetic differences (Croft*, et al* 2001). The GPVI gene has 2 common alleles, with differences in the glycosylated stem and the cytoplasmic domain. These alleles account for much of the differences between individuals in platelet response measured by aggregation, fibrinogen binding, PS exposure, P-selectin expression and thrombin generation (Joutsi-Korhonen*, et al* 2003), and this variation in GPVI accounts for ~30-35% in response to CRP-XL response (Jones*, et al* 2007, Joutsi-Korhonen*, et al* 2003), while additional 10-20% of variability can be attributed to other genetic polymorphism

(Jones, *et al* 2009). Other factors including antioxidant capacity and reactive oxygen species (ROS) maybe have additional regulation of GPVI.

#### ***1.4.1.2.2. Regulation of GPVI***

Accumulating evidence indicates that the redox state ($E_h$), antioxidant capacity and ROS (discussed in section 1.7 and chapter 7) regulate cell function and their signalling pathways (Jones*, et al* 2004, Noble*, et al* 2003), including in platelets (Murphy*, et al* 2010). Disruption of the balance of redox state contributes and has an impact on platelet function. While the underlying mechanism is not fully understood, it is thought to be mediated by the thiol and disulphide bonds. Thiol groups in platelets can be found as free thiol, disulphide or mixed disulphide when conjugated with glutathione or cysteine (Essex and Li 2003). In the plasma (the surrounding environment) there are various redox couples, including the reduced glutathione/oxidised glutathione (GSH/GSSG) and cysteine/cystine (Cys/CySS), which have been shown to be involved in regulating early events of atherosclerosis (Go and Jones 2005) and platelet reactivity in response to collagen (Murphy*, et al* 2010), with data emerging of a coupling of the redox state to platelet surface integrins, including α2β1 (Murphy*, et al* 2010), and GPs, including GPVI and GPIb-IX-V (Berndt*, et al* 2010). Indeed, extracellular Cys/CySS has been shown to stimulate intracellular $H_2O_2$ production in endothelial cells (Go and Jones 2005).

The main source of ROS in non-phagocytic and phagocytic cells is the NAD(P)H oxidase pathway complex, which has various subunits, one of which is the $p47^{phox}$ (discussed below). The TNF-receptor associated receptor 4 (TRAF4) is linked to the redox state and has been shown to be linked to the $p47^{phox}$. In addition, TRAF4 is linked to GPVI

receptor (Berndt*, et al* 2010). The presence of antioxidant enzymes not only prevents deleterious effects of ROS, but also alters the redox state in platelets. The relationship between redox state, antioxidant and platelet function systems is not straightforward and data contradicting. An antioxidant such as quercetin was found to inhibit GPVI phosphorylation and collagen-induced platelet aggregation (Hubbard*, et al* 2003, Hubbard*, et al* 2006, Hubbard*, et al* 2004). This section has shown briefly that the redox state and antioxidants regulate platelet functions for recent review see Freedman (2008) and the redox regulation of platelet receptors was also reviewed by (Arthur*, et al* 2008), but the exact mechanisms and relationship between redox state, antioxidant and ROS in platelets need further elucidation.

### 1.4.1.3. G-protein coupled receptors (GPCRs)

GPCRs are seven transmembrane receptors, and in platelet comprise receptors for thrombin, ADP and $TxA_2$, and other soluble agonists. GPCRs activate associated heterotrimetric guanine nucleotide-binding proteins leading to the activation of PLCβ, which hydrolyses PIP2 to generate IP3 and DAG. IP3 leads to transient spiking elevation of $[Ca^{2+}]i$. Also it activates PKC and PI3K. In addition, phosphatidyl-inositol-3,4,5 triphosphate (PIP3) is generated by the action of PI3K on PIP2. All of these mechanisms lead to cytoskeleton organization, shape change and inhibition of cyclic adenosine monophosphate (cAMP) synthesis, a major inhibitor of platelet activation, for review see (Stegner and Nieswandt 2010).

#### 1.4.1.3.1. Thrombin receptors

Thrombin activates platelets by acting on PAR-1 and PAR-4 in concert. PAR-1 (~2,500 copies per platelets) mediates activation at low thrombin concentrations and PAR-4

mediates activation at high thrombin concentrations (Hung*, et al* 1992, Kahn*, et al* 1998). In addition, thrombin has the ability to bind to other receptors, including GPIbα.

#### ***1.4.1.3.2. ADP receptors***

ADP is a secondary agonist released from activated platelets which affects platelets in an autocrine manner. It is released from the dense granules and potentiates aggregation and platelet responses induced by other agonists (Trumel*, et al* 1999). ADP is a weak agonist causing full platelet aggregation and spiking elevation of $[Ca^{2+}]i$ (Arthur*, et al* 2006, Hollopeter*, et al* 2001, Lenain*, et al* 2003, Nurden and Nurden 2003), but has no effect on PS exposure and causes little degranulation (Janes*, et al* 1994). ADP primarily activates integrins. The main receptors of ADP on platelets are $P2Y_1$ (~250 per platelet) and $P2Y_{12}$ (~500 per platelet). $P2Y_1$ acts via $G\alpha_q$-coupled receptor causing shape change, which is needed for aggregation and leads to $Ca^{2+}$ mobilization, while $P2Y_{12}$ acts via $G\alpha_i$-coupled receptor leading to a decrease in cAMP. A lack of $P2Y_1$ has been reported to lead to reduced platelet aggregation for all agonists (Leon*, et al* 1999). On the other hand, mice who over express P2Y1 have shorter bleeding time and more aggregation induced by collagen and epinephrine when compared to a control (Hechler*, et al* 2003). ADP receptors are important in haemostasis, which are targeted by the antithrombotic drugs, clopidogrel and ticlopidine (Michelson 2010).

#### ***1.4.1.3.3. $TxA_2$ receptor***

Arachidonic acid (AA) is released from the membrane PLs by the activation of PLA2 by the action of platelet agonist such as thrombin and collagen. $TxA_2$ is then generated from the AA by cyclooxygenase-1 (COX-1). This pathway can be irreversibly inhibited

by aspirin (Vane and Botting 1998). $TxA_2$ is an important secondary wave platelet agonist. It is a weak inducer of PS exposure and MP formation. The main receptor of $TxA_2$ is TP, of which there is ~2000 per platelet, and which exists in two variant forms (TPα and TPβ). A lack of TP has been reported to be related to delayed aggregation induced by collagen, prolonged bleeding time and thrombus instability (Thomas*, et al* 1998).

#### 1.4.1.4. Calcium "$Ca^{2+}$" Signalling pathway

Platelet activation with all agonists is a $Ca^{2+}$ dependent process, which leads to increases in cytosolic $Ca^{2+}$ concentrations, but only GPVI and the outside-in signalling activation by integrins give sustained increases in cytosolic $Ca^{2+}$, for review see (Varga-Szabo*, et al* 2009). $[Ca^{2+}]i$ plays an important role in PS exposure and MP formation, which both need sustained increases in cytosolic $Ca^{2+}$ concentrations; this explains why there is a little effect of PS exposure and MP formation with GPCR's agonists, as they only induce transient spiked increases in $[Ca^{2+}]i$.

### 1.4.2 Platelet activation response

#### 1.4.2.1 Adhesion, aggregation and degranulation

Resting platelets show no interaction with the blood vessel wall unless it is injured or altered (Gawaz*, et al* 2005). At the site of a vascular injury, the first step in the haemostatic response is the adhesion of platelet receptors to the subendothelium on the injured vessel surface. The adhesion process is largely influenced by the rheological conditions; at low shear stress the binding is mainly dependent on collagen, while at high shear stress it is dependent on vWF. This binding is mediated by the interaction of the multimeric vWF and collagen on the subendothelium with the

platelet GPIbα and GPVI receptors, respectively (Ruggeri 1997, Ruggeri 2002, Ruggeri 2003, Weiss 1995), which induce morphological changes allowing them to roll and make contact with the subendothelial surface. The formation of this adhesion plug "tethers" can be dissociated by the haemodynamic forces of the blood (Maxwell*, et al* 2007, Yuan*, et al* 1999). However, this is not a firm adhesion, but this binding triggers the activation of the intracellular signalling pathway known as inside-out signalling, which leads to the activation and other integrins that bind to their ligands in the vessel wall (e.g. fibronectin, vitronectin, etc) and increased expression of additional adhesion receptors, including GPIIb/IIIa (integrin $\alpha IIb\beta_3$), a receptor that binds fibrinogen, which largely mediates platelet aggregation by forming stable bridges between platelets (Clemetson 1995, Kroll*, et al* 1991, Nesbitt*, et al* 2002, Zaffran*, et al* 2000). Fibrinogen has a dual binding site which allows it to form a bridge between activated platelets. GPIIb/IIIa also binds to other molecules, such as fibronectin, vWF and vitronectin (Plow*, et al* 1985, Plow*, et al* 1984); this binding triggers the outside-in signalling pathway. The platelets then spread and are stabilized by the binding of GPIa-IIa (integrin $\alpha_2\beta_1$), α5β1 and α6β1 on platelets to collagen, fibronectin and laminin receptors on ECs (Monnet*, et al* 2000). After the inside-out signalling, GPIa-IIa possesses a high affinity binding to collagen. The adhesion process lead to trigger a number of signalling pathways within platelets, including cytoskeletal reorganization, $Ca^{2+}$mobilization (Kroll*, et al* 1996), tyrosine phosphorylation of platelet proteins (Asazuma*, et al* 1997, Razdan*, et al* 1994), activation of PKC (Kroll*, et al* 1996) and PI3K (Jackson*, et al* 1994) and "second wave mediators" from the dense granules (ADP and serotonin), as well as synthesising $TxA_2$ to reinforce the haemostatic response by

recruitment and activation of additional platelets into the growing thrombus (Lorant*, et al* 1991, McEver*, et al* 1989). The GP Ib-V-IX receptor complex is very important in haemostasis, a lack of or dysfunctional in this receptor leads to a congenital bleeding disorder known as Bernard Soulier Syndrome. The important of GPIIb/IIIa is illustrated by the effectiveness of GPIIb/IIIa inhibitors *in vitro* (Kereiakes*, et al* 1997) or in clinical interventions (Boersma*, et al* 2002, Coller 1997, Lefkovits*, et al* 1995, Quinn*, et al* 2002, Topol*, et al* 2001), as well as in reducing mortality in patients with MI (Boersma*, et al* 2002) or ACS (Roffi*, et al* 2001).

#### 1.4.2.2 Phosphotidylserine (PS) exposure and microparticle (MP) formation

The late stage of platelet activation is PS exposure and MP formation (Bevers*, et al* 1991), which is a $Ca^{2+}$-dependent process, therefore agonists that induce sustained elevations in $[Ca^{2+}]i$ levels are capable of inducing the loss of membrane asymmetry, which is normally accompanied by membrane budding and release. The most potent inducer of PS and MP formation is the non-physiological agent calcium ionophore, A23187, followed by a combination of the physiological agonists thrombin and collagen, and then collagen alone, while the rest of the soluble agonists are weaker inducers of PS exposure and MP formation. ADP, $TxA_2$ and epinephrine are not capable of inducing PS exposure and MP formation alone, but potentiate the effect of collagen (Appleby*, et al* 2006, Bode*, et al* 1985, Comfurius*, et al* 1990, Reininger*, et al* 2006, Sandberg*, et al* 1985). In addition, the complement complex (C5b-9) has been found to induce PS exposure and MP formation (Sims*, et al* 1988). Furthermore, Karpatkin's group have shown that MP formation can be induced by an unique auto-Ab in HIV patients targeted at β3 (GPIIIa49-66) in GIIb-IIIa, which induces the activation of

platelet and MP formation independently of the complement system, but which is dependent on ROS generation mediated by the NAD(P)H oxidase pathway (Li*, et al* 2008, Nardi*, et al* 2001, Nardi*, et al* 2007).

Oxidative substances have also been shown to induce the release of endothelial-derived MPs (EMPs) (Leroyer*, et al* 2008) and platelet-derived MPs (PDMPs) (Nardi*, et al* 2007). The mechanism of PS exposure and MP formation will be discussed in section 1.6.2. Loss of membrane asymmetry is a feature of both platelet activation and cell apoptosis (Bennett*, et al* 1995, Casciola-Rosen*, et al* 1996, Martin*, et al* 1995), observed in physiological and pathological conditions, is found in tumorigenic cells (Utsugi*, et al* 1991) and has also been linked to thrombosis (Zwaal 1978, Zwaal*, et al* 1977). The procoagulant surface, mainly PS, regulates the coagulation process by enhancing the catalytic activity of the TF/FVIIa complex, the coagulation complexes, tenase and prothrombinase (as discussed previously in section 1.2). It has been shown that the catalytic activity tenase and prothrombinase complexes increased 60-fold on the soluble form of PS known as C6PS. The binding of FXa to its cofactor FVa is weak in solution (Boskovic*, et al* 2001), but not on the PS surface (Krishnaswamy*, et al* 1989). The latter also suggests that PLs induce conformational changes in the protein binding of FX and FVa.

## 1.5 Microparticles “microvesicles”

MPs are small membrane vesicles or fragments, which were described by Wolf in 1967 as “platelet dust”. Circulatory MPs are defined as <1µm, are heterogonous in size and content. In addition, MPs carry the antigenic features of their parent cells and express PLs, mainly negatively charged PLs and oxidized lipids (Jimenez*, et al* 2003, Morel*, et al*

2006). MPs can be released from activated platelets or cells undergoing apoptosis (Essayagh*, et al* 2007). The majority of circulatory MPs in healthy individuals (70-90%) are derived from platelets. MP levels can be elevated in various physiological and pathological conditions and have been shown to play a role in signal transduction between cells, promoting haemostasis and coagulation, inflammation (Morel*, et al* 2006), waste removal (Abid Hussein*, et al* 2007), thrombosis, vascular injury and dysfunction, and may be a useful biomarker for clinical studies. In haemostasis, PDMPs have been shown to support thrombin generation in healthy individuals (Berckmans*, et al* 2001), but also they are elevated in thrombotic conditions (Lee*, et al* 1993). Their procoagulant activity comes from PS exposed on their outer surface that results from the loss of lipid asymmetry, and which has been shown to bind FVa and to assemble the prothrombinase complex. Indeed, synthetic PLs containing PS also support clotting assays in plasma (Jones*, et al* 1985). It has been confirmed that PDMPs express adhesion molecules such as P-selectin (Sandberg*, et al* 1985) and can bind to leukocytes (Jy*, et al* 1995).

### 1.5.1 Loss of membrane asymmetry and MP formation

The localization and translocation ("flip-flop") of the PLs across the cell membrane is controlled and regulated by three specific channels, two of which are ATP-dependent (Seigneuret and Devaux 1984, Zwaal and Schroit 1997). The inward movement of PS and PE (PS is preferred over PE) is rapidly and actively mediated by the ATP-dependent 'flippase' (translocase), and the outward movement of all membrane PLs is mediated by the slow continuous ATP-dependent 'floppase'. Meanwhile, scramblase is responsible for the bi-directional movement of all PLs across the cell membrane.

Scramblase is not active in resting platelets, but switches on following activation. Depletion of ATP reduces the activity of the flippase and floppase channels (Fig 1.6) (Bevers*, et al* 1999).

Once a platelet becomes activated, it loses the asymmetrical distribution of the PLs across the cell membrane due to the reorganization of the flip-flop mechanism, leading to increased PS exposure on the outer surface of the cell membrane (Bevers*, et al* 1982, Bevers*, et al* 1983). This process is often accompanied by the release of MPs, which involves the disruption of the membrane cytoskeleton and protein reorganization. Increased [Ca2+]i leads to the activation of kinases and calpain and inhibition of phosphatase. Calpain subsequently destroys the structural stability of the membrane cytoskeleton by degrading talin. Actin is also cleaved by gelosin. In addition, albeit the two processes (MP formation and PS exposure) are linked together, MP formation, but not PS exposure, is calpain dependent (Pasquet*, et al* 1998). The exact mechanism of MP formation *in vivo* is not fully understood, but the increase in $[Ca^{2+}]i$, and the loss of membrane integrity both play a major role in membrane budding (release of MPs). This process can be inhibited by the use of either EGTA, a $Ca^{2+}$ chelator (Miyoshi*, et al* 1996) or an arg-gly-asp (RGD) sequence, suggesting MP formation is linked to GPIIb-IIIa (inside-out signalling). This finding has been confirmed by the study of patients with Glanzmann's thrombasthenia who suffer from reduced or non-functional GPIIb-IIIa (Gemmell*, et al* 1993).

MPs can also be released from apoptotic cells, and the composition of MPs is different in both processes (activation and apoptosis) (apoptosis is discussed below)

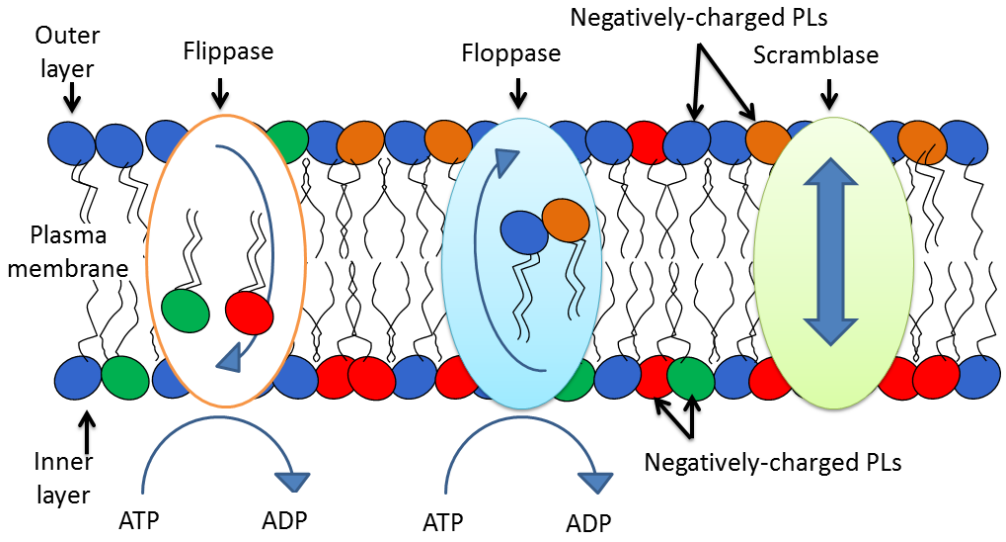



**Fig 1.6 :Flip-flop of the membrane phospholipids in platelets.** In the resting state, the aminophospholipids (phosphotidylserine (PS) and phosphatidylethanolamine (PE) are transported inward by the flippase in an ATP-dependent manner, while the floppase are responsible for the slow outward transportation of both the Choline-PLs (Sphingomylein (SM) and Phosphotidylcholine (PC)) and the aminophospholipids in an ATP-dependent manner. After activation, scramblase becomes activated leading to a bi-directional movement of PLs across the cell membrane. Adapted from Bevers*, et al* (1999).

(Jimenez*, et al* 2003). It is suggested that MP release from cells undergoing apoptosis occurs by abdifferent mechanism to platelet activation (Fig 1.7). Apoptosis is caspase dependent, which cleaves and activates the Rho-associated kinase (ROCK I). ROCK I in turn generates a contractile force on the actin-myosin filaments leading to membrane blebbing (Maekawa*, et al* 1999).

## 1.6 Apoptosis

Apoptosis, a form of programmed cell death, is an important, controlled, physiological process to remove unwanted cells, including platelets, in order to maintain body homeostasis, mainly by macrophages (Danial and Korsmeyer 2004, Kerr*, et al* 1972, Raff 1992) and the reticuloendothelial (RE) system, in a process that does not involve the release of inflammatory mediators. The recognition of apoptotic cells is mediated through PS exposure via scavenger receptors (Fadok*, et al* 1992a, Fadok*, et al* 1992b, Gardai*, et al* 2006, Savill*, et al* 1993). Apoptosis in nucleated cells is accompanied with distinct characteristics of morphological and chemical changes, including cell shrinkage, nuclear fragmentation, chromatin condensation, mitochondrial swelling, disruption of mitochondrial membrane potential (ΔΨm), cytochrome C release, loss of cell membrane asymmetry leading to PS exposure, release of apoptotic bodies procoagulant MPs, caspase activation and ROS production (Fadok*, et al* 1992a, Martin*, et al* 1995, Mower*, et al* 1994, Wolf and Green 1999). There are two apoptotic pathways — the extrinsic and intrinsic — which converge to activate the common effector (executioner) pathway, which is mainly driven by caspases, which can cleave over 500 cellular targets (Thornberry and Lazebnik 1998, Vaux and Strasser 1996). The extrinsic pathway is triggered by the death receptors on the cell surface upon engaging

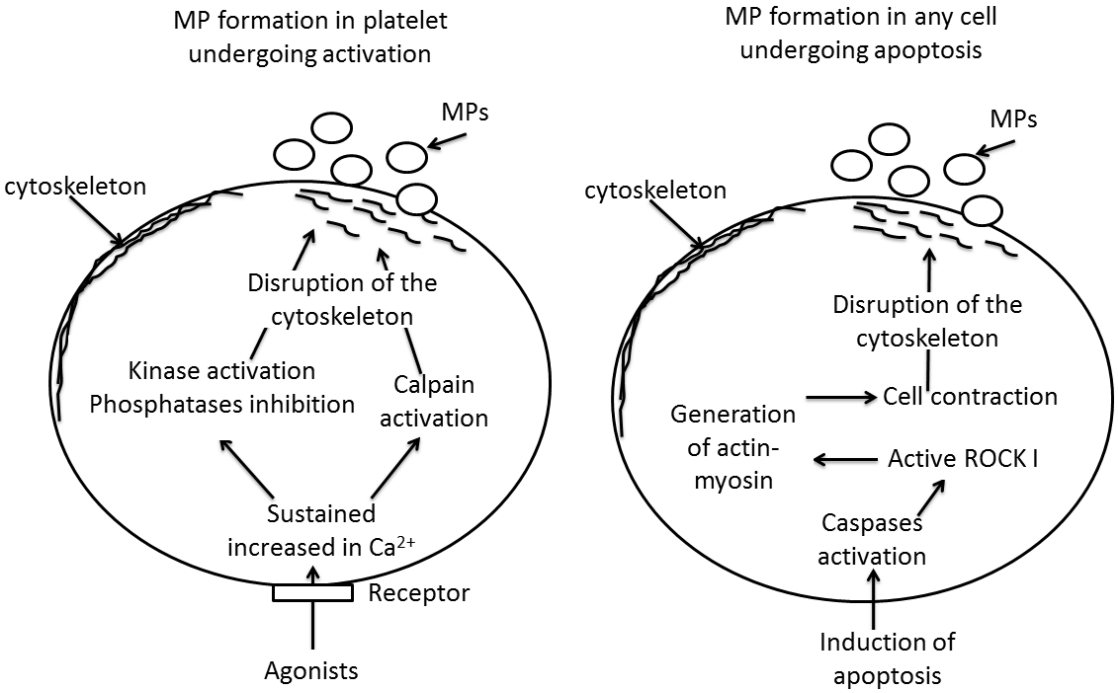



**Fig 1.7: Microparticle formation in activated platelet compared to cell undergoing apoptosis.** Both activation in platelets and any cell undergoing apoptosis induce PS exposure and MP formation through different signalling pathways. Adapted from VanWijk*, et al* (2003).

with their specific receptor, such as the tumour necrosis factor (TNF) which leads to signal transduction, while the intrinsic pathway is tightly controlled by a balance between the pro- and anti-apoptotic proteins of the Bcl-2 family, to regulate the central step in this pathway, which is maintaining the mitochondrial membrane potential. Bcl-2 family proteins are the regulators of apoptosis.

The anti-apoptotic proteins (Bcl-2, Bcl-$x_L$, Bcl-w, Mcl-1 and Bcl-2A1) restrain the pro-apoptotic proteins — mainly Bak, Bad and Bax — to prevent apoptosis. Signals are transmitted to Bak, Bad and Bax by the pro-apoptotic proteins of the multidomain (BH-1,-2,-3 homology domain); Bak, Bad and Bax also contain a hydrophobic domain that is able to bind to BH3 pro-apoptotic proteins. Once Bak and Bax become activated, thus leads to mitochondrial damage. As a consequence, cytochrome C is released from the mitochondria into the cytosol, which then interacts with apoptotic protease-activating factor-1 (APAF-1) to activate caspase 9. Activated caspase 9 cleaves downstream caspases, ultimately leading to cell death reviewed by (Ashkenazi and Dixit 1998, Green and Reed 1998, Gross*, et al* 1999).

## 1.7 Lipid peroxidation

PLs and cholesterol are susceptible to oxidative stress. Free radicals degrade the cell membrane, particularly the polyunsaturated fatty acids (PUFAs) in the cell membrane, by a process of lipid peroxidation. In some situations, oxidized PLs are truncated, leading to a protrusion of oxidized fatty acids on the outer cell membrane, which form so-called lipid whiskers (Greenberg*, et al* 2008). Lipid peroxidation is of significance because it damages the cell membrane and is likely to occur upon free radical production (Tuma 2002). Lipid peroxidation has been linked to apoptosis in different

cells, including platelets (Sener*, et al* 2005), and has been implicated in various diseases and tissue injuries (Picklo*, et al* 2002). ROS has been shown to cause lipid peroxidation in platelets measured using the thiobarbituric acid reactive substances (TBARS) assay (Nowak*, et al* 2003) or accurately by mass spectrometry (Yu*, et al* 2009).

## 1.8 Reactive oxygen species (ROS)

ROS production is well defined in phagocytic cells as a defence mechanism against microbes (Segal 2005) or as a secondary messenger for signal transduction (Lambeth 2004). ROS are highly reactive molecules that can react with lipids (inducing peroxidation), proteins (inducing oxidation or nitration of aa residues), or induce DNA damage, all of which are irreversible changes. ROS have the ability to alter and modify the lipid structure, particularly the unsaturated fatty acid residues of the membrane PLs in cells. NAD(P)H oxidase complex is the major producer of extracellular ROS in phagocytic cells, and has recently been found to be one of the major sources of intracellular ROS in non-phagocytic cells (Lambeth 2004) including platelets. NAD(P)H oxidase is a multicomponent enzyme consisting of cytosolic and membrane subunits (Fig 1.8). The cytosolic components $p47^{phox}$, $p67^{phox}$, $p40^{phox}$ and Rac, are all regulatory subunits, while the membrane-bound components are the regulatory subunit $p22^{phox}$ and the catalytic subunit $gp91^{phox}$ (also known as NOX2). NAD(P)H becomes activated to produce ROS upon PKC phosphorylation of the cytoplasmic regulatory component $p47^{phox}$, which co-assembled with other cytosolic subunits and translocates to activate the membrane-bound regulatory subunit $p22^{phox}$ (mediated by the SH3 domain), and with membrane bound $PIP_2$, which is in turn a product of PI3K.

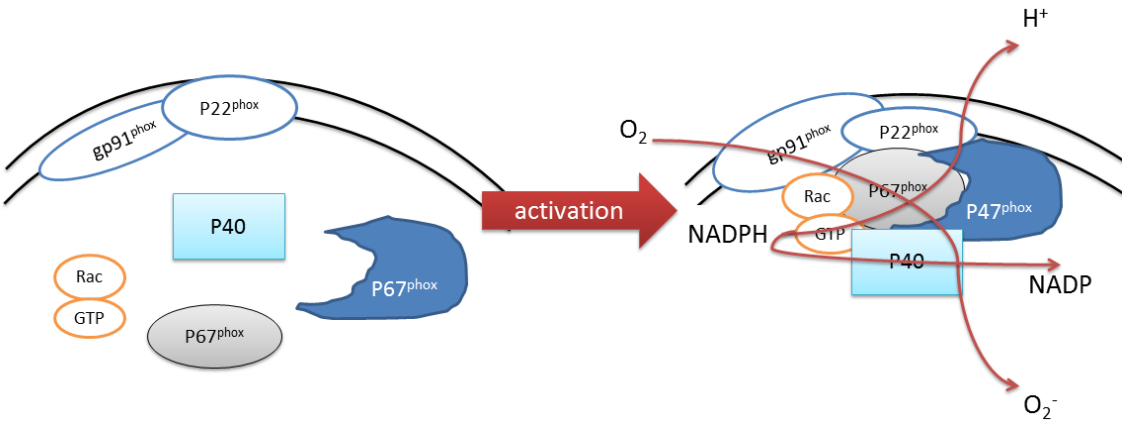



**Fig 1.8. Co-assembled of NAD(P)H oxidase subunits for intracellular ROS generation in platelet.** Adapted from (Dusting, *et al* 2005).

## 1.9 Measuring the ability of PS to generate thrombin

All of these changes in platelets including activation, apoptosis, and lipid peroxidation lead to exposure of the negatively charged surface and there are different ways to measure this procoagulant surface. Indeed, there are several methods for the detection of PS exposure in platelets and MPs, as well as the quantification and detection of the phenotypic characteristics of MPs (for a recent review see (Yuana*, et al* 2010). The phenotypic analysis of MPs can be achieved using an antigen specific to the parent cells (for example by using CD42b for detection of platelet-derived MPs, CD144 for endothelial cell-derived MPs or CD14 for monocyte-derived MPs) and the procoagulant surface (the negatively charged PLs) can be detected using annexin-V, which binds avidly to anionic PLs (Burnier*, et al* 2009, Jy*, et al* 2004, Mobarrez*, et al* 2010). The functional activity of MPs — mainly procoagulant MPs and TF-bearing MPs — can be measured by different methods, including thrombin generation (prothrombinase) and FXa generation. Thrombin generation is widely used nowadays, measuring the endogenous thrombin potential (ETP) (which is also known as the Calibrated Automated Thrombography (CAT)) (Hemker*, et al* 2003), or in modified enzyme-linked immunosorbent assays (ELISAs) (capture based assay) (Ay*, et al* 2009, Bal*, et al* 2009, Steppich*, et al* 2009). The latter can also be used for phenotypic detection (Nomura*, et al* 2009, Ueba*, et al* 2008). Electron microscopy (Heijnen*, et al* 1999) and fluorescence confocal laser scan microscopy (Dale*, et al* 2005) have also been used to visualize MPs. In addition, with developments in technology, new approaches have been developed, including dynamic light scattering (Lawrie*, et al* 2009) and nanoparticle tracking analysis (NTA) (Harrison*, et al* 2010), both of which are

based on Brownian motion. Other techniques including atomic force microscopy (Yuana*, et al* 2009), impedance-based flow cytometry (Hoffman*, et al* 1981) and proteomic analysis using mass spectrometry (Garcia*, et al* 2005, Smalley*, et al* 2007) have been used as well. However, the most commonly used technique for PS exposure detection is the annexin-V binding assay, and for the functional activity is the CAT.

### 1.9.1 Flow cytometry

Flow cytometry is widely and extensively used to study both PS exposure and MPs (Jy, *et al* 2004). It allows the quantification of cell surface antigens and intracellular molecules in single platelets. This was achieved thanks to the ability of flow cytometry to distinguish cells according to their cellular origin using specific fluorescence labelled Abs (Sims*, et al* 1988) or the detection of their procoagulant surface by the use of annexin-V, which is a generic marker that binds to the negatively-charged surface and can be fluorescently labelled (Andree*, et al* 1990). Thus annexin-V provides a measure of relative proportion of cells or particles expressing a procoagulant surface. However, flow cytometry fails to detect small MPs that are less than 0.5µm, due to wavelength limitations (Lacroix*, et al* 2010a) and even with lower wavelength lasers, size is still a limitation. Many approaches have been developed to overcome the current limitation, such as the use of calibrated beads to detect MP size and count (Kim*, et al* 2002, Lacroix*, et al* 2010a). Most of the published studies on MPs focus on the detection of PS exposure, cellular origin, size and count using flow cytometric analysis, rather than on the functional activity of the PS on MPs (Jy, *et al* 2004).

### 1.9.2 Thrombin generation assay

The thrombin generation assay using CAT is a promising tool to investigate the hypo- and hyper-coagulability activity of platelets and plasma, which has been developed by Hemker and colleagues over the last 3 decades. It started from an existing subsampling method using a chromogenic substrate and has become a continuous measurement method using the slow reactant chromogenic and more recently fluorogenic substrate (Hemker*, et al* 2003). It is increasingly considered to be a promising tool for predicting the actual status of the haemostatic system because it describes all the phases of thrombin generation and the balance between the procoagulant and anticoagulant factors. It is supposed to be more physiologically relevant compared to other assays, such as the routine clotting-based assays (i.e. prothrombin time and aPTT) detect only a small fraction (3-5%; 10nM) of thrombin formed from prothrombin and ignore the remaining 95%. In addition, while prothrombin time and aPTT assays are useful in the diagnosis of critical changes in the normal balance of haemostasis; they also have limitations as they cannot predict subtle changes in the haemostatic mechanism. The CAT assay not only predicts the hypercoagulability and hypocoagulability of the sample (Joop*, et al* 2001, Livnat*, et al* 2006), but also the contribution of the antithrombotic drugs (Gerotziafas*, et al* 2004), warfarin therapy and efficiency of replacement factor deficiencies in haemophiliac patients (Gatt*, et al* 2008), which can be achieved by using different reagents.

There are different reagents for thrombin measurement in the CAT; the most widely used reagents are 5pM TF/4μM PL and 1pM TF/4μM PL for plasma, and 1pM TF for PRP analysis. According to Hemker, *et al* (2003), the measurement of thrombin

generation in plasma using 5pM TF/4μM PL (standard condition) is sensitive to the levels of coagulation factors FVIII and FIX, as well as procoagulant and anticoagulant factors; and this sensitivity is increased by using the 1pM TF/4μM PL for FXI and the previous coagulation factors (FVIII and FIX) in the plasma (Beltran-Miranda*, et al* 2005, Dargaud*, et al* 2005, Lewis*, et al* 2007, van Veen*, et al* 2009). The 1pM TF reagent is normally used to test the procoagulant activity for platelets, but its use for measuring the procoagulant activity of PDMPs has not been fully evaluated. Using a low concentration of TF, 1pM TF but not 5pM, makes the assay sensitive to contact pathway activation, mainly FXIIa, which significantly influences the measurement of thrombin by enhancing thrombin formation; therefore increasing the assay's variability. To overcome this false overestimation, corn trypsin inhibitor (CTI) has been found to be useful to eliminate the effect of contact pathway activation and to improve the measurement of thrombin generation, either in normal plasma or patients' plasma (Dargaud*, et al* 2005, Luddington and Baglin 2004, Van Veen*, et al* 2008).

## 1.10 Hypothesis

That the procoagulant activity of platelets and PDMPs can be analysed by measuring their ability to enhance thrombin generation, which is increased in patients with prothrombotic disease states, and regulated by ROS generation in platelets undergoing activation and apoptosis.

**Specific aims:**

- To optimize the analysis of PDMPs by CAT focusing on the sensitivity of detection with different reagents and on minimizing artefacts due to pre-and analytical processing.
- To determine whether procoagulant MPs are present in plasma from patients at risk of cardiovascular disease (CVD) focussing on patients with CAD and ESRD.
- To compare the regulation of thrombin generation and MP formation by platelets and PDMPs using a range of methodologies.
- To determine the role of ROS production on the procoagulant surface generation from platelets measured by thrombin generation in platelets and PDMPs.
- To compare the phenotypic and functional characteristic of activated platelets and PDMPs with those of apoptotic platelets.

# Chapter 2: Materials and Methods

## 2.1 Materials

### 2.1.1 General reagents

The following reagents were all obtained from Sigma (Gillingham, Dorset, UK); bovine serum albumin (BSA), calcium chloride ($CaCl_2$), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), phosphate buffered saline (PBS), hydrogen peroxide ($H_2O_2$), dimethyl sulphoxide (DMSO), sodium chloride (NaCl), tri-sodium citrate, citric acid, glucose, magnesium sulphate ($MgSO_4$), potassium chloride (KCL), absolute ethanol, sodium bicarbonate ($NaHCO_3$), sodium phosphate dibasic heptahydrate ($Na_2HPO_4$)$7H_2O$, calcium nitrate tetrahydrate ($Ca(NO_3)_2.4H_2O$), potassium dihydrogen phosphate ($KH_2PO_4$) anhydrous, phenol red, absolute ethanol, ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), 1,1,3,3-tetramethoxypropane (malonaldehyde bis-dimethyl acetal; MDA), sulphuric acid ($H_2SO_4$), glutaraldehyde, sodium acetate, thiobarbituric acid, sodium dodecyl sulphate, ferric chloride ($FeCl_3$), and prostaglandin $I_2$ sodium salt ($PGI_2$). 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-$H_2$DCFDA; an intracellular ROS probe) was purchased from Molecular Probes, Invitrogen (UK).

## 2.2 Buffer and reagent preparation

### 2.2.1 General buffers

The buffer of choice, which was used as a universal diluent in all of the experiments unless otherwise stated, was HEPES buffer saline (HBS; 150mM NaCl, 1mM MgSO4 and

10mM HEPES in 500ml Milli.Q.$H_2O$). The pH of the buffer was adjusted to 7.2 and filtered through a 0.2µm filter. The buffer was then aliquoted and stored at 4°C for up to 6 weeks. HBS/$Ca^{2+}$ at 2mM final concentration was prepared on the day of analysis from 200mM $Ca^{2+}$ stock; this concentration of $Ca^{2+}$ was used for the whole study. In some experiments, Tyrode's buffer (134mM NaCl, 2.9mM KCl, 0.34mM $NaH_2PO_4$, 1.0mM $MgCl_2$, 12.0mM $NaHCO_3$, HEPES 10mM and 5mM glucose into 1L of Milli.Q.$H_2O$; pH 7.4) was used to dilute platelets. Washing buffer (2.22g $CaCl_2$ anhydrous in 200ml Milli.Q.$H_2O$) was used for washing the Fluoroskan plate reader, and was stored at 4°C for up to 1 week. Acid-Citrate-Dextrose (ACD) anticoagulant solution was prepared by dissolving 0.085M trisodium citrate, 0.071M citric acid and 0.11M glucose in 1L of Milli.Q.$H_2O$. The pH of the ACD anticoagulant solution was adjusted to 6.3. A 0.2% solution of formyl-saline for fixing samples for flow cytometric analysis was prepared by adding 0.2% v/v formaldehyde in 0.85% w/v isotonic saline and then filtered through a 0.2µm filter.

### 2.2.2 Thrombin generation reagents

A thrombin calibrator, a fluorogenic substrate (FluCa kit; Z-Gly-Gly-Arg-AMC substrate with $Ca^{2+}$) and thrombin generation reagents with different concentrations of PLs and TF (known as PRP and PPP reagents, Table 2.1) and Thermo-Immulon 2HB 'u' bottom 96-well plates were obtained from Stago Diagnostica (Asnières sur Seine, France). A synthetic bovine brain PL vesicle preparation (91/542) was obtained from the National Institute for Biological Standards and Control (NIBSC) (Potters Bar, Hertfordshire, UK). NIBSC PL (10mg/ml; comprising 36.6% PC, 28.1% PE, 17.1% SM, 12.0% PS and 6.2% PI)

was reconstituted with 1ml Milli.Q.$H_2O$ until the powder dissolved and was then divided into aliquots of 25µl and stored at -80°C. NIBSC PL working stock for the thrombin generation assay was made on the day of the assay by adding 25µl of stock concentration (10mg/ml) to 4.75ml filtered HBS buffer to obtain a working stock of 50µg/ml concentration. Other reagents, such as thrombin calibrator and PPP and PRP reagents, were reconstituted with 1ml Milli.Q.$H_2O$ and pre-warmed for 10 minutes and used within the 3 hours of preparation.

| | Composition | | To measure TG in |
|---|---|---|---|
| Reagent | Tissue Factor (pM) | Phospholipid (µM) | |
| PPP high reagent | 20 | 4 | Plasma |
| PPP normal reagent | 5 | 4 | Plasma |
| PPP low reagent | 4 | 4 | Plasma |
| PRP reagent | 1 | 0 | PRP |
| PRP/NIBSC PL reagent | 1 | ~2 | Plasma |

**Table 2.1: details of reagents used in thrombin generation assay.** Thrombin generation (TG)

## 2.2.3 Reagents for TBARS assay

TBARS assay was used for the detection of lipid peroxidation in platelets. The TBARS assay will be described in detail later in this chapter. The thiobarbituric acid (TBA) reagent was prepared by adding 1.23g sodium acetate, 188mg TBA and 75mg SDS to 50ml of Milli.Q$H_2O$. A solution of BHT was prepared by dissolving 22mg of BHT and 27.0mg of $FeCl_3$ to 50ml of absolute ethanol.

### 2.2.4 Reagents for flow cytometric

Annexin V-Fluorescein isothiocyanate (FITC) was obtained from BD PharMingen Biosciences (Oxford, UK). Monoclonal mouse anti-human CD42b R-Phycoerythrin (RPE) (platelet glycoprotein Ibα, clone AN51), negative RPE conjugated isotype control (clone # MOPC-21PE) for CD42b-RPE and polyclonal rabbit anti-human Fibrinogen-FITC were obtained from DakoCytomation (High Wycombe, UK). Monoclonal anti-human P-selectin (CD62P-FITC) and mouse $IgG_1$ isotype control FITC conjugated were obtained from R&D System (Abingdon, UK).

### 2.2.5 Agonists

The following agonists were used to activate platelets. CRP-XL (Morton*, et al* 1995) was obtained from Dr Richard Farndale, University of Cambridge, the calcium ionophore, A23187, and the PAR-1 thrombin receptor activator peptide (TRAP) with the sequence SFLLRN were obtained from Sigma. The BCL-2/BCL-xL inhibitor (ABT-737 and ABT-263) used to initiate apoptosis in platelets, were obtained from Selleck Chemicals (Houston, TX, USA) and ABT-737 was also obtained from Chemietek (Indianapolis, IN, USA). TRAP was prepared at different concentrations and then these stocks were aliquoted in Milli.Q.$H_2O$ prior to storage at -80°C. A23187 and ABT-737 were prepared as 1000X stocks and aliquoted in HBS buffer prior to storage at -20°C. For CRP-XL, a working CRP-XL stock (100µg/ml) was made from the main stock (10.0mg/ml) on the day of analysis using HBS/$Ca^{2+}$, which was discarded after the analysis. The main stock of CRP-XL (10.0mg/ml) was stored at 4°C. All aliquots were discarded after use.

### 2.1.6 Inhibitors

The inhibitors used were the contact factor pathway inhibitor, CTI, which was obtained from Cambridge Biosciences (UK) and Enzyme Research (Swansea, UK), hirudin (a direct thrombin inhibitor) was obtained from Sigma, Gly-Pro-Arg-Pro peptide (GPRP; a fibrin polymerization inhibitor) were obtained from both Sigma and Peptide Protein Research Ltd. (Hampshire, UK). The PI3K inhibitor wortmannin, NAD(P)H oxidase complex inhibitors, apocynin (4'-Hydroxy-3'-methoxyacetophenone) and diphenyleneiodonium (DPI), the cell permeable $O^{\bullet}_{2}{}^{-}$ scavenger, tiron (4,5-Dihydroxy-1,3-benzenedisulphonic acid disodium salt), a superoxide dismutase mimetic (tempol), a nitric oxide synthase inhibitor (L(G)-nitro-L- arginine methyl ester; L-NAME), BHT antioxidant, the vitamin E analogue, trolox, (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), and an N-acetyl-L-cysteine (NAC), antioxidant, 12-lipoxygenase (12-LOX) inhibitor, baicalein (5,6,7-trihydroxyflavone) were all obtained from Sigma. Esculetin, another 12-LOX inhibitor, cyclosporine (Cys) A and dexamethasone (a glucocorticoid drug) were obtained from Enzo-Life Sciences (Exeter, UK). The pan caspase inhibitors, z-VAD.fmk and Q-VD-OPh, were from MP biomedicals (UK).

## 2.3 Methods of blood collection

### 2.3.1 Blood collection from normal healthy subjects

Venous citrated blood was obtained from normal healthy individuals who had not taken any medication for at least two weeks prior to donation, and who had signed an informed consent form approved by the Ethics Committee of the University of Leicester. Blood was drawn from the antecubital vein into vacutainer tubes (Becton-

Dickinson, Oxford, UK) by venipuncture via a 21 gauge butterfly needle, using a lightly applied tourniquet which was removed after the insertion of the needle. The collection procedure was performed by a trained nurse/phlebotomist under laboratory guidelines to avoid the activation of platelets. The first tube of blood was collected into a vacutainer EDTA tube and was used to obtain a full blood count throughout the study. Subsequent tubes were collected into vacutainers containing 0.105mol/L sodium citrate (4.5ml capacity). The proportion of sodium citrate anticoagulant to blood was 1:9v/v. Immediately after blood collection, unless otherwise stated, CTI was added to the whole blood at a final concentration of 18.3µg/ml to prevent contact factor activation. The blood was processed within 10 minutes of collection.

### 2.3.2 Blood collection from BLOODOMICS cohort

Whole blood was collected from Caucasian patients with MI and matched Caucasian controls by Dr Unni Krishnan. Patients who had suffered an MI episode before the age of 60 were identified from the Myocardial Infarction National Audit Project (MINAP). The blood was collected from the cases at least 3 months after their MI episode so that all patients were in a stable condition at the time of blood collection. The control group were subjects with no personal or family history of MI. The cohort was matched for age, gender and smoking status. The procedure for blood collection described in section 2.3.1 was used. Venous citrated blood samples were processed after 10 minutes of collection. The study was approved by the Derbyshire Research Ethics Committee (#06/Q2401/134) and all participants signed an informed consent.

### 2.3.3 Renal Cohort

Twenty haemodialysis (HD) patients and seventeen peritoneal dialysis (PD) patients were recruited from the relevant HD and PD programmes at the Leicester General Hospital. A matched group of twenty healthy individuals with no reported kidney disease, matched for age and gender, were recruited from the University of Leicester, UK. HD patients had native arterio-venous fistulae and were dialysed for 3.5 hours three times per week. The majority of these patients had been treated with recombinant human erythropoietin. Blood samples from the HD patients were collected at the time of cannulation immediately before the start of treatment. Blood was collected from PD patients and healthy subjects using a loosely fastened tourniquet and 21-gauge needle into 3mL S-monovette vacutainer sodium citrate tubes (Sarstedt AG, Nümbrecht, Germany). The same blood collection procedure was performed to collect blood from nine end-stage renal patients. The study was approved by the Leicestershire Local Research Ethics Committee (#05/Q2502/80) and all the participants signed an informed consent. All blood samples were collected by Dr James Burton.

### 2.3.4 Whole blood count (WBC)

Cell counts were measured using an automated cell counter, a Coulter Act-diff haematology analyzer (Beckman Coulter™ Ltd, High Wycombe, UK), from the first EDTA tube. Various parameters were obtained, including red cell, white cell and platelet counts. The Act-diff haematology analyzer was also used to count platelets

after preparing PRP or washed platelets. The Act-diff haematology analyzer was checked daily using coulter 4C-EC cell control panel.

### 2.3.5 Preparation of platelet-poor plasma (PPP)

*Normal healthy subjects*: citrated whole blood or adjusted PRP was centrifuged at 1,800x*g* (3,000rpm) for 30 minutes to obtain PPP at RT. This is the procedure used to obtain PPP throughout the study, unless stated otherwise. In some experiments plasma was separated at 4°C instead of RT. For comparison study on thrombin generation between protocols of plasma preparation, PPP was prepared using two centrifugation steps; 1,500x*g* for 15 minutes, followed by 13,000x*g* for 2 minutes, and stored at -80°C until analysed for thrombin generation.

*Bloodomics cohort:* citrated whole blood was centrifuged at 1800x*g* (3000rpm) for 30 minutes at 4°C to obtain PPP and stored at -80°C until analysed for thrombin generation.

*Renal cohort:* PPP was prepared using two centrifugation steps; one of 1,500x*g* for 15 minutes, followed by one of 13,000x*g* for 2 minutes. PPP was aliquoted and stored immediately at -80°C for thrombin generation measurement.

Although, there are different methods used to prepare plasma in the study, these methods were compared to determine if they had any influence on thrombin generation assay (Chapter 3).

## 2.3.6. Preparation of platelet-rich plasma (PRP)

Platelet-rich plasma (PRP) was prepared by centrifugation of citrated whole blood at 160x*g* (900rpm) for 20 minutes. Centrifugation was performed at RT unless otherwise stated. PRP was gently aspirated from the upper layer of the blood; about 75% of the PRP was taken without any disturbance to the buffy coat layer. Unless otherwise stated, the PRP count was adjusted with autologous PPP to $150X10^9$/L.

## 2.3.7 Preparation of the plasma pool (PP)

Citrated whole blood was collected within an hour period from 20 healthy donors as described above with the addition of CTI. The blood was centrifuged at 1,800x*g* (3,000rpm) for 30 minutes at RT, the plasmas were then pooled and aliquoted. Aliquots were immediately stored at -80°C. The whole process of plasma collection, preparation and preparing the PP took less than 2 hours.

## 2.3.8 Preparation of washed platelet

After collecting venous whole blood as described above, ACD solution was added immediately at a ratio of 6:1 blood: ACD prior to centrifugation at 160x*g* for 20 minutes at RT to obtain PRP. Platelets were then pelleted by centrifugation of PRP at 750x*g* for 15 minutes in the presence of 200ng/ml of freshly prepared $PGI_2$. The supernatant was removed and the pellet was gently resuspended in HBS pH 6.0 and recentrifuged at 750xg for 15 minutes at RT, again in the presence of 200ng/ml of freshly prepared $PGI_2$. Washed platelets were again gently resuspended in HBS pH 7.2 and the platelet count was adjusted to $150x10^9$/L with HBS/$Ca^{2+}$ buffer. Platelets were

left for 30 minutes prior to use to allow the effect of $PGI_2$ to decay. In some experiments, Tyrode's buffer and $Ca^{2+}$ were used instead of HBS and HBS/$Ca^{2+}$.

### 2.3.9 Preparation of PDMP-rich samples

PRP or washed platelets adjusted to $150x10^9$/L were activated with CRP-XL (2.0µg/ml, unless otherwise stated) for 10 minutes at 37°C to generate PDMPs. For each experiment a control PRP or washed platelet preparation was treated in the same way, but without addition of CRP-XL. Both activated and control samples were then centrifuged at 1,800x*g* (3000rpm) for 30 minutes at RT to obtain MP-rich plasma from PRP, or MP-rich supernatant from washed platelets, which was used for measurement of thrombin generation, 12-hydroxyeicosatetraenoic acid (12-HETE) and thromboxane $B_2$ ($TxB_2$).

## 2.4 Methods

### 2.4.1 CAT assay

Thrombin generation was measured in this study using CAT as described previously (Hemker*, et al* 2003), with some modifications. The reaction is dependent on TF to initiate the reaction, and on procoagulant PLs to support generation of thrombin, as well as coagulation factors. In the analysis of plasma by CAT, both factors (TF/PL) are normally added, as normal plasma lacks sufficient levels of both factors to initiate or support the reaction. For CAT analysis in PRP, only TF is added, as the platelets provide the procoagulant PLs, once they are activated.

Thrombin generation was continuously measured in PRP, PPP, platelet suspension (washed platelets resuspended in autologous filtered plasma) or MP-rich supernatant (supernatant from washed platelets resuspended in autologous filtered plasma) in a 96-well plate (Immulon 2HB, U bottomed plate) using a slow reactant fluorogenic substrate. To summarise the process, 80µl of the sample was added to the wells, which already contained 20µl of the appropriate reagent (Table 2.1). Then, 20µl of the fluorogenic substrate with $Ca^{2+}$ was added to initiate the reaction. This was followed by a 10-second shake of the plate. The assay plate was run in a Fluoroskan Ascent plate reader equipped with Thrombinoscope software, which has excitation/emission filters of 390nm and 460nm, respectively. Thrombin calibrator wells were run in parallel for each donor. All samples were analysed in duplicate.

Thrombin generation analysis shows four main parameters (Fig 2.1): (i) the lag time (LT; equivalent to the clotting time and representing the time in minutes from initiating the reaction until the thrombin burst), the time to peak (ttP; the time to maximum thrombin generation in minutes, which provides a representation of the rate of the initial reaction), peak thrombin (PT; the maximum amount of thrombin generated in nmol at any one time), and the ETP (the area under the curve in nmol in minutes, which represents a measure of the total amount of thrombin generated).

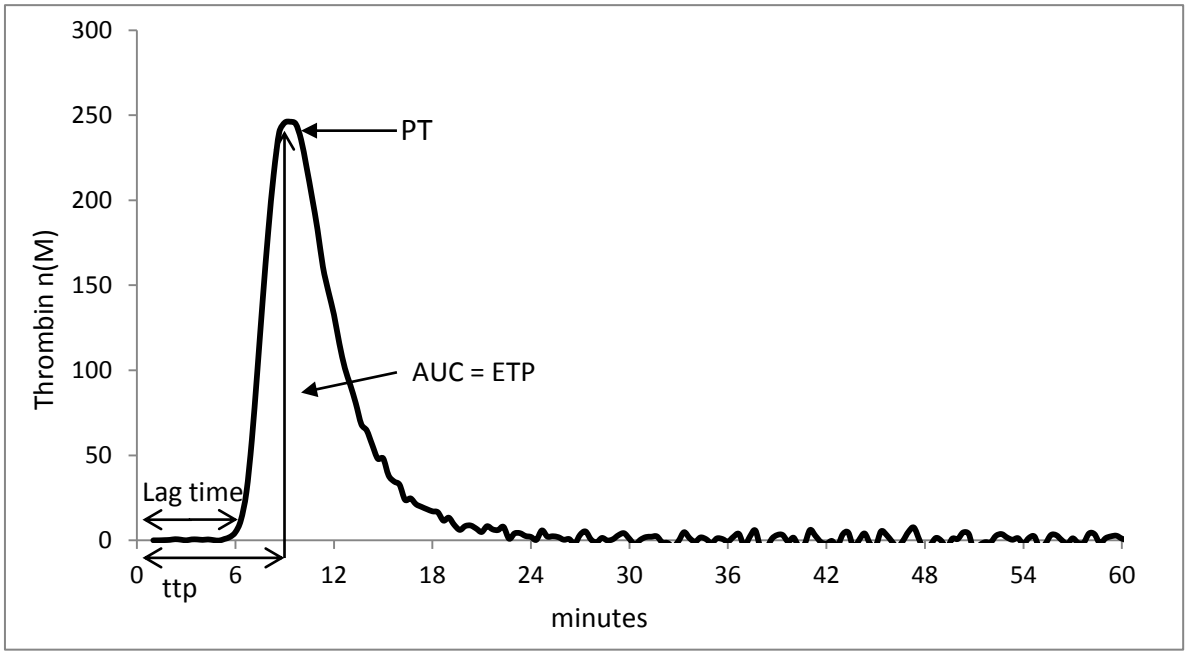



**Fig 2.1: A representative example of a thrombin generation curve.** The curve shows four main parameters: the lag time, endogenous thrombin potential (ETP), peak thrombin (PT) and time to peak (ttP).

## 2.4.2 Flow Cytometric Analysis

Measurement of the platelet procoagulant surface and activation markers by flow cytometry provides a sensitive, rapid and quantitative analysis, which allows detection of even small changes in the platelet status. In this study, flow cytometry was used to assess the effect of CRP-XL on platelets and PDMPs which was assessed by using annexin-V binding for the detection of the procoagulant surface, the platelet activation markers, P-selectin expression and fibrinogen binding.

### 2.4.2.1. General methods

The analysis of all samples by flow cytometry was performed using a Coulter Epics XL-MCL™ analyser (Beckman Coulter, High Wycombe, UK). The flow cytometer is

equipped with a 15mW argon-ion laser with minimum wavelength excitation at 488nm. The detection bandpass filtered of the fluorescence is 525±20nm for FITC and 575±20nm for R-PE. The signals of FITC and PRE were corrected electronically for any overlap, when both were used with two colours. The flow cytometer was aligned daily by running flow-check bead fluorospheres (Beckman Coulter). Signals were detected at logarithmic gain.

All antibodies were titrated to select the optimum concentration before using them in the study. The negative controls in the analysis (fluorescence-conjugated-matched isotype/species nonspecific for each of the antibodies used) were used for each experiment to set at 2% positive. The flow rate of the analysis was medium for all protocols. The sheath fluid used in the analysis was isoton (Beckman Coulter). The discriminator value was 1 in this analysis in order to eliminate background noise and debris, while still detecting platelets and PDMPs. The results of the fluorescence measurement were expressed as the percentage of positive events and the median fluorescence intensity (MFI) of positive events, with MFI being an arbitrary unit.

The optimum concentration of CRP-XL was determined by running samples incubated with serial dilution of CRP-XL in the annexin-V binding assays, P-selectin expression and fibrinogen binding assays. For this, PRP was incubated with different concentrations of CRP-XL (0-10µg/ml) to determine the concentration of CRP-XL that is optimum for inducing the highest effect. This was carried out for each new batch of CRP-XL and at intervals for each batch to check for deterioration.

### 2.4.2.1 Identification of platelet population

Platelet population in the flow cytometric analysis was identified on the basis of forward scatter (FS; measures cell size) and side scatter (SS; measures cell granularity). The subsequent gating on the fluorescence-labelled antibody was performed according to the assay of interest using monoclonal antibodies targeting the cell surface, namely CD42b (a pan platelet marker), and markers expressed during the activation of platelets which target PS exposure, P-selectin expression, and fibrinogen binding using annexin-V, CD61P and polyclonal antibodies, respectively.

### 2.4.2.2 Annexin-V binding assay

A two-colour flow cytometric assay was used to detect PS exposure on activated platelets and the generation of PDMPs as described previously (Goodall and Appleby 2004) with slight modification. Platelets were activated with either CRP-XL or the $Ca^{2+}$ ionophore, A23187. This process is normally accompanied by PS exposure and PDMP formation. The negatively charged PLs surface was detected with fluorescently conjugated annexin-V (a universal generic marker) on the surface of the platelets or PDMPs. Annexin-V has a high binding avidity for anionic negatively-charged PLs. This process is calcium dependent, so HBS/$Ca^{2+}$ buffer was used in all the experiments. Monoclonal antibody CD42b-PRE was used to identify platelets and PDMPs.

5µl of PRP was added into an LP3 polystyrene tube containing HBS/$Ca^{2+}$ buffer, hirudin (10U.$mL^{-1}$), GPRP (0.5mg.$ml^{-1}$), diluted CRP-XL (0-10µg.$ml^{-1}$ final concentration), CD42b-PRE, and annexin V-FITC to bring the volume to 50µl. In parallel, a negative FITC control tube was set up in exactly the same way as the above setup except for HBS

buffer (no $Ca^{2+}$) being used. This tube was set up to show that the binding of annexin V-FITC to the negatively-charged PLs is $Ca^{2+}$ dependent, and that in the absence of $Ca^{2+}$ no binding can be achieved. This tube was used for the FITC control (to determine non-specific binding). The second control tube was for a PRE-conjugated isotype, in which 5µl of the sample was added into LP3 polystyrene tube containing HBS buffer (no calcium) and mouse IgG1 monoclonal antibody for the RPE isotype control. After incubation for 20 minutes at RT or 37°C, samples were further diluted 1:10 in HBS/$Ca^{2+}$ or with HBS alone for the FITC and RPE isotype controls. Then, 50µl of each tube was transferred into a further 450 µl of the appropriate buffer; either HBS/$Ca^{2+}$ or HBS. Therefore, the overall dilution of the samples in every tube was 1/1000. The samples were then run immediately in the flow cytometer.

Subsequent experiments which used PRP and washed platelets with inhibitors were slightly modified; platelets were pre-treated with inhibitors for 30 minutes and then either activated for 10 minutes, or treated with ABT-737 or ABT-263 for 2h at 37°C instead of RT. Then 5µl of treated and untreated platelets were processed using the same protocol described above for the annexin-V binding assay.

### 2.4.2.2 Measurement of platelet activation markers

#### *2.4.2.2.1 Measurement of P-selectin expression*

The surface expression of P-selectin was assessed in either PRP or washed platelets following CRP-XL, TRAP or A23187 activation, or in apoptotic platelets treated with ABT-737 or ABT-263 as described previously (Goodall and Appleby 2004) with slight modification. Five µl of each sample (either PRP or washed platelets) was incubated

with P-selectin FITC antibody (2µl) or FITC–conjugated mouse IgG1 isotype control (2µl) for 10 minutes at RT in 50µl of HBS. 450µl of 0.2% formal-saline was then added to bring the total volume to 500µl. The samples were incubated for a further 10 minutes, then 50µl were transferred into a further 450 µl 00.2% formal-saline and analysed by flow cytometry. The platelets were identified according to their forward scatter and side scatter, which was then analysed for percentage positivity for P-selectin expression.

#### ***2.4.2.2.2 Fibrinogen binding assay***

The binding of platelets to fibrinogen was assessed in PRP following CRP-XL and TRAP activation, or in apoptotic platelets treated with ABT-737 or ABT-263 as described previously (Goodall and Appleby 2004) with slight modification. Five µl of PRP was added to LP3 tubes containing 45µl HBS buffer and 2µl FITC-conjugated rabbit polyclonal antibody to fibrinogen. The negative control was prepared in exactly the same way as the rabbit polyclonal antibody, but with the addition of 6mM EDTA, which chelates $Ca^{2+}$ and prevents the binding of fibrinogen to αIIbβ3, which is a calcium dependent process. After, a 10 minute incubation at RT, 450µl of 0.2% formal-saline was added to bring the total volume to 500µl. The samples were incubated for a further 10 minutes, then 50µl of each sample was transferred into 450µl 0.2% formal-saline and analysed by flow cytometry.

### 2.4.3 Aggregation studies

Platelet aggregation was measured in PRP ($150x10^{-9}$/L) using a PAP-8E aggregometer (Bio-Data Corporation®, UK), according to the turbidmetric method described by Born

and Cross (1963). The PRP was pre-warmed to 37°C for 2 minutes in a siliconised tube containing a magnetic stirrer. The full transmittance of 100% light transmission (blank) of the aggregometer was set up using autologous PPP. The baseline was set using 250µl PRP. PRP aggregation was then measured immediately after the addition of agonists for 6 minutes, under continuous stirring at 1,200 rpm at 37°C.

Primary aggregation (PA) and area under the curve (AUC) parameters were used to evaluate the percentage of aggregation in the study. Briefly, 250 µl of stirred PRP was stimulated with CRP-XL or TRAP. Initially, for each donor the maximum concentration of TRAP and CRP-XL-induced full aggregation was determined by running a dose-response for the agonist. To study the effect of inhibitors, PRP was incubated with inhibitors for 30 minutes at 37°C prior to the addition of the agonist. In experiments using the ABT compound, ABT-737 and ABT-263 were added to the whole blood or PRP for 2 hours at 37°C. After that, PRP was then obtained and used in the aggregation assay, as previously described.

### 2.4.5 Assessment of ROS by CM-H2DCFDA

Intracellular ROS was detected using fluorescence probe by flow cytometric analysis as described previously (Roberts*, et al* 1999) with s. 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCFDA) is a ROS-sensitive fluorescent probe which diffuses easily into platelets. The intracellular esterases cleave the acetate groups of the probe preventing it from diffusing out of the cell. ROS production results in fluorescence which is detected by flow cytometry in the FL1 channel. Freshly-isolated washed platelets were incubated with CM-H2DCFDA (25µM

final concentration) for 30 minutes in the dark at 37°C; because the probe was dissolved in DMSO, a DMSO control vehicle was run in parallel. Next, the washed platelets were activated with CRP-XL for 10 minutes at 37°C. After the incubation time, the samples were immediately placed in the flow cytometer. Fluorescence was measured as the MFI of the positive population.

### 2.4.6 Detection of lipid peroxidation by TBARS

Lipid peroxidation was evaluated in platelets before and after CRP-XL treatment, and the degree of peroxidation was assessed by measuring TBARS using an absorbance plate reader for monitoring the level of MDA as described previously (Pickering*, et al* 2004) with slight modification. Washed platelets were incubated for 30 and 60 minutes with and without CRP-XL and in two concentrations of $H_2O_2$ at 37°C. After that, washed platelets were diluted in PBS to a final volume of 500µl. 50µl of BHT (100mM, an antioxidant to prevent lipid peroxidation during the assay) and 1 ml of TBA reagent were added to each tube. All samples were boiled for 60 minutes at 90°C and then were cooled on ice. The samples were centrifuged at 13,000 rpm for 10 minutes to clear and sediment the cell debris. The supernatants were obtained and the absorbance was read at 532nm using an absorbance plate reader. An MDA standard curve was created by diluting MDA in 1% (v/v) $H_2SO_4$ to give a standard curve of 0, 5µM, 10µM, 20µM, 40µM and 80µM and treated in the same way as the sample. MDA was prepared and diluted in 1% (v/v) $H_2SO_4$ 2 hours prior to assay under continuous stirring at RT.

## 2.5 Statistics

Statistical analysis was performed with Graph Pad Prism software version 5.0 for Windows (GraphPad Software Inc., San Diego, CA, USA). Unless otherwise stated, results are presented as mean ± standard error (SEM). The use of SEM is mainly due to the inter-individual variability both in response to CRP-XL and in thrombin generation. A Student's t-test analysis for paired/unpaired data was used to compare the differences between two groups when data were normally distributed. An analysis of variance (ANOVA) was used to give a comparison between two or more groups, followed by the appropriate post-test. P-values were considered statistically significant if $< 0.05$.

# Chapter 3: Analysing the effect of PDMPs by the CAT: assay and pre-analytical variables

## 3.1 Introduction

The procoagulant activity of the MP surface is well studied in supporting the binding of coagulation complexes, which accelerates the formation of thrombin (Berckmans*, et al* 2001, Sims*, et al* 1989, Sinauridze*, et al* 2007). PDMPs are the most abundant type of MPs in the circulation and can be easily generated upon activation with an agonist such as collagen, and their level is often elevated in thrombotic disorders (Morel*, et al* 2006). Despite, the agreement on the role of MPs as useful biomarkers in physiological and pathological conditions, there is no agreement on pre-analytical and analytical procedures for their analysis, which is a significant drawback and source of potential variation between studies (Jy*, et al* 2004, Yuana*, et al* 2011, Zahra*, et al* 2011).

MP analysis is affected by different pre-analytical factors including the effects of blood sampling, the type of anticoagulant used for blood collection, the needle bore size (Shah*, et al* 2008), the centrifugation protocol, the freezing-thawing temperature (Dey-Hazra*, et al* 2010, Shah*, et al* 2008, Trummer*, et al* 2009), and analytical factors including the use of calibrated beads for MP detection (Lacroix*, et al* 2010b), when studied by the common method, flow cytometry. In addition, very few attempts have been made to look at the influence of the pre-analytical and analytical conditions on MP detection using functional assays including the thrombin generation assay (Chantarangkul*, et al* 2004, Gerotziafas*, et al* 2005, Hemker*, et al* 2003, Vanschoonbeek*, et al* 2004), which makes comparing these studies and determining

their clinical importance difficult. However, thrombin generation assay is important because it has been proposed as a tool for investigating plasma hypercoagulability and hypocoagulability (Hemker*, et al* 2003), which could be a clinically-useful, globally-available test of coagulability (Hemker and Beguin 2000) and recently has been used for MP detection in thrombotic diseases (Bidot*, et al* 2008, Hron*, et al* 2007). Keuren *et al* (2006) have looked at the effects of storage on MPs obtained from platelet concentrates using flow cytometry, and on thrombin generation initiated with 1pM TF in plasma and other have looked at pre-analytical variables, particularly the interference of contact factor activation with thrombin generation in plasma (Luddington and Baglin 2004, Van Veen*, et al* 2008). Other groups have also looked at the influence of TF and PL on the analysis (Gerotziafas*, et al* 2005, Hemker*, et al* 2003, Vanschoonbeek*, et al* 2004).

Therefore the aim of this study is to comprehensively investigate the pre-analytical and analytical variables to evaluate the CAT assay for detection of MPs, and specifically PDMPs, using the four commonly-used reagents (PPP normal [5pM TF/4µM PL], PPP low [1pM TF/4µM PL], PRP/NIBSC [1pM TF/~2µMNIBSC PL] and PRP [1pM TF]), because of their clinical importance. This was achieved by (i) using different reagents of CAT to trigger thrombin generation; (ii) investigating the influence of pre-analytical conditions, including freeze-thaw cycles, temperature and centrifugation speeds on the activity of PDMPs in the plasma; and (iii) confirming the dependency of thrombin generation assay on the presence of PDMPs.

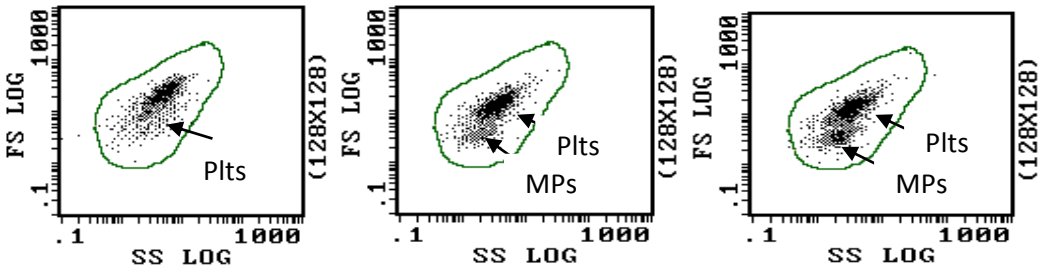
## 3.2 Results

### 3.2.1. The effect of PDMPs on thrombin generation measured with different reagents.

The following preliminary experiments were conducted to determine the optimum concentration of CRP-XL for inducing maximum MP generation from PRP using flow cytometry to measure annexin-V binding (Fig 3.1), and thrombin generation by the PDMPs using the CAT assay (Fig 3.2). PRP was prepared from citrated blood samples from ten healthy donors in the presence of CTI (18.3μg/ml). Then, PPP was separated, with or without stimulation of the platelets with CRP-XL (0-10.0μg/ml).

Representative histograms of the flow cytometric analysis (Fig 3.1a) illustrate that activated PRP with CRP-XL has a small MP population (middle and right histograms) that has a lower size (FS) and granularity (SS) than the platelet population, which is not seen in the unactivated (resting) platelets (left histogram). In a concentration-dependent analysis, CRP-XL increased annexin-V percentage positivity significantly (Fig 3.1b; $p<0.0001$) and (MFI data not shown). PS exposure increased with increasing concentrations of CRP-XL (one-way ANOVA <0.05), and reached a plateau at 2.0μg/ml. CRP-XL at a higher concentration (10.0μg/ml) induced a further slight increase in annexin-V positive events, but was not statistically different from that observed at 2.0μg/ml ($p>0.05$ using paired t-test). Therefore, CRP-XL at 2.0μg/ml was selected as the optimal concentration in this study, and all subsequent experiments were performed with CRP-XL at a concentration of 2.0μg/ml, unless otherwise stated.

A) Resting sample CRP-XL (2.0 µg/ml) CRP-XL (10.0 µg/ml)




B)

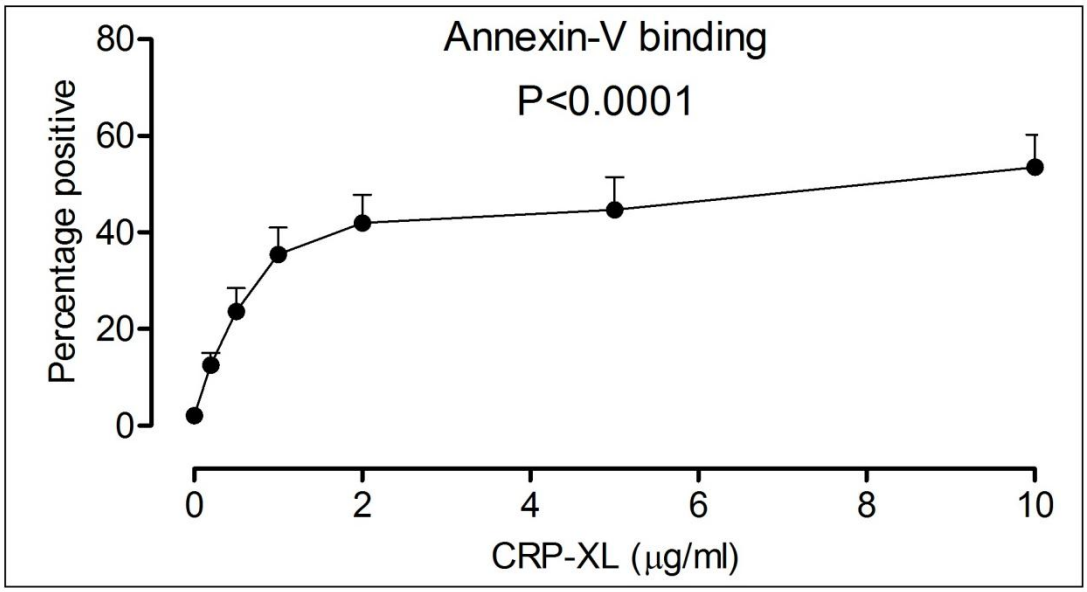
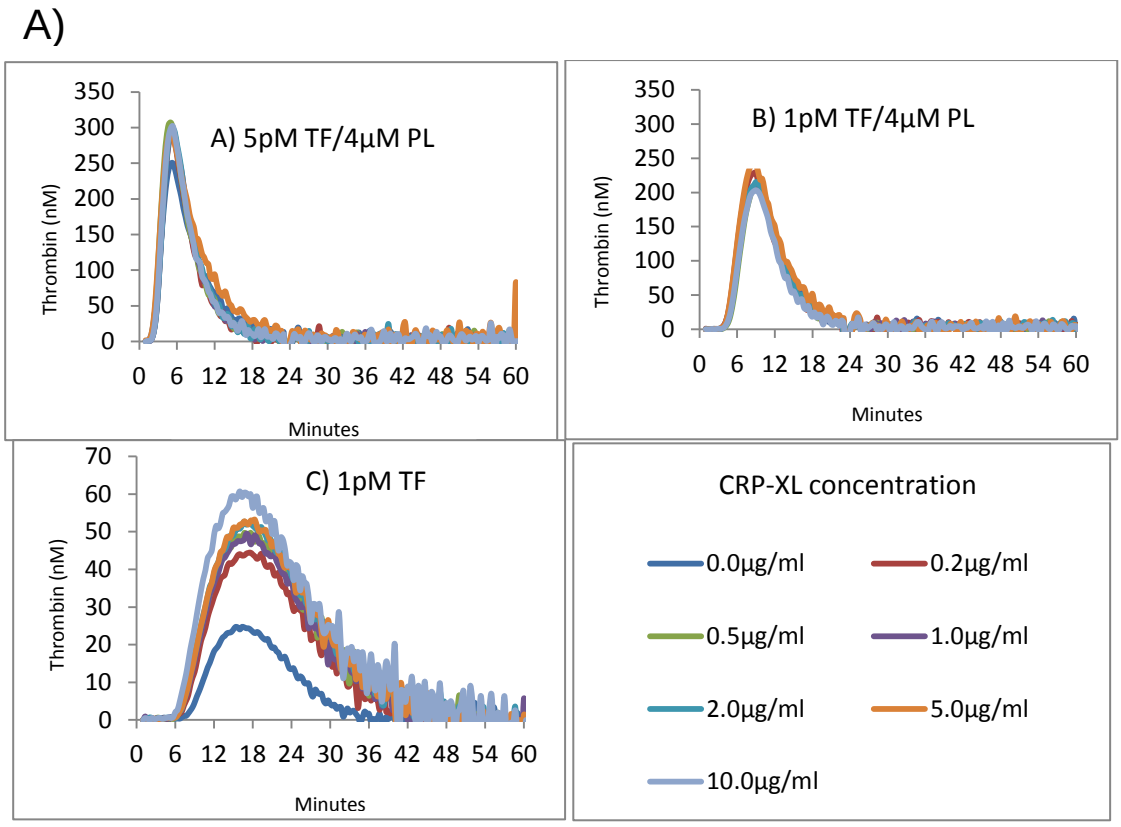



**Fig 3.1. Effect of CRP-XL (dose-response) on PS exposure assessed by annexin-V binding assay.** (A) Representative histograms of the presence of MPs in PRP**.** (B) Numerical data from 10 healthy individuals. Platelets (Plts) were incubated with increasing concentrations of CRP-XL for 10 min at RT and then PS exposure was assessed by annexin-V binding assay using flow cytometry. Microparticles (MPs). Results are shown as mean±SEM; (One-way ANOVA).

Also, PPP separated, with or without stimulation of the platelets with CRP-XL (0-10.0µg/ml) was analysed in the CAT assay with the PPP normal (5pMTF/4µMPL), PPP low (1pMTF/4µMPL), and PRP (1pM TF) (n=3-9) reagents (Fig 3.2). Representative thrombin generation curves are shown in Fig 3.2a, which illustrates the differences in the curves obtained for the three different reagents, and the influence of the preformed procoagulant PDMP in the plasma obtained by dose-response of CRP-XL on the measurements of the four recorded parameters; namely the LT, ttP, PT and ETP. In line with previous studies, the three reagents produced markedly different curves, reflecting the relative concentrations of TF and PL, with time parameters (LT and ttP) increasing as the concentrations of both TF and PL decreased, and the amount of thrombin parameters (shown by PT and ETP) decreasing as the concentrations of both TF and PL decreased. In general the LT and ttP are mainly affected by TF concentration, while the PT and ETP and mainly affected by PL concentration. In addition, the analysis showed that maximum thrombin was generated with the lowest concentration of CRP-XL used (Fig 3.2b). It is clearly observed from the analysis that the 1pM TF reagent was the only reagent that is most sensitive to the level of the preformed PDMPs in the PPP, generated by the dose-response activation of the PRP with CRP-XL (Fig 3.2b), this will be confirmed later in this chapter. The 2.0µg/ml CRP-XL was also selected as the optimal concentration in the generation of thrombin in plasma driven by preformed procoagulant PDMPs in the CAT assay.



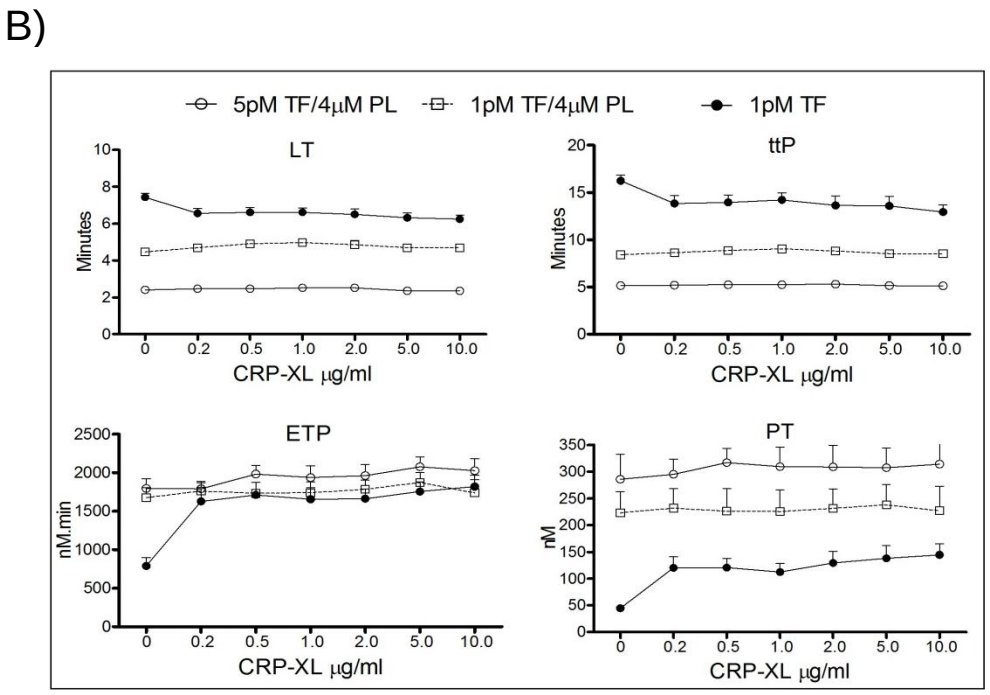



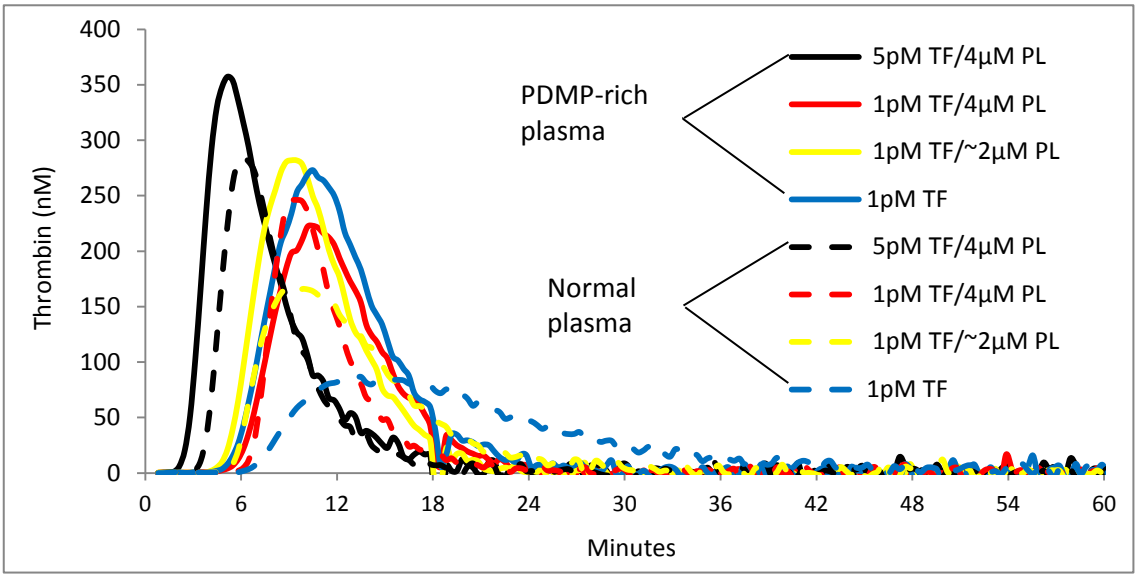

**Fig 3.2. Does-dependency effect of CRP-XL activation on thrombin generation measured in PPP.** PRP was stimulated with CRP-XL (dose-response) for 10 minutes at 37°C, then PDMP-rich plasma was prepared following centrifugation of PRP at 1800 g for 30 minutes. Thrombin generation was triggered with PPP normal (5pMTF/4µMPL) reagent (open circles; n=3), PPP normal (1pMTF/4µMPL) reagent (open squares; n=3) or PRP (1pMTF) reagent (closed circles; n=9). Panels A: Representative example of thrombin generation curves from a single run with each of the three reagents. Panel B: Numerical data are shown as mean±SEM.

Then, thrombin generation was measured in PPP separated with and without stimulation of the platelets with CRP-XL (2.0µg/ml); those PPP separated after CRP-XL will have preformed procoagulant PDMPs (Fig 3.3). Representative thrombin generation curves are shown in Fig 3.3a, which illustrates both the differences in the curves obtained for the four reagents (PPP normal [5pM TF/4µM PL], PPP low [1pM TF/4µM PL], PRP/NIBSC PL [1pM TF/2µMNIBSC PL] and PRP [1pM TF]), and the influence of PDMP-rich plasma (filled bars and solid lines) on the measurements of thrombin generation as compared to normal plasma (open bars and dashed lines) as described previously.

In the absence of exogenous PL (i.e. using the PRP reagent containing only 1pMTF), the thrombin generation curve was flatter and gave significantly lower PT and ETP and longer LT and ttP values compared to reagents that contained exogenous PL (PPP normal, PPP low and PRP/NIBSC reagents). When comparing thrombin generation by the different reagents with either normal plasma or PDMP-rich plasma, in the presence of exogenous PL (PPP normal, PPP low and PRP/NIBSC reagents) the level of TF (5pM vs. 1pM) had a marked effect on the rate of the reaction (both the LT and ttP, comparing 5pMTF vs. 1pMTF, paired t-test $p<0.001$) and a slight effect on amount of thrombin generated (both ETP and PT). The PRP reagent (1pM TF) gave significantly longer LT and ttP and lower ETP and PT compared to the other reagents. When the number of PDMPs in the plasma was increased by activating the platelets with CRP-XL prior to separation of the plasma, the reactions with all four reagents were greater. The extent of the effect is shown clearly in Fig 3.3b, which gives the numerical data from samples taken from six donors. Here it can be seen that although the PDMPs

(filled bars) decreased the LT and ttP, the effect was small and, apart from the ttP measured with the PPP low (1pM TF/4μM PL) reagent, was not statistically different ($p>0.05$) to normal plasma (open bars) with PPP reagent with exogenous PL. Only the PRP (1pM TF) reagent gave a clear and significant separation of the PDMP-rich plasma compared to normal plasma in all parameters. The LT and ttP were significantly shorter, and the PT and ETP values were significantly higher in PDMP-rich plasma (filled bars) compared to normal plasma (open bars), demonstrating the sensitivity of the CAT assay with this reagent to the endogenous PL in the form of PDMPs. It can be observed from the previous analysis that the best reagent than can identify the contribution of PDMPs on thrombin generation is the PRP (1pM TF) reagent.

A)




B)

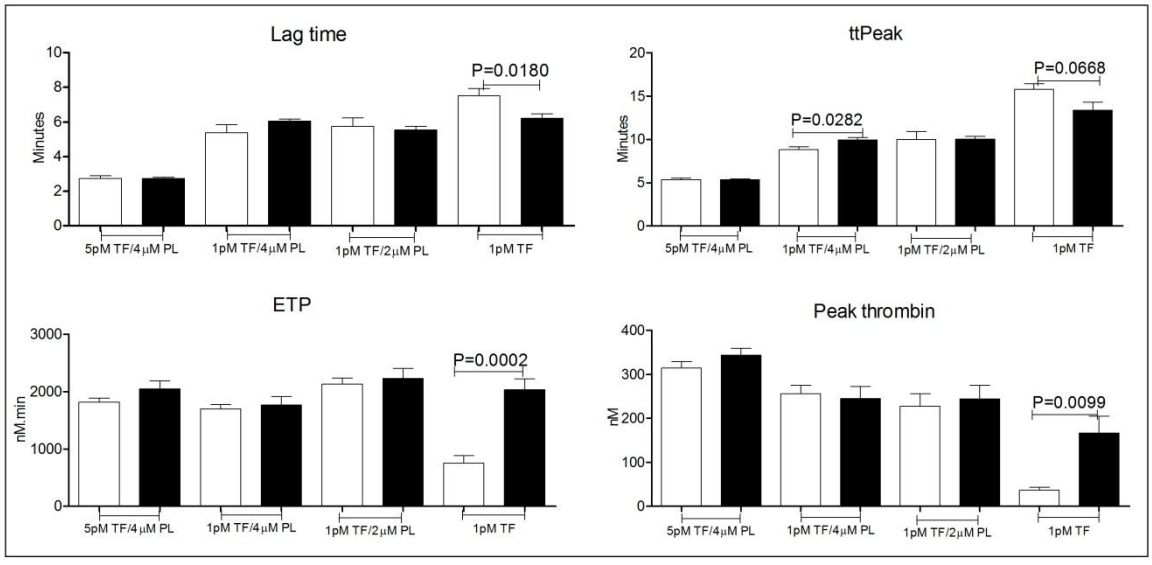



**Fig 3.3. Thrombin generation measured in normal and PDMP-rich plasma.** Thrombin generation was initiated with PPP normal (5pMTF/4µMPL) reagent, PPP low (1pMTF/4µMPL) reagent, PRP/NIBSC (1pMTF/~2µMPL) reagent and PRP (1pMTF) reagent in normal (open bars and dashed lines) and PDMP-rich plasma (filled bars and solid lines). A) Representative example of thrombin generation curves obtained from the same donor with and without CRP-XL stimulation. B) The numerical data ( mean±SEM from 6 donors). Differences between plasma and PDMPs in each reagent were determined using paired t-test analysis.

## 3.2.2. Assay precision

### 3.2.2.1. Inter-assay precision

The inter-assay variability of thrombin generation (between-run imprecision) was assessed using the PPP normal (5pM TF/4µM PL), PPP low (1pM TF/4µM PL) and PRP (1pM TF) reagents by repeating the analysis of pooled plasma 15 times on different days. The %CV values were relatively similar between the reagents, and were all below 13%, with the exception of the PT measurement with the PPP low (1pM TF/4µM PL) reagent (18.8%) (Table 3.1). Overall, the LT and ttP had marginally lower %CVs compared to the PT and ETP measurements.

### 3.2.2.2. Intra-assay (within run) precision

The focus was on the PRP reagent (1pM TF) as it is the reagent of choice, so the intra-assay variability of thrombin generation with the PRP reagent was assessed by repeating the measurement of three different plasma preparations on the same 96-well plate; namely pooled plasma (22 times), a normal plasma (11 times) and a PDMP-rich plasma (24 times). The intra-assay CV was less than 5% for all parameters of thrombin generation, with the exception of the measures of ETP and PT in normal plasma which were both <10% (Table 3.2).

| Inter-assay %CV | 5pM TF/4µM PL | 1pM TF/4µM PL | 1pM TF |
|---|---|---|---|
| L T (min) | 10.6% | 10.7% | 10.2% |
| ttP (min) | 10.5% | 9.6% | 8.4% |
| ETP(nM*min) | 12.3% | 12.2% | 10.6% |
| PT (nM) | 7.5% | 18.8% | 12.9% |

**Table 3.1. The inter-assay variability of thrombin generation.** Inter-assay variability of assay was assessed by repeating the measurement of pooled plasma 15 times on different days and at different times of the day with the PPP normal (5pM TF/4µMPL) reagent, the PPP low (1pM TF/4µMPL) reagent or the PRP (1pMTF) reagent. CV: coefficient of variation.

| Intra-assay %CV | Pooled plasma 22 times | Normal plasma 11 times | PDMP-rich plasma 24 times |
|---|---|---|---|
| L T (min) | 3.9% | 3.4% | 2.5% |
| ttP (min) | 2.8% | 9.6% | 3.2% |
| ETP(nM*min) | 2.4% | 7.5% | 1.6% |
| PT (nM) | 3.9% | 3.4% | 2.5% |

**Table 3.2**. **The intra-assay variability of thrombin generation measured using the 1pMTF reagent**. The intra-assay variability of the assay was assessed by repeating the measurement of pooled plasma 22 times, normal 11 times and PDMP-rich plasma 24 times on the same plate. Thrombin generation was triggered with the PRP (1pMTF) reagent. CV: coefficient of variation.

### 3.2.3. Effect of contact factor pathway activation

The contact factors, including FXIIa, have been shown to influence (Rand*, et al* 1996) and increase the variability of thrombin generation; this is seen most at low TF (<5pM) concentration. The addition of CTI, improves the measurement of thrombin generation in clinical samples (Luddington and Baglin 2004, Van Veen*, et al* 2008). Whole blood was therefore collected with and without the addition of CTI to confirm the effect of contact factor activation on thrombin generation. A concentration of 18.3µg/ml was used, based on previous studies (Luddington and Baglin 2004, Van Veen*, et al* 2008).

#### 3.2.3.1. Effect of CTI on pooled plasma

Pooled plasma with and without CTI was analysed using the PPP normal (5pM TF/4µM PL; table 3.3a), PPP low (1pM TF/4µM PL; table 3.3b) and PRP (1pM TF; table 3.3c) reagents. The analysis showed higher thrombin generation in pooled plasma without CTI (PP-CTI) compared to that with CTI (PP+CTI) for all reagents (table 3.3). In the absence of CTI, thrombin generation was faster (short LT and ttP) with all reagent but this was only statistically significant with the PRP (1pM TF) reagent ($p \leq 0.001$) or the PPP low (1pM TF/4µM PL) for ttP ($p=0.0094$). Similarly ETP and PT were slightly higher but with only PT reaching statistical significance with the PPP low reagent ($p=0.0006$).

A)

| 5pM TF/4µM PL | Pooled plasma | | | %CV | |
|---|---|---|---|---|---|
| **Parameter** | **PP-CTI** | **PP+CTI** | **P value** | **PP-CTI** | **PP+CTI** |
| L T (min) | 2.9+0.1 | 2.8+0.1 | 0.3559 | 10.6 | 10.9 |
| ttP (min) | 5.6+0.2 | 6.5+0.1 | 0.5835 | 10.5 | 7.6 |
| ETP(nM*min) | 2010+62 | 1935+51 | 0.2044 | 10.5 | 12.3 |
| PT (nM) | 341.2+6.6 | 334.2+6.6 | 0.1649 | 7.5 | 7.9 |

B)

| 1pM TF/4µM PL | Pooled plasma | | | %CV | |
|---|---|---|---|---|---|
| **Parameter** | **PP-CTI** | **PP+CTI** | **P value** | **PP-CTI** | **PP+CTI** |
| L T (min) | 6.8+0.2 | 7.2+0.2 | 0.1376 | 9.4 | 10.7 |
| ttP (min) | 10.6+0.3 | 12.2+0.3 | **0.0004** | 9.6 | 9.6 |
| ETP(nM*min) | 1640+75.7 | 1627+51.4 | 0.7645 | 17.9 | 12.2 |
| PT (nM) | 208.8+12.9 | 178.5+9.3 | **0.0006** | 9.3 | 8.7 |

C)

| 1pM TF | Pooled plasma | | | %CV | |
|---|---|---|---|---|---|
| **Parameter** | **PP-CTI** | **PP+CTI** | **P value** | **PP-CTI** | **PP+CTI** |
| L T (min) | 7.6±0.2 | 8.4±0.2 | **0.0007** | 11.0 | 10.2 |
| ttP (min) | 16.4±0.7 | 18.9±0.4 | **0.0010** | 13.3 | 8.4 |
| ETP(nM*min) | 1595±66 | 1558±43 | 0.4091 | 16.0 | 10.6 |
| PT (nM) | 81.2 | 71.3 | 0.0899 | 22.3 | 12.9 |

**Table 3.3. Comparison of thrombin generation measurement in pooled plasma with and without CTI.** Pooled plasma with (+) and without (-) CTI (18.3µg/ml) were analyzed by CAT using PPP normal (5pMTF/4µMPL; table a), PPP low (1pMTF/4µMPL; table b) and PRP (1pMTF; table c) reagents. Data are shown as mean±SEM from 15 measurement of pooled plasma. Differences between pooled plasma with (PP+CTI) and without (PP-CTI) CTI in each reagent were determined using paired t-test analysis. %CV: coefficient of variation.

### 3.2.3.2. Effect of CTI on individual plasma

To further assess the influence of contact pathway activation on thrombin generation triggered with low concentrations of TF (1pM TF) in fresh samples, whole blood was collected from six healthy subjects with (+) and without (-) the addition of CTI (18.3µg/ml) (table 3.4). The plasma was analysed in the CAT using 1pMTF and different concentration of PL [4µM PL (PPP low reagent), ~2µMNIBSC PL (PRP/NIBSC reagent) or no PL (PRP reagent)]. The higher concentration of TF (5pM) was not used because the contribution of the contact factor pathway was seen only at low concentrations of TF (1pM) in both the previous analysis of the pooled plasma (section 3.2.3.1) and in the data in the literature (Luddington and Baglin 2004, Van Veen*, et al* 2008). In this analysis, slightly elevated thrombin generation was seen in the absence of CTI (-) using all three reagents (table 3.4), which reached statistical significance for ttP ($P<0.05$) using the PPP low reagent and in PT ($p<0.05$) using either the PPP low reagent or the PRP/NIBSC PL reagent.

A)

| 1pM TF/4µM PL | Individual plasmas (n=6) | | | %CV | |
|---|---|---|---|---|---|
| **Parameter** | **plasma-CTI** | **Plasma+CTI** | **P value** | **plasma-CTI** | **Plasma+CTI** |
| L T (min) | 5.4±0.3 | 5.5±0.4 | 0.1386 | 16.9 | 18.1 |
| ttP (min) | 9.7±0.5 | 10.4±0.6 | **0.0201** | 13.1 | 16.3 |
| ETP(nM*min) | 1498±164 | 1429±169 | 0.0942 | 29.0 | 31.2 |
| PT (nM) | 177±22.4 | 147±21.3 | **0.0231** | 33.5 | 38.4 |

B)

| 1pM TF/2µM NIBSC PL | Individual plasmas (n=6) | | | %CV | |
|---|---|---|---|---|---|
| **Parameter** | **plasma-CTI** | **Plasma+CTI** | **P value** | **plasma-CTI** | **Plasma+CTI** |
| L T (min) | 9.6±0.9 | 10.5±1.1 | 0.1311 | 21.6 | 25.5 |
| ttP (min) | 18.5±1.0 | 19.5±1.2 | 0.2848 | 12.9 | 15.2 |
| ETP(nM*min) | 1293±129 | 1208±186 | 0.1895 | 24.4 | 37.7 |
| PT (nM) | 74.5±7.6 | 68.2±9.8 | **0.0458** | 24.9 | 35.0 |

C)

| 1pM TF | Individual plasmas (n=6) | | | %CV | |
|---|---|---|---|---|---|
| **Parameter** | **plasma-CTI** | **Plasma+CTI** | **P value** | **plasma-CTI** | **Plasma+CTI** |
| L T (min) | 11.0±1.0 | 11.0±1.0 | 0.939 | 21.1 | 28.8 |
| ttP (min) | 20.3±1.3 | 21.3±1.1 | 0.1251 | 12.9 | 15.1 |
| ETP(nM*min) | 855±154 | 799±188 | 0.7121 | 44.0 | 58.00 |
| PT (nM) | 45±8.3 | 40±8.8 | 0.5447 | 45.4 | 53.7 |

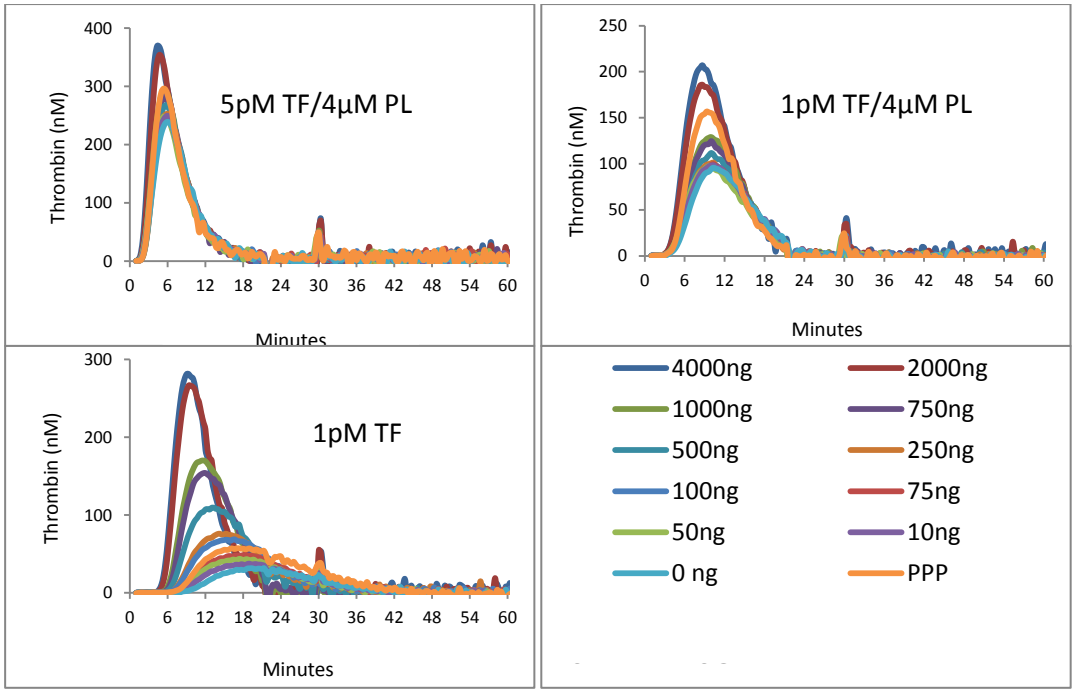
**Table 3.4**. **Effect of CTI on thrombin generation measured in individual plasma.** Fresh plasma with (+) and without (-) CTI (18.3µg/ml) was prepared from whole blood from 6 healthy individuals. Thrombin generation was analyzed using PPP low (1pMTF/4µMPL) reagent, PRP/NIBSC (1pMTF/2µMPL) reagent and PRP reagent (1pMTF). Data are shown as the mean±SEM and the difference between pooled plasma with (+) and without (-) CTI in each reagent was determined using paired t-test analysis. %CV: coefficient of variation.

### 3.2.4. Sensitivity of CAT to PL

To confirm the sensitivity of CAT for PL, thrombin generation was measured with three different reagents (PPP normal [5pM TF/4μM PL], PPP low [1pM TF/4μM PL] and PRP [1pM TF]) in plasma, with a titration of exogenous NIBSC PL. First, a standard preparation of micellar NIBSC PL was diluted in filtered pooled plasma to give a final concentration ranging between 10ng/ml and 4μg/ml PL. Representative curves from one experiment using the three reagents (Fig 3.4a) illustrate that as the concentration of exogenous PL increased, the thrombin generation curve increased in height and shifted to the left, indicating a faster rate and greater level of thrombin generation; this was clearly observed with the PRP reagent (1pM TF), but less clear with PPP normal (5pM TF/4μM PL) and PPP low (1pM TF/4μM PL) reagents. The numerical data for one experiment is shown in Fig 3.4b, and it can be seen that with the PRP (1pM TF) reagent, all four parameters measured were sensitive to the concentration of exogenous PL, with LT and ttP showing a relatively linear relationship, and the PT and ETP values increasing when the PL concentration was above 0.5μg/ml. However, there was little effect on the PL concentration on any of the parameters of thrombin generation when measured with the two PL-containing reagents, regardless of TF concentration.

To test whether the same was true for PL in the form of PDMPs, plasma obtained from PRP that had been maximally stimulated with CRP-XL (2μg/ml) was diluted with autologous filtered plasma to remove endogenous MPs and then analysed with all three reagents. Representative thrombin generation curves for dilutions of PDMP-rich plasma between neat and 1:1000, using all three reagents are illustrated in Fig 3.5a.

A)




B)

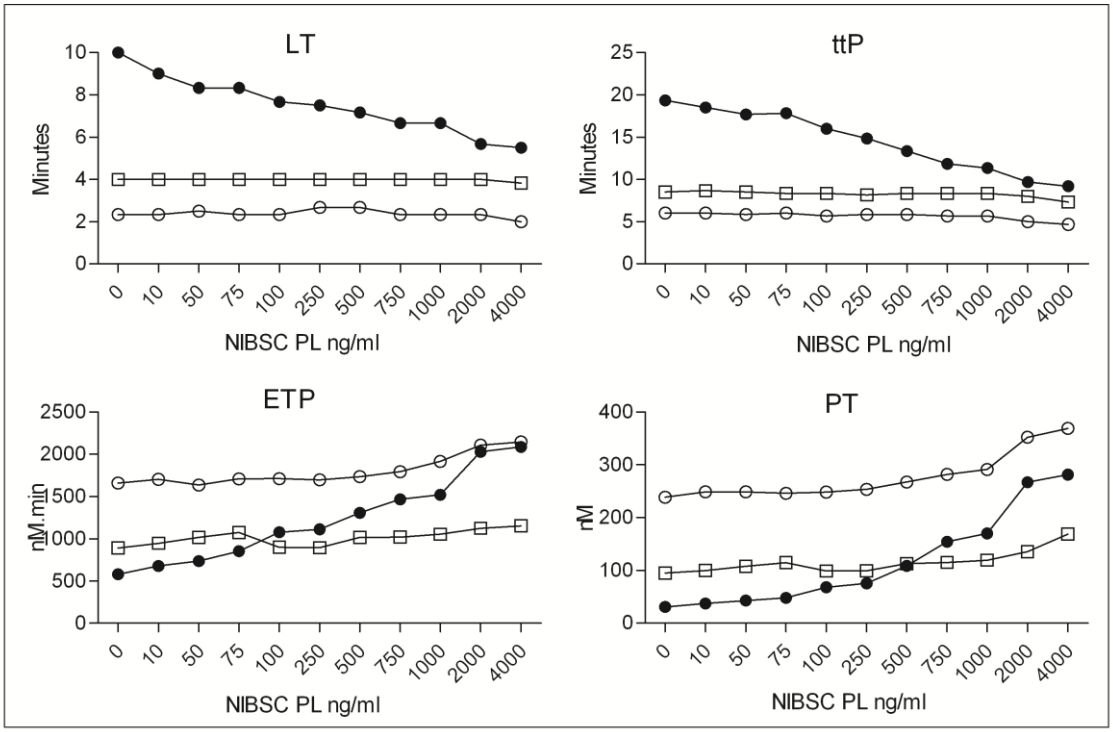
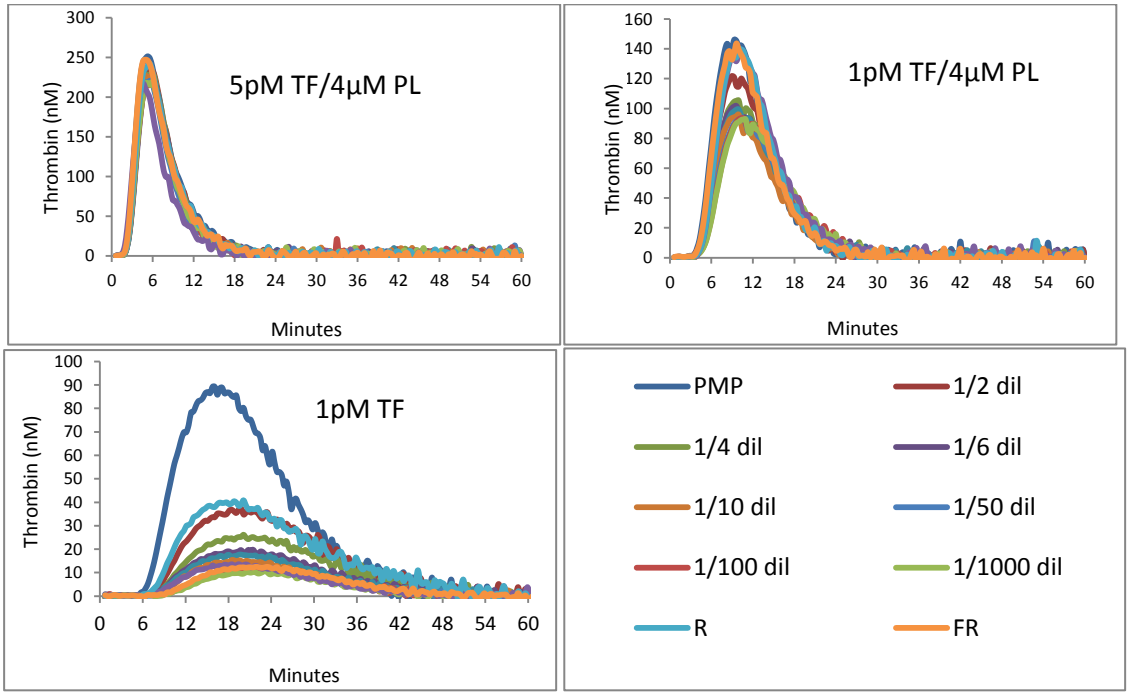



**Fig 3.4. Effect of exogenous phospholipids on thrombin generation in filtered pooled plasma.** Filtered pooled plasma was prepared by filtering pooled plasma through a 0.22µm filter. Different concentrations of PL (0, 10, 50, 75, 100, 250, 500, 750, 1000, 2000, 4000ng/ml) were added to the FPP. Then, the reaction was triggered with PPP normal (5pMTF/4µMPL) (open circles; two independent runs), PPP low (1pMTF/4µMPL) (open squares; two independent runs) or PRP (1pMTF) reagent (closed circles; three independent runs). Panels A: Representative example of thrombin generation curves from a single run. Panel B: Numerical data from one single run for PPP normal, PPP low and PRP reagents. Data are mean±SEM

A)




B)

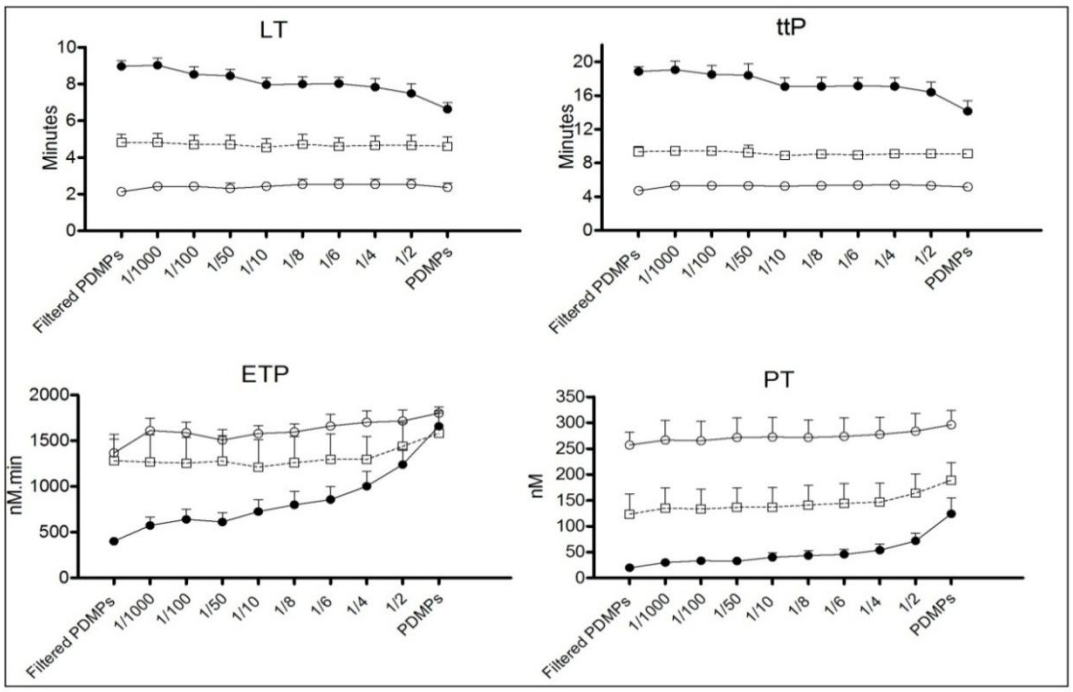



**Fig 3.5. Effect of diluting PDMP-rich plasma on thrombin generation.** PDMPs were generated from PRP stimulation with CRP-XL (2.0µg/ml) and then PPP was obtained by centrifugation at 1800*xg* for 30 minutes. PDMP-rich plasma was diluted with autologous filtered plasma. Thrombin generation was initiated with PPP normal (5pMTF/4µMPL) reagent (open circles; n=3); PPP low (1pMTF/4µMPL) reagent (open squares, n=3); and PRP (1pMTF) reagent (closed circles; n=6). Panels A: Representative example of thrombin generation curves from one donor with the PRP reagent only. Panel B: Numerical data from 3 separates run shown as mean±SEM. Resting (R), filtered resting (FR) dilution (dil).

As observed with the PL preparations, higher levels of PDMPs were associated with a faster and greater reaction, but this was seen only with the PRP (1pM TF) reagent (Fig 3.5b), and over the whole range of dilutions of the PDMP preparation. In addition the data shows that both ETP and PT are sensitive to the level of PL, with the suggestion that the most sensitive parameter of CAT for PL is PT.

### 3.2.5. Demonstration that the procoagulant effect is dependent on MPs

To demonstrate that the assay is sensitive to the procoagulant PL derived from activated platelets in the form of preformed MPs, plasma samples were analysed in the CAT assay before and after the removal of MPs. The most commonly used methods of MP removal are filtration and centrifugation. The effectiveness of these two was compared in normal plasma samples (open bars) (n=6) and in plasma samples rich in PDMPs (filled bars) (n=7), prepared in parallel from the same donors following CRP-XL stimulation of PRP (Fig 3.6 and Fig 3.7). Filtration through a 0.2µM acrodisc filter was compared with a second centrifugation step (13,000x*g* for 10 minutes), and thrombin generation was measured with the PRP reagent (1pM TF) (Fig 3.6), PPP normal (5pM TF/4µM PL) reagent, PPP low (1pM TF/4µM PL) reagent and PRP/NIBSC (1pM TF /2µMNIBSC PL) reagent (Fig 3.7).

Both methods - filtration and double centrifugation - significantly altered all four parameters of thrombin generation in both the normal (which contains MPs from circulation in the "resting" normal plasma that were removed by filtration/centrifugation) and PDMP-rich plasma samples (Fig 3.6; $p<0.05$ for all) with the 1pMTF, increasing the LT and ttP and reducing the levels for PT and ETP, which is consistent with a reduction in thrombin generation. A similar but smaller effect was

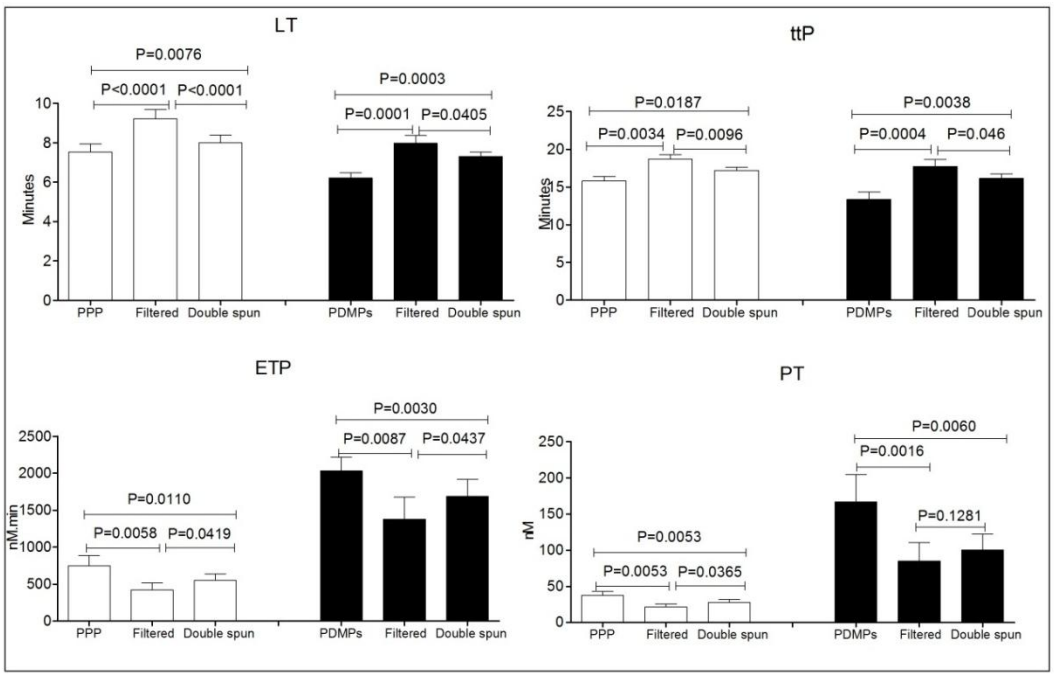
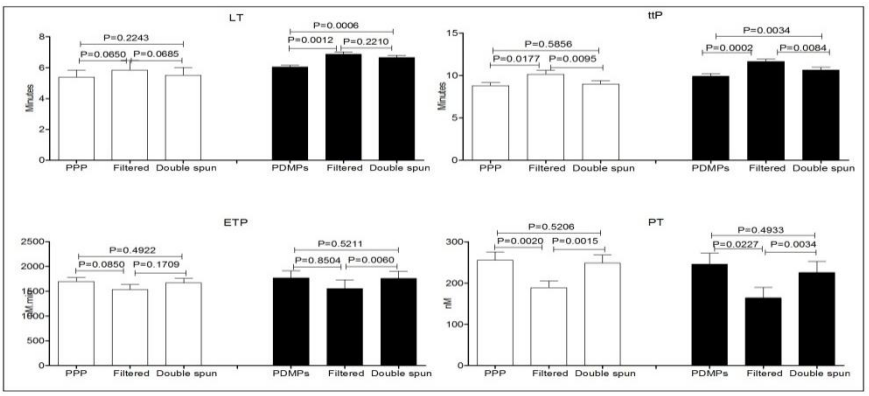



**Fig 3.6. Comparison between filtration and double centrifugation of plasma on thrombin generation measurement using the 1pM reagent.** Thrombin generation was measured in normal plasma (open bars) and PDMP-rich plasma (filled bars). Normal plasma (n=6) and PDMP-rich plasma (n=7) were either filtered through a 0.2µm acrodisc filter or centrifuged at 13000*xg* for 10 minutes and then analysed with the PRP (1pMTF) reagent. Data are shown as mean±SEM and differences in each method were determined using paired t-test analysis.

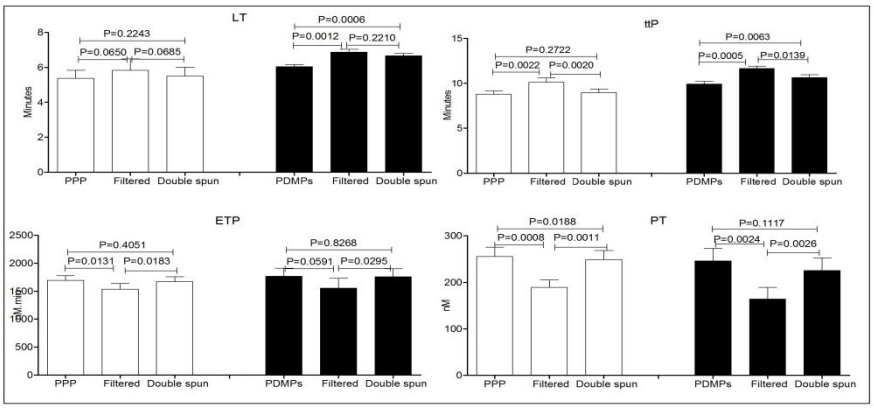
observed using the other reagents, which contained exogenous PL (Fig 3.7); this effect was expected because those reagents are less sensitive to procoagulant MPs in the plasma. Of the two methods, filtration had the greatest effect in both types of sample, which had a significant effect ($p<0.05$) for all measures with the exception of the PT value for the PDMP-rich plasma measured with the PRP reagent (1pMTF).

We then compared the effectiveness of two filtration devices: (i) the Pall acrodisc filter, and (ii) a 96-well manifold device (Ceveron MFU 500 filtration unit). Again both normal plasma (open bars, n=6) and PDMP-rich (filled bars, n=7) plasma was analysed using the PRP (1pMTF) reagent (Fig 3.8). Both methods of filtration significantly reduced both the rate and the amount of thrombin generated ($p< 0.05$). Of the two methods, the Ceveron unit had the greatest effect with the normal plasma (open bars; Fig 3.8), although this was not statistically significant for any of the parameters ($p>0.05$) apart from the ETP ($p=0.0165$, open bars). In PDMP-rich plasma (filled bars), the acrodisc filtration unit had the greatest effect on PT ($p=0.0012$) and ETP ($p=0.0042$). The analysis confirms that both filtration devices are effective in removing the effect of PDMPs on thrombin generation, which can be seen in the significant reduction in the ETP and PT with prolonged LT and ttP, with the Acrodisc filter being more effective.

A) 5pM TF/4µM PL




B) 1pM TF/4µM PL




C) 1pM TF/2µM NIBSC PL

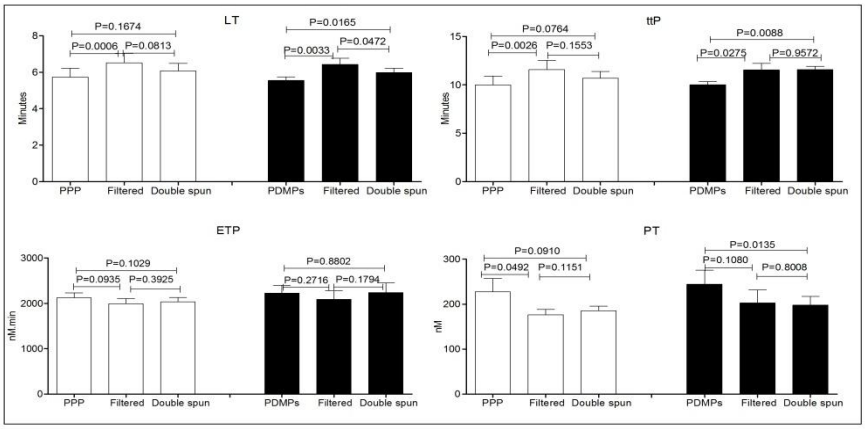



**Fig 3.7. Comparison between filtration and double centrifugation of plasma on thrombin generation measurement.** Thrombin generation was measured in normal plasma (open bars) and PDMP-rich plasma (filled bars). Normal plasma (n=6) and PDMP-rich plasma (n=7) were either filtered through a 0.2µm acrodisc filter or centrifuged at 13000 xg for 10 minutes and then analysed with PPP normal (5pMTF/4µMPL) reagent (A), PPP low (1pMTF/4µMPL) reagent (B) and PRP/NIBSC (1pMTF/2µMNIBSC PL) (C). Data are shown as mean±SEM and Difference in each method was determined using paired t-test analysis.

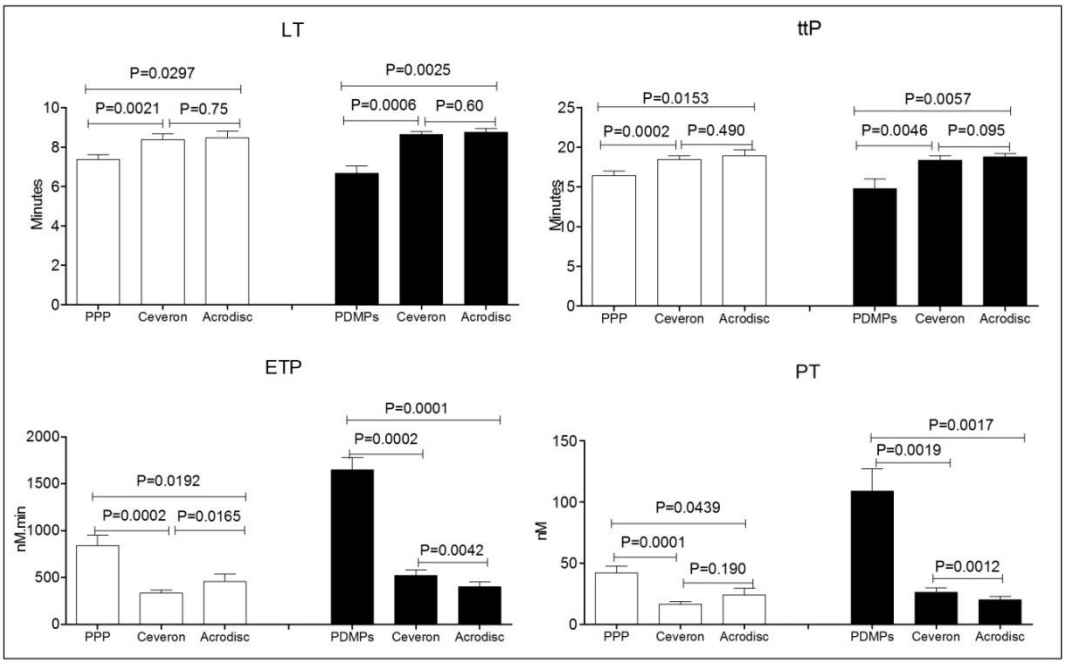



**Fig 3.8. A comparison between methods of filtration on thrombin generation using the 1pM TF reagent.** Thrombin generation was measured in pre-filtered plasma normal plasma (open bars A; n=6) and PDMP-rich plasma (filled bars B; n=7) compared to filtrated plasma with either ceveron or acrodisc. Data are shown as mean±SEM. Difference between plasma and PDMPs were determined using paired t-test analysis.

### 3.2.6. The effect of separating the plasma at different centrifugation speeds

There is no consensus on the exact method of MP preparation [for recent reviews see (Jy*, et al* 2004, Yuana*, et al* 2011)]. Therefore, methods of plasma separation were explored comparing the lab's method (1,800x*g* for 30 minutes) (open bars) with a widely used method of obtaining plasma for MP analysis using flow cytometry (1,500x*g* for 15 minutes followed by a second sharp spin at 13,000x*g* for 2 minutes) (filled bars) (Fig 3.9) (Jy*, et al* 2004) to determine their effect on the thrombin generation triggered with the PRP (1pM TF) reagent in both normal plasma and PDMP-rich plasma. Thrombin generation from normal plasma and PDMP-rich plasma showed slight differences with different donors and speeds (Fig 3.9). These slight differences were seen in all of the measures taken between the two separation speeds, and reached statistical significance in normal plasma for ttP ($p=0.0159$), and in PDMP-rich plasma for both ttP and PT, ($p=0.0189$ and $p=0.0342$ respectively). The two-step centrifugation retained more MPs, which resulted in a shorter time, and generated more thrombin. More activity was seen at the lower speed before and after CRP-XL stimulation.

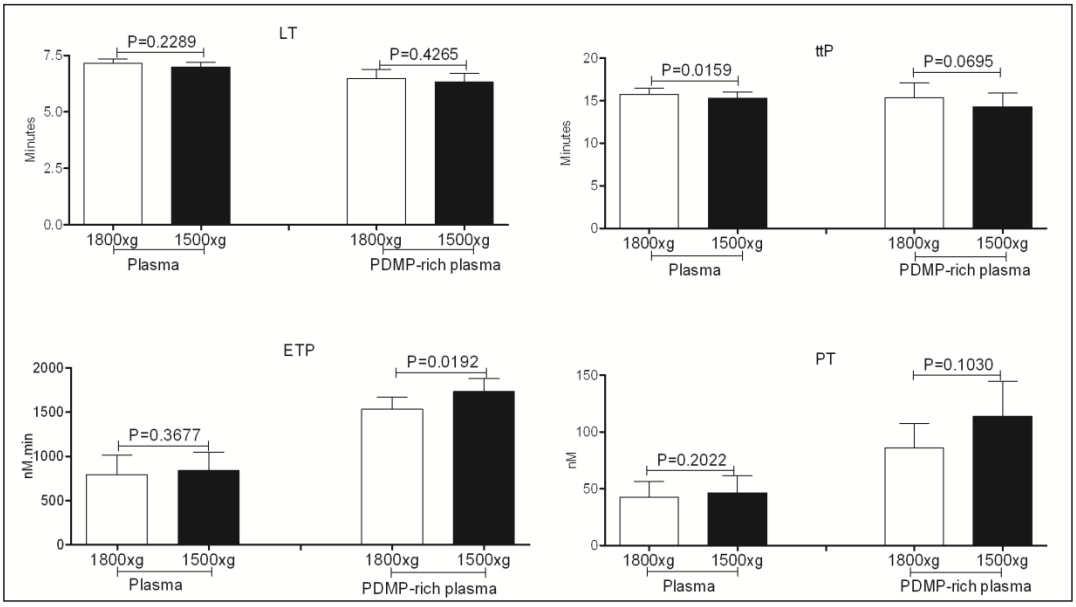



**Fig 3.9.The effect of different centrifugation methods on thrombin generation assay in normal plasma and PDMP-rich plasma triggered with the PRP (1pMTF) reagent.** Normal and PDMP-rich plasma were prepared by centrifugation at 1800*xg* (open bars) and 1500*xg* /followed by a sharp spin at 13,000*xg* for 2 minutes (filled bars). Data shown as mean±SEM (n=3) using paired t-test.

### 3.2.7. Effect of freezing and thawing on thrombin generation

Plasma separated from citrated blood or from PRP following stimulation with CRP-XL by a single centrifugation at 1800x*g* for 30 minutes at RT will be free of cells, but the majority of MPs should remain in suspension. If MPs are present, whether of platelet origin or from other cell types, this will affect thrombin generation. Removal of MPs, either by filtration through a 0.2µm filter, or by a second, more rigorous centrifugation step at 13,000x*g* for 10 minutes, should remove most if not all of the MPs. Similarly, plasma may be affected by freezing and thawing, producing either more reactive plasma, due to disruption of the membrane of MPs that may be present, or less, due to the denaturation of coagulation factors, particularly labile factors such as FVIII and FV. Finally, if the initial plasma separation is performed at 4°C, many coagulation factors will be preserved, but others may become activated, particularly FVII and FVIII (Favaloro*, et al* 2004), leading to altered values in the thrombin generation assay.

In addition, there are contrasting reports in the literature of the effect of freezing and thawing of plasma samples on MP integrity (Dey-Hazra*, et al* 2010, Trummer*, et al* 2009) and the subsequent effect on thrombin generation (Keuren*, et al* 2006). This study therefore investigated these questions in samples of normal and PDMP-rich plasma, with and without removal of the MPs by either filtration though a 0.2µm filter or by a second centrifugation at 13,000x*g* for 10 minutes before freezing. These samples were analysed in the CAT assay using the four reagents — PPP normal (5pMTF/4µMPL), PPP low (1pMTF/4µMPL), PPP normal (1pMTF/2µMNIBC PL) and PRP (1pMTF) — either immediately or after one or two cycles of freezing and thawing at -80°C. Separation of the plasma from whole blood at RT or 4°C had no effect compared

to RT on thrombin generation using the PPP normal reagent (data not shown); therefore, in the following experiments the separation of the plasma at 4°C was not included.

### 3.2.7.1. Effect of freeze/thaw cycles on normal and PDMP-rich plasma

Freezing and thawing had no effect on the measures of thrombin generation in the normal plasma, apart from a small, non-significant reduction in thrombin generation after 2 cycles using the PPP normal (5pMT F/4μM PL), PPP low (1pM TF/4μM PL) and PRP/NIBSC (1pM TF/2μM PL) reagents (table 3.5; one-way ANOVA) and a significant effect on (longer) ttP ($p=0.0409$) with the PPP low reagent. However, when analysed with the PRP reagent, the two measures of the amount of thrombin generated (PT and ETP) were significantly affected after freezing and thawing ($p<0.05$; one-way ANOVA); both ETP and PT significantly ($p<0.005$; paired t-test) went up after 1 freeze/thaw then fell after 2-but not back down to fresh level, which is indicative of either an effect on coagulation factors or a disruption of the level of MPs or apoptotic bodies if present from circulation in the plasma, leading to increase membrane permeability. To confirm if the effect is on MPs or coagulation, plasma with no MPs (they were removed either by filtration or a second rigorous centrifugation step) was analysed in parallel (described in the next sections 3.2.7.2 and 3.2.7.3).

Freezing and thawing had only a modest effect on PDMP-rich plasma (table 3.5). The LT and ttP increased slightly on repeated freezing and thawing, and these differences were significant ($p<0.05$) with all four reagents apart from in the ttP measured with the PRP reagent. However, the PT and ETP measures were not statistically altered. This

A)

| 5pM TF/4uM PL | Plasma (n=6) | | | | PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 2.7±0.2 | 2.8±0.2 | 2.9±0.2 | 0.8581 | 2.7±0.1 | 2.8±0.1 | 3.0±0.1 | **0.0222** |
| ttP (min) | 5.4±0.2 | 5.5±0.1 | 5.7±0.1 | 0.3291 | 5.4±0.1 | 5.7±0.1 | 5.9±0.2 | **0.0369** |
| ETP (nM.min) | 1816±72 | 1729±74 | 1567±93 | 0.1182 | 2046±137 | 2125±159 | 2140±161 | 0.8984 |
| PT(nM) | 315±14 | 306±12 | 269±21 | 0.1354 | 343±15 | 348±20 | 345±20 | 0.9843 |

B)

| 1pM TF/4uM PL | Plasma (n=6) | | | | PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 5.4±0.5 | 5.3±0.4 | 5.6±0.5 | 0.9086 | 6.0±0.1 | 6.6±0.2 | 6.9±0.1 | **0.0008** |
| ttP (min) | 8.8±70.4 | 9.0±20.3 | 9.9±0.2 | **0.0409** | 9.9±0.3 | 11.0±0.4 | 11.7±0.4 | **0.0067** |
| ETP (nM.min) | 1695±81 | 1532±80 | 1414±107 | 0.1200 | 1769±142 | 1759±152 | 1771±172 | 0.9984 |
| PT(nM) | 256±20 | 234±24 | 188±32 | 0.1936 | 246±27 | 213±29 | 205±28 | 0.5629 |

C)

| 1pM TF/2uM NIBSCPL | Plasma (n=6) | | | | PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 5.7±0.5 | 6.1±0.3 | 6.8±0.4 | 0.1938 | 5.6±0.2 | 6.3±0.2 | 7.2±0.3 | **0.0006** |
| ttP (min) | 10±0.9 | 10.8±0.5 | 12.1±0.7 | 0.1503 | 10.0±0.3 | 11.4±0.6 | 12.6±0.8 | **0.0305** |
| ETP (nM.min) | 2128±104 | 2066±99 | 1820±84 | 0.0888 | 2230±173 | 2259±168 | 2104±237 | 0.8362 |
| PT(nM) | 227.5±29.1 | 205.8±14.1 | 159.7±9.0 | 0.0706 | 244.3±31 | 230.1±28 | 206.3±35 | 0.6998 |

D)

| 1pM TF | Plasma (n=6) | | | | PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 7.5±0.4 | 7.2±0.2 | 8.1±0.5 | 0.2556 | 6.2±0.2 | 7.2±0.2 | 7.9±0.4 | **0.0027** |
| ttP (min) | 15.8±0.6 | 15.8±0.7 | 17.6±0.9 | 0.1873 | 13.4±1.0 | 14.8±0.9 | 15.7±1.3 | 0.3171 |
| ETP (nM.min) | 751±137 | 1446±127 | 1252±194 | **0.0178** | 2032±187 | 2065±179 | 2000±234 | 0.9740 |
| PT(nM) | 37.5±5.9 | 81.1±10.4 | 68.8±12.4 | **0.0202** | 166.8±37.9 | 161.5±32.9 | 157.9±39.3 | 0.9854 |

**Table 3.5: Effects of freeze/thaw cycles on normal and PDMP-rich plasma on thrombin generation.** Thrombin generation was measured either in normal plasma or PDMP-rich plasma after subjecting 2 freeze/thaw cycles with (A) 5pM TF/4µM PL, (B) 1pM TF/4µM PL, (C) 1pM TF/2µM NIBSC PL and (D) 1pM TF. Data are shown as mean±SEM. Differences between the three cycles were determined by One-WAY ANOVA.

suggests that the effect of freezing and thawing masked by the high level of MPs in the plasma. In addition, the data suggest that the MPs in normal plasma might behave differently from PDMPs generated from platelets by CRP-XL stimulation. It should, however, be emphasized that the changes seen were small and some of the statistical differences could be due to false positive associations caused by multiple testing. It is therefore reasonable to suggest that accurate estimates of thrombin generation can be obtained from stored plasma samples, provided they are treated in the same way.

### 3.2.7.2. Effect of freeze/thaw cycles on filtered normal and PDMP-rich plasma

The influence of freezing and thawing was then investigated using the same samples of plasma and PDMP-rich plasma following filtration before freezing and thawing cycles. Filtration was used to remove most, if not all, of the MPs from the plasma to determine if the changes seen in stored samples are mainly due to the deterioration in coagulation factors. Freezing and thawing had little effect on the measures of thrombin generation in the normal plasma, apart from a small but significant reduction in thrombin generation after 2 cycles using the PRP/NIBSC (1pM TF/2μM PL) reagent (table 3.6). The pattern of reduction was similar to that previously seen in the unfiltered normal plasma (table 3.5), where the main effect was seen in the ETP and PT. This was observed by more ETP/PT after 1 cycle of freeze/thaw and then felt after 2 cycles. In filtered PDMP-rich plasma, freezing and thawing also had a significant ($p<0.05$) effect only on the time parameters (LT and ttP) of thrombin generation, but not on the amount (ETP and PT) (Table 3.6), for all reagents except for the ttP with the PRP reagent and the LT with the PPP normal reagent. Again, this is similar to the observation with unfiltered PDMP-rich plasma (table 3.5).

A)

| pM TF/4uM PL | Filtered Plasma (n=6) | | | | Filtered PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 2.9±0.2 | 2.9±0.3 | 2.9±0.2 | 0.9704 | 2.9±0.1 | 3.0±0.1 | 3.2±0.1 | 0.0516 |
| ttP (min) | 5.9±0.3 | 6.2±0.3 | 6.3±0.3 | 0.6387 | 6.1±0.1 | 6.4±0.2 | 6.8±0.2 | **0.0287** |
| ETP (nM.min) | 1771±69 | 1669±75 | 1541±93 | 0.1576 | 2033±132 | 2127±146 | 2047±159 | 0.8860 |
| PT(nM) | 277.4±10.5 | 262.6±12.3 | 232.6±16.2 | 0.0816 | 303±15.3 | 302.7±14.5 | 286.2±17.3 | 0.6892 |

B)

| 1pM TF/4uM PL | Filtered Plasma (n=6) | | | | Filtered PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 5.9±0.6 | 6.0±0.6 | 6.0±0.6 | 0.9736 | 6.9±0.2 | 7.3±0.3 | 7.7±0.2 | **0.0461** |
| ttP (min) | 10.1±0.5 | 11.1±0.2 | 11.6±0.6 | 0.1005 | 11.7±0.3 | 12.7±0.4 | 13.3±0.3 | **0.2837** |
| ETP (nM.min) | 1535± 102 | 1400± 104 | 1227±94 | 0.1241 | 1552±177 | 1432±160 | 1360±159 | 0.7144 |
| PT(nM) | 189±16 | 157.8±20 | 126.6±21 | 0.1025 | 164.3±25 | 129.1±19 | 119.1±17 | 0.2837 |

C)

| 1pM TF/2uM PL | Filtered Plasma (n=6) | | | | Filtered PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 6.5±0.5 | 7.0±0.4 | 7.7±0.5 | 0.2142 | 6.4±0.3 | 7.6±0.4 | 8.2±0.4 | **0.0180** |
| ttP (min) | 11.6±1.0 | 12.3±0.8 | 13.8±1.0 | 0.2400 | 11.6±0.7 | 14.0±0.7 | 14.9±0.7 | **0.0105** |
| ETP (nM.min) | 1996± 114 | 1947±121 | 1612±83 | **0.0462** | 2091±193 | 1961±209 | 1858±262 | 0.7790 |
| PT(nM) | 176.3±12 .0 | 167.5±12.0 | 129.4±10.0 | **0.0249** | 202.6±29.0 | 156.6±20.8 | 143.6±23.8 | 0.2427 |

D)

| 1pM TF | Filtered Plasma (n=6) | | | | Filtered PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 9.2±0.5 | 9.6±0.7 | 10.9±0.9 | 0.2351 | 8.0±0.4 | 9.2±0.5 | 10.1±0.6 | **0.0251** |
| ttP (min) | 18.7±0.6 | 19.6±0.8 | 20.3±0.8 | 0.9853 | 17.7±0.9 | 19.8±0.9 | 20.8±1.4 | 0.1623 |
| ETP (nM.min) | 421±96 | 442±142 | 412±137 | 0.9792 | 1379±302 | 1202±326 | 1098±359 | 0.8327 |
| PT(nM) | 20.7±4.4 | 22.5±7.2 | 21.2±6.8 | 0.3447 | 85.2±25.7 | 70.7±23.5 | 69.3±27.4 | 0.8887 |

**Table 3.6: Effects of freeze/thaw cycles on filtered normal and filtered PDMP-rich plasma on thrombin generation.** Thrombin generation was measured either in normal plasma or PDMP-rich plasma after subjecting 2 freeze/thaw cycles with 5pM TF/4μM PL, 1pM TF/4μM PL and 1pM TF. Data are shown as mean±SEM. Differences between the three cycles were determined by One-WAY ANOVA.

### 3.2.7.3. Effect of Freeze/thaw cycles on double spun normal and PDMP-rich plasma

Again, the influence of freezing and thawing was then investigated on samples of plasma and PDMP-rich plasma prepared from the same plasma but this time following a second centrifugation of the plasma and PDMP-rich plasma at 13,000x*g* for 10 minutes (table 3.7) before freeze/thaw cycles. Similar observations were observed in the second centrifuged plasma as for the normal plasma (table 3.5) and the filtered normal plasma (table 3.6). In the double-spun normal plasma, freezing and thawing had a small effect, but only reached a statistically significant effect on ETP and PT with the PRP/NIBSC PL reagent and on PT with PRP low ($p<0.05$) (table 3.7). Again, as with the previous observations in PDMP-rich plasma and filtered PDMP-rich plasma, in the double-spun PDMP-rich plasma the effect of freezing and thawing was only seen on time parameters, with statistically significant effects with PPP normal, PPP low and PRP reagents ($p<0.05$; table 3.7) and no effect on ETP or PT.

These observations suggest that the effect seen in normal and PDMP-rich plasma was largely due to a disruption of MPs in the 1$^{st}$ cycle of freeze/thaw and on coagulation factors may be after 2 cycles. The effect of the 1$^{st}$ cycle was clearly seen in normal plasma, which few MPs from circulation, but was masked by the large level of MPs in the PDMP-rich plasma. Furthermore, the data suggest a unique effect on the normal plasma and suggest that the MPs in the normal plasma behave differently from MPs generated from platelets.

A)

| 5pM TF/4uM PL | Double spun Plasma (n=6) | | | | Double spun PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 2.8±0.2 | 2.9±0.3 | 2.9±0.2 | 0.9694 | 2.9±0.1 | 3.1±0.1 | 3.1±0.1 | **0.0069** |
| ttP (min) | 5.4±0.2 | 5.6±0.2 | 5.7±0.2 | 0.6770 | 5.7±0.1 | 6.0±0.1 | 6.3±0.2 | **0.0123** |
| ETP (nM.min) | 1795±73 | 1706±77 | 1536±96 | 0.1119 | 2098±132 | 2143±146 | 2090±158 | 0.9617 |
| PT(nM) | 313.3±12.7 | 302.5±13.7 | 268±20.6 | 0.1482 | 336.2±14 | 334.4±14 | 324.2±16 | 0.8237 |

B)

| 1pM TF/4uM PL | Double spun Plasma (n=6) | | | | Double spun PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 5.5±0.5 | 5.7±0.5 | 5.7±0.5 | 0.9503 | 6.7±0.1 | 7.2±0.2 | 7.4±0.1 | **0.0195** |
| ttP (min) | 9.0±0.4 | 9.5±0.3 | 10.1±0.2 | 0.0586 | 10.6±0.3 | 11.8±0.5 | 12.1±0.5 | 0.0530 |
| ETP (nM.min) | 1669±90 | 1589±102 | 1337±110 | 0.0831 | 1758±146 | 1673±143 | 1645±162 | 0.8603 |
| PT(nM) | 237.8±16 | 212.9±19 | 164±22 | **0.0426** | 225.4±27 | 188.3±28 | 179.4±26 | 0.4577 |

C)

| 1pM TF/2uM PL | Double spun Plasma (n=6) | | | | Double spun PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 6.1±0.4 | 6.5±0.4 | 7.0±0.4 | 0.3039 | 6.0±0.2 | 6.8±0.4 | 7.4±0.4 | 0.0549 |
| ttP (min) | 10.7±0.7 | 11.2±0.6 | 12.1±0.7 | 0.3256 | 11.6±0.4 | 12.6±0.8 | 13.4±0.8 | 0.2229 |
| ETP (nM.min) | 2033±101 | 2057±118 | 1700±72 | **0.0380** | 2242±211 | 2199±178 | 2070±212 | 0.8197 |
| PT(nM) | 182.1±112 | 188.6±15 | 148.4±6 | **0.0525** | 197.5±20 | 195.4±25 | 174.5±23 | 0.7343 |

D)

| 1pM TF | Double spun Plasma (n=6) | | | | Double spun PDMP-rich plasma (n=7) | | | |
|---|---|---|---|---|---|---|---|---|
| | Fresh | Once | Twice | P value | Fresh | Once | Twice | P value |
| LT (min) | 8.0±0.4 | 8.5±0.5 | 9.2±0.6 | 0.2809 | 7.3±0.2 | 8.2±0.4 | 8.8±0.4 | **0.0368** |
| ttP (min) | 17.2±0.4 | 17.5±0.5 | 18.3±0.8 | 0.8673 | 16.2±0.6 | 17.9±0.9 | 18.7±1.1 | 0.1359 |
| ETP (nM.min) | 599±102 | 681±116 | 614±128 | 0.6632 | 1689±227 | 1647±231 | 1571±259 | 0.9393 |
| PT(nM) | 27.2±4 | 34.2±5 | 31.5±6 | 0.4294 | 100±22 | 121±34 | 114±33 | 0.8842 |

**Table 3.7. Effects of freeze/thaw cycles on double spun normal and filtered PDMP-rich plasma on thrombin generation.** Thrombin generation was measured either in normal plasma or PDMP-rich plasma after subjecting 2 freeze/thaw cycles with 5pM TF/4μM PL, 1pM TF/4μM PL and 1pM TF. Data are shown as mean±SEM. Differences between the three cycles were determined by One-WAY ANOVA.

### 3.2.8 Influence of TF in plasma from normal individuals

The measurement of thrombin generation in the previous experiments was carried out with added, exogenous TF. However, blood borne TF is reported in the circulation (Eichinger*, et al* 1995, Giesen*, et al* 1999, Morrissey*, et al* 1993) either as soluble or TF-bearing MPs (Giesen*, et al* 1999). To investigate the influence of endogenous TF, thrombin generation was triggered in plasma from healthy subjects (n=13) with the PRP (1pMTF) reagent and without any reagent in order to make the assay dependent on the endogenous TF and PL (Ollivier*, et al* 2010). Twenty µl of HBS was added to the reaction mixture instead of the PRP reagent to maintain the total volume. In the plasma, more activity was observed with the PRP (1pM TF) reagent than without a reagent (no TF/PL) (Fig 3.10), this activity was seen in only 4/13 subjects with very long LT and ttP and very low ETP and PT ($p<0.0001$; Fig 3.10).

To investigate if CRP-XL (2.0µg/ml) stimulation of PRP induces TF-bearing PDMPs, PDMP-rich plasma was then compared to normal plasma from 9 healthy subjects, and thrombin generation was initiated with no reagent (no TF) (Fig 3.11). Thrombin generation was not detected in 6/9 in normal plasmas and in 3/9 in PDMP-rich plasmas. PDMPs enhanced thrombin generation compare to the normal plasma, which might indicate the presence of active TF in form of soluble or TF-bearing MPs upon CRP-XL stimulation of PRP.

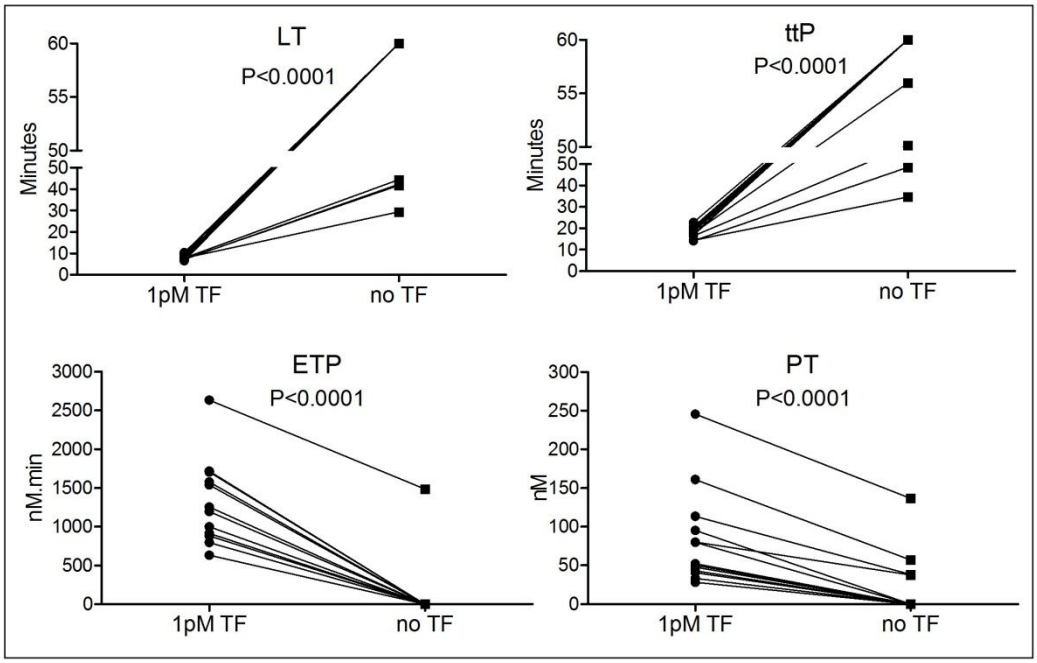



**Fig 3.10. Dependency of thrombin generation on TF in plasma.** Thrombin generation was triggered in normal plasma with and without the PRP (1pMTF) reagent. Data are shown as individuals points, n=13. Statistical analysis using paired t-test.

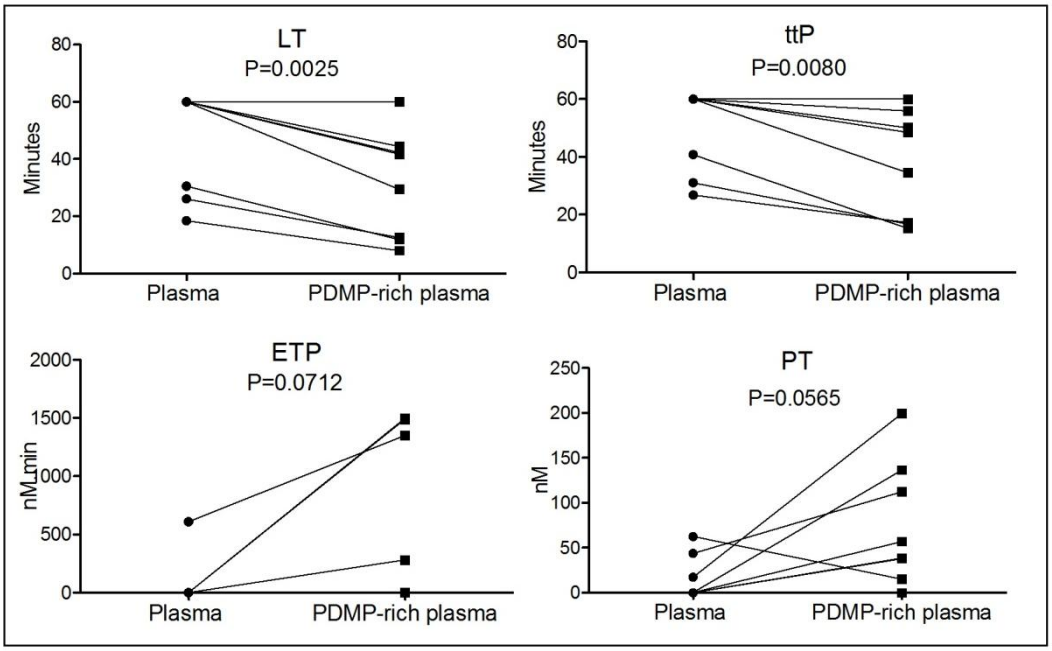



**Fig 3.11. PDMPs enhance thrombin generation independently of exogenous TF.** Thrombin generation was triggered in normal plasma and PDMP-rich plasma without exogenous TF. Plasma was obtained after CRP-XL (2.0µg/ml) stimulation of PRP. Data are shown individuals points (n=9) Statistical analysis using paired t-test.

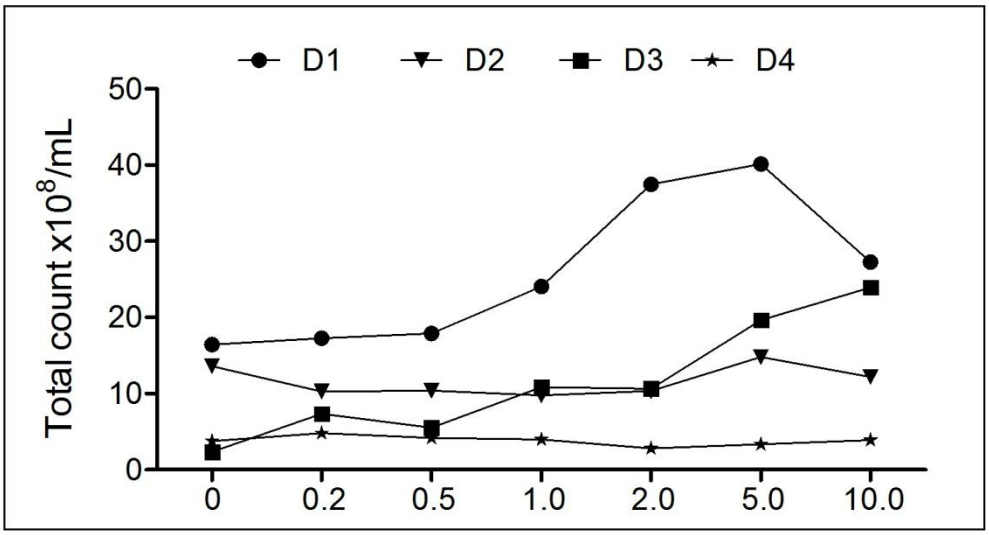
## 3.2.9 Preliminary study of MP size and count using nanoparticle tracking analysis (NTA)

This is a preliminary study of MP size and count using NTA, which tested whether there is a relationship between MP count and/or size and thrombin generation. NTA provides a new method for the detection and counting of MPs based on Brownian motion. Washed platelets were stimulated with different concentrations of CRP-XL (0, 0.2, 0.5, 1.0, 2.0, 5.0 and 10.0µg/ml) for 10 minutes at 37°C, PDMP-rich supernatants were obtained and then stored in aliquots at -80°C for NTA analysis. Then PDMP-rich supernatants were analysed by Dr Chris Gardiner in Oxford University using Nanosight, LM 1.0 (NTA software2.0).

### 3.2.9.1 MP size and count using NTA

The analysis showed an increased in PDMP count with increasing concentrations of CRP-XL (Fig 3.12a shows the total count of PDMPs and in Fig 3.12b shows PDMPs >200nM in size), because the previous analysis showed that plasma supported thrombin more before filtration compared filtered plasma through a 0.2µm filter. The count of PDMPs varied between individuals. Three donors (D1, D2 and D3) showed a dose-dependent increase in PDMP count with increasing CRP-XL concentration. However, in D1 the count of MP reduced at 10.0µg/ml CRP-XL for both total and MP>200nm (Fig 3.12), as well as D3 for MP>200nm only. D4 showed no increase in the total count of PDMP count (Fig 3.12a), with slight increase in the count of PDMP >200nM (Fig 3.12b). It can also be noticed that the count of large MPs (>200nm) is almost half the total count of all particles count (Fig 3.12a). It was also observed that at 10.0µg/ml CRP-XL the count of the larger (>200nm) MPs was reduced in two donors

A)




B)

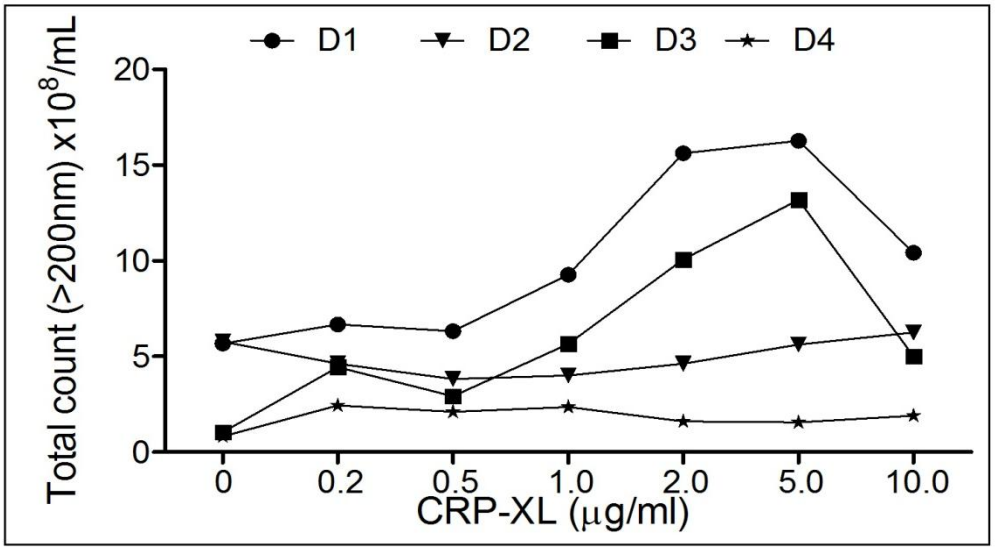
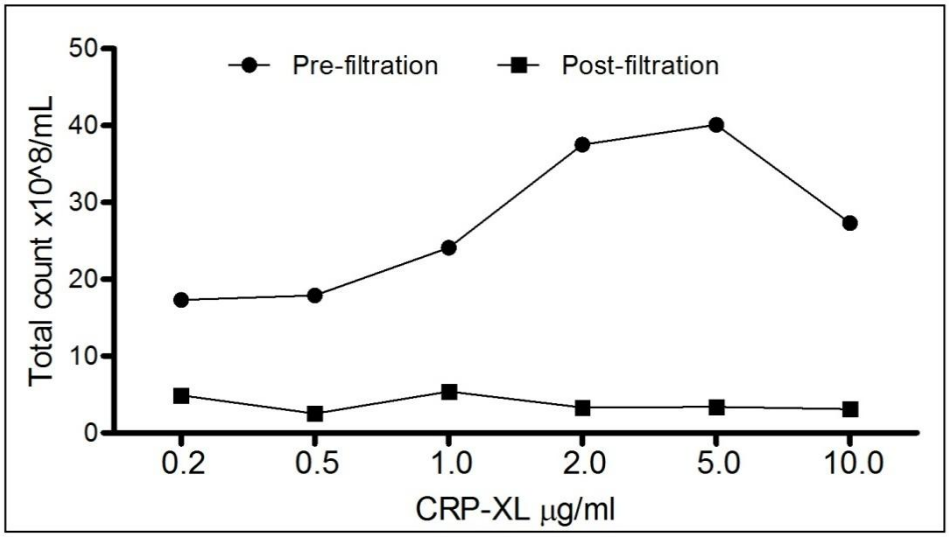



**Fig 3.12. Number of PDMPs in the supernatant of activated washed platelets with dose response CRP-XL.** A) Total PDMPs and B) PDMPS size >200nm. Washed platelets were activated with increasing concentration of CRP-XL (0-10μg/ml) for 10 minutes at 37°C. Then the supernatant was obtained by centrifugation of platelet suspension at 1800xg for 30 minutes. Supernatant was obtained and stored at -80°C for analysis.

A)




B)

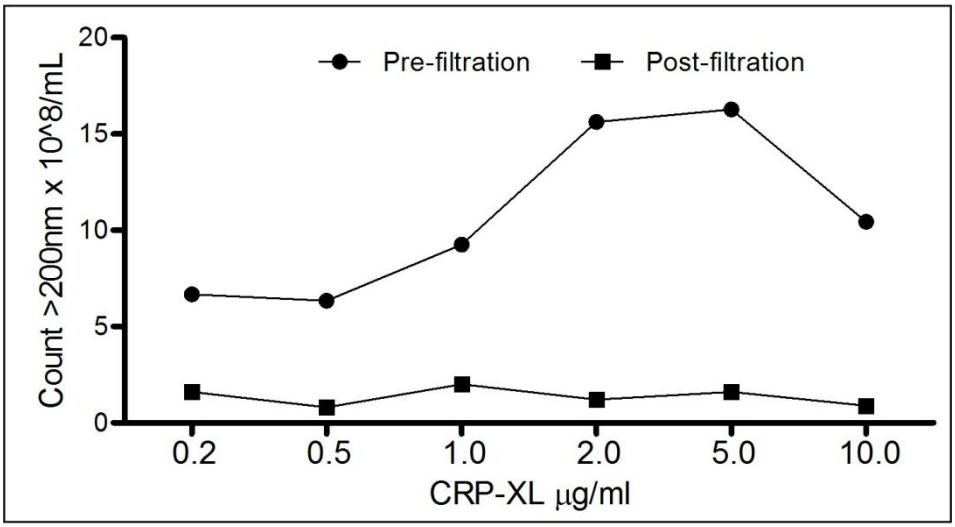



**Fig 3.13. Effect of filtration through a 0.2µm filter on PDMP count.** (A) Shows the effect of filtration on the total count of PDMPs (B) Shows the effect of filtration on the count of PDMPs >200nm. Data are from one donor.

(D1 and D3) (Fig 3.12b). Filtration through a 0.2µm filter had a significant effect on MP numbers, removing up to 60-90% of all MPs and even those MP of >200nm (Fig 3.13; n=1).

### 3.2.9.2 Correlation between thrombin generation and PDMP count

Thrombin generation was measured in 3 of the 4 donors shown in Fig 3.12. The data for each donor were analysed individually because the inter-individual response to CRP-XL varies considerably- making it hard to combine the data. Given the small number of samples, two donors showed strong correlation of thrombin generation with PDMP count (Figs 3.14 and 3.15) and also with PDMP >200nm (data not shown) but one did not (Fig 3.16). The strong correlation was mainly with the ETP and PT, and an inverse modest correlation was found with LT and ttP for both donors. The strong correlation was determined by r value above 0.5 and significant P value (not shown).

Donor 3

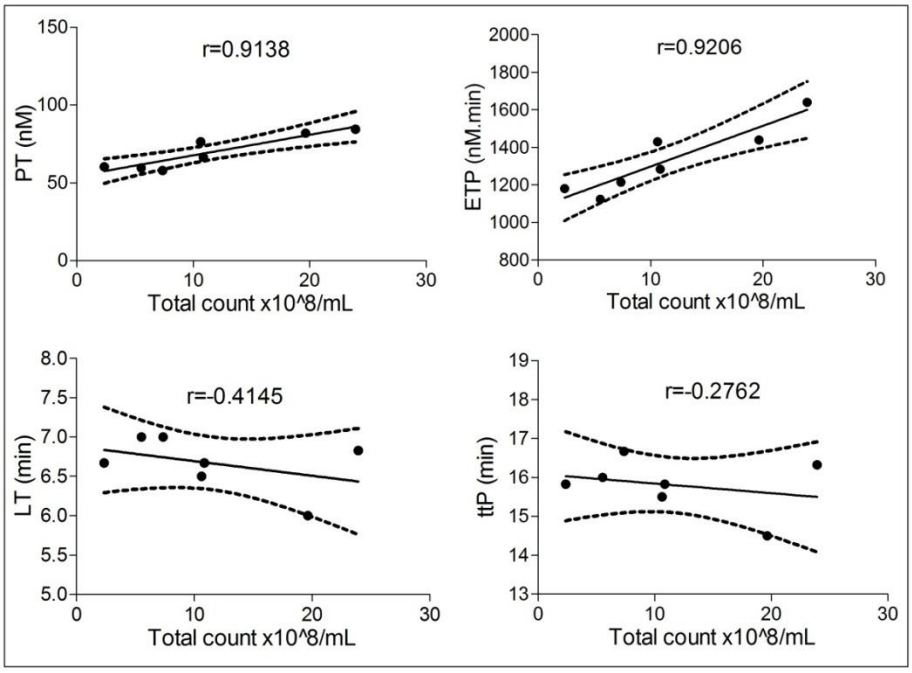



**Fig 3.14: A strong correlation was observed between total PDMP count and thrombin generation parameters in donor 3.**

Donor 4

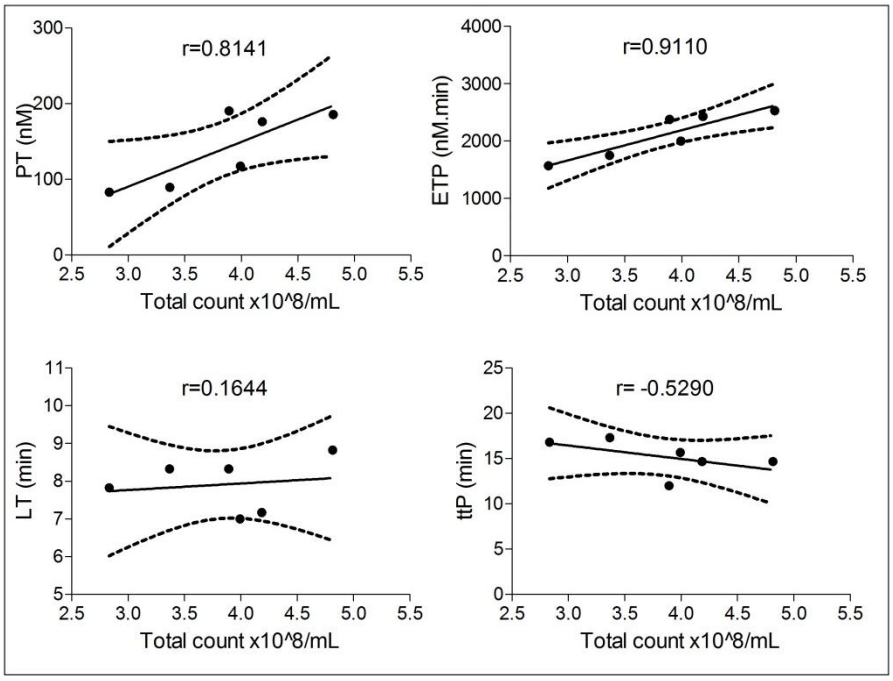



**Fig 3.15: A strong correlation was observed between total PDMP count and thrombin generation parameters in donor 4.**

Donor 2

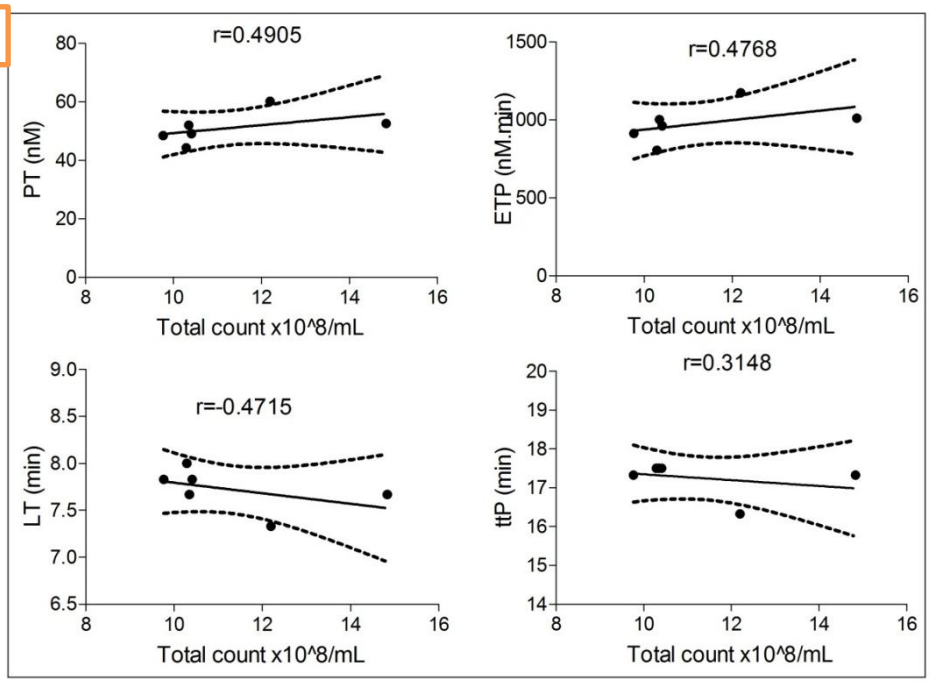



**Fig 3.16: No correlation was observed between total PDMP count and thrombin generation parameters in donor 2.**

## 3.3. Discussion

Activation of platelets with CRP-XL leads to the release of MPs that support thrombin generation through PS exposure. PDMPs may also be elevated in the blood in a variety of atherothrombotic disease states. Measurement of PDMPs in plasma from patients may have diagnostic and/or predictive value. The thrombin generation assay developed by Hemker group is a tool for investigating the hypercoagulability and hypercoagulability of plasma, so different reagents exist (Hemker*, et al* 2003). It has been used for the detection of procoagulant MPs in the plasma of healthy subjects and patients with thrombotic diseases, but there have been no critical studies on the best reagent for measurement of the procoagulant activity of MPs. Macey, *et al* (2011) used the PPP normal (5pMTF/4µMPL) reagent, and others (Bidot*, et al* 2008, Pereira*, et al* 2006b) have used the PRP (1p MTF) reagent, but it has not been fully evaluated. This makes a comparison between studies difficult and indicates no agreement among laboratories on the best reagent for MP detection and also no agreement on the pre-analytical conditions. This why the Vascular Biology group of the Scientific and Standardization Committee of the International Society for Thrombosis and Haemostasis (ISTH) is addressing these issues in MP detection.

Different commercial reagents exist for thrombin generation measurement in plasma, with TF as the trigger and PL as the platelet substitute (known as PPP reagents) with the PRP (1pM TF) reagent primarily used for thrombin generation driven by platelets (Hemker*, et al* 2003). When this study began in late 2007, there were limited data, and almost no comprehensive studies, that investigate the effect of pre-analytical and analytical conditions on thrombin generation in normal and PDMP-rich plasma. For

example, there were studies on the effect of residual platelets in the plasma on thrombin generation after storage (Chantarangkul*, et al* 2004), the effect of storage on MPs on thrombin generation (Keuren*, et al* 2006), the effect of contact factor activation in plasma (Luddington and Baglin 2004, Van Veen*, et al* 2008), but none of these studies agreed on the best reagents to use to detect procoagulant activity of MPs by thrombin generation, and most of the studies used flow cytometry for evaluating those pre-analytical and analytical variables (Kim*, et al* 2002, Lacroix*, et al* 2010a, Mobarrez*, et al* 2010, Shah*, et al* 2008).

The current study has demonstrated that the PRP (1pM TF) reagent is the most sensitive reagent for MP detection in the plasma, which significantly discriminates between PDMP-rich plasma and normal plasma. The PRP (1pM TF) reagent makes assay dependent on the level of endogenous PL, while the other reagents with exogenous PL, failed to discriminate between the unstimulated plasma and PDMP-rich plasmas, or to vary with varying concentration of PL of PDMPs, mainly because they contain the rate limiting factor "PL" and because of the effect of endogenous PL on thrombin generation (Figs 3.3, 3.4 and 3.5). The assay with PPP reagents are considered to be mainly dependent on the level of coagulation factors in the samples, with less sensitivity to the levels of TF and procoagulant MPs, while the PRP reagent depends mainly on the procoagulant PL and coagulation factors. Once one is omitted then the assay becomes dependent on the missing factor. To confirm this sensitivity for PL, the CAT assay was run with different concentrations of exogenous PL (NIBSC) and the sensitivity was observed for the 1pM reagent only, similar to the observation for the titration of the PDMPs. This is in agreement with Gerotziafas *et al* (2005), who

showed significant dependency between the amount of thrombin generated in terms of ETP and PT and the level of exogenous PL at 0-3pM TF. In addition, this is in agreement with what is mentioned, but not clearly demonstrated in the literature, that triggering thrombin generation with <1pMTF in the plasma is the best reagent to differentiate between hyper- and hypercoagulable conditions. Also, our findings illustrate that the best, most sensitive, parameter for PL detection is PT, while ETP is less sensitive because it needs small amount of PL to generate maximum thrombin, however it can be a parameter of how plasma is thrombogenic.

With the 1pMTF the sensitivity of the thrombin generation assay to coagulation factors - mainly FVIII, FIX and FXI - is high (Beltran-Miranda*, et al* 2005, Dargaud*, et al* 2005, Hemker*, et al* 2003, Lewis*, et al* 2007, van Veen*, et al* 2009). Therefore, using the 1pMTF reagent for procoagulant MP detection could raise the question of whether the effect is due to PL dependency of the assay, but instead due to variations between levels of individual coagulation factors. Our data with pooled plasma, where all the coagulation factor levels were the same, show that the assay is sensitive to the level of the synthetic NIBSC PL or PDMPs which was not seen using the other two reagents. To confirm the dependency of the thrombin generation assay on PDMPs, and also to investigate the best method of removing the effect of PDMPs, filtration via a 0.2µM filter was compared with a double centrifugation step (1,800x*g* for 30 minutes followed by 13,000x*g* for 10 minutes). Both methods significantly reduced the procoagulant activity of PDMPs on the thrombin generation assay. This finding is in agreement with previous studies, which showed the removal of MP activity, measured by thrombin generation, either in normal plasma or in fresh frozen plasma using either

a PPP normal reagent or a PRP reagent (Lawrie*, et al* 2008, Macey*, et al* 2011). In addition, we found the ceveron filter device is better at removing MPs from normal plasma than a normal acrodisc filter; however, in the presence of a high level of PDMPs in the plasma it becomes saturated, so the acrodisc is better to remove MPs in plasma with a high level of MPs. With the finding that the most sensitive parameter for MPs detection is PT, while for thrombotic plasma it is ETP.

The current study demonstrate that our single spin protocol in plasma separation (1800x*g* for 30 minutes) had similar results compared to commonly used protocol using 2 steps of centrifugation (1,500x*g* for 15 minutes followed by 13,000xg for 2 minutes) for detection of MPs (Jy*, et al* 2004), with the latter retained more activity than the single step centrifugation, which contradict the data the single spin protocol retain residual platelets, because they used lower speed and less time (1500*xg* for 20 minutes) (Ayers*, et al* 2011) compared to our protocol (18,00x*g* for 30 minutes). The single step spin protocol (one centrifugation step) is much better because it prevents the loss of MPs from the samples (18,00x*g* for 30 minutes), and also does not retain residual platelets, as 2 steps protocol led to a loss of up to 80% of MPs (Horstman*, et al* 2004).

Using low TF (<1pM) to trigger thrombin generation makes the assay susceptible to contact factor activation (mainly FXIIa), which can be easily activated by sample transportation and blood collection. To avoid this, blood should be drawn without a tourniquet, or with only a minimal tourniquet applied, and the blood should be transported as quickly as possible. However, these conditions cannot be met for all

studies, especially in a hospital setting. Contact factor activation significantly influences the measurement of thrombin by increasing the assay's variability and impression plasma (Luddington and Baglin 2004). To overcome this false overestimation of the assay, which affects the assay reproducibility, CTI has been found to be useful in eliminating the effect of contact pathway activation and improving the measurement of thrombin generation in plasma from both healthy subjects and patients (Dargaud*, et al* 2005, Luddington and Baglin 2004, Van Veen*, et al* 2008). The results of the present study are in agreement with the previous publications, with an emphasis on the use of CTI at low concentrations of TF (<1pM) to trigger the reaction. The effects seen here were relatively small reflecting the immediacy of the same processing, however, this may be more apparent for remotely-collected and stored plasma.

The current analysis of the precision of the CAT assay ,CV, was found to be within an acceptable limit in the current study. The inter-assay variation CV (table 3.1), was assessed using four reagents. The results revealed good precision (<12.0%) that was not affected by the low concentration of TF, as has been published previously. Also, the intra-assay CV (table 3.2) was very low. This study also agrees with the findings in the literature on the variability of thrombin measurement, mainly ETP and PT, between individuals. Our study confirms that these factors are increased due to the MPs in PDMP-rich plasma and it is suggested that this is due to the levels of PS (Gerotziafas*, et al* 2005, Vanschoonbeek*, et al* 2004) and indicating that the assay is reproducible.

There is no agreement on the best method of storing plasma or whether to run it as a fresh sample for subsequent analysis; the common practise is to freeze plasma to be analysed on a later date, because it is cost-effective and better to eliminate batch-to-batch variability of MP analysis to run a number of samples at the same time; however, this conflicts with the view that it is best to run fresh sample*s.* Previous studies mainly on the effect of storage on MPs used flow cytometric analysis and few have used thrombin generation. The common understanding is that freezing of plasma generates MPs from platelets (Xiao*, et al* 2002) and affects MP count (Keuren*, et al* 2006). Our results showed an effect of freezing and thawing of the plasma on thrombin generation was mainly due to the effect on MPs on the first cycle of thawing and on coagulation factors on the second cycle of freeze/thaw, as the effect was clearly seen in normal, with a small effect was seen on the plasma with MPs removed by either filtration or double spinning. This is in agreement with the observations of Keuren *et al* (2006) who found an effect on the level of MPs after one freeze-thaw cycle at -80°C using both flow cytometry analysis and thrombin generation; they found an increase in the MP count and an enhancement of thrombin after freezing compared to fresh samples. However, the effect observed on Keuren *et al* (2006) was high; the possible explanation for these is that Keuren *et al* (2006) had looked at the effect of freezing on isolated MPs from a platelet concentrate diluted in a HBS buffer instead in the plasma, which is likely to provide a protective environment for the MPs.

In addition, our study in agreement with the following studies (Dey-Hazra*, et al* 2010), who showed there was an effect of storage over time (freezing) at -80°C on MPs in increasing the MPs in the plasma, as measured by annexin-V binding and the MP count

using flow cytometry. With flow cytometric analysis, van Lerssel, *et al* (2010) found the same result: an increase in the MP count after snap freezing followed by storage at -80°C and the same was found after at -80°C and thawed on ice (Ayers*, et al* 2011). Other groups have systematically studied the effect of the thawing procedure after snap freezing, thawing samples on ice, at RT or 37°C, and made the recommendation that samples should be thawed at RT or 37°C in a water bath, and that thawing on ice should be avoided (Trummer*, et al* 2009). We also investigated the effect of storing normal and PDMP-rich plasma on the procoagulant activity of MPs, and after removal of MPs either by filtration through a 0.2µm filter or by a hard spin at -80°C by comparing thrombin generation in fresh plasma and after subjecting the samples to 2 cycles of freeze-thaw. Chantarangkul *et al* (2004) studied the effect of snap freezing plasma in liquid nitrogen followed by storage at -70°C on thrombin generation using 1pMTF with three concentrations of PL (0, 0.5, 1µM). They looked at the effect of the remaining residual platelets in plasma prepared by centrifugation at 1,200x*g* for 12 minutes. Indeed, the current study demonstrate that filtration would remove MPs as well as residual platelets, which this group recommended filtering the plasma before or after storage are the best methods to remove the effect of residual platelets.

We found in the current analysis the presence of TF-bearing MPs in a few subjects, and the number of subjects with TF-containing MPs was increased after CRP-XL stimulation. This is based on the finding that only 4/13 subjects supported thrombin generation dependent on endogenous TF, compared to thrombin generation driven by 1pMTF in the same subjects (Fig 3.10), while PDMP-rich plasma in 3 of them supported thrombin generation after CRP-XL of the PRP (Fig 3.11). Indeed, TF circulates in the

blood of healthy individuals either in soluble form or in the form of TF-bearing MPs derived from a range of blood cells; the role of these MPs in the haemostatic response remains to be elucidated. Indeed, MPs are widely described as the main carrier of TF in the circulation (Panes, *et* al 2001). TF has also been found on platelets and MPs in the circulation (Eichinger*, et al* 1995, Giesen*, et al* 1999, Morrissey*, et al* 1993). TF is thought to be found in the circulation in an encrypted "inactive" form, which can be de-encrypted into "active TF" upon oxidation or stimulation with agonists.

The same observation on the presence TF-containing MPs in few individuals has also been reported by Keuren *et al* (2006) who found TF-bearing MPs isolated from platelet concentrates could not initiate thrombin generation, mainly because of the action of TFPI, although MPs have been found to play a positive role in functional TF using a FXa generation assay. The current observations of endogenous TF and those of Keuren *et al* (2006) on the effect of TFPI on thrombin driven by endogens TF might explain the contradiction between studies on the activity of endogenous TF (Bogdanov*, et al* 2003, Butenas and Mann 2004).

It is well established that the flow cytometers fails to detect smaller MPs below 0.5µm due to the wavelength of the laser (488nm). Although there are sizing calibration beads "Megamix", these still not allow flow cytometry is to detect all the MPs, meaning flow cytometry is not an ideal technique for MP detection (Lacroix 2010). This explains why SSC of the ISTH is collaborating with many laboratories to standardize the analysis of MP by flow cytometry and pre-analytical and analytical variables (Jy*, et al* 2004, Lacroix 2010). Therefore, promising techniques have been developed to

overcome the limitations of flow cytometry for accurate sizing and counting of MPs, particularly the NTA. In the current study, as expected the NTA analysis showed increased in MP count with increasing concentration of CRP-XL, but surprisingly 2/4 donors had less count at 10.0µg/ml CRP-XL. In addition, a strong correlation in 2/3 donors was observed between thrombin generation parameters and PDMP count and size. Strong correlations between MP count and thrombin generation parameters have been reported by others using Megamix in flow cytometry (Bidot*, et al* 2008, Pereira*, et al* 2006a, Sossdorf*, et al* 2008), EDMP and PDMP relative level and thrombin generation (Burton*, et al* Submitted). However, other studies have showed no correlation between MP functional assays and counts of MPs using either flow cytometry or the ELISA techniques (Haubold*, et al* 2009, Tesselaar*, et al* 2007).

In conclusion, the CAT assay has an acceptable precision with low inter- and intra-assay measuring of the plasma using the four different reagents described in this study and the CAT assay is sensitive to the activity of PDMPs in the plasma using the 1pM TF reagent. The most sensitive parameter for MPs detection is PT, while for thrombotic plasma is ETP. The effect of MP activity is completely removed by filtration and double centrifugation step. Filtration with the Ceveron MFU 500 filtration unit is potentially limited by the amount of MPs in the plasma. In addition, it is recommended to analyse fresh samples in the assay, and that caution should be taken when analysing frozen samples in that they should be compared to a reference sample (pooled plasma) treated in the same way. This study also highlights on the importance of MPs in supporting coagulation and their important role in haemostasis, which could have

clinical relevance in terms of using them as factors for restoring the haemostatic balance in patients with thrombocytopenia or other diseases, but further studies are needed to elucidate that.

## 3.4. Further work

- Measure the level of coagulation factors in pre- and post-filtered plasma, to ensure that these are not lost during filtration which might be a source of variability on the CAT assay.
- The current study has looked only at PDMPs generated from platelets stimulated by CRP-XL. Whilst this is a potent stimulator of MP generation , MPs from platelets stimulated with the more physiologically relevant fibrillar collagen (which will also interact with the GPIa/IIa collagen receptor), or by combinations of CRP-XL with other agonists (ADP, TRAP etc) may behave differently in terms of procoagulant activity, either from anionic PLs or from TF.
- MPs from other cellular sources (e.g. endothelial cells or monocytes) may behave differently in the thrombin generation assay, or in stored samples.
- Studying MP level by NTA measurement in more donors to allow a more comprehensive correlation between both count and size and thrombin generation, and with even flow cytometry using annexin-V binding.

# Chapter: 4 Procoagulant activity of plasma from patients' samples

## 4.1. Introduction

Having characterized the CAT assay it was then used to study plasma from subjects with, or at risk of CVD, which is the most common cause of death and disability worldwide. CVD is a complex pathophysiological process involving genes and gene products, a variety of environmental influences, and biochemical changes of the haemostatic system, which all lead to increased thrombotic risk. Arterial thrombosis is mainly due to rupture in vulnerable atherosclerotic plaques, which gradually develop in the arterial wall through the accumulation, with age, of lipid (mainly cholesterol and oxidized low density lipoprotein, LDL), leukocytes (mainly monocytes/macrophages) and platelets (Fitzgerald*, et al* 1986, Robbie and Libby 2001, Ruggeri 2003, Thaulow*, et al* 1991, Trip*, et al* 1990, Tschoepe*, et al* 1993). The migration and infiltration of monocytes to the sub-endothelial matrix is triggered by chemotactic, chemokines and cytokines released by the damaged endothelium and by activated platelets. The subsequent internalization of lipid droplets by macrophages turns them into foam cells in the artery wall. The plaques that accumulate in some conditions are fragile and can be easily ruptured, especially lipid-rich plaques. Once a plaque is ruptured, procoagulant TF and MPs are exposed into the circulation. Both TF and MPs are originally derived from activated platelets, damaged endothelium and apoptotic macrophages, and smooth muscle cells within

the plaque (Mallat*, et al* 1999, Tedgui and Mallat 2001), which their release lead to a thrombus and may be subsequently to MI (Thim*, et al* 2008).

Patients with chronic kidney disease (CKD) have a high prevalence of cardiovascular complications (Drueke 2000, Go*, et al* 2004). Uremic patients develop vascular disease involving endothelium dysfunction, damage and arterial stiffening, a key player in the development of arteriosclerosis (Dursun*, et al* 2009, Nakanishi*, et al* 2002, van Guldener*, et al* 1998). Renal failure as a disease is associated with defects impaired platelet function and defects in coagulation function, therefore leading to the development of a dysfunctional haemostatic system (Gawaz*, et al* 1994). On the other hand, uremic toxins in renal disease reduce platelet aggregation as they affect the binding of fibrinogen to GP IIb-IIIa (Escolar*, et al* 1990, Gawaz*, et al* 1994, Rysz*, et al* 1997). Although these factors contribute to bleeding episodes in renal patients, those patients also have a high risk of developing CVD.

In both diseases, CAD and CKD, a high level of circulatory MPs compared to normal individuals have been reported (Amabile*, et al* 2005, Ando*, et al* 2002, Bernal-Mizrachi*, et al* 2004, Faure*, et al* 2006, Mallat*, et al* 2000), which are procoagulant and MPs have been associated with thrombotic diseases (Bidot*, et al* 2008, Hron*, et al* 2007). Although the pathophysiology between CAD and CKD is different, hypercoagulability is seen in both diseases. Therefore, does the pre-existing hypercoagulability due to procoagulant MPs in the plasma from those subjects can be detected by thrombin generation assay.

As described in chapter 3, the CAT assay is sensitive to the level of procoagulant MPs in the plasma using the 1pMTF reagent, so this study aimed to measure the pre-existing hypercoagulability of plasma from patients with the two different diseases (pre-mature MI and ESRD). Each group was matched to a control group for comparison. A filtration step was included with each reagent to determine whether the effect was seen due to MPs in the plasma.

## 4.2. Results

The plasma used in the present study had been prepared at different speeds. In the previous chapter, it was shown that there were no significant differences observed between the different protocols used. The protocol was used to prepare plasma from the MI cohort used centrifugation at 1,800x*g* for 30 minutes at 4°C. The renal cohort plasma was prepared by the centrifugation of the blood at 1,500x*g* for 15 minutes at RT, followed by a second step of centrifugation at 13,000x*g* for 2 minutes. The MI cohort was recruited by Dr Unni Krishnan (Department of Cardiovascular Sciences, University of Leicester). The renal cohort was recruited by Dr James Burton (Renal unit, University of Leicester). All samples were thawed and analyzed in the CAT assay with and without filtration performed through a 96-well manifold vacuum filtration device (Ceveron MFU 500 filtration unit) to remove the effect of MPs on thrombin generation.

### 4.2.1. MI cohort

Twenty patients, all North European Caucasians, who had an MI episode before the age of 60 were recruited. The blood was collected at least 3 months after the MI

episode so that the acute effect of their cardiovascular event would have diminished. A control group matched for age, gender and smoking status was also recruited. Those two groups will be called the MI case and control cohorts. Table 4.1 shows the demographic data for the cohorts, which shows the significant differences between the two groups in terms of many of the risk factors associated with MI.

## Circulatory MPs show comparable thrombin generation in MI cases compared to controls

The plasma from the cases and controls gave the same thrombin generation; LT, ttP and ETP were almost similar in the cases compared to the controls; ETP was 1945±387nM.min vs. 1935±308nM.min; LT was 6.5±0.9min vs. 6.1±1.0min and ttP was 12.7±2.4min vs. 11.3±2.2min (all $p>0.05$). Although, PT was lower in the MI cases compared to the controls (160.4±60.5nM vs. 194.2±8.3nM), no statistical significance was observed ($p=0.10$). Removal of MPs by filtration caused a significant reduction in thrombin generation by procoagulant MPs ($p<0.0001$ with all parameters). After filtration, PT was statistically significantly higher in the control group compared to the cases ($p=0.0451$), but no statistically significant differences were observed for the other parameters ($p>0.05$), indicating the presence of a soluble factor that enhances the procoagulant activity in the controls.

| | Cases (n=20) | controls (n=20) | P value |
|---|---|---|---|
| Age (years) | 54.6±6.7 | 51.3±7.0 | 0.11 |
| Gender female | 4/20 | 3/20 | 0.7273 |
| BMI | 29.5±4.2 | 26.5±2.8 | **0.0081** |
| Total cholesterol | 4.1±0.7 | 5.6±0.8 | **<0.0001** |
| HDL | 1.0±0.2 | 1.2±0.3 | **0.0025** |
| LDL | 2.4±0.5 | 3.6±1.0 | **<0.0001** |
| TriG | 1.6±0.9 | 1.5±0.8 | 0.49 |
| Smoker (n) | 15/20 | 8/20 | 0.2419 |

**Table 4.1. Demographic data for MI and matched controls.** MI subjects had an MI episode before the age of 50 and samples were collected at least 3 months after the MI episode. Data are shown as mean±SD. Differences between continuous variable determined using unpaired t-test and between variables using $X^2$ test. Body Mass index (BMI), triglyceride (TriG), low density lipoprotein (LDL) and high density lipoprotein (HDL)

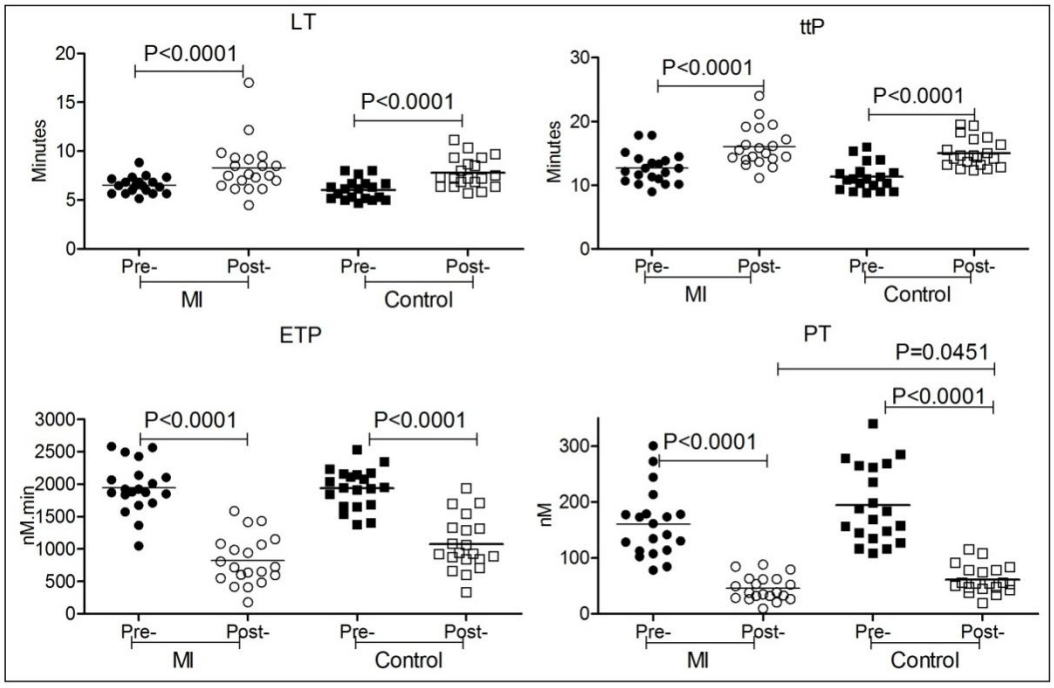



**Fig 4.1: Thrombin generation in pre- and post-filtered plasma from MI cases compared to controls using the PRP (1pMTF) reagent.** Analysis was performed using unpaired t-test, for comparison between MI cases compared to controls and paired t-test for comparison between pre- and post-filtered plasma either within MI cases or controls.

## 4.2.2. Renal cohort

Thirty-seven CKD (Twenty HD patients and seventeen PD patients) were recruited, as well as twenty matched healthy individuals as a control group. The demographic data of these subjects are shown in table 4.2, showing they were matched for age, gender and most risk factors apart from diabetes.

### Circulatory MPs in renal cases are more procoagulant than matched control

Plasma from end stage renal patients (HD and PD) had increased thrombin generation compared to the controls. PT ($p=0.0111$) and ETP ($p=0.0004$) were significantly higher in CKD patients compared to the controls (Fig 4.2). The initiation of thrombin generation as measured by LT was significantly slower in CKD patients compared to the controls ($p=0.0077$), but no statistical significant difference was observed in ttP ($p>0.05$). Filtration had a significant effect on thrombin generation in the CKD and healthy control groups. PT and ETP were markedly reduced in the filtered plasma compared with the unfiltered plasma in all groups ($p<0.0001$), and LT and ttP were significantly prolonged after filtration in the CKD group only. In controls, filtration had no effect on the LT ($p>0.05$) but significantly on ttP ($p=0.0011$). After filtration, PT still more in the CKD patients compared to controls ($p=0.0210$) but not ETP ($p>0.05$). The LT and ttP were significantly shorter in the controls compared CKD ($p<0.001$). This indicates the presence of TF-bearing MPs that have been removed by filtration in the CKD patients that might contribute to their prothrombotic phenomena. In a comparison of the subgroup of CKD patients, HD and PD; although PD had more thrombin generations compared to HD, no statistical significant differences were observed between them ($p>0.05$).

| | CKD | | | |
|---|---|---|---|---|
| | HD group (n=20) | PD group (n=17) | Control (n=20) | P value |
| Age (years) | 54.2±8.4 | 52.9±11.8 | 52.6±7.9 | 0.84 |
| Male gender (n) | 10 | 6 | 10 | 0.59 |
| Hypertension (n) | 5 | 4 | 2 | 0.42 |
| IHD (n) | 7 | 2 | 2 | 0.09 |
| CVD (n) | 3 | 1 | 0 | 0.17 |
| PVD (n) | 1 | 1 | 0 | 0.57 |
| Hypercholesterolaemia (n) | 3 | 4 | 2 | 0.53 |
| Diabetes | 10 | 2 | 1 | **<0.01** |
| Smoker (n) | 0 | 1 | 1 | 0.57 |

**Table 4.2. Demographic data for CKD (HD and PD) and healthy controls.** All patients were matched for all variables factors expect diabetes. HD, haemodialysis; PD, peritoneal dialysis; IHD, ischaemic heart disease; CVD, cerebrovascular disease; PVD, peripheral vascular disease.

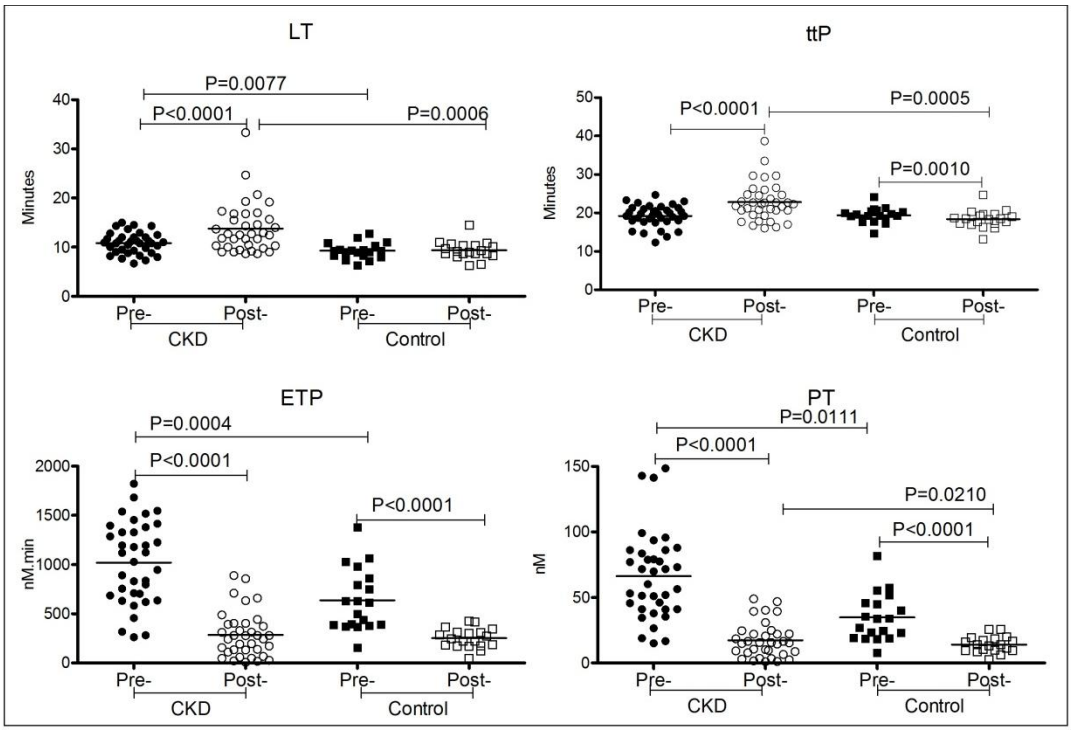



**Fig 4.2. Thrombin generation in pre- and post-filtered plasma from CKD compared to controls using the PRP (1pMTF) reagent.** A) Panel A for post-filtered plasma and B) panel B for post-filtered plasma. Thrombin generation was triggered with the 1pM TF reagent in pre-filtered plasma and post-filtered plasma via 0.2µm filter. Data are from healthy control (n=20) and CKD patients (n=37) and. Analysis between CKD and control was performed using un-paired t-test. Comparison of pre- and post-filtration plasma was performed using paired t-test

### 4.2.3. Filtration removes MP, the main source of the procoagulant entity in the plasma

It is now necessary to explore whether filtration only removes MPs and has no effect on coagulation factors, because some data in the literature showed significant reductions, although its small, in coagulation factors following filtration —mainly FVIII (Lawrie*, et al* 2008). Thrombin generation was measured in plasma from a normal healthy control and from patients with CKD, both with and without filtration (n=6; Fig 4.3). In addition, MPs recovered from a pellet of plasma centrifuged twice at 17,000*xg* for 30 minutes was added back to the filtered plasma to confirm that MPs are mainly responsible for the procoagulant activity, rather than loss of coagulation factors with filtration. Pelleted MPs were resuspended in 20µl of HBS buffer then added to 180µl of filtered autologous plasma. As shown previously, filtration significantly removed the effect of MPs on thrombin generation ($p<0.05$), but recovered MPs reversed this effect bringing thrombin generation back to the level observed in the pre-filtered plasma ($p>0.05$).

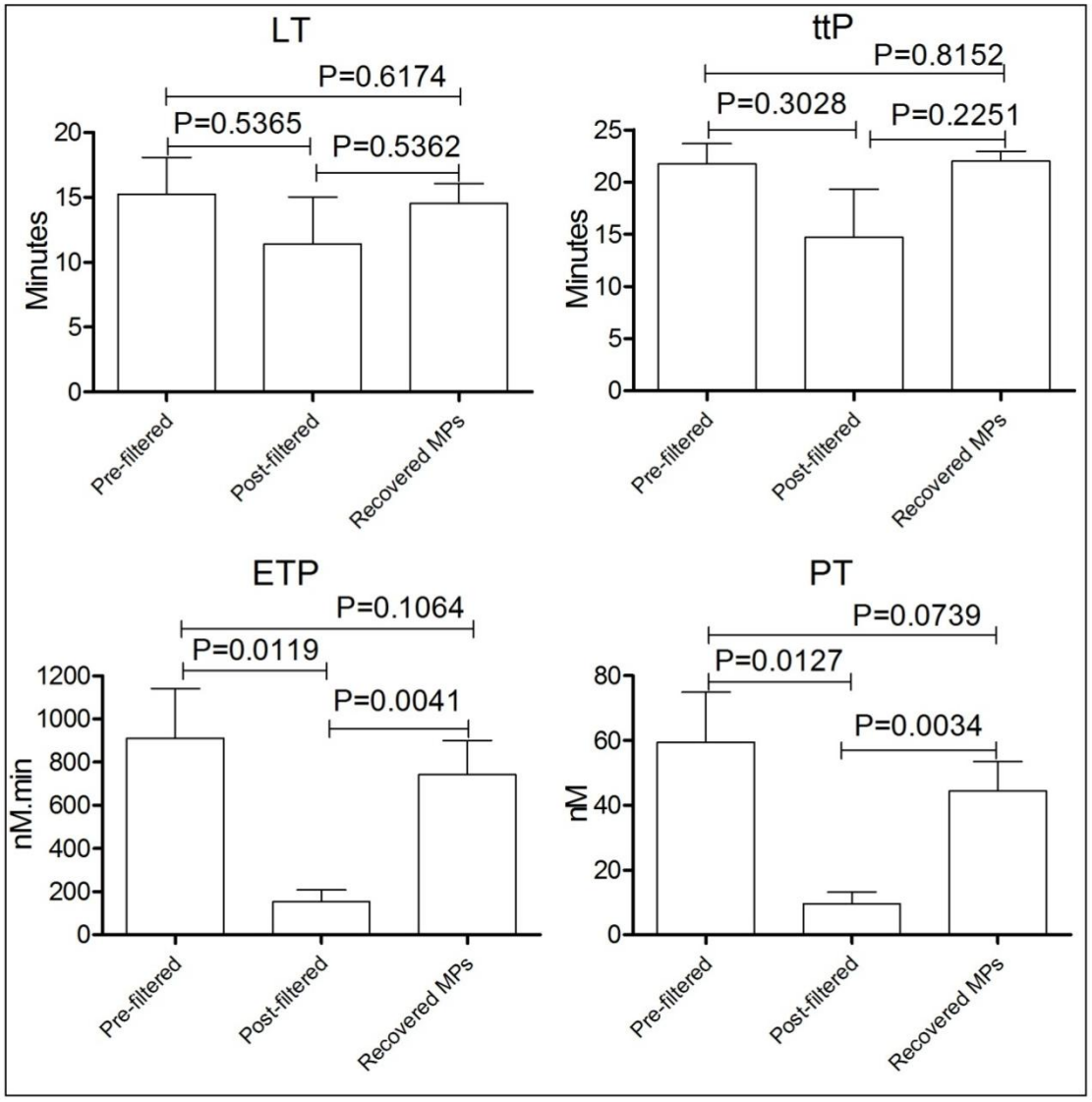



**Fig 4.3. Recovered MPs reversed the effect of filtration on thrombin generation triggered with the PRP (1pMTF) reagent.** Thrombin generation was measured in plasma from normal healthy control and patients with end stage renal disease with and without filtration. In addition, recovered MPs from a pellet of centrifuged plasma twice at 17,000*xg* for 30 minutes were added to the filtered plasma. Data shown as mean±SEM from 6 individuals, statistical analysis was performed using paired t-test.

## 4.3. Discussion

The disruption of vulnerable atherosclerotic plaque with subsequent thrombin generation leads to triggering of the haemostatic response that could determine the outcome. Cardiovascular risk is associated with many diseases, and plasma from subjects with (i.e. MI), or at risk (i.e. CKD) of CVD are reported to have markedly more ability to generate thrombin, and a greater subsequent ability to produce a thrombus, due to increased levels of coagulation factors, procoagulant MPs and TF compared to healthy subjects (Aras*, et al* 2004, Bidot*, et al* 2008, Biro*, et al* 2003, Hron*, et al* 2007, Nieuwland*, et al* 1997, Wright*, et al* 2005). The most common measures for the detection of thrombotic events is prothrombin fragment 1+2 (a specific marker for endogenous thrombin generation), but this measures thrombin after generation (recorded an event) (Li*, et al* 1999). Conversely the pre-existing hypercoagulability of the plasma in patients with cardiovascular risk may determine the outcome and tendency of the haemostatic response and this can be measured using CAT assay (Aras*, et al* 2004, Bidot*, et al* 2008, Biro*, et al* 2003, Hron*, et al* 2007, Leroyer*, et al* 2007, Nieuwland*, et al* 1997).

The current data clearly demonstrate the presence of procoagulant MPs in the plasma of CKD patients, as they supported more thrombin generation in terms of ETP and PT compared to match healthy controls and their effect was completely removed by filtration. However, this prothrombotic activity was not observed in patients with previous premature MI, who were in a stable condition. Although, CAD and CKD have an increased prevalence of thrombotic events, with a common feature in both diseases being endothelial damage, the majority of MPs in CAD patients are from ruptured

plaques, while in CKD the MPs originate from endothelial damage. Both diseases have elevated levels of circulatory MPs compared to healthy controls (Mallat*, et al* 2000, Nieuwland*, et al* 1997); renal disease (Amabile*, et al* 2005) and CAD (Bernal-Mizrachi*, et al* 2003) and MPs have also been associated with prothrombotic condition (Aras*, et al* 2004, Biro*, et al* 2003) and with single and recurrent venous thrombosis (Bidot*, et al* 2008, Hron*, et al* 2007, van Hylckama Vlieg*, et al* 2007).

In the current analysis, thrombin generation driven by MPs was higher in CKD patients compared to matched healthy controls, which their effect is removed by filtration indicting that the effect is not due to soluble factors. In the same plasma, CKD patients have been shown to contain significantly higher levels of EDMPs and PDMPs compared to healthy matched control using platelet (CD42b) and endothelial (CD144) markers measured by western blotting, but no other markers have been used for MP-derived from other cell types (Burton*, et al* Submitted). This is in agreement with previously reported findings that patients in end stage renal failure have higher level of MPs (Amabile*, et al* 2005, Ando*, et al* 2002, Faure*, et al* 2006), who also shown that these MPs are highly procoagulant (using annexin-V binding) and can be derived from platelet, ECs and also erythrocyte. In addition, our data shows greater procoagulant activity in PD patients compared to HD; this is in line with reported observation of higher level of MPs in PD compared HD (Burton, *et al* submitted) using western blotting. We (Burton, *et al* submitted) observed a strong correlation between the level of PDMPs and EDMPs and thrombin generation parameters. *In vivo*, the mechanism of MP formation and release in CKD patients is still unclear, but *in vitro* uremic toxins have been shown to increase MP formation from endothelial cells (Faure*, et al* 2006),

which might suggest that those uremic toxins contribute to MP formation *in vivo* in those patients. The increase in the procoagulant activity of MPs in CKD patients might contribute to the tendency to prothrombotic events when encountering stimuli (Bonderman*, et al* 2002, Leroyer*, et al* 2007, Mallat*, et al* 1999) and also increase level of EDMPs in the plasma correlates with severity of endothelial dysfunction (Amabile*, et al* 2005).

In addition, the current result demonstrates that well-managed premature MI patients in a chronic, stable, stage have comparable thrombin generation driven by MPs in terms of ETP compared to the controls, and slightly lower PT values than the control group. In line with previous report by Mallat, *et al* (2000) who found no differences in circulatory procoagulant MPs between patients with stable angina and non-coronary patients. The same group found more circulatory procoagulant MPs in ACS patients compared both to patients with stable angina and non-coronary individuals. This might suggest that well managed patients with chronic stable CVD have fewer procoagulant MPs than those in an acute condition. A recent study by Sinning, *et al* (2010) suggest the MPs could be used as an independent marker of cardiovascular outcome. The possible explanation for comparable thrombin generation between MI and controls this is that MI patients is in a chronic state and were on antiplatelet drugs including statin, which might affect MPs procoagulant activity, as well as thrombin generation. According to Brodin *et al* (2009), MI patients on antithrombotic treatment, including warfarin alone or a combination of aspirin and warfarin, but not aspirin alone, have an 18-28% reduction in thrombin generation in terms of ETP and PT in plasma using the 5pMTF/4uMPL reagent, although we have shown that this reagent is not mainly

sensitive to procoagulant MPs. On the other hand, Bandi *et al* (2007) observed a significant reduction in thrombin generation in stable CAD patients on aspirin and clopidogrel compared to a healthy control. Other groups have mentioned similar effects on thrombin generation (Altman*, et al* 2007, Wielders*, et al* 1997).

In addition, the current data showed that filtration significantly prolonged the initiation (LT) of thrombin generation in MI and their matched control and CKD patients, but not their matched healthy controls. This suggests the presence of high level of TF-bearing MP in CKD patients but not MI patients, which contribute to the procoagulant activity in the plasma. In addition, soluble TF and TF-bearing MPs have been detected in the circulation in healthy subjects (Giesen*, et al* 1999) and patients. Indeed, the level of TF-bearing MPs increased significantly in the plasma with different diseases compared than in a normal people including ACD (Mallat*, et al* 2000) and cancer associated thrombosis (Kasthuri*, et al* 2009), which has been shown to be is associated with poor prognosis. This mechanism of how TF can enhances the thrombotic diseases is clearly understood.

The current data shows that filtration is mainly removing procoagulant MPs, and the reduction observed on thrombin generation after filtration is not due to coagulation factors. MPs were recovered from a pellet obtained from plasma centrifuged at 17,000x*g* for 30 minutes and then added back into filtered plasma. The recovered MPs reversed the effect of filtration significantly, thus indicting the effect observed with filtration is not due to the effect on coagulation factors (Fig 4.3). Lawrie, *et al* (2008) showed that filtration using Ceveron unit significantly reduced the level of FVIII in the

plasma and to emphasise that the effect on thrombin generation is mainly driven by procoagulant MPs, but not due to soluble factors.

The main limitations of this study are the lack of characterisation of the type, and absolute levels of MPs in the MI and CKD, with only a partial analysis of the type MPs in CKD patients using a western blot based on the use of markers for endothelial and platelet-derived MPs, but not for other cell types such as monocytes. Also, the contribution of contact coagulation factors, mainly FXII, in these plasmas cannot be ruled out as the samples were not collected in CTI (Dargaud*, et al* 2007, Luddington and Baglin 2004, Ollivier*, et al* , Spronk*, et al* 2009, Van Veen*, et al* 2008).

The overall conclusion is that our findings indicate that plasma from CKD patients are more prothrombotic than matched healthy control, which is mainly due to circulatory procoagulant TF-bearing MPs assessed by thrombin generation. In addition, pre-mature MI patients have comparable thrombin generation. In both diseases filtration significantly reduced thrombin generation indicting that this is not dependent on soluble factors. After filtration in CKD, the initiation (LT) of thrombin generation was prolonged indicates the presence of active TF-bearing MPs that was removed by filtration. This study describes a novel way to measure the thrombotic response of the plasma, which can be used as a useful biomarker to predict the increased risk of CVD.

## 4.4 Further work

- Measurement of thrombin generation in a larger cohort, and with different reagents to make the assay sensitive to the level of coagulation factors, MPs and

either soluble or TF-bearing MPs, because TF enhances the procoagulant activity of MPs.

- Direct measurement of TF in the plasma either using FXa generation assay or commercial ELISA, because the current data from CKD suggest the presence of TF-bearing MPs, because the data suggest the presence of endogenous TF-bearing MP. TF enhances the procoagulant activity of the plasma.
- Identifying the cell origin of MPs in the cohorts using flow cytometry and possibility counting them using NTA.

# Chapter 5: Standardization of the analysis of platelet procoagulant activity

## 5.1. Introduction

The procoagulant surface on activated platelets and MPs has been well studied using different approaches, including flow cytometry and prothrombinase assay. Flow cytometry is widely used in laboratories for PS detection using annexin-V binding and MP count (Abrams*, et al* 1990, Jy*, et al* 2004, Thiagarajan and Tait 1990), but fails to provide functional measure, or to detect smaller (<0.5µm) MPs. The prothrombinase assay relies on the addition of exogenous TF and coagulation factors, making the rate-limiting factor the availability of PS, but this may not be physiologically relevant. In more physiological-relevant conditions, the CAT assay provides a continuous measurement of the contribution of the procoagulant surface from platelets and/or MPs in a suspension to generate thrombin (Hemker*, et al* 2003). However, the CAT and prothrombinase assays only give information on the functional activity but not count and size of MPs. This chapter investigates some of pre-analytical and analytical variables of annexin-V binding and thrombin generation measurement in platelets. In addition, it demonstrates the differences between the flow cytometric assay and the thrombin generation assay in comparing platelets and PDMPs, and tests whether there is a relationship between annexin binding and thrombin generation.

# 5.2 Results

## 5.2.1. Characterisation of procoagulant surface in PRP suspension

A two-colour flow cytometric assay was used to detect PS exposure on the surface of platelets and PDMPs using FITC-labelled annexin-V and PE-labelled CD42b as a pan platelet marker that binds to GPIbα. Annexin-V has a high avidity to bind the anionic negatively-charged PLs in a $Ca^{2+}$ dependent process (Andree*, et al* 1990, Tait*, et al* 1989, Thiagarajan and Tait 1990). Therefore, in all of the experiments HBS buffer/$Ca^{2+}$ (HBS/$Ca^{2+}$ final concentration 2mM) was used unless otherwise stated. In this study, freshly isolated PRP was analysed with and without the stimulation with CRP-XL.

Stimulation of platelets with CRP-XL caused PS exposure and MP formation as previously shown (Appleby*, et al* 2006) in our laboratory. The presence of a platelet population was identified according to its size using FS, and granularity using SS, as illustrated in Fig 5.1 (histogram A). The population of resting (unstimulated) platelets form a single, diffuse population (histogram A left panel), but upon platelet stimulation with CRP-XL (10.0μg/ml) (histogram A right panel) a new, smaller population appears which is less granular, than the population of platelets, these are the PDMPs. The annexin-V positive (AxV+ve) events and negative (AxV-ve) events are plotted against PE positive (CD42b) events, which show three regions: FITC negative events, weakly positive FITC events and strongly positive FITC events (histogram B). Then the whole population in histogram B (either FITC +ve or -ve) is also shown as size (FS) vs. count in histogram C (red box). The resting (unstimulated) platelet population (histogram C left panel) showed only one population on the basis of size, while the CRP-XL stimulated population (histogram C right panel) shows two populations; the

large particles are unactivated and activated platelets, and the smaller population are the PDMPs. Histogram D shows size (FS) against AxV+ve events only; here the CRP-XL stimulated population shows 3 regions: (i) weak AxV+ve events are large particles on the basis of FS and are therefore mainly platelets; (ii) intermediate annexin-V positive events (AxV++ve) events are small particles on the basis of FS (mainly PDMPs); and (iii) strong annexin-V positive events (AxV+++ve) events are small particles on the basis of FS (mainly PDMPs), which are similar to the particles in the AxV++ve in size. This analysis shows that both platelets and PDMPs can bind annexin-V, but that PDMPs are smaller in size and bind more annexin-V than the activated platelet population, suggesting that PDMPs are more procoagulant than the platelets.

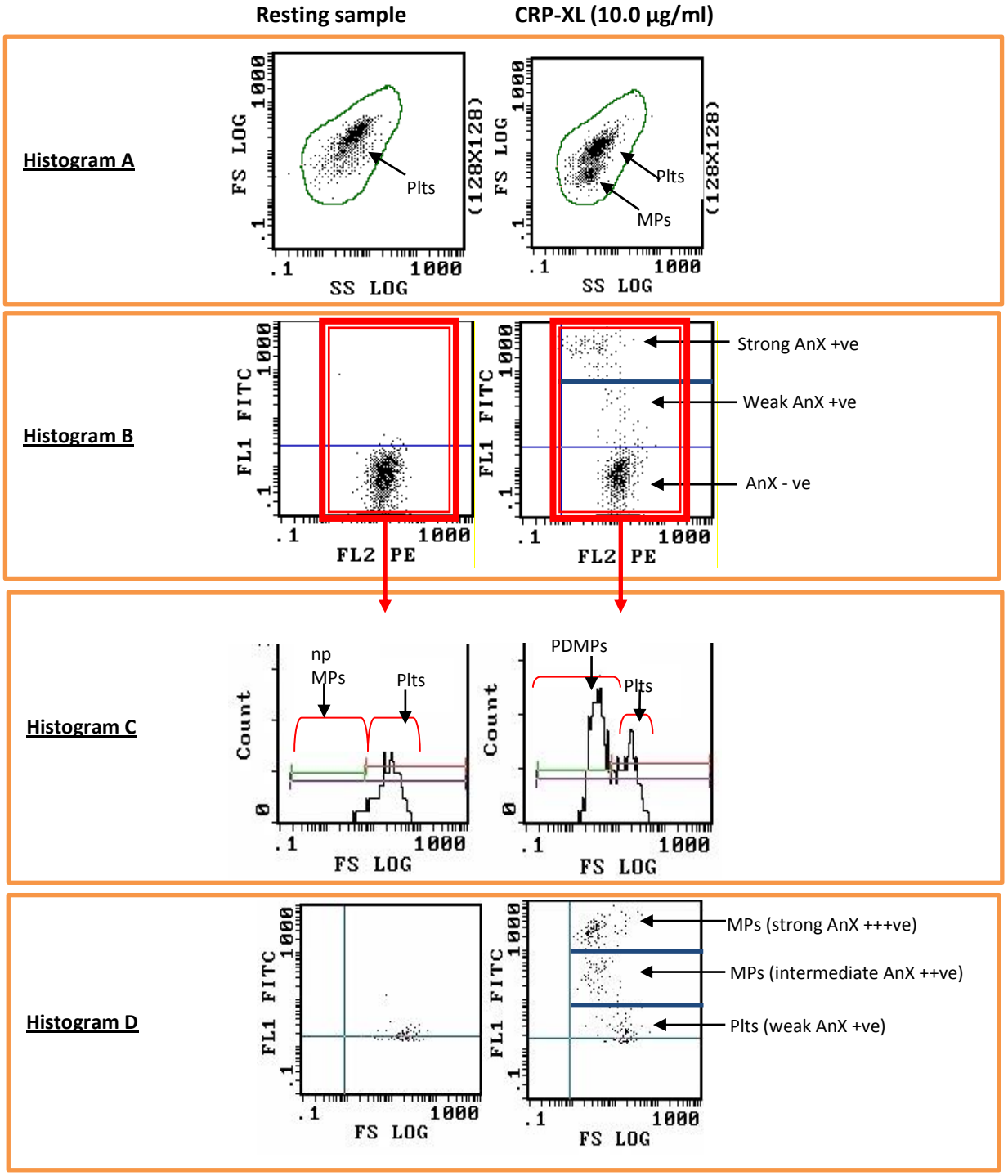



**Fig 5.1: Flow cytometric analysis of the procoagulant surface on platelets (Plts) and MPs using annexin-V.** PRP was incubated with RPE-conjugated CD42b (pan platelet marker for GPIbα) (FL2) and annexin-V FITC (FL1). Left histograms show resting platelets and right histograms show CRP-XL (10.0µg/ml) stimulated platelets. The analysis is based on identifying platelet populations on their forward scatter (size) and side scatter (granularity) in histogram A. Histogram B shows the distribution of FITC versus PE positive events. Histogram C shows the forward scatter (FS) distribution of total population (red box). Histogram D shows the distribution of FS positive events with FITC. Platelet-derived microparticles (PDMPs).

## 5.2.2. Optimal dose of CRP-XL to induce full platelet responses

To determine the best concentration of CRP-XL for inducing the maximum procoagulant surface on platelets and the release of PDMPs, a dose response of CRP-XL between 0.2 to 10.0μg/ml was carried out in 10 healthy individuals (Fig 5.2). CRP-XL significantly increased both the percentage of AxV+ve cells and the MFI significantly in all donors; PS exposure increased with increasing concentration of CRP-XL (one-way ANOVA $p<0.05$), reaching a maximum at 2.0μg/ml. The weak AxV+ve population appears first and plateaus at 15%, whereas the strongly AxV+ve population continues to increase in number, but not in fluorescence intensity. From these studies, CRP-XL at 2.0μg/ml was selected as the optimal concentration for all subsequent experiments, unless otherwise stated.

Then, the same dose-response was investigated on other platelet activation markers such as aggregation, degranulation and thrombin generation. Platelet degranulation, as assessed by P-selectin expression, reached a maximum at the lowest concentration of CRP-XL used (0.2μg/ml) (Fig 5.3a). Similarly, both fibrinogen binding (Fig 5.3b) and aggregation (Fig 5.4) also reached a maximum at the lowest concentration of CRP-XL. In addition, P-selectin and fibrinogen binding are seen on ~100% of platelets whereas annexin-V binding varies between 20-60%, never reaching the maximum (100%), as previously shown (Appleby*, et al* 2006) in our laboratory. Similar finding with thrombin generation by platelets (in PRP), which is different from chapter 3. The lowest concentrations of CRP-XL induce the maximum thrombin generation triggered by the PRP (1pMTF) reagent (Fig 5.5). In more detail, resting (no CRP-XL) PRP had a longer LT and ttP and generated less ETP and PT to compared the stimulated platelet (contains

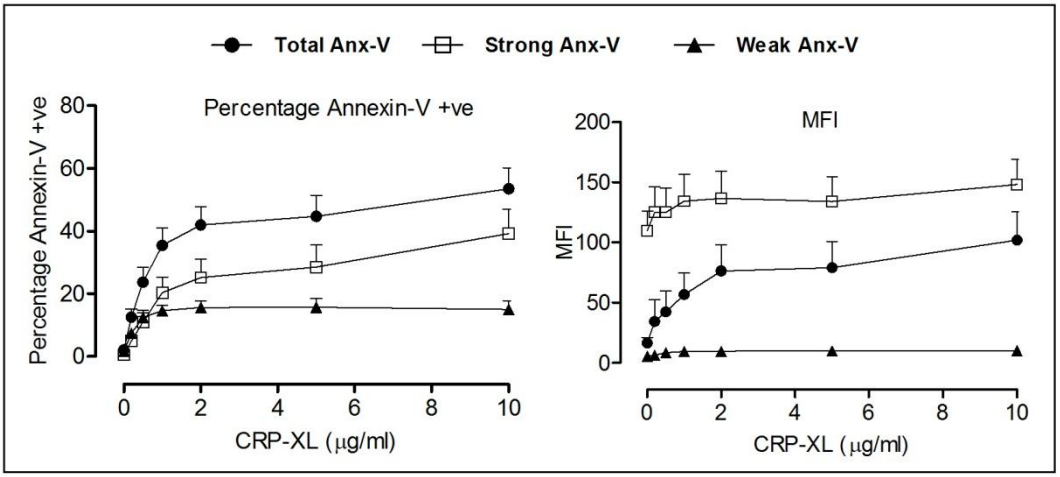



**Fig 5.2 CRP-XL induced PS exposure in dose-response manner.** Platelets were incubated with an increasing concentration of CRP-XL and then PS exposure was measured by flow cytometry using an annexin-V binding assay. Results are shown as mean±SEM from 10 healthy individuals. (One-way ANOVA <0.05 considered significant).

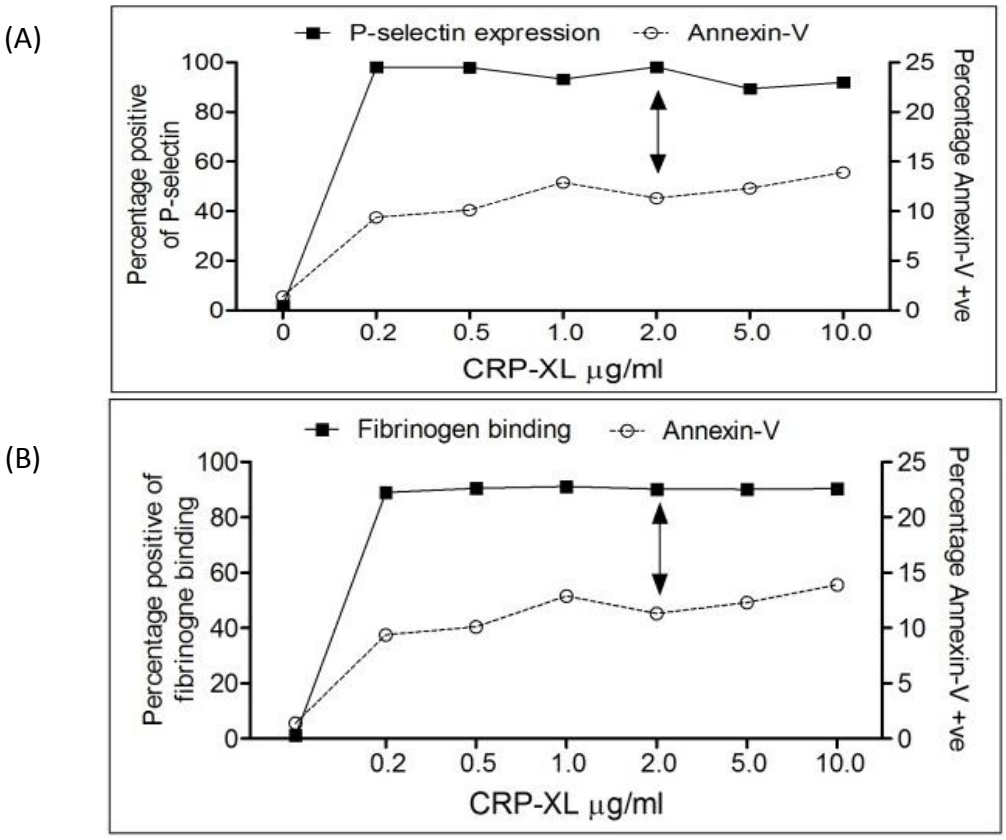



**Fig 5.3. Dose-dependent effect of CRP-XL on platelet activation marker using flow cytometry.** Panel A shows P-selectin expression. Panel B shows fibrinogen binding assay). PRP was obtained for three healthy donors. Data shown as mean±SEM from 3 healthy individuals.

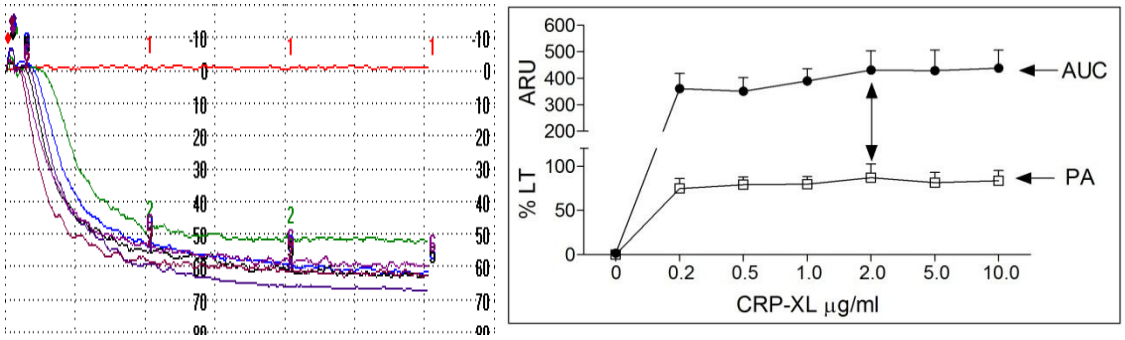
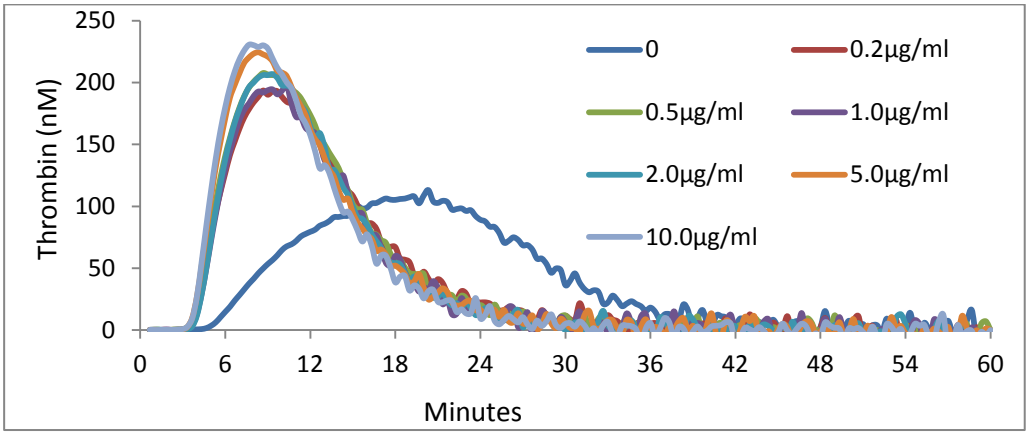



**Fig 5.4. Dose-dependent effect of CRP-XL on platelet aggregation.** Left panel representatives data of the aggregation from one run. Right panel shows primary aggregation (PA) and area under the curve (AUC). PRP was obtained for three healthy donors. Data shown as mean±SEM.

A)




B)

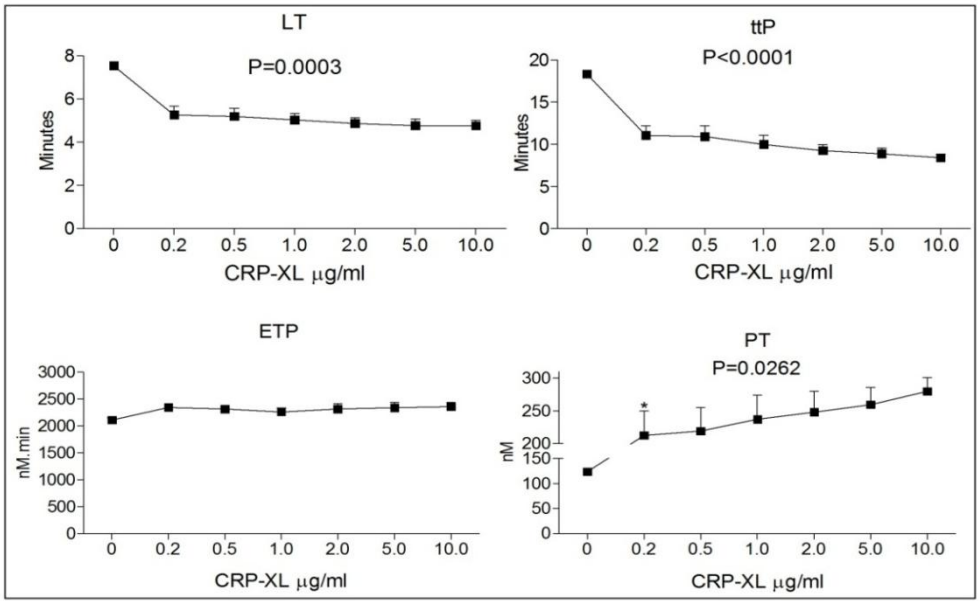



**Fig 5.5. Influence of dose-response of CRP-XL on thrombin generation measured in PRP.** (A) Representative example of thrombin generation curves from one donor. (B) The mean of the numerical data (n=5). PRP was stimulated with CRP-XL for 10 minutes at 37°C, and then thrombin generation was triggered by the PRP (1pM TF) reagent. Data is shown as mean±SEM, One-way ANOVA.

activated platelet and MPs) in any concentration of CRP-XL (0.2-10.0µg/ml). A significant (one-way ANOVA $p<0.05$) difference was observed in all thrombin generation parameters except for ETP. Although there was an increase in PT with increased doses of CRP-XL it was not statistically significant. However, when the highest concentration of CRP-XL (10µg/ml) was compared to any of the other concentrations of used it was not significant except CRP-XL at 0.2µg/ml (*).

### 5.2.3. Effect of platelet count on thrombin generation

The effect of platelet count on thrombin generation was studied using the PRP (1pM TF) reagent and the PRP/NIBSC (1pM TF/2.0µMNIBSC PL) reagent. The PRP reagent is the recommended reagent for thrombin generation measurement by platelets (Hemker*, et al* 2003). The PRP/NIBSC reagent was used to investigate whether the addition of exogenous PL would affect the measurement of thrombin generation. The PRP sample was diluted in autologous filtered PPP to reach the desired count. Using the PRP reagent, the PT and ETP were affected by the platelet count but the LT and ttP were not (Fig 5.6a and c). PT in the presence of platelet counts of $10x10^9$/L and below was similar to that in the filtered plasma, but increased linearly with increasing platelet counts up to a count of $200x10^9$/L. ETP also increased with increasing platelet counts from $5x10^9$/l, reaching a plateau at $50x10^9$/L and above, as previously reported by (Vanschoonbeek*, et al* 2004). When the samples from two of the donors were run in the presence of exogenous PL, the effect of the platelet count on thrombin generation was abolished for all parameters (Fig 5.6b and d). In all subsequent analyses the procoagulant activity of platelets, the count was adjusted to $150x10^9$/L and thrombin generation was triggered using the 1pMTF reagent.

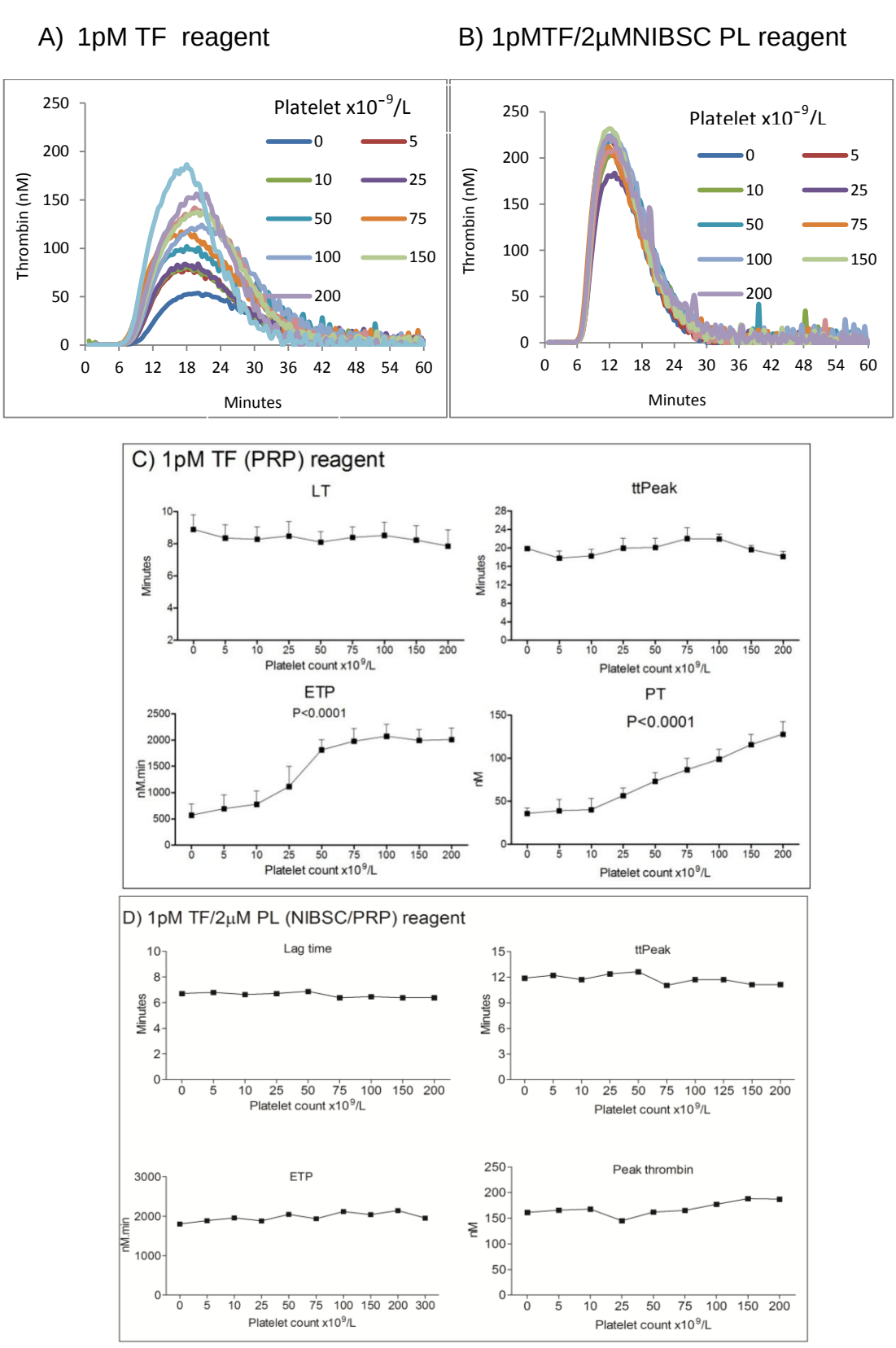



**Fig 5.6. Influence of platelet count on thrombin generation measured in PRP.** Thrombin generation was triggered with (panels A/C) 1pM TF (n=4) or (panels B/D) 1pM TF/2µMNIBSC PL (n=2). Panels A and B are representative examples and C and D are the mean values. PRP was stimulated with CRP-XL for 10 minutes at 37°C. Data are shown as mean±SEM, One-way ANOVA in panel C. Zero (0) count is PPP.

### 5.2.4. Effect of time on PS exposure

The time of the assay can vary considerably when large numbers of samples are being analyzed; therefore, the stability of the annexin-V binding assay was investigated. PRP was incubated with annexin-V and CD42b and then stimulated with 0, 0.2, 1.0 and 10.0µg/ml CRP-XL for 20 minutes at RT, then the samples were diluted 1:1000 according to the protocol outlined in section 2.4.2.1. The diluted samples were then incubated for 0, 30, 60, 90, 120 minutes at RT, Prior to the analysis of the sample using flow cytometry. This analysis was performed for two separate donors. The data for 0 and 10.0 CRP-XL only are shown (Fig 5.7). PS exposure increased in the first 30 minutes, but after that neither the percentage of AxV+ve particles nor the MFI changed over the subsequent hour. PS exposure then increased in the activated samples between 90 and 120 minutes, but almost no changes were seen in the resting sample over the whole two hours apart from the MFI that increased after 1 hour. Similar results were observed in the strongly AxV+ve events. The weakly AxV+ve events reached a maximum by 90 minutes, followed by a reduction in the percentage of both annexin-V positive and MFI. It can be concluded that the assay is stable after 30 minutes and for up to 1 hour, so for all of the subsequent experiments all samples were analyzed within this time window. The same results were observed for the other two CRP-XL concentrations, 0.2 and 1.0µg/ml and for the second donor (data not shown).

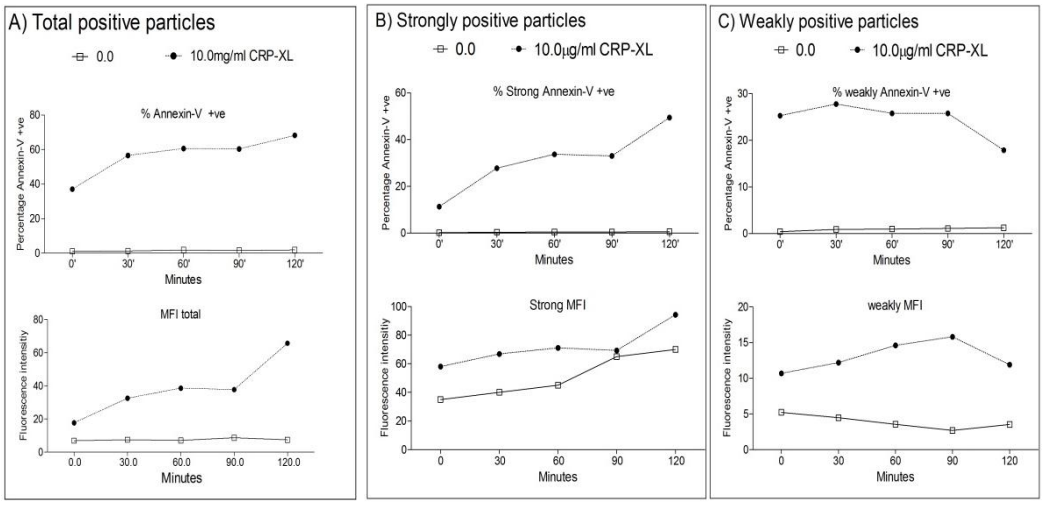



**Fig 5.7. Effect of time on PS exposure.** A) the effect of incubation time on the total annexin-V percentage positive and MFI, B) the effect of incubation time on strong percentage positive particles and strong MFI and C) the effect of incubation time on weak percentage positive particles and strong MFI. PRP was analyzed at the given time for PS exposure using 0 (□) and 10.0ug/ml (●) CRP-XL. Results are shown from one experiment.

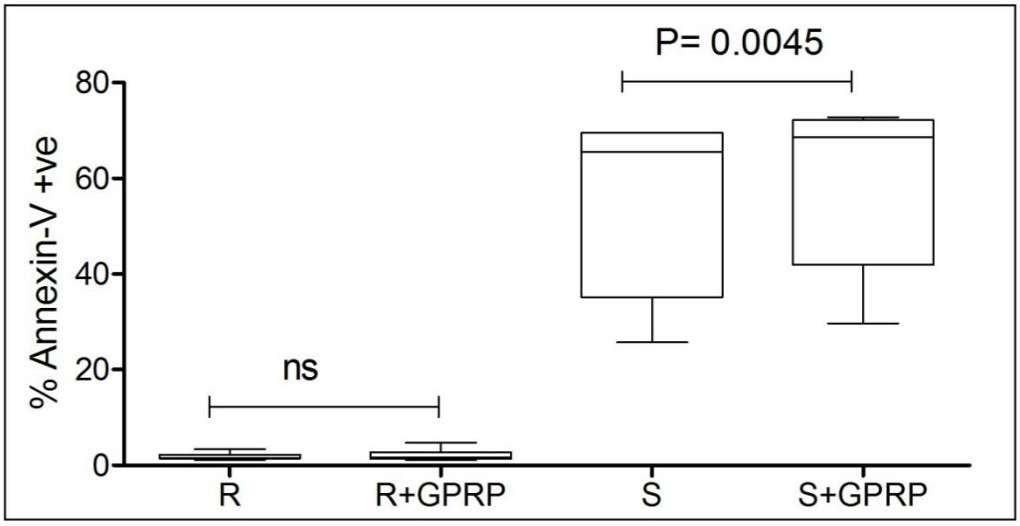



**Fig 5.8. Influence of fibrin polymerization inhibitor on PS assessment by annexin-V binding.** PRP was incubated with and without GPRP (0.5mg/ml) before CRP-XL stimulation for 10 minutes at RT. Data shown from 6 health subjects (n=6), paired t-test) with and without GPRP.

### 5.2.5. Effect of fibrin polymerization inhibitor on Annexin-V binding

This experiment addresses whether the presence of GPRP, a fibrin polymerization inhibitor, affects the percentage of annexin-V positive platelets or PDMPs in the PRP or thrombin generation by plasma. Therefore, GPRP was added at a 0.5mg/ml final concentration to PRP before stimulation with CRP-XL (2.0µg/ml) (Fig 5.8) from six healthy individuals. Resting and CRP-XL stimulated PRP were analysed using annexin-V binding, while plasma and PDMP-rich plasma were obtained from the same samples and analysed in the CAT assay using the 1pM TF reagent (data not shown). The addition of GPRP slightly, but significantly, increased the percentage of AxV+ve platelets with GPRP (59.6±17.7) in the activated sample compared to activated PPR without GPRP (55.7±18.8) (p=0.0045). No significant change was observed in the resting sample. For thrombin generation measurement driven by MP; the results show no significant differences between the measurement of thrombin in normal plasma or in PDMP-rich plasma with and without GPRP (data not shown).

### 5.2.6. Inter-individual variability to CRP-XL

The analysis of the CRP-XL dose-response from the 10 healthy subjects showed a wide range of responses to CRP-XL in the annexin-V binding (Fig 5.9). For example, it can be seen that at high concentrations of CRP-XL (e.g. 10.0µg/ml) there is at least a three to four fold variation between normal subjects in the maximum amount of annexin-V binding. This supports previous observations in this lab (Appleby*, et al* 2006) and elsewhere (Joutsi-Korhonen*, et al* 2003), and demonstrates there is an inherent inter-individual variability, as these differences in response are consistent over time (Appleby*, et al* 2006). Similar inter-individual variability has been also observed in the

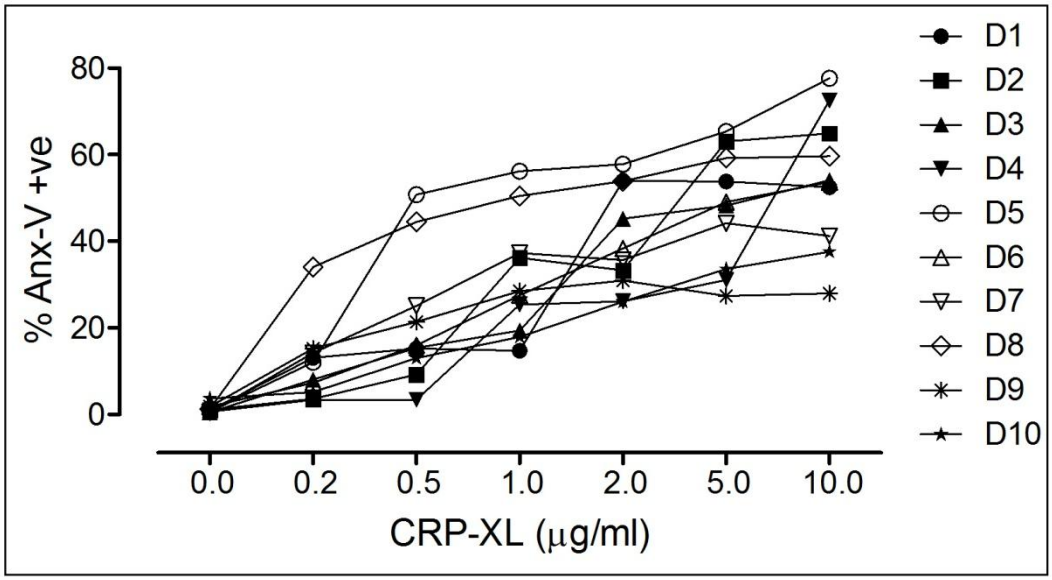



**Fig 5.9. Variability in individual response for CRP-XL (dose-response) assessed by annexin-V.** Platelets from ten healthy subjects were activated with a CRP-XL at dose ranging from (0-10.0µg/ml) for 20 minutes at RT, and PS was analysed by flow cytometry. Each symbol represents an individual donor.

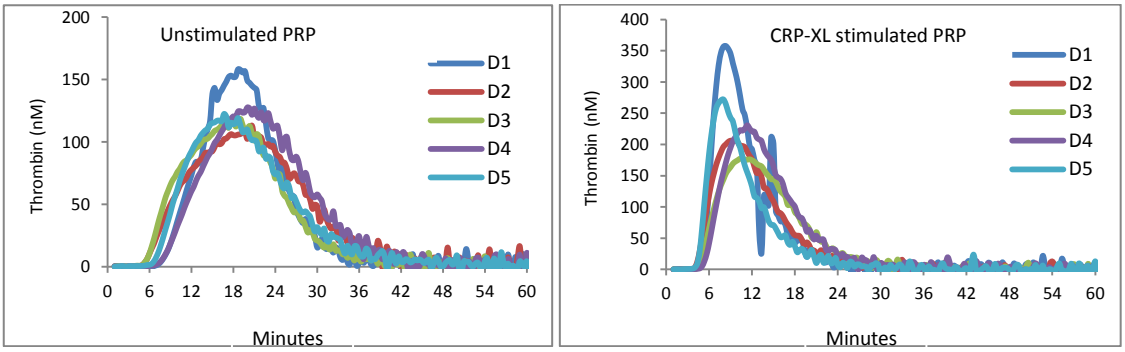



**Fig 5.10. Representative examples of the variability among individual in response to CRP-XL in thrombin generation.** Platelets from five healthy subjects were activated with and without CRP-XL (2.0µg/ml) for 10 minutes at 37°C, and thrombin generation was analysed with the PRP (1pMTF) reagent. Each symbol represents an individual donor.

thrombin generation assay (representative examples are shown in Fig 5.10).

### 5.2.7. Comparison of thrombin generation in PRP before and after CRP-XL stimulation

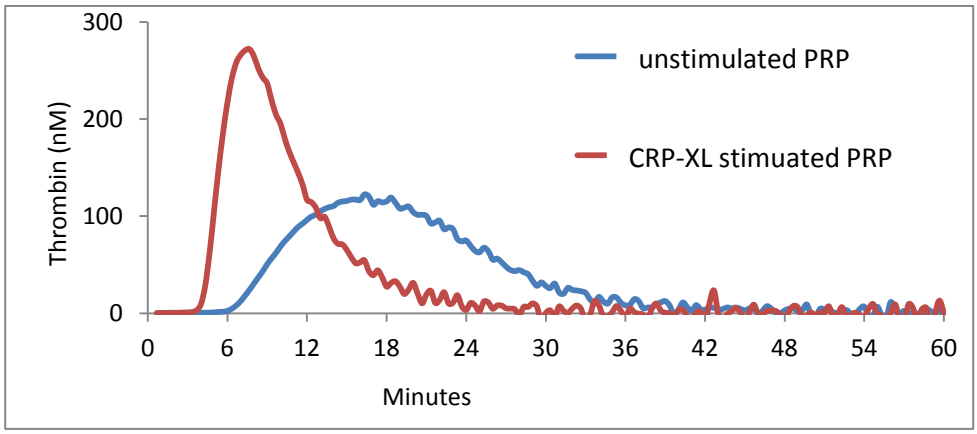
After determining the standard platelet count analysis in the thrombin generation and the optimum dose of CRP-XL, as well as confirming the best CAT reagent, the current analysis compared the analysis of PRP with and without stimulation with CRP-XL (2.0µg/ml). Normal PRP contains platelets and a few MPs from circulation, while CRP-XL stimulated PRP will have both activated platelets and PDMPs in the suspension. Triggering the reaction with the 1pMTF reagent gave significant enhancement of thrombin generation in CRP-XL stimulated PRP over and above the unstimulated sample, with more significant ETP and PT, and shorter LT and ttP compared to unstimulated PRP (Fig 5.11, all parameters $p<0.05$). Of the four parameters, ETP was the least affected by activation-because it is a reflection of the whole process interaction i.e. coagulation factors and PL levels whereas the PT reflects PL and LT and ttP reflect TF.

### 5.2.8. Comparison of thrombin generation between PRP and PPP

Plasma was obtained from the same samples (described in 5.2.7) to analyse thrombin generation in parallel. The analysis compared unstimulated PRP (which contains both platelets and MPs) to plasma (which contains only MPs). In addition, the analysis compared CRP-XL-stimulated PRP (which will contain both activated platelets and be rich in PDMPs) to PDMP-rich plasma (without platelets) from the same samples. Representative examples of thrombin generation curves are shown in Fig 5.12a.

Comparing unstimulated PRP (green line) and plasma (blue line) from the same individuals, which showed that PRP had significantly higher amount of thrombin generated compared to plasma, as measured by ETP ($p=0.0002$) and PT ($p=0.0003$) (Fig 5.12b) with a faster LT and ttP in the PRP compared to PPP ($p>0.05$). After CRP-XL activation, the CRP-XL-stimulated PRP (red line) had significantly more thrombin generation, in terms of ETP ($p=0.0332$) and PT ($p=0.0044$), and a faster LT ($p=0.0024$) and ttP ($p=0.0053$) when compared to PDMP-rich plasma (black line) from the same donor (Fig 5.12c).

A)




B)

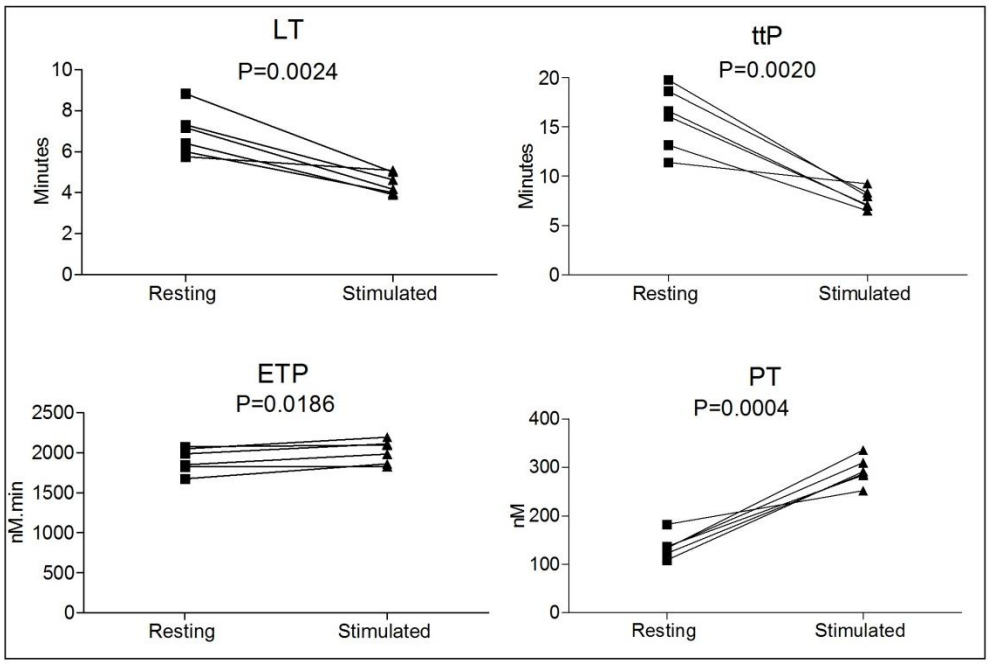



**Fig 5.11. Thrombin generation measurement in PRP before and after CRP-XL stimulation from healthy individuals.** A) Representative examples of thrombin generation curve from one donor. B) Numerical data from 6 health donors (n=6). Thrombin was triggered with the 1pMTF in PRP before and after CRP-XL (2.0µg/ml) stimulation. Paired t-test analysis was used and $p<0.05$ was considered significant.

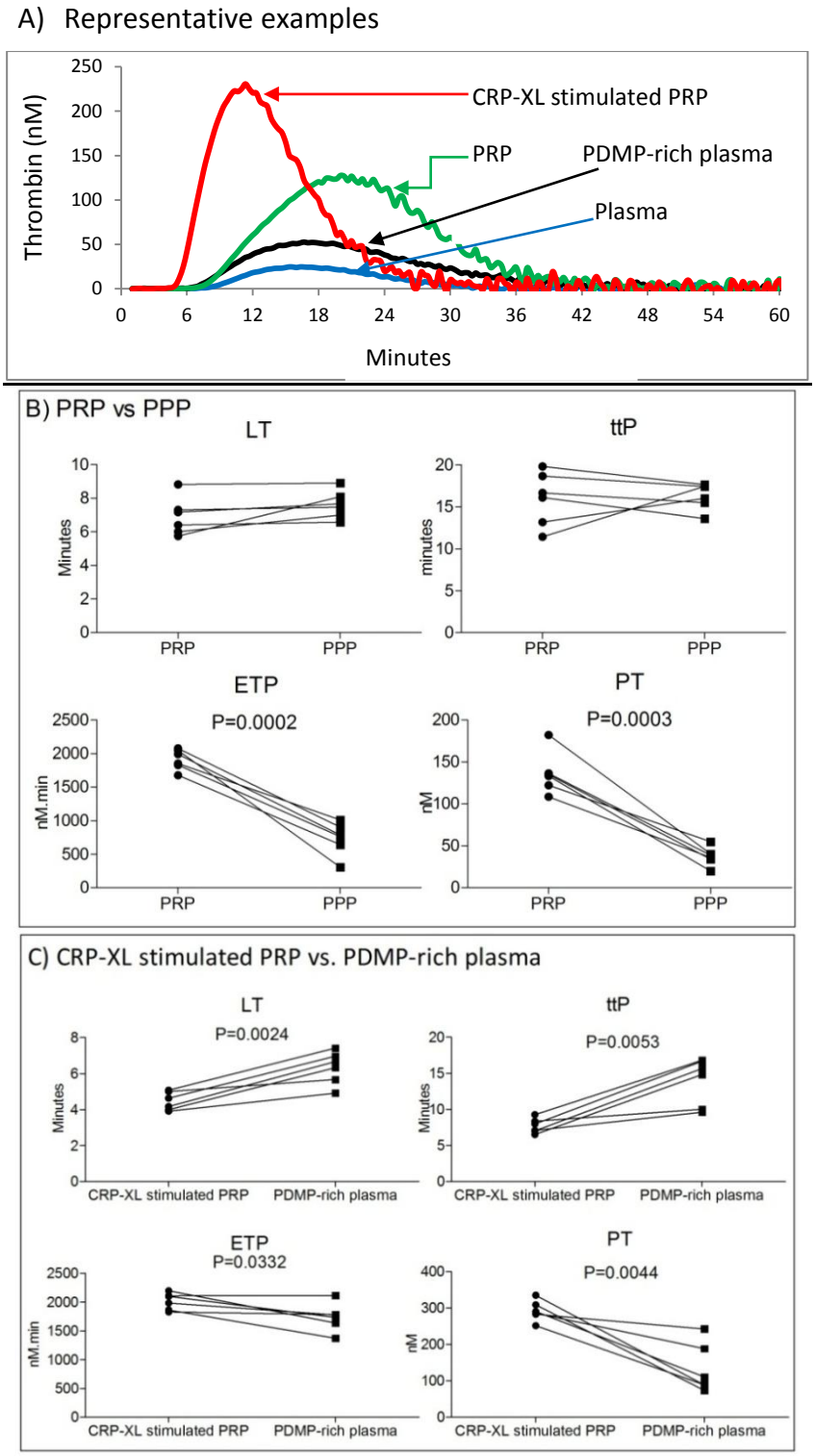



**Fig 5.12. Measurement of thrombin generation in PPP and PRP before and after CRP-XL stimulation from 6 healthy.** A) Representative example of thrombin generation curves from one healthy donor B) Resting PRP compared to PPP samples C) CRP-XL stimulated PRP compared to PDMP-rich plasma sample. PPP was obtained from either PRP with and without CRP-XL stimulation for 10 minutes at 37°C in the presence of GPRP (0.5mg/ml), then samples were centrifuged to obtain either PPP or PDMP-rich plasma at 1800xg for 30 minutes at RT. Thrombin generation was triggered with the 1pMTF reagent. Data shown from 6 healthy individuals (paired t-test analysis and $p<0.05$ was considered to be significant).

### *5.2.9.* Correlation between thrombin generation parameters and PS exposure

Fig 5.1 shows that platelets and PDMPs can be identified and their fluorescence can be analysed. In addition, by gating on the different AxV populations (weak, intermediate and strong) this can give a measure of their size. Fig 5.13 is similar to Fig 5.1 but with all the original histograms of the flow cytometric analysis using 2.0μg/ml CRP-XL. Briefly, Fig 5.13 Histogram A shows platelet population. The analysis is based on identifying platelet population (gate A) on their FS (size) and SS (granularity) in histogram A. Then the platelet population was analysed for expression of PE-CD42b in histogram B. Any AxV+ve event was gated from histogram B into histogram C. FITC AxV+ve events in histogram C were further divided into weak and strong positive populations. Histogram D shows the distribution of FITC versus RPE positive events. Histogram E shows the forward scatter distribution of total FITC positive events in the histogram "C". Histogram F shows the weak positive events. Histogram G shows strong positive events. Histogram H shows the distribution of FS positive events with FITC. The overall message is that the flow cytometric analysis allow to identify the AxV+ve (weak), AxV++ve (intermediate) and AxV+++ve (strong) populations and also can identify their size.

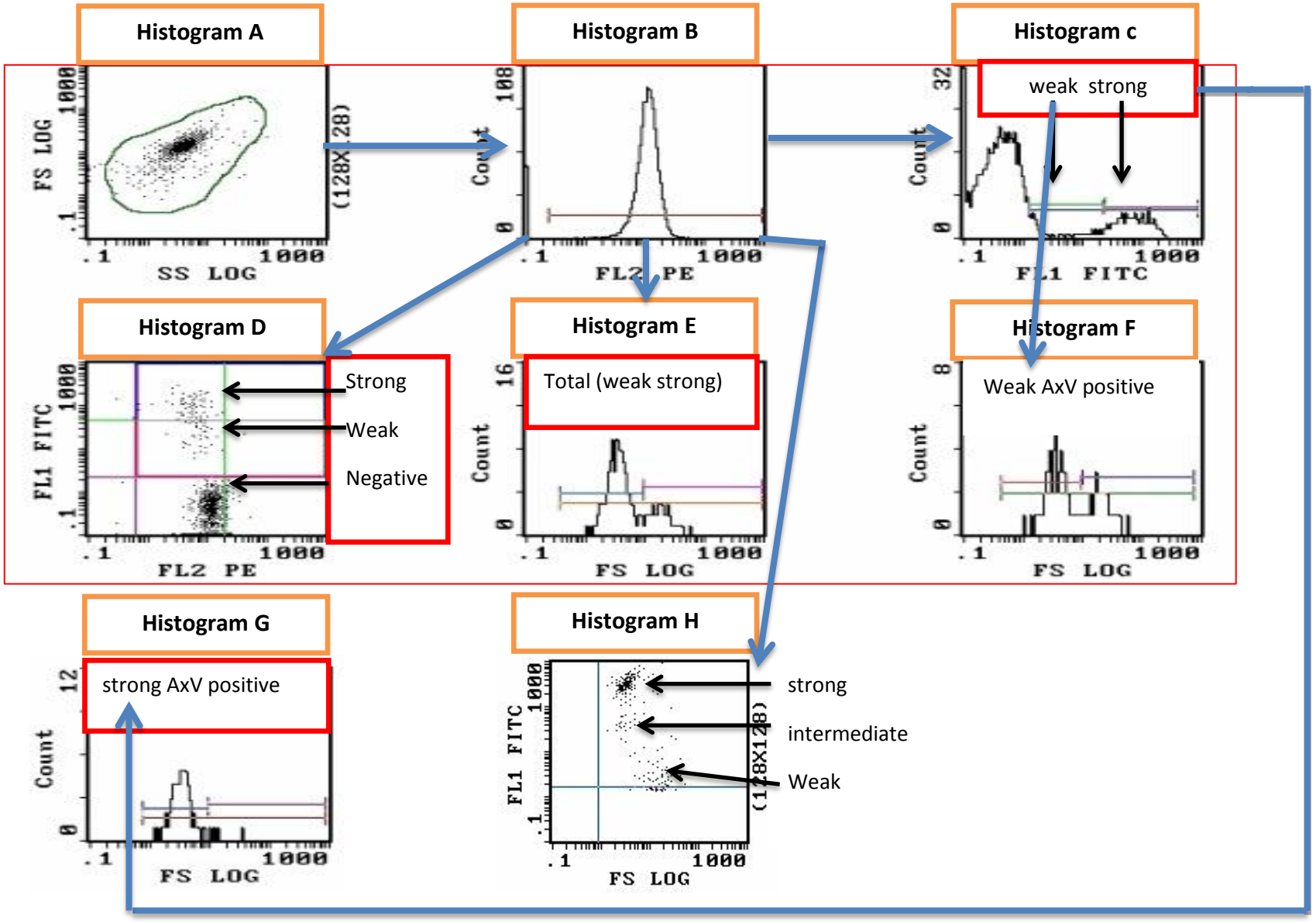

Histogram A
FS LOG
SS LOG
(128X128)
Histogram B
Count
FL2 PE
Histogram c
weak strong
Count
FL1 FITC
Histogram D
FL1 FITC
FL2 PE
Strong
Weak
Negative
Histogram E
Total (weak strong)
Count
FS LOG
Histogram F
Weak AxV positive
Count
FS LOG
Histogram G
strong AxV positive
Count
FS LOG
Histogram H
FL1 FITC
FS LOG
strong
intermediate
Weak

**Fig 5.13: Flow cytometric analysis of the procoagulant surface in CRP-XL activated PRP using annexin-V.** PRP was incubated with RPE-conjugated CD42b (pan platelet marker for GPIbα) (FL2) and annexin-V FITC (FL1), then was activated with CRP-XL (2.0μg/ml) and analysed using flow cytometry, resulting in eight histograms from one run.

### *5.2.9.1.* Correlation between thrombin generation parameters and PS exposure

The analysis in this chapter confirms the findings in the literature that activated platelets and PDMPs expose negatively-charged PL; this procoagulant surface can be detected by annexin-V binding and the activity can be measured by thrombin generation assay. Therefore, the current analysis tests whether there is a relationship between annexin-V binding and thrombin generation. PRP was stimulated with a dose range of CRP-XL for 10 minutes, then thrombin generation and annexin-V binding were measured in these samples in parallel.

There was a strong and significant positive correlation between the total % of AxV+ve particles or of MFI with the amount of thrombin generated in these samples (the cut-off were r and p values) (Fig 5.14), PT was strongly correlated with both the total % +ve particles ($r=0.8557$; $p<0.0001$) and MFI ($r=0.7501$; $p<0.0001$) for annexin-V binding. A similar correlation was observed with ETP ($r=0.\ 8015$; $p<0.0001$) for % AxV+ve and ($r=0.6794$; $p=0.007$) for MFI. Neither the total % of AxV+ve particles ($r=0.3839$; $p>0.05$) nor total MFI were correlated ($r=0.1671$; $p>0.05$) with LT, but ttP was strongly inversely correlated with the total % AxV+ve particles ($r=-0.5591$; 0.0084), but not the MFI ($r=-0.3648$; $p>0.05$).

When analysing the strongly (AxV+++ve) particles, PT ($r=0.7975$; $p<0.0001$) and ETP ($r=0.7590$; $p<0.0001$) showed a significant correlation with the % of AxV+++ve particles, but not the MFI ($p>0.05$) (Fig 5.15). The LT and ttP showed no correlation with either the % of AxV+++ve particles, but inverse strong correlation with the strong MFI ($p<0.05$). For the weakly AxV+ve particles, PT ($p<0.0001$) and ETP ($p<0.0001$)

showed a strong correlation with the MFI, with only PT (p=0.0060) showed a strong association with the weak % of AxV+ve particles, whereas the LT (p=0.0022) and ttP (p=0.0072) showed a strong inverse correlation with weak % of AxV+ve particles, but only ttP had a strong inverse correlation with weak MFI (p=000255) but not LT (Fig 5.16).

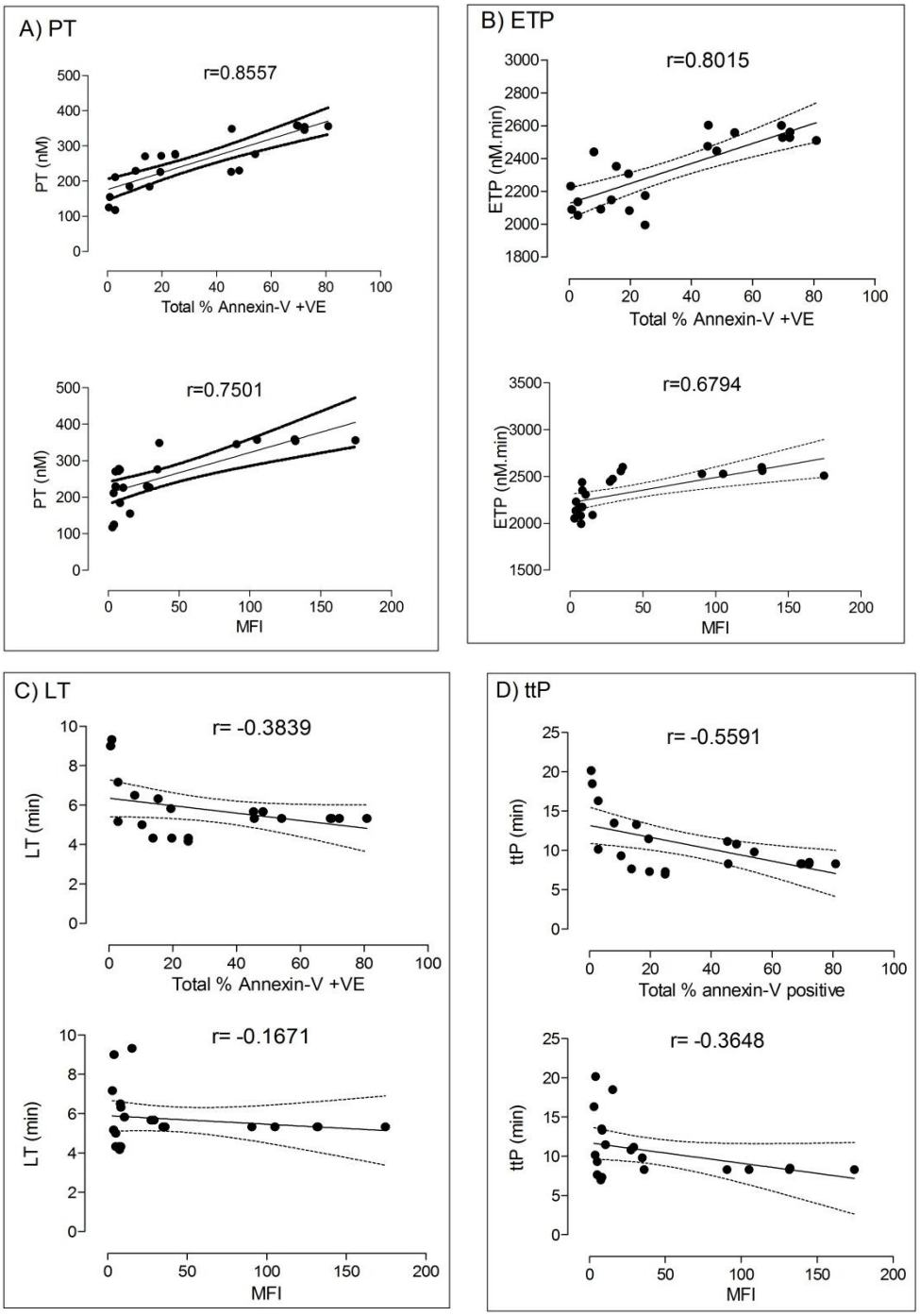



**Fig 5.14: Correlation of thrombin generation parameters and total annexin-V positive events CRP-XL stimulated PRP.** (A) PT, (B) ETP, (C) LT and (D) ttP with total percentage annexin-V positive (upper panel) and with total MFI (lower panel). Data are taken from histogram C in Fig 5.13. PRP was stimulated with different concentrations of CRP-XL (0-10.0µg/ml). PRP was analysed on CAT assay with the 1pM TF and was analysed using annexin-V binding (n=3), numbers of XY analysis were 21 pairs.

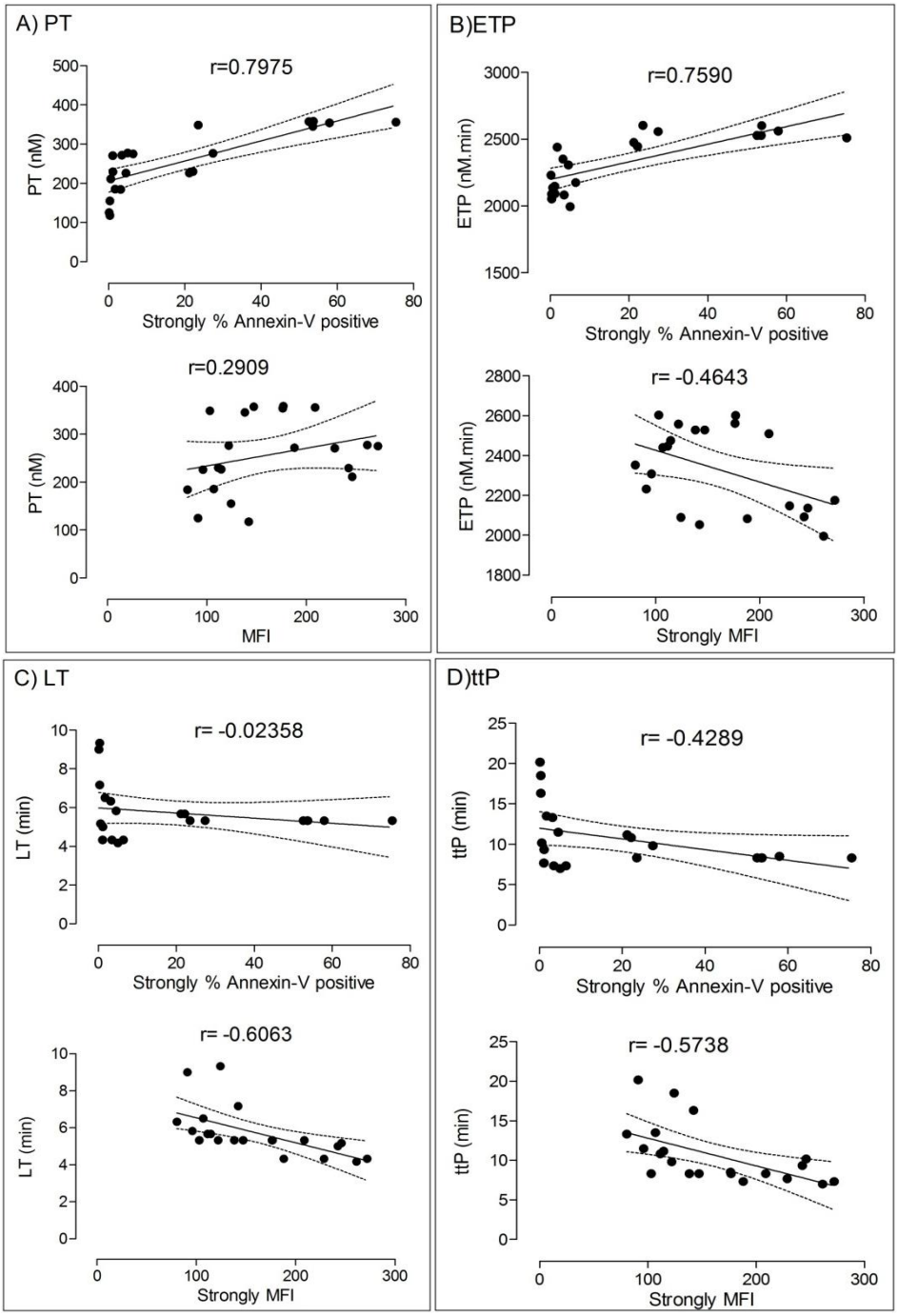



**Fig 5.15: Correlation of thrombin generation parameters and strong annexin-V positive events CRP-XL stimulated PRP.** (A) PT, (B) ETP, (C) LT and (D) ttP with strong annexin-V positive events (upper panel) and with strong MFI (lower panel). Data are taken from histogram C in Fig 5.13. PRP was stimulated with different concentrations of CRP-XL (0-10.0µg/ml). PRP was analysed on CAT assay with the 1pM TF and was analysed using annexin-V binding (n=3), numbers of XY analysis were 21 pairs.

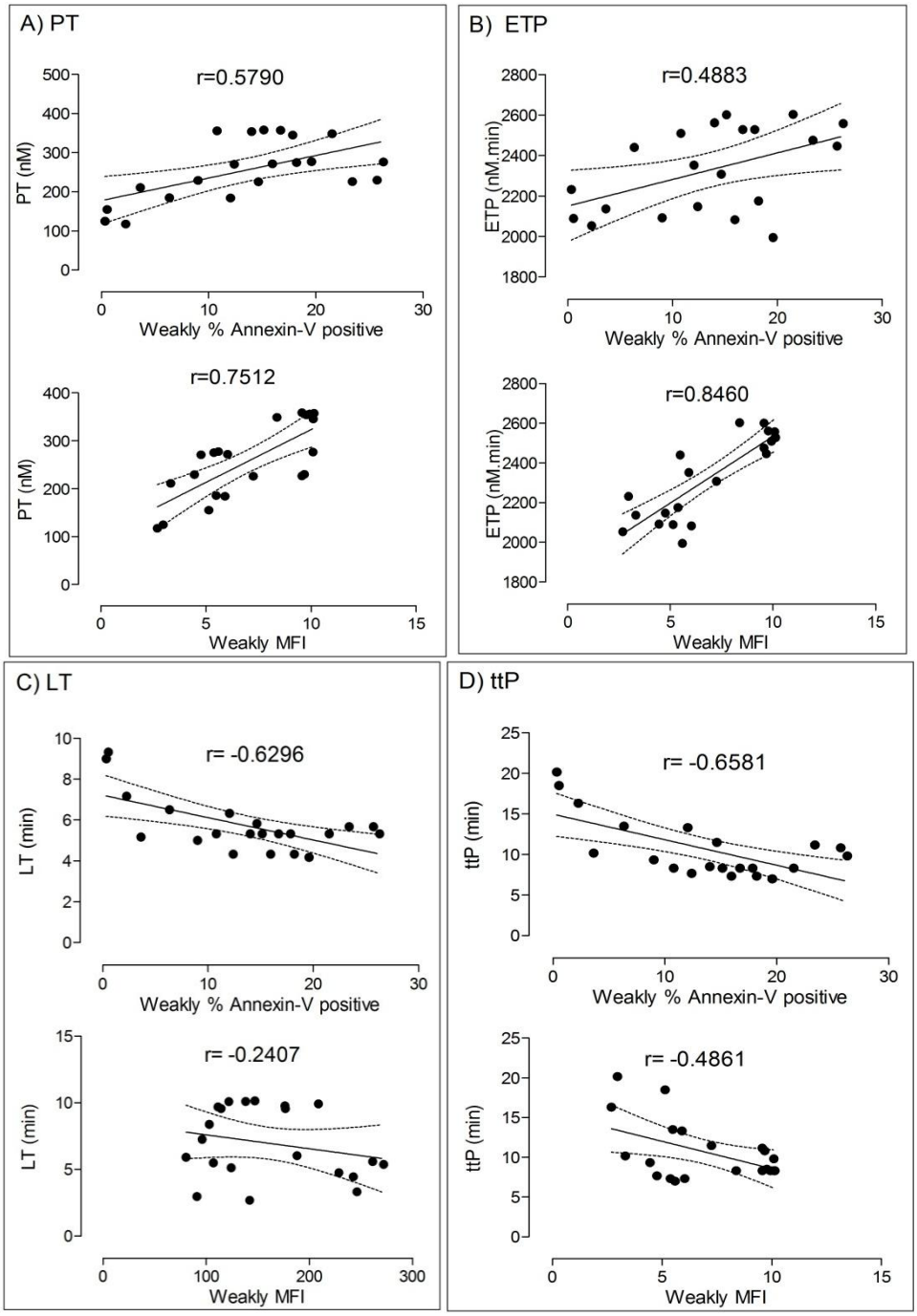



**Fig 5.16: Correlation of thrombin generation parameters with weak annexin-V positive events CRP-XL stimulated PRP.** (A) PT, (B) ETP, (C) LT and (D) ttP with weak annexin-V positive events (upper panel) and with weak MFI (lower panel). Data are taken from histogram C in Fig 5.13. PRP was stimulated with different concentrations of CRP-XL (0-10.0µg/ml). PRP was analysed on CAT assay with the 1pM TF and was analysed using annexin-V binding (n=3), numbers of XY analysis were 21 pairs.

## 5.3. Discussion

Annexin-V is widely used for the detection of PS exposure on activated platelets and MPs using flow cytometry (Abrams*, et al* 1990, Jy*, et al* 2004, Thiagarajan and Tait 1990), and the procoagulant activity is commonly detected using a prothrombinase assay and, more recently, by the CAT assay. Each method has advantages and disadvantages. Flow cytometry provides rapid analysis, but fails to detect functional activity, while the CAT assay developed by Hemker, *et al* (2003) provides only a measurement of the procoagulant activity provided by platelets and/or MPs in the plasma. This mimics the actual physiological conditions, but does not differentiate between platelets and MPs.

In the present study, CRP-XL induced PS exposure and MP formation from platelets (Fig 5.1 and Fig 5.2) in a dose dependent manner, reaching a plateau at 2.0µg/ml. This concentration of CRP-XL and above only induced PS exposure ~40-60% of the platelets, as detected by flow cytometry, but this concentration and even lower (0.2µg/ml) induced the maximum (~90-100%) activation of platelets assessed by aggregation, fibrinogen binding and P-selectin expression and thrombin generation. This result supports previous findings from this laboratory (Appleby*, et al* 2006). In addition, the current study confirms that PS exposure is distinct from other platelet responses including degranulation and integrin activation, in agreement with previously published finding (Delaney*, et al* 2010).

The current analysis shows that annexin-V binding increased over time for the first half hour and then the assay was stable for 1 hour at RT (Fig 5.7). This is very important to

determine the best analytical conditions affecting PS exposure and MP formation, which have been under discussion and investigation for many years (Jy*, et al* 2004, Mobarrez*, et al* 2010, Ramstrom*, et al* 2010, Shah*, et al* 2008, Yuana*, et al* 2010). Ramstorm *et al* (2010) found the assay is stable for up to one hour. The slight difference between the results might be due to the time studied and the type of agonist(s) used in each study, because Ramstorm *et al* (2010) used a combination of collagen and thrombin rather than CRP-XL alone. A combination of collagen and thrombin a potent induced of both PS exposure and MP formation compared to any other physiological agonist (Sims*, et al* 1989).

The current study showed the addition of GPRP (0.5mg/ml), a fibrin polymerisation inhibitor, (Michelson 1994) has no effect on thrombin generation driven by MPs in the plasma using the 1pMTF reagent compared to thrombin generation without GPRP (data not shown) and this observation was supported by observing a slight but significant enhancement of PS exposure in the activated PRP with CRP-XL, but no differences were seen in the unstimulated platelets. In plasma with high levels of MPs, clot formation might trap some MPs, which might lead to an underestimation of their procoagulant activity. Previously, GPRP has been shown to increase thrombin time and abolishes the platelets' secondary response, but to have no effect on thrombin-induced aggregation (Ilveskero*, et al* 2001, Jarvis*, et al* 2003, Soslau*, et al* 2001).

The role of platelets in thrombin generation has been revised since the proposals for the cell-based model of haemostasis (Hoffman and Monroe 2001), which replaced the old view of the coagulation cascade. The procoagulant surface provided by platelets is

needed for efficient generation of thrombin. In the current, thrombin generation is significantly reduced when the platelet count is less than $50x10^9$/L, using low physiological concentration of TF (1pM) to trigger the reaction with an effect seen mainly on the PT, a parameter that is sensitive to the procoagulant surface. PT increased with increasing platelets count (0-$200x10^9$/L), but the amount of thrombin generated (ETP) had already reached its maximum at $50x10^9$ platelets/L (Fig 5.6). This indicates that platelet count has the biggest effect on the rate of the reaction and that a small percentage of PS exposure is needed to get the maximum amount of thrombin generation (ETP); again this is in agreement with Vanschoonbeek *et al* (2004) and Gerotziafas *et al* (2005). The effect of platelet count on thrombin generation disappeared when exogenous PL was added (Fig 5.6). The normal platelet count is between 150-$400x10^9$/L and a platelet count in patients less than $50x10^9$/L is considered thrombocytopenic, which could put the patients at risk of bleeding; however, this risk level has recently been reduced to less than $10x10^9$/L, because serious bleeding is rare at higher counts (Greinacher and Selleng 2010, Rebulla 2001).

The current study also shows that CRP-XL stimulation of PRP increased thrombin generation by 3-4 fold, which is in agreement with Vanschoonbeek *et al* (2004) who found that even weak agonists such as ADP and epinephrine, which normally do not induce PS exposure in platelets measured by annexin-V binding, shorten the onset of thrombin generation and shift the reaction curve to the left (Vanschoonbeek*, et al* 2004) and also induces PS exposure. In the current study, PS exposure was detected by the generic marker annexin-V. Although the analysis showed that both activated platelet and MPs were positive for annexin-V binding, MP were smaller in size and

can bind to annexin-V more strongly than the larger-sized activated platelets, which activated platelets exhibited both strong and weak binding of annexin-V (Fig 5.1 and Fig 5.13). This analysis suggests that MPs are more procoagulant than activated platelets, in line with finding that MPs have 50-100 procoagulant activity than platelets (Sinauridze*, et al* 2007). This is apparently contradicted by the observation that although both activated platelets and PDMPs (Fig 5.12) were able to support thrombin generation, but in our experimental condition platelets supported more thrombin than MPs. This is because platelets in the reaction milieu are continuously activated by the feedback loop of thrombin being generated, while the MPs are preformed, and cannot be further activated.

CRP-XL stimulated platelets in the reaction milieu with the feedback of generated thrombin resemble so-called COAT platelets **(co**llagen **a**nd **t**hrombin-activated), which have been described in recent years (Alberio*, et al* 2000). COAT (or ”Coated”) platelets have the ability to bind higher levels of FVa and other coagulation factors following dual activation of platelets by collagen and thrombin (Dale*, et al* 2002).

Finally, the current study, showed strong correlation between annexin-V binding and thrombin generation in PRP. The amount of thrombin generated, in terms of ETP and PT, were strongly correlated with total AxV+ve events (percentage and MFI) and with the AxV+++ve (strongly) positive events. However, no correlation was observed between thrombin parameters measured in normal and PDMP-rich plasma with flow cytometry analysis of PS exposure (data not shown). This is because of the limitations of the flow cytometers which has been discussed previously in Chapter 3.

The inter-individual variability between donors in terms of PS exposure, as assessed by annexin-V binding assay, or in terms of thrombin generation, as assessed by CAT assay, was remarkable. The platelet procoagulant response to CRP-XL varies among individuals (Fig 5.9 and 5.10), some have low response and some have a high response, which is in agreement with previous results in this lab (Appleby*, et al* 2006). This variability has also been reported using Xase and prothrombinase assays (Sumner*, et al* 1996), and it has been linked to the GPVI receptor. Indeed, the GPVI haplotype accounts for 30-35% of this variability in response between individuals to CRP-XL (Jones*, et al* 2007, Joutsi-Korhonen*, et al* 2003). The inter-individual variability in thrombin generation has been previously reported by many groups in healthy individuals (Gerotziafas*, et al* 2005, Vanschoonbeek*, et al* 2004) and patients (Chantarangkul*, et al* 2003). These studies show that the variability is not only dependent on native PS exposure in thrombin generation between individuals, because when exogenous procoagulant PL (in term of synthetic PL or platelet count) was added, did eliminates this variability between individuals, but still occurred, suggesting other factors play a role in the enhancement of the procoagulant activity.

In conclusion, this study confirms that PS exposure and MP formation from CRP-XL activated platelets are procoagulant and variable. CRP-XL at low concentration induces modest PS exposure, but maximum activation of platelets measured by aggregation, P-selectin and fibrinogen binding. Our data supports that annexin-V binding give identification of the procoagulant surface and thrombin generation showed accelerating thrombin generation. PDMPs are more procoagulant than activated

platelets, but generates less thrombin compared to platelets, because of the feedback of thrombin in the reaction milieu in the CAT assay.

## 5.3. Further work

- Examining the MP population with a method that enables more accurate determination of size and number; for example using specific calibrated beads for accurate identification by flow cytometry.
- Measuring the binding of the tenase and prothrombinase complexes to the surface of unstimulated and stimulated platelets with CRP-XL and the surface of MPs, to demonstrate whether MPs have more capacity to bind coagulation factors.
- Annexin-V is a probe for negatively-charged PLs but a lactadherin probe is more specific for PS exposure. The level of PS exposure may be underestimated by the use of annexin-V.

# Chapter 6: The role of ROS in the generation of platelet-procoagulant phospholipids activated through GP-VI

## 6.1. Introduction

Platelet activation with collagen through the GPVI pathway is different from the other agonists pathways; it is the only pathway that, on its own is able to generate PS exposure and MP budding. GPVI stimulation also leads to the generation of ROS (Chlopicki*, et al* 2004), including superoxide ($O^{\bullet-}_2$), hydrogen peroxide ($H_2O_2$), peroxynitrite ($ONOO^-$) hydroxyl radical ($^{\bullet}OH$) and nitric oxide (NO) (Krotz*, et al* 2004). Platelets can also produce ROS upon stimulation with other agonists such as thrombin, TRAP, the $TxA_2$ analog (U46619), convulxin (Begonja*, et al* 2005), 12-HETE and A23187 (Nardi*, et al* 2007, Seno*, et al* 2001). Although the exact sources of ROS production in platelets are not fully determined, there are various potential pathways that may lead to ROS generation: (i) the NAD(P)H oxidase complex (Begonja*, et al* 2005, Chlopicki*, et al* 2004, Krotz*, et al* 2002, Nardi*, et al* 2007, Seno*, et al* 2001), (ii) the AA pathway; 12-lipoxynase (12-LOX) (Nardi*, et al* 2007) and COX-1 (Caccese*, et al* 2000), (iii) mitochondrial respiration, (iv) xanthine oxidase, and (v) nitric oxide synthase (NOS).

ROS have the ability to alter and modify the structure of PLs; mainly the unsaturated fatty acid residues of the membrane PLs in the cell membrane. Indeed, membrane PLs are the preferred targets for ROS, particularly PS, which is susceptible to the oxidation burst leading to lipid peroxidation (Lamba*, et al* 1994, Lamba*, et al* 1991). Pickering *et al* (2008) have demonstrated the involvement of lipid peroxidation in enhancing the

procoagulant activity of nucleated cells undergoing apoptosis measured using FXa and thrombin generation assays, which was dependent on the oxidation of PS. This effect was inhibited by the addition of an antioxidant, BHT. The Kagan group also demonstrated the selective oxidation of PS during apoptosis (Kagan*, et al* 2000, Tyurina*, et al* 2004, Tyurina*, et al* 2000). In addition, oxidized PS vesicles enhance prothrombinase activity six fold compared to the control un-oxidized vesicles. Again, this effect was inhibited by the addition of antioxidants such as α-tocopherol, γ-tocopherol or ascorbate (Weinstein*, et al* 2000). Furthermore, Rota et al (1998b) have shown that oxidized PLs within LDL increased thrombin generation, whereas the addition of vitamin E reduced it. Nardi et al (2007) have shown the involvement of ROS in MP formation and that this mediated by the NAD(P)H oxidase complex and the 12-LOX pathways following platelet stimulation by A23187, phorbol myristate acetate (PMA) and Abs against platelet integrin GPIIIa that recognize a.as 49-66. This raises the question of whether ROS production enhances the exposure of the procoagulant surface on the surface of activated platelets and/or in the form of PDMPs upon CRP-XL activation.

The data in the previous chapters demonstrate that platelets and PDMPs from normal healthy individuals are able to support thrombin generation, and that this ability is significantly enhanced after activation with the collagen mimetic peptide, CRP-XL. PS exposure is a key feature of the procoagulant response, but accumulating data indicates the participation of ROS generation and oxidized PLs in the process. Thus, this chapter investigates whether intracellular ROS generation can enhance the

procoagulant activity of the CRP-XL stimulated platelets and the PDMPs, and the possible sources of this intracellular ROS.

## 6.2 Results

In this chapter, washed platelets were used at $150x10^9$/L with or without CRP-XL (2.0µg/ml) stimulation for 10 minutes at 37°C; this concentration of CRP-XL was chosen as it was previously found to give the maximum generation of PS, PDMPs and other platelet responses, including aggregation, fibrinogen binding, and P-selectin expression. The effects on both platelets and PDMPs were investigated in the current analysis. PDMP-rich supernatants were obtained by the centrifugation of either unstimulated or CRP-XL-stimulated washed platelets for 30 minutes at 1,800x*g*. For thrombin generation measurement, washed platelets or PDMP-rich supernatants were mixed with an equal volume of autologous filtered PPP (100µl of the sample + 100µl of the plasma) to restore coagulation factors. Thrombin generation was then measured with the 1pMTF reagent, as at this concentration of TF the assay is dependent on the procoagulant PLs provided either by platelets and/or PDMPs.

### 6.2.1. Comparison between thrombin generation by platelets in washed platelet preparation vs. PRP

The analysis began with a comparison between washed platelets and PRP before and after CRP-XL activation from six healthy donors, to investigate whether washed platelets support thrombin generation in a similar way to PRP (Fig 6.1). The overall results show that thrombin generation was supported by both PRP and washed platelets before and after CRP-XL stimulation, indicating that procoagulant activity can

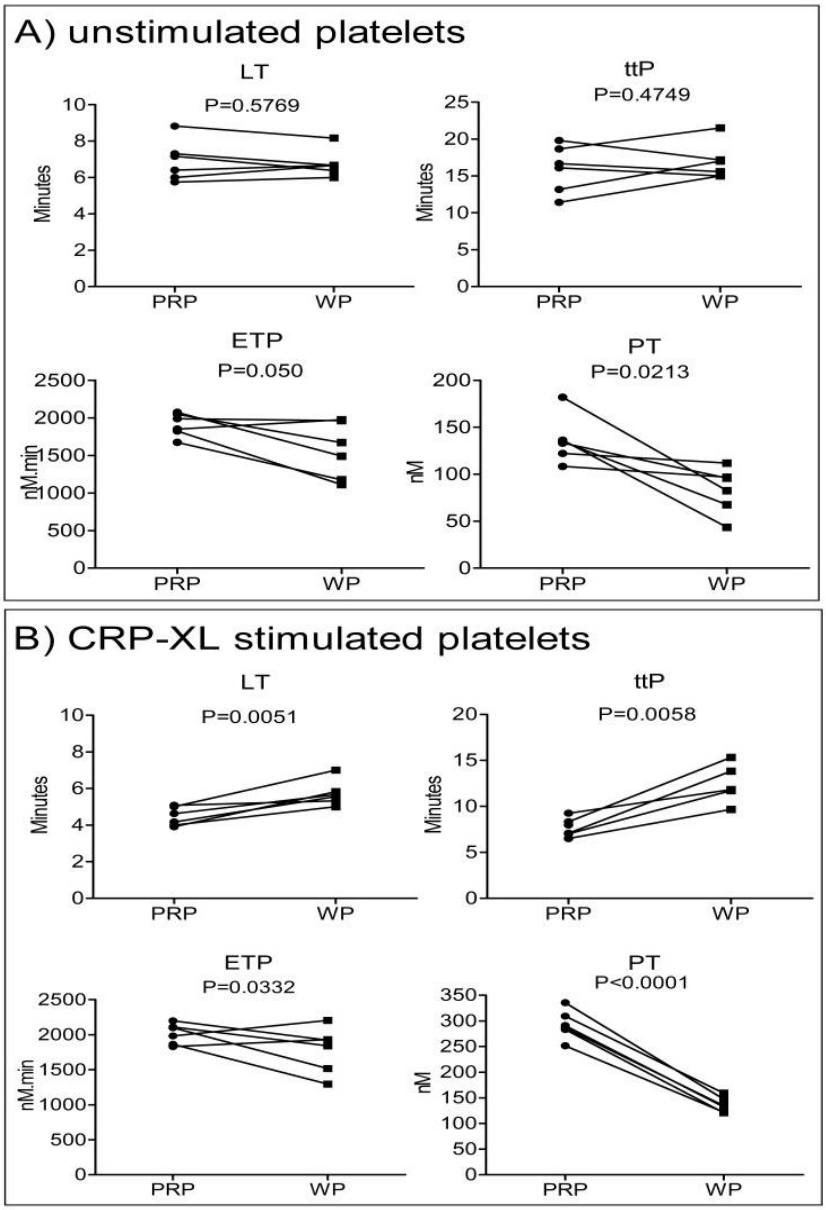



**Fig 6.1: Comparison between thrombin generation in PRP and washed platelets before and after CRP-XL activation.** (A) Thrombin generation measurement in resting (unstimulated) PRP and resting (unstimulated) washed platelets. (B) Thrombin generation measurement in CRP-XL (2.0µg/ml) stimulated PRP and CRP-XL stimulated washed platelets. Platelets were stimulated for 10 minutes at 37°C. Washed platelets were mixed with equal autologous filtered PPP. Thrombin generation was trigged by the PRP (1pMTF) reagent in washed platelets and PRP. Data are from 6 healthy individuals; unpaired t-test.

be measured in the washed platelets. The overall amount of thrombin generated (PT and ETP) was higher before and after CRP-XL stimulation in PRP than in washed platelets, which was a consequence of the 1:1 dilution of the plasma. The rate of the reaction measured by the LT and ttP were similar in the unstimulated samples ($p>0.05$), but significantly faster after CRP-XL stimulation in PRP compared to washed platelets ($p<0.05$). The overall thrombin generation was profoundly enhanced after CRP-XL stimulation in both PRP and washed platelets.

### 6.2.2. Effect of CRP-XL stimulation on the procoagulant activity of platelets and PDMPs

The effect of CRP-XL activation was studied in the supernatant of unstimulated washed platelets and PDMP-rich supernatant obtained from unstimulated and CRP-XL stimulated washed platelets, respectively. In this analysis there were fifteen donors, six of whom were included in the data shown in Fig 6.1. As observed in the previous chapters for PRP, CRP-XL (2.0µg/ml) activation significantly enhanced thrombin generation in washed platelets compared to unstimulated platelets ($p<0.05$) (Fig 6.2). PDMPs released into the supernatant of platelets by CRP-XL activation generated significantly more procoagulant activity compared to the supernatant of unstimulated platelets ($p<0.05$ all parameters). As shown previously, the activity of PDMPs was lower than that of platelets. PS exposure was detected by annexin-V binding from the same samples, which was also significantly increased upon CRP-XL activation of platelets compared to unstimulated platelets ($n=8$; $4.6\pm1.4$ vs. $21.3\pm11.7$; $p=0.0052$) (Fig 6.3), this is in line with previous experiments

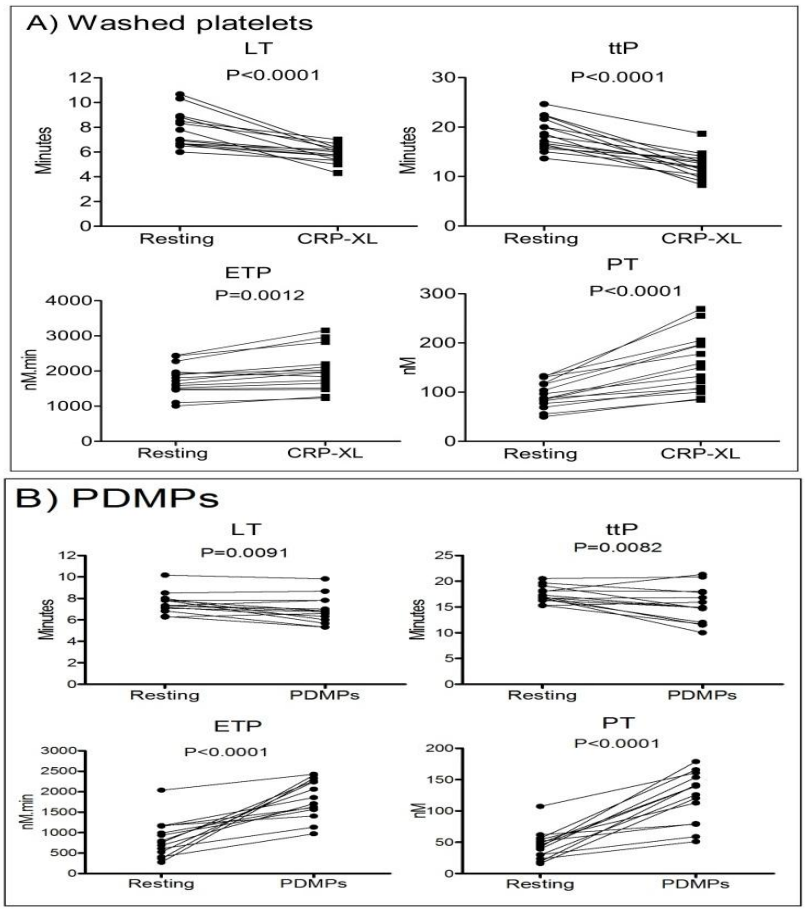



**Fig 6.2. Effect of CRP-XL activation on thrombin generation triggered by the 1pMTF measured in washed platelets and their supernatants.** (A) Thrombin generation measurement in washed platelets. (B) Thrombin generation measurement by PDMPs obtained from the supernatants of unstimulated washed platelets and CRP-XL stimulated washed platelets. Washed platelets were activated by CRP-XL for 10 minutes at 37°C. PDMP-rich supernatant was obtained by centrifugation of washed platelet at 18000xg for 30 minutes, and 90% of the supernatant was removed and called PDMP-rich supernatant. Thrombin generation was triggered with 1pM TF in washed platelets and PDMP-rich supernatant. Data are from fifteen healthy individuals; paired t-test.

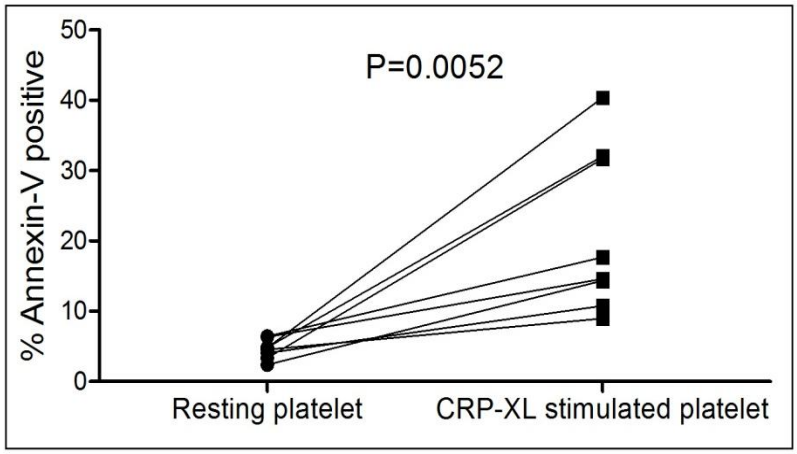



**Fig 6.3. CRP-XL induces PS exposure in washed platelets.** Washed platelets were stimulated with and without CRP-XL (2.0µg/ml) for 10 minutes at 37°C. PS exposure was assessed using annexin-V binding assay. Data shown are from 8 healthy subjects (paired t-test).

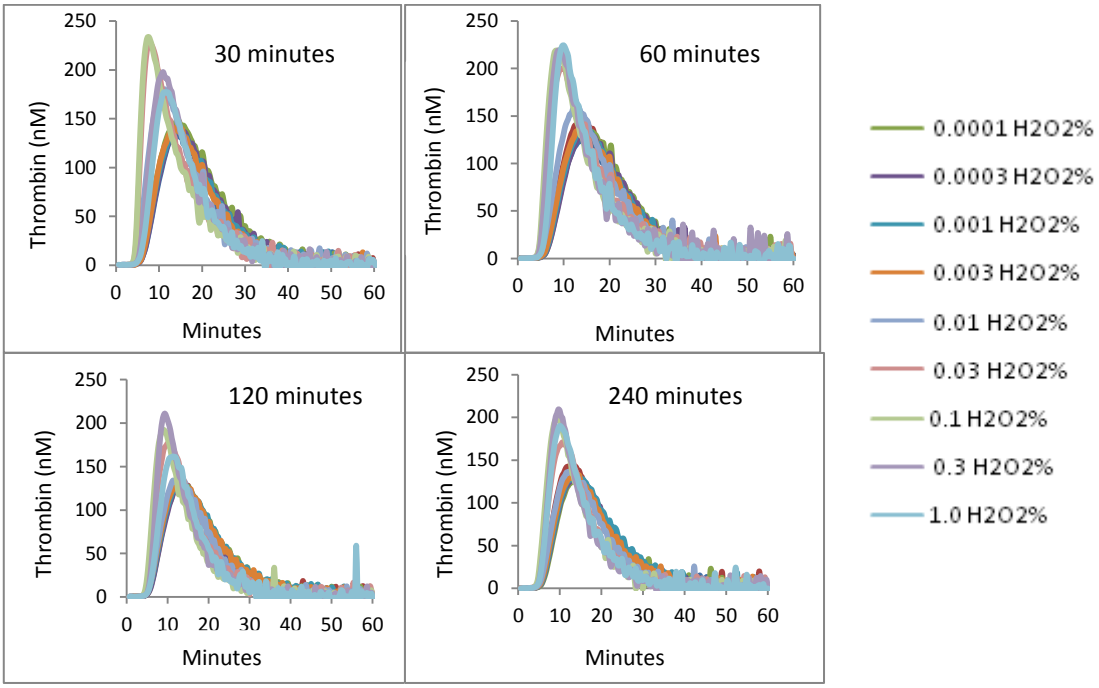
## 6.2.3. Lipid peroxidation induced by $H_2O_2$

### 6.2.3.1. Effect of lipid peroxidation platelet procoagulant surface

There are contrasting reports in the literature on the effect of exogenous ROS on platelet functions, with almost no studies on the procoagulant activity of platelets or thrombin generation. In addition, it is well documented that $H_2O_2$ induces lipid peroxidation of the membrane PLs of platelets. The following initial studies were carried out to explore the effect of lipid peroxidation on thrombin generation and PS exposure. Platelets were exposed to increasing concentrations of $H_2O_2$ (0 up to 1.0% [v/v]) for 30, 60, 120 and 240 minutes. Representative examples of thrombin generation are shown in Fig 6.4a. $H_2O_2$ showed a dose-dependent enhancement (30%-50% fold) of thrombin generation at all time points above 0.003% and up to 0.1%, but fell at 0.3% and 1.0% (Fig 6.4b). Almost no effect was seen in any parameters of thrombin generation at concentrations below 0.001% $H_2O_2$.

The effect of the highest concentration of $H_2O_2$ (1.0%) was used to illustrate the effect on PS exposure in platelets in comparison to CRP-XL (Fig 6.5). Although both induced PS exposure, $H_2O_2$ induced markedly greater PS exposure than CRP-XL ($p=0.0502$).

A)




B)

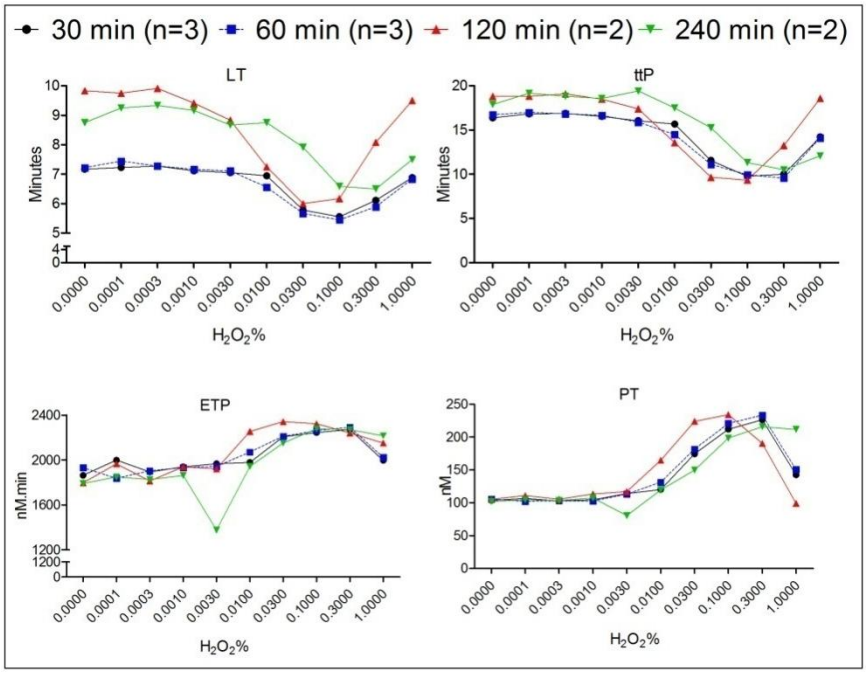



**Fig 6.4. Lipid peroxidation induced by $H_2O_2$ enhances thrombin generation in platelets.** A) Represents a single run from one donor. B) 3 independent runs unless indicated in the figure. Washed platelets were pre-incubated with increasing concentrations of $H_2O_2$ from 0 to 1.0% for 30 minutes (●, black line), 60 minutes (■, blue line), 120 minutes (▲, red line) and 240 minutes (▼, green line). Thrombin generation was initiated by the 1pMTF reagent.

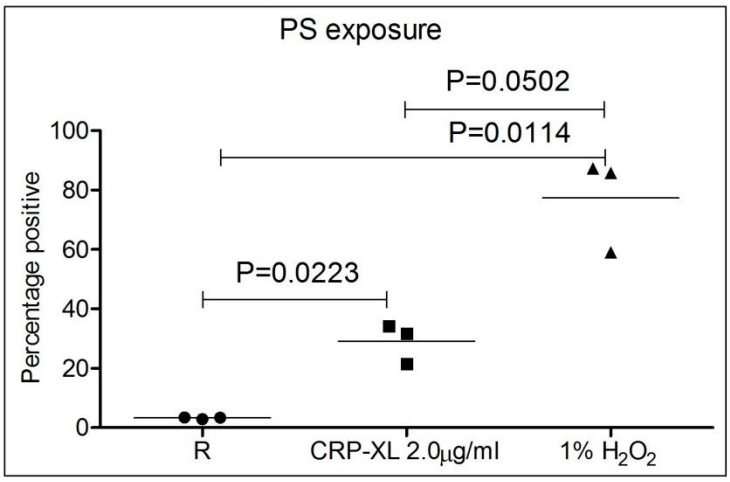



**Fig 6.5. $H_2O_2$ induced PS exposure assessed by annexin-V binding than CRP-XL.** Washed platelets were pre-incubated with CRP-XL or $H_2O_2$ for 10 minutes at 37°C. Data shown as 3 independent runs (n=3; paired t-test; *p<0.05*).

### 6.2.3.2. Lipid peroxidation

It is well documented that $H_2O_2$ induces lipid peroxidation which can be measured by TBARS assay. Therefore, the following experiment examined whether CRP-XL (2.0µg/ml) can also induce lipid peroxidation, comparing this with two concentrations of $H_2O_2$ (0.1% and 1.0%) as a positive control. Washed platelets were pre-incubated for 30 minutes and 60 minutes with either CRP-XL or $H_2O_2$, in the absence or presence of four ROS inhibitors, apocynin (100µM), DPI (10µM), tiron (100µM) and esculetin (25µM) (table 6.1). The concentration of TBARS in the experiments was calculated as equivalents of MDA. Lipid peroxidation was detectable in washed platelets treated with both concentrations of $H_2O_2$ after 30 minutes and 60 minutes, but was not detectable with CRP-XL (n=1; Fig 6. 6). In addition, nothing was detectable in the resting platelets. The additions of the four inhibitors virtually abolished lipid peroxidation induced by $H_2O_2$ at both time points.

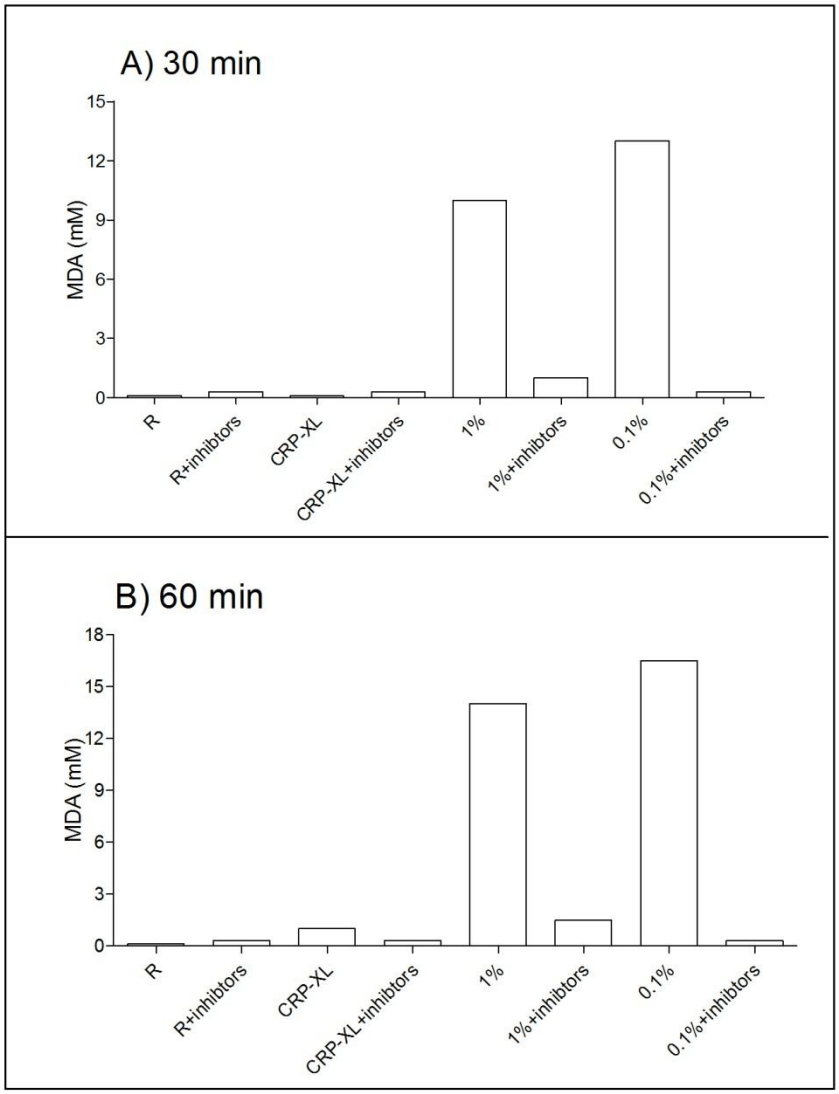



**Fig 6.6: Lipid peroxidation assessment in CRP-XL stimulated platelets compared to $H_2O_2$.** Washed platelets were either incubated with or without esculetin (25µM), apocynin (300 µM), DPI (10 µM) and tiron (100 µM) for 30 min at 37°C and then followed by either activation with CRP-XL (2.0µg/ml) or $H_2O_2$ (0.1% and 1.0%) or nothing (resting, R) for (A) 30 minutes or (B) 60 minutes at 37°C. Data are shown from one run.

### 6.2.3.3. ROS measurement

Intracellular ROS production was detected using the CM-$H_2$DCFDA probe, which is an a fluorescence intracellular probe that rapidly diffuses across the cell membrane and is subsequently retained in the cells following desertification by endogenous esterases. A high dose of CRP-XL (10µg/ml) was selected to measure intracellular ROS generation in platelets and this was compared with agents that known to induce ROS production in platelets including A23187 (10µM), PMA (1µM) (Nardi*, et al* 2007) and exogenous $H_2O_2$ (1%). The ROS probe (25µM) was incubated with washed platelets for 30 min at 37°C, and then the platelets were challenged with the various agents alone for 10 min at 37°C. All agents induce a marked shift in fluorescence to the right in the flow cytometer indicative of ROS generation, with the highest shift observed with A23187 followed by PMA and then CRP-XL and $H_2O_2$ (Fig 6.7a). At different doses of CRP-XL, ROS production increased in a dose-dependent manner ($p>0.05$; n=4; Fig 6.8); the maximum dose of CRP-XL inducing a 1.6-fold increase in ROS generation compared to the resting sample. In a separate experiment, CRP-XL at 2.0µg/ml induced ROS production (as evidenced by a fluorescence shift to the right) compared to the resting sample, which was inhibited by apocynin (300µM) shifting it to an even lower level than in the resting sample (n=1; Fig 6.7b). This indicates that resting platelets generate low levels of ROS. Similar inhibition by apocynin was observed with A23187 (data not shown; n=1).

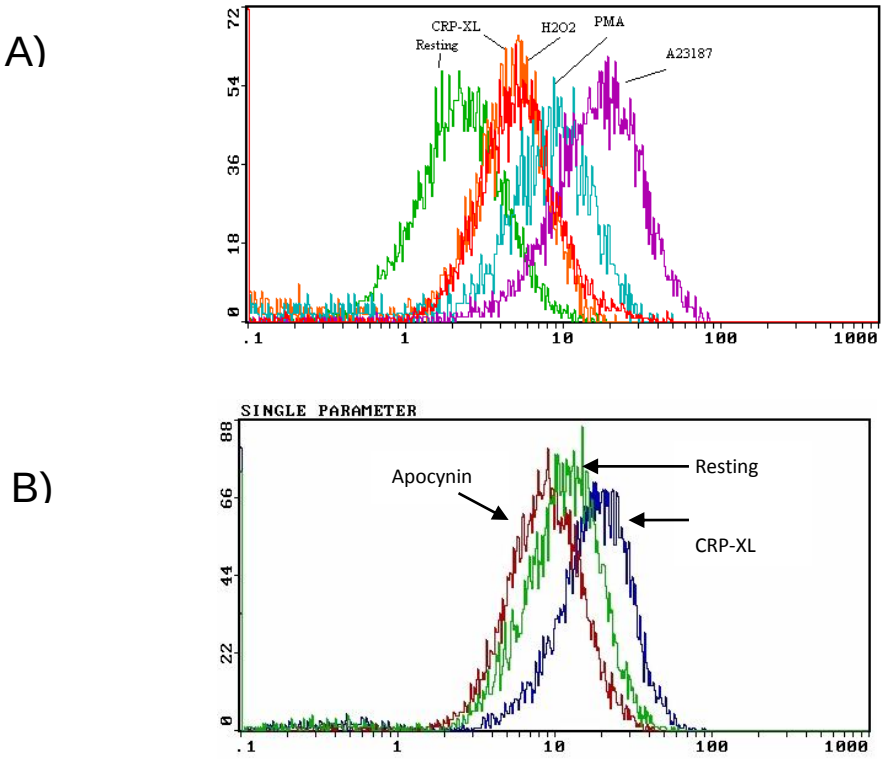



**Fig 6.7. Flow cytometric analysis of ROS generation by CM-$H_2$DCFDA-loaded washed platelets**. A) Fluorescence peak of unstimulated platelets (green), CRP-XL (10µg/ml; amber), H2O2 (1%; red), PMA (1µM; light blue) and A23187 (10µM, lavender). B) Washed platelets stimulated with CRP-XL (2.0µg/ml; blue) in the presence and absence of apocynin (300µM; red) compared to resting platelets (green).

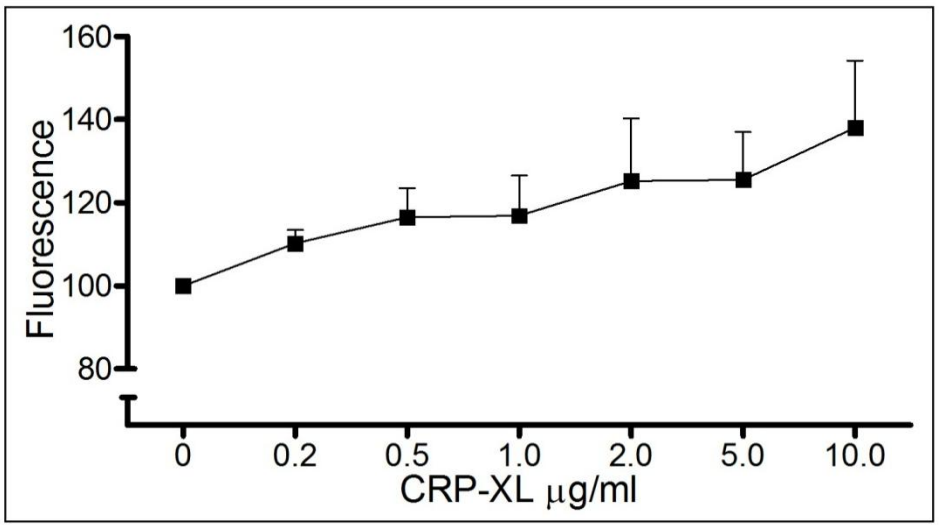



**Fig 6.8. CRP-XL induces dose-dependent increase in ROS generation by platelets detected by CM-$H_2$DCFDA**. Washed platelets were pre-loaded with the intracellular probe CM-$H_2$DCFDA for 30 minutes at 37˚C, and then stimulated for 10 minutes with CRP-XL. Data are the mean±SEM from 4 healthy individuals; one-way ANOVA.

## 6.2.4. Effect of non-physiological agonist, A23187, on the procoagulant surface

A23187 is a known inducer of PS exposure and MP formation that is dependent on ROS generation, which the process of MP formation was inhibited using ROS inhibitors (DPI, dexamethasone and 12-LOX (Nardi*, et al* 2007). A23187 is much more potent than CRP-XL as it causes ~100 of the platelets to form MPs, and its mode of action is different. Therefore, the current experiment investigates whether if A23187 could enhances thrombin generation and could be inhibited by using known ROS inhibitors. Washed platelets were challenged with A23187 (10µM), CRP-XL (2.0µg/ml) or no agonist for 10 min at 37°C, and then thrombin generation and PS exposure were assessed using CAT and annexin-V binding, respectively.

Both, CRP-XL and A23187 induced significant PS exposure ($P<0.05$), but A23817 induced more than 85% of platelets compared to CRP-XL (<40%) (Fig 6.9; n=4). Washed platelets were pre-incubated with either DPI (10µM) or dexamethasone (4.6µM) for 30 minutes and then stimulated with A23187 for 10 minutes at 37°C. These inhibitors have been shown to reduce MP formation in A23187 treated platelets. A23187 induced 86.9% annexin-V in platelets, which was reduced by dexamethasone alone to 67.9% and by DPI to 37.4% (Fig 6.10; n=1). Thrombin generation was enhanced by both A23817 and CRP-XL, more than the unstimulated platelets to a similar extent (Fig 6.11). A23187 had a slightly greater effect, but was not significant ($p>0.05$; n=4). Apocynin (300µM) significantly reduced ETP ($p=0.0112$) by A23187, but not PT and the time parameters (LT and ttP) (Fig 6.12). Tempol had no effect on A23187-induced thrombin generation in platelets (Fig 6.13a), while tiron

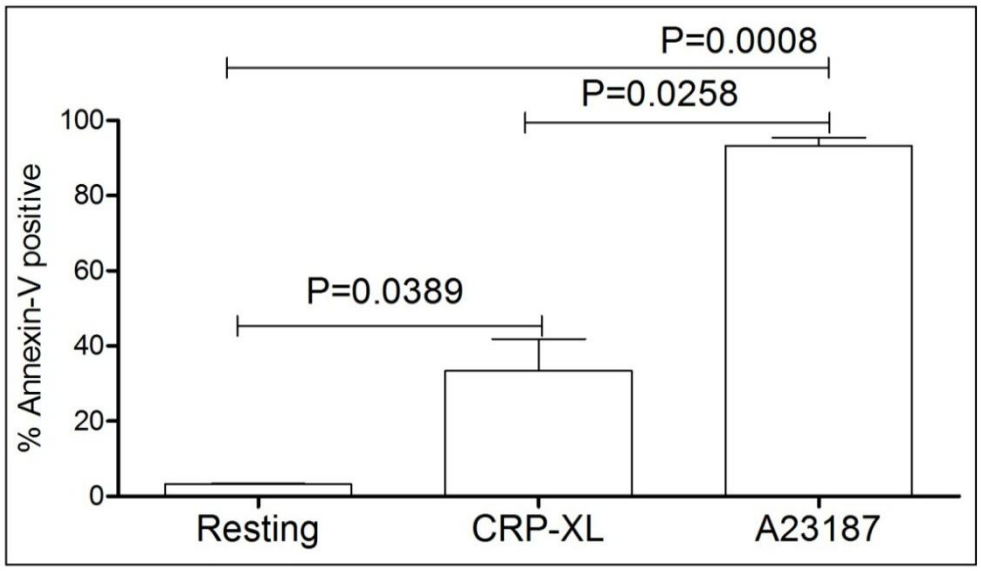



**Fig 6.9: Effect of A23187 on PS exposure in platelets compared CRP-XL.** Washed platelets were activated by A23817 (10µM), CRP-XL (2.0µg/ml) or nothing for 10 minutes at 37°C. Annexin-V exposure was detected by flow cytometry.. Data are from four healthy individuals; paired t-test.

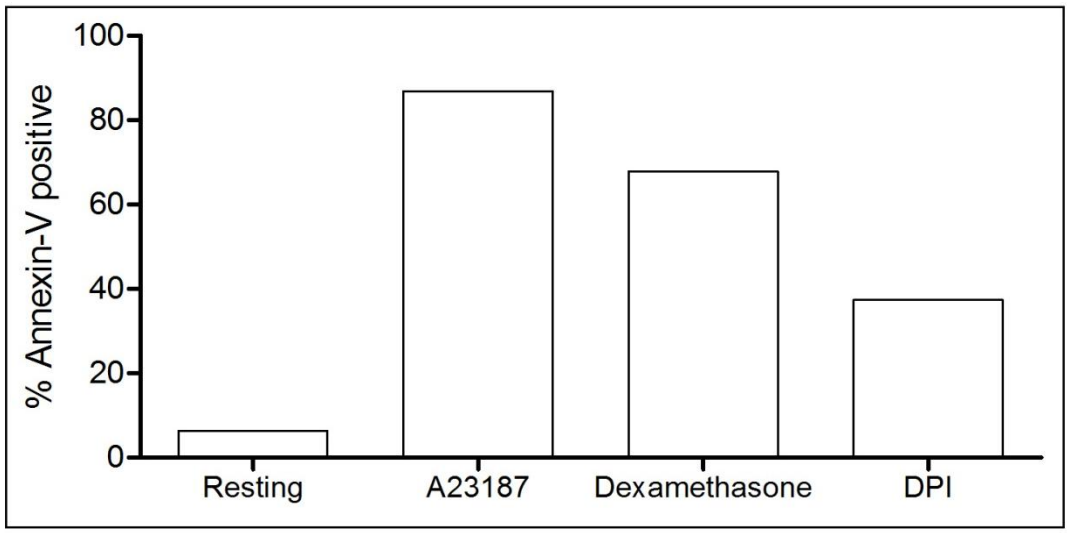



**Fig 6.10: Effect of dexamethasone (4.6µM) and DPI (10µM) on A23187 induced PS exposure in platelets compared CRP-XL.** Washed platelets were pre-incubated with dexamethasone (4.6µM) and tiron (10µM) for 30 minutes at 37°C then were activated by A23817 (10µM) for 10 minutes at 37°C. Data are from one run.

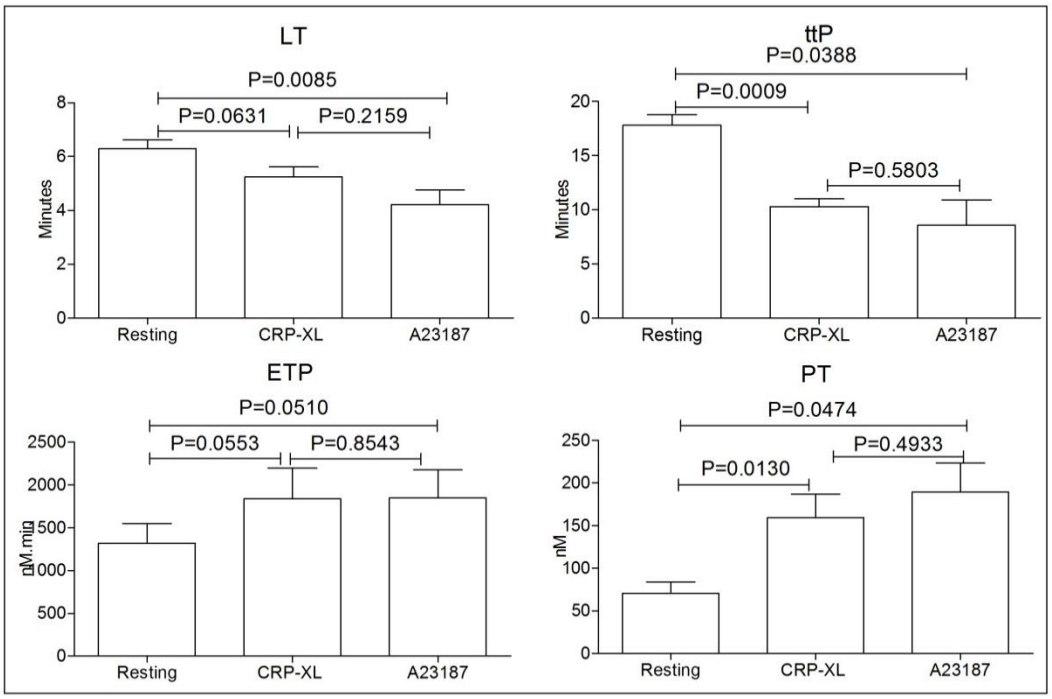



**Fig 6.11: Effect of A23187 on thrombin generation measured in platelets compared CRP-XL.** Washed platelets were activated by A23817 (10µM), CRP-XL (2.0µg/ml) or nothing for 10 minutes at 37°C. Thrombin generation was triggered with 1pM TF in washed platelets. Data are from four healthy individuals; paired t-test.

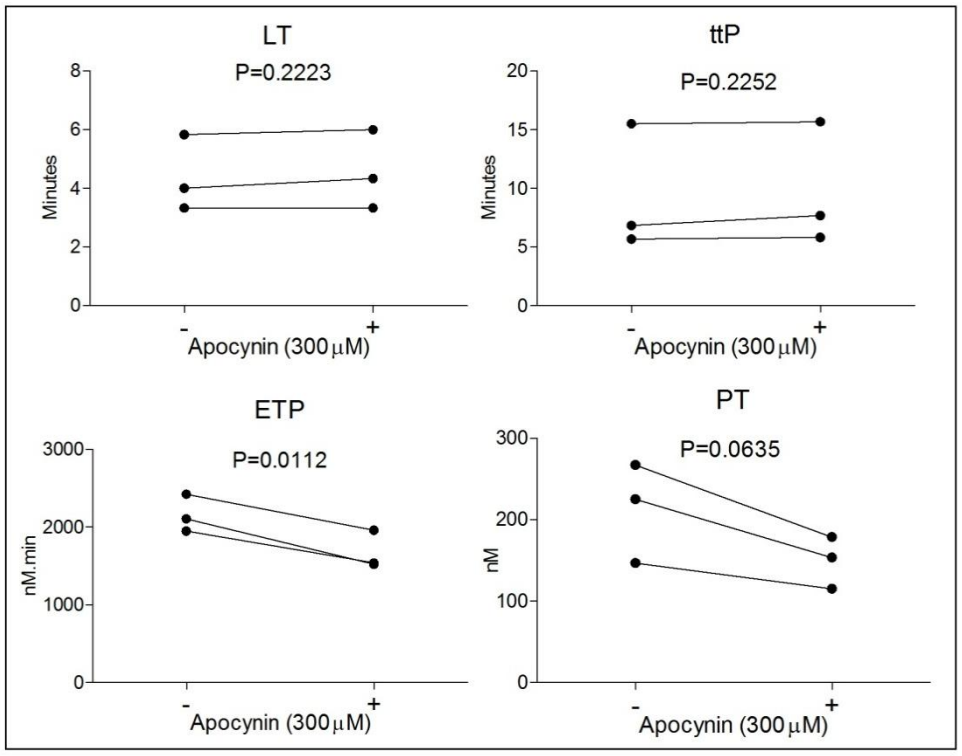



**Fig 6.12: Effect of apocynin on A23187 induced thrombin generation in platelets.** Washed platelets were pre-incubated with apocynin for 30 minutes at 37°C then were activated by A23817 (10µM) for 10 minutes at 37°C. Thrombin generation was triggered with 1pM TF. Data are from three healthy individuals; paired t-test.

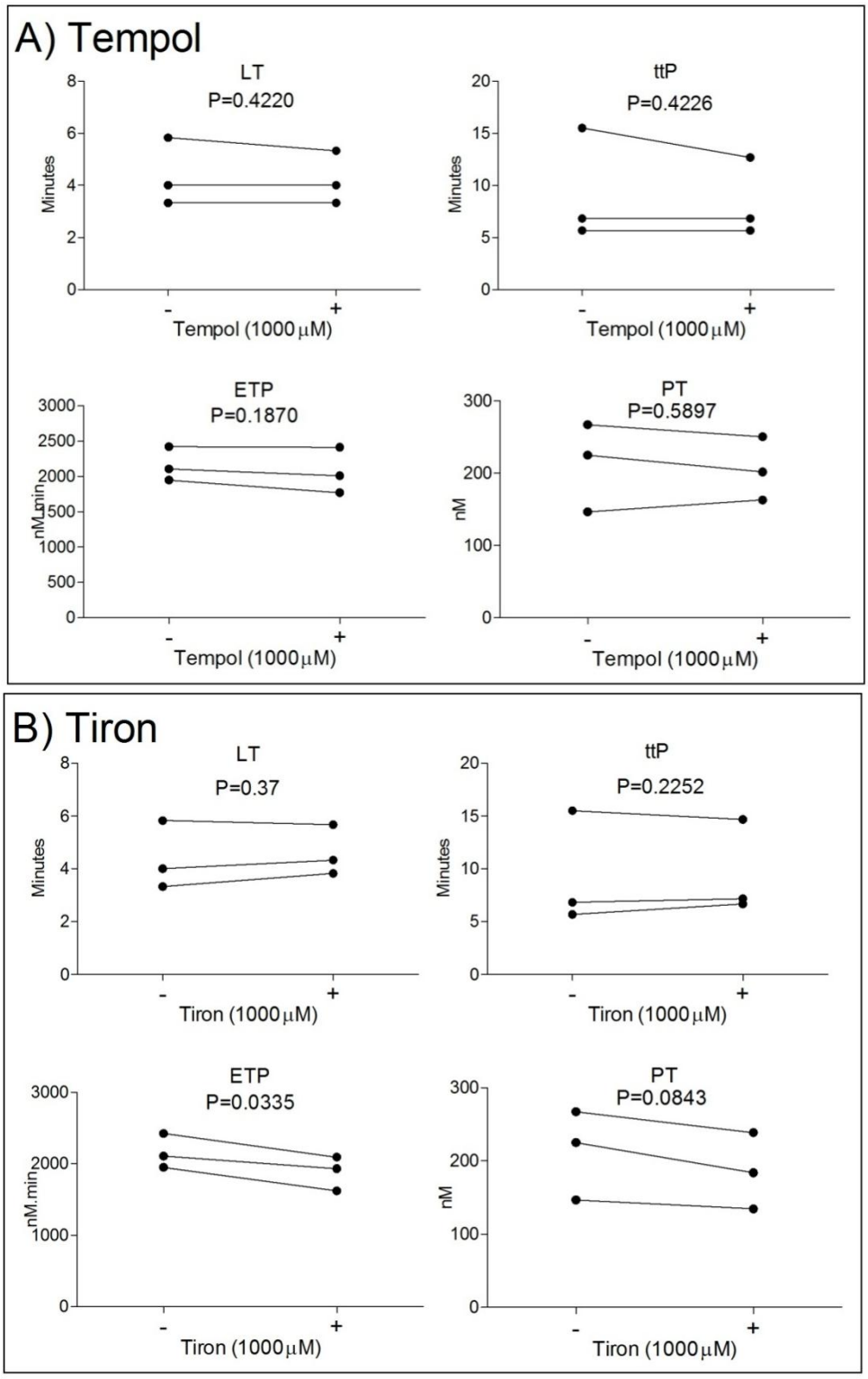



**Fig 6.13: Effect of ROS scavengers on A23187 induced thrombin generation in platelets.** Washed platelets were pre-incubated with tempol (1000μM; panel A) and tiron (1000μM; panel B) for 30 minutes at 37°C then were activated by A23817 (10μM) for 10 minutes at 37°C. Thrombin generation was triggered with 1pM TF. Data are from three healthy individuals; paired t-test.

reduced the amount of thrombin generated (ETP; $p<0.05$ and PT; $p>0.05$) but not the time parameters (LT and ttP) (Fig6.13b). Wortmannin and NAME also reduced A23187-induced thrombin generation (n=1; data not shown). Again, these preliminary findings, although there number is low, support the participation of ROS generation described by Nardi, *et al* (2007) in the procoagulant activity of platelets.

## 6.2.5. Effect of ROS inhibitors on thrombin generation

The effect of CRP-XL in inducing the procoagulant activity of platelets and PDMPs was measured in the thrombin generation assay in the absence and presence of the inhibitors listed in table 6.1 in order to investigate the role of ROS on the procoagulant activity measured by CAT. In these experiments, washed platelets were pre-incubated with the different inhibitors that inhibited ROS generated from either the AA pathway (aspirin, esculetin and baicalein), and NADP(H) oxidase pathway, or using ROS scavengers (tempol and tiron), antioxidants (BHT, NAC and trolox), and an anti-inflammatory drug that affects PLCγ production (dexamethasone) for 30 minutes before stimulation with CRP-XL for 10 minutes at 37°C. As control of the inhibition of the GPVI signalling pathway, the PI3k inhibitor, wortmannin, was used. The concentrations for these inhibitors were chosen because they had been shown to affect ROS generation or aggregation induced by collagen in platelets in the literature.

Before showing the effect of the inhibitors, experiments were performed to test the effects of the solvent used to dissolve the inhibitors on thrombin generation in CRP-XL treated platelets. Different solvents (DMSO, ethanol and water) were added to the platelets for 30 minutes before stimulation with CRP-XL (n=1; Fig 6.14). DMSO and ethanol at high concentrations (>10%) inhibited thrombin generation; this can be

| inhibitor | Targeted pathway in platelets |
|---|---|
| Apocynin | NAD(P)H oxidase complex (Begonja*, et al* 2005) |
| DPI | NAD(P)H oxidase complex (Begonja*, et al* 2005, Nardi*, et al* 2007) |
| Esculetin | A selective inhibitor of platelet 12-LOX (Sekiya*, et al* 1982b) |
| Baicalein | A selective inhibitor of platelet 12-LOX (Sekiya and Okuda 1982a) |
| Tempol | A general ROS scavenger (Chakrabarti*, et al* 2004) |
| Tiron | A specific superoxide ROS scavenger (Begonja*, et al* 2005) |
| Aspirin | Cyclooxygenase-1 (Begonja*, et al* 2005) |
| BHT | Antioxidant |
| NAC | Antioxidant; a glutathione reductase substrate |
| Trolox (a vitamin E analog) | Antioxidant |
| L-NAME | NO synthase inhibitor that Inhibits NO synthesis |
| Dexamethasone | a glucocorticoid class steroid drug (Nardi*, et al* 2007) |
| Wortmannin | phosphoinositide 3-kinase (PI3K) (Pasquet*, et al* 1999, Pearce*, et al* 2002, Wu*, et al* 2010, Wymann*, et al* 1996) |

**Table 6.1. Details of the inhibitors used in the study and their targeted pathway in platelets.** Diphenyliodonium (DPI), $N^G$-Nitro-L-Arginine-Methyl Ester. (L-NAME)

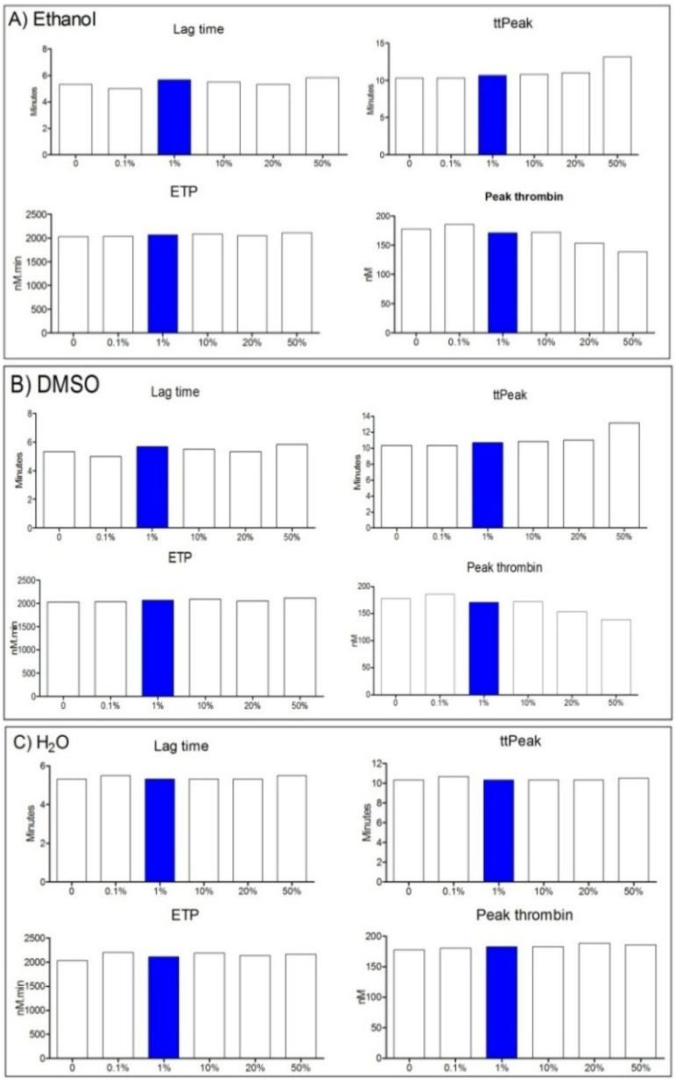



**Fig 6.14. Dose-dependent effect of the control-vehicles (solvents) on thrombin generation measured in activated platelets with CRP-XL.** Washed platelets were pre-incubated with ethanol (A), DMSO (B) or $H_2O$ (water; C) for 30 minutes, followed by 10 minutes activation of CRP-XL. Filled bars show the maximum level of each solvent present in the assay with inhibitors. Thrombin generation was initiated by the 1pMTF reagent. Data are from one run. Solvents were diluted in HBS buffer so expressed in percentage.

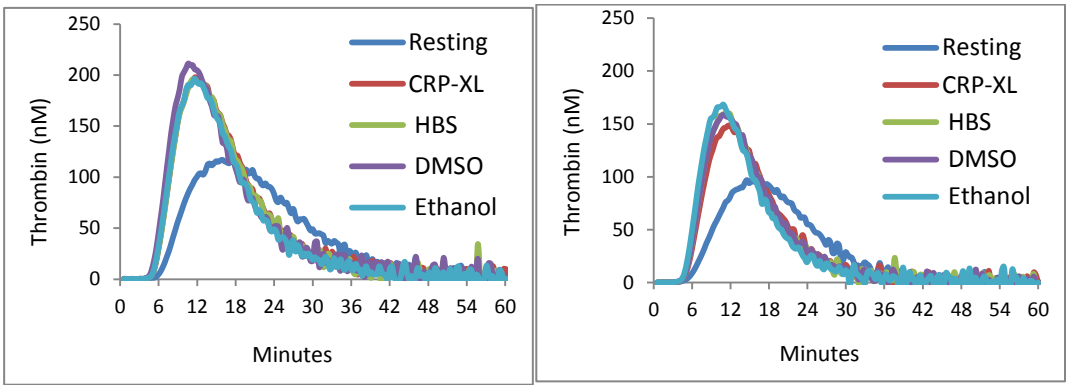



**Fig 6.15: Representative example of the effect of the control vehicles on thrombin generation.** Thrombin generation was measured in platelets (left panel) and by PDMPs (right panel). Thrombin generation was initiated by the 1pMTF reagent.

clearly seen by increasing the ttP and reducing the PT. Water had no effect on thrombin generation at any of the concentrations used. However, the maximum concentration of the solvents used in the presence of the inhibitor did not exceed 1% (blue colour in Fig 6.14) and this had no effect on thrombin generation. Subsequently a control vehicle (C) was included in each experiment in a CRP-XL-stimulated sample and gave similar results to the CRP-XL stimulated platelets or PDMPs, Representative examples of the control vehicles (<1%) are shown in Fig 6.15.

### 6.2.5.1. Effect of NAD(P)H pathway

Two structurally unrelated NAD(P)H oxidase complex inhibitors, apocynin (100µM and 300µM) (Fig 6.16) and DPI (10µM) (Fig 6.17) significantly attenuated CRP-XL-induced thrombin generation driven by platelets (all parameters $p<0.05$ except ETP with DPI $p=0.3145$). Thrombin generation by PDMPs was not affected by the low dose of apocynin (100µM) and DPI (10µM) ($p>0.05$), but was significantly reduced by apocynin at higher doses (300µM). This inhibition was only observed in the amount of thrombin generated (ETP and PT; $p<0.005$), but had no effect on the rate of the reaction (LT and ttP). In line with these findings, apocynin (300µM) significantly reduced PS exposure measured in the annexin-binding flow cytometric assay ($p=0.0183$) (Fig 6.18a), but DPI had no effect on PS exposure ($p>0.05$) (Fig 6.18b).

### 6.2.5.2. Effect of AA pathway

PLA2 releases AA from platelet cell membranes upon CRP-XL activation, which results in 12-HETE and $TxA_2$ production mediated by 12-LOX and COX-1 respectively. The 12-LOX inhibitor, esculetin reduced thrombin generation by platelets significantly (all parameters ($p<0.05$) (Fig 6.19). However, the COX-1 inhibitor, aspirin had no effect on

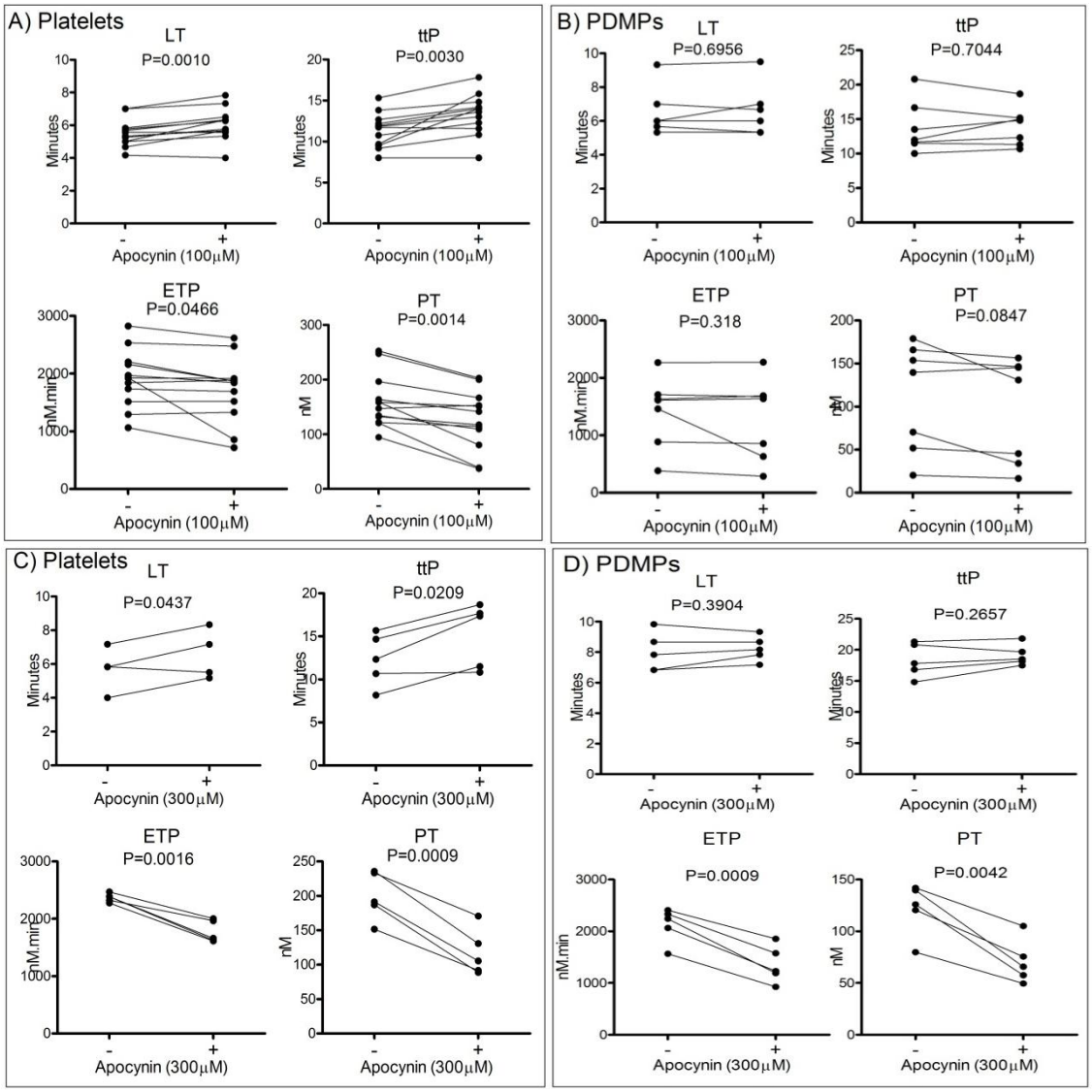



**Fig 6.16: Effect of apocynin on thrombin generation.** Thrombin generation was measured by CRP-XL stimulated platelets (A and C) or PDMPs (B and D). Washed platelets were incubated with apocynin (100μM, panels A and B) and (300μM, panels C and D) for 30 min at 37°C followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 5-12 healthy individuals. Paired t-test ($p<0.05$).

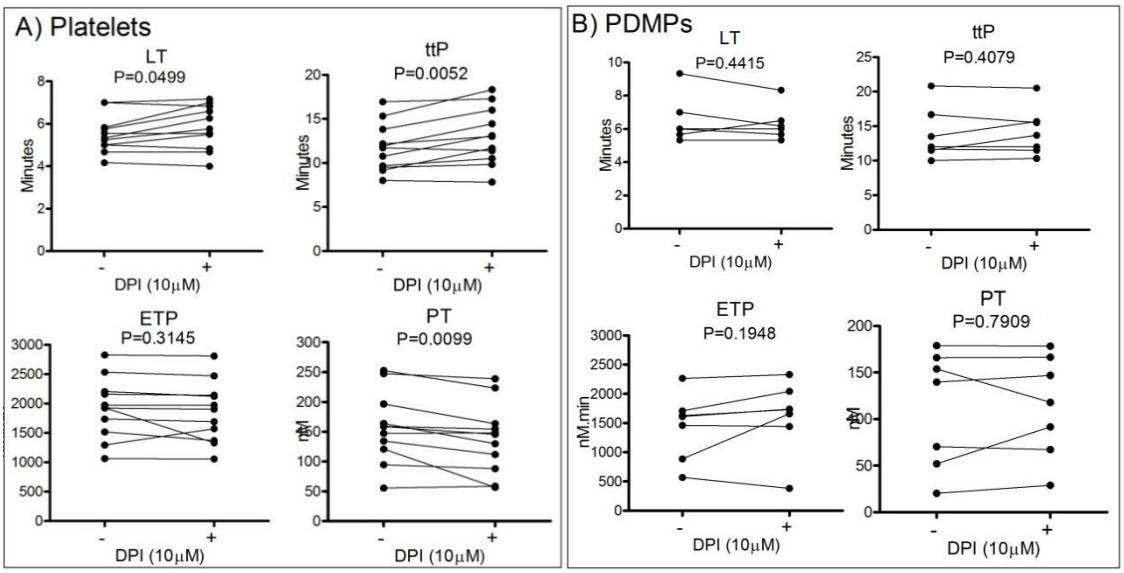



**Fig 6.17: Effect of DPI on thrombin generation.** Thrombin generation was measured by CRP-XL stimulated platelets (A) or PDMPs (B). Washed platelets were incubated with DPI (10μM) for 30 min at 37°C followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 5-12 healthy individuals. Paired t-test ($p<0.05$).

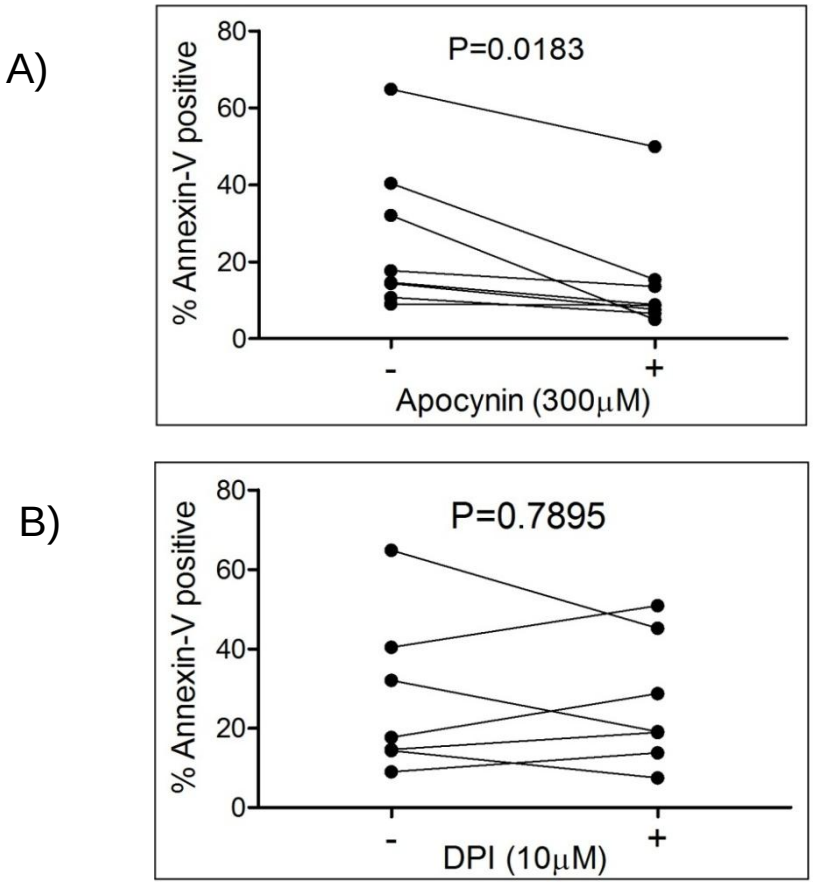



**Fig 6.18. Effect of NAD(P) oxidase inhibitors on CRP-XL induced PS exposure in platelets.** Washed platelets were pre-treated with apocynin (300μM; panel A) or DPI (10μM; panel B) for 30 minutes at 37°C followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes. PS exposure was detected by annexin-V using flow cytometry. Data shown are from 7-8 healthy subjects (paired t-test).

platelet-induced thrombin generation ($p>0.05$) (Fig 6.20). Neither esculetin (Fig 6.19) nor aspirin (Fig 6.20) had any inhibitory effect on thrombin generation by PDMPs. In line with the effects of esculetin on platelet thrombin generation by platelets, esculetin reduced PS exposure significantly ($p<0.05$) (Fig 6.21).

### 6.2.4.3. Effect of ROS scavengers

Tempol (300µM), a general ROS scavenger, significantly inhibited all thrombin generation parameters by platelets ($p<0.05$ except ETP $p>0.05$; Fig 6.22), while a superoxide scavenger, tiron, had no effect at a low dose (100µM) on either platelet- or PDMP-mediated thrombin generation ($p>0.05$) (Fig 6.23). In line with this, tiron has no effect on PS exposure (Fig 6.24). At a higher dose, 1000µM, tiron significantly inhibited ($p<0.05$) the amount of thrombin generated (ETP and PT) but not the rate of the reaction ($p>0.05$) by platelets (Fig 6.23). In PDMPs, a small effect was seen in the rate of reaction of thrombin generation (only LT $p<0.05$), but no effect was seen on the ETP or PT.

### 6.2.5.4. Effect of antioxidant

BHT had no effect on thrombin generation by platelets (Fig 6.25). In fact in 2/5 subjects thrombin generation was enhanced. However, the overall results did not reach statistical significance ($p>0.05$). On the other hand, BHT inhibited thrombin generation by PDMPs in four donors out of five, and only one donor showed enhancement. Again, the overall results did not reach significance ($p>0.05$).

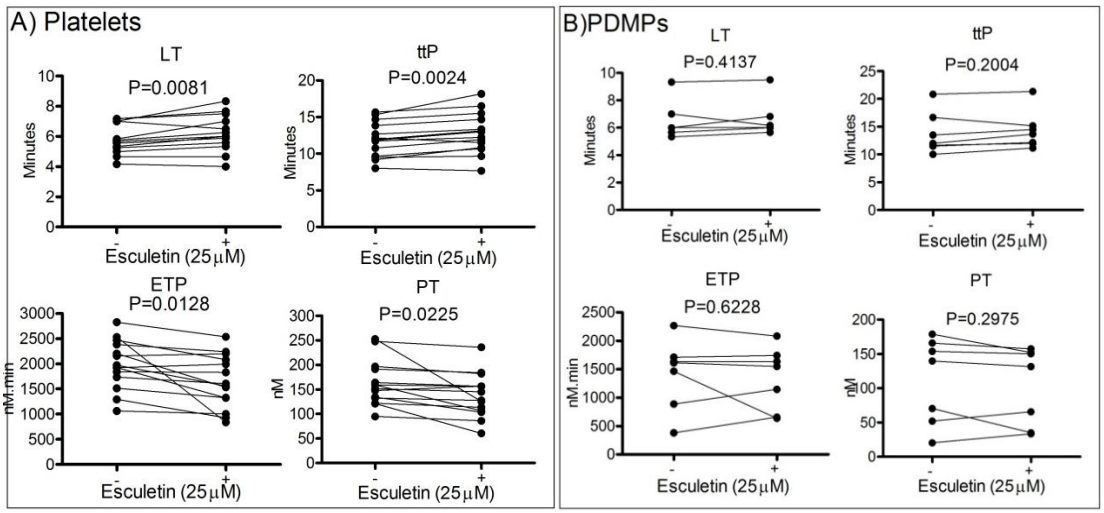



**Fig 6.19. Effect of esculetin on thrombin generation.** Thrombin generation was measured in CRP-XL stimulated platelets (A) or PDMPs (B). Washed platelets were incubated with esculetin (25μM) for 30 min at 37°C followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 5-12 healthy individuals. Paired t-test ($p<0.05$).

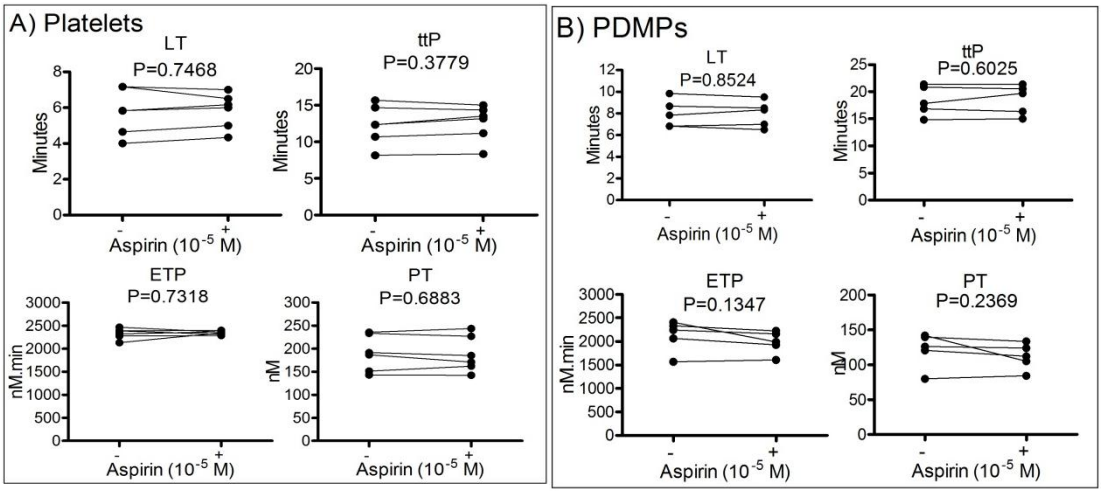



**Fig 6.20: Effect of aspirin on thrombin generation.** Thrombin generation was measured by CRP-XL stimulated platelets (A) or PDMPs (B). Washed platelets were incubated with aspirin ($10^{-5}$M) for 30 min at 37°C followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 5-7 healthy individuals. Paired t-test ($p<0.05$).

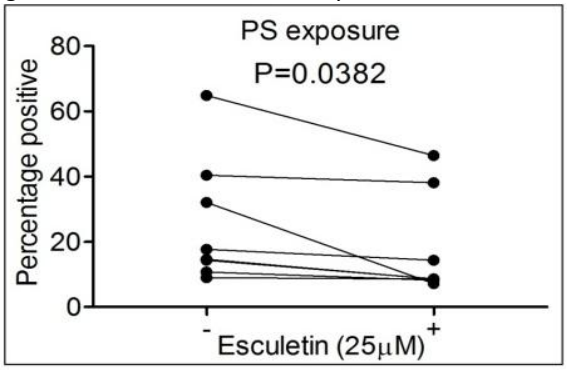



**Fig 6.21: Effect of esculetin on CRP-XL induced PS exposure in platelets.** Washed platelets were pre-treated with esculetin (25μM) for 30 minutes followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes at 37°C. PS exposure was detected by annexin-V using flow cytometry. Data shown are from 7 healthy subjects (paired t-test).

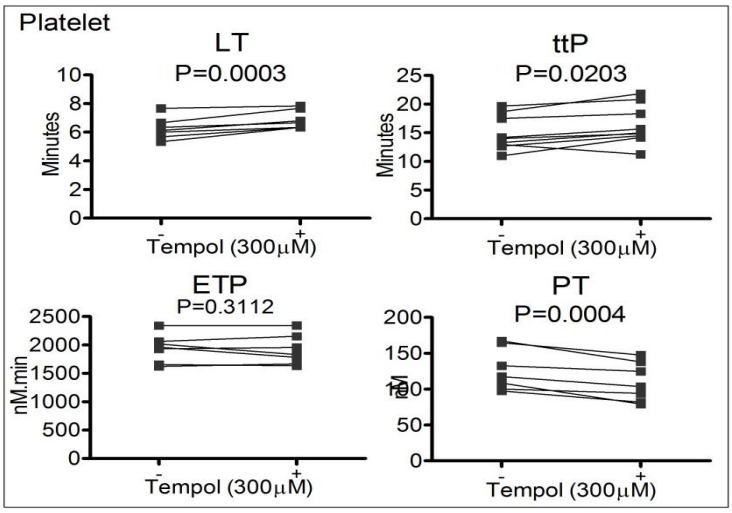



**Fig 6.22. Effect of tempol on thrombin generation measured in platelets.** Washed platelets were incubated with tempol (300µM) for 30 min at 37°C followed by CRP-XL (2.0µg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. PS exposure was detected by annexin-V binding using flow cytometry. Data are from 8 healthy individuals. Paired t-test ($p<0.05$).

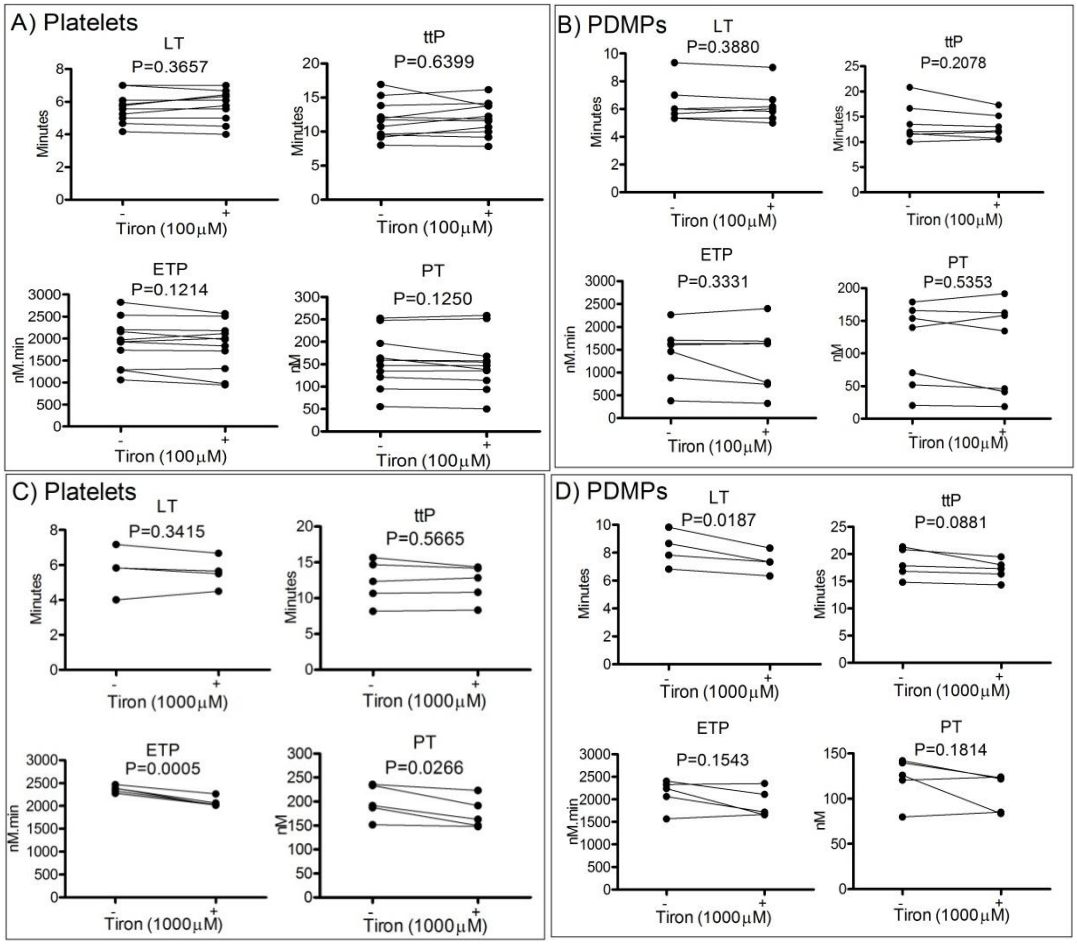



**Fig 6.23. Effect of tiron on thrombin generation.** Thrombin generation was measured by CRP-XL stimulated platelets (A and C) or PDMPs (B and D). Washed platelets were incubated with tiron (100µM, panels A and B) and (1000µM, panels C and D) for 30 min at 37°C followed by CRP-XL (2.0µg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 4-12 healthy individuals. Paired t-test ($p<0.05$).

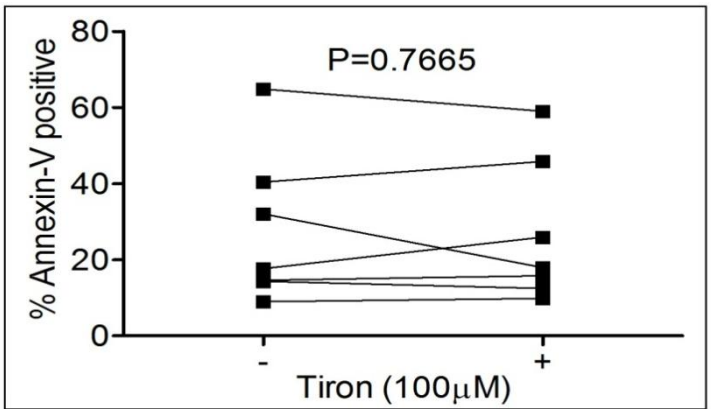



**Fig 6.24. Effect of tiron on CRP-XL induced PS exposure in platelets.** Washed platelets were pre-treated with tiron (100μM) for 30 minutes followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes at 37°C. PS exposure was detected by annexin-V binding using flow cytometry. Data shown are from 7 healthy subjects (paired t-test).

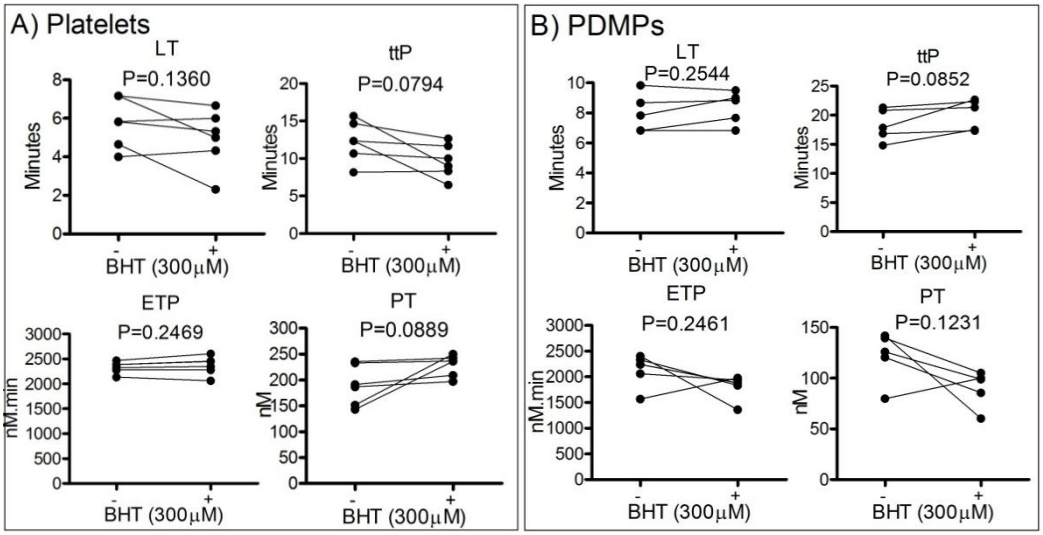



**Fig 6.25: Effect of BHT on thrombin generation.** Thrombin generation was measured by CRP-XL stimulated platelets (A) or PDMPs (B). Washed platelets were incubated with BHT (300μM) for 30 min at 37°C followed by CRP-XL (2.0μg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 5-7 healthy individuals. Paired t-test ($p<0.05$).

### 6.2.5.5. NOS inhibitor

L-NAME showed a small reduction in thrombin generation by platelets and PDMPs, reaching statistical significance ($p<0.05$) in the PT of platelets and in the ETP of PDMPs (Fig 6.26).

### 6.2.5.6. PI3K inhibitor

Wortmannin at 100nM significantly reduced the rate and the amount of thrombin generated in washed platelets (all parameters $p<0.05$) and in PDMPs (all parameters <0.05; except ttP; $p>0.05$) (Fig 6.27).

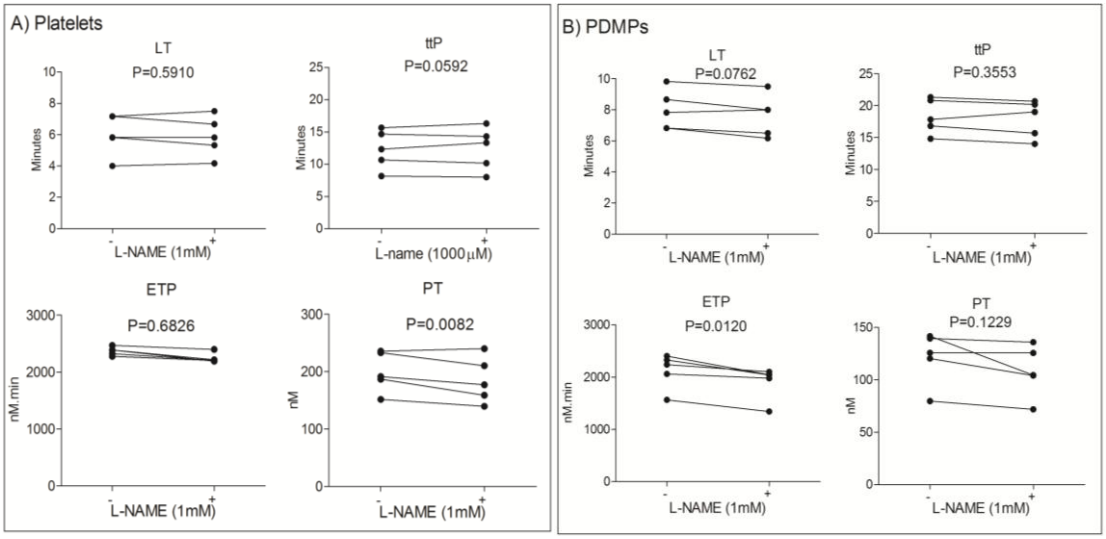



**Fig 6.26. Effect of L-NAME on thrombin generation.** Thrombin generation was measured by CRP-XL stimulated platelets (A) or PDMPs (B). Washed platelets were incubated with L-NAME (1mM) for 30 min at 37°C followed by CRP-XL (2.0µg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 5-7 healthy individuals. Paired t-test ($p<0.05$).

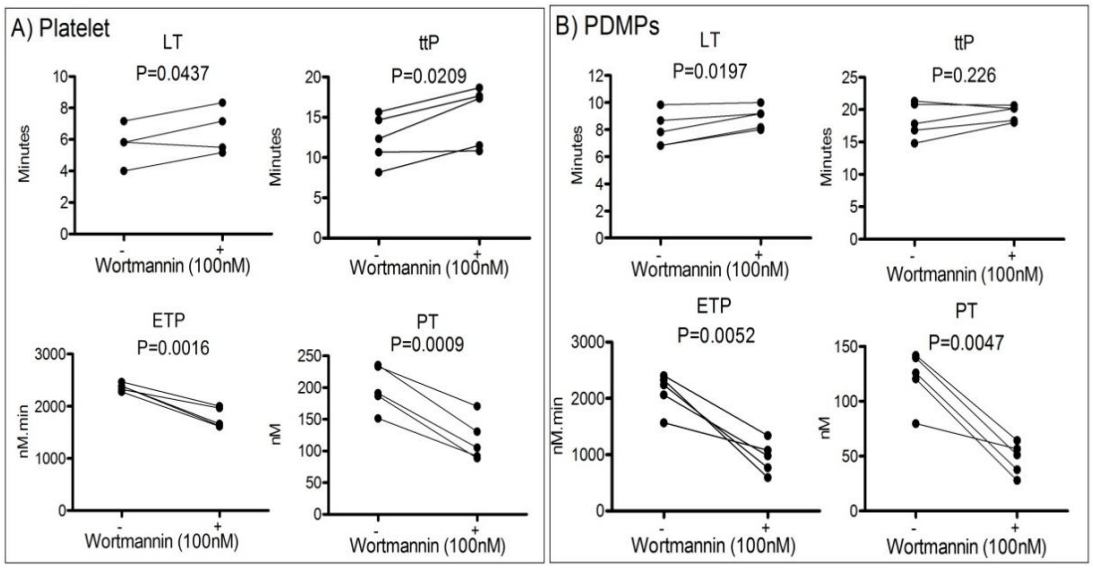



**Fig 6.27: Effect of wortmannin on thrombin generation.** Thrombin generation was measured by CRP-XL stimulated platelets (A) or PDMPs (B). Washed platelets were incubated with wortmannin (100nM) for 30 min at 37°C followed by CRP-XL (2.0µg/ml) stimulation for 10 minutes at 37°C. Thrombin generation was initiated by the 1pMTF reagent. Data are from 5-7 healthy individuals. Paired t-test ($p<0.05$).

## 6.2.6. Effect of the inhibitors on the CRP-XL induced platelet responses

To further investigate the effect of the previous inhibitors, a dose-response for each inhibitor was carried out on thrombin generation by platelets and PDMPs, and one or two doses of the inhibitors were used to investigate the effect of CRP-XL-induced aggregation, P-selectin expression and fibrinogen binding in PRP. In addition, the generation of 12-HETE and $TxB_2$ in the supernatant of washed platelets was measured. For this washed platelets ($150x10^{-9}$/L), after platelets were incubated with and without CRP-XL for 10 minutes at 37°C in the presence and absence of the inhibitor, the supernatant was obtained by centrifugation for 30 minutes at 1,800x*g*. 12-HETE and $TxB_2$ were determined using reverse phase liquid chromatography-tandem mass spectrometry on a 4000 Q-Trap instrument in the laboratory of Professor Valerie O'Donnell, Cardiff University. Eicosanoids were extracted from platelet supernatants using a liquid extraction method. 12HETE-d8 and PGE2-d4 were used as internal standards for 12-HETE and TxB2, respectively, and quantitation used standard curves generated by varying the primary standard versus deuterated as described by O'Donnell, *et al* (2009) and Thomas, *et al* (2010).

Aggregation was measured in PRP adjusted to $150x10^{9}$/L, pre-incubated at 37°C with and without the inhibitors for 30 minutes. A CRP-XL dose response was performed for each donor (6 different subjects) to select a dose that induced full aggregation. In the absence of the inhibitors, CRP-XL induced full aggregation in a dose-dependent manner; although different responses were observed in different donors, CRP-XL at 2.0µg/ml induced full aggregation in all donors (Fig 6.28). The PA and AUC both showed significant increases with CRP-XL ($p<0.001$; n=6; One-way ANOVA). Therefore,

in the subsequent analysis, two doses of CRP-XL were used (0.5 and 2.0µg/ml) unless otherwise stated. In addition, CRP-XL at 2.0µg/ml induced significant P-selectin expression (6.0±1.7 vs. 86.0±0.8; P=0.0004; Fig 6.29 left panel) and fibrinogen binding (6.0±1.7 vs. 86.0±0.8; P=0.0004; Fig 6.29 left panel) compared to resting PRP, and significant production of 12-HETE and $TxB_2$ was measured in the supernatants of washed platelets (Fig 6.30).

### 6.2.6.1. Effect of NAD(P)H oxidase complex pathway inhibitors

In line with previous results, apocynin and DPI reduced the thrombin generation measured in platelets or PDMPs in a dose-dependent manner but at different magnitudes; the inhibitory effect of apocynin (Fig 6.31) was more marked than DPI (Fig 6.36). Apocynin had a pronounced inhibition on all parameters of thrombin generation in both platelets and PDMPs. In platelets the effect was significantly observed for all parameters ($p<0.005$) except for ETP ($p=0.0591$). In PDMPs, apocynin inhibited thrombin generation, with the most effect observed on PT ($p=0.0035$) and ETP ($p>0.05$). The inhibitory effect on PT increased with increasing concentrations of apocynin. In addition, apocynin dose-dependently inhibited PS exposure in washed platelets (n=2, Fig 6.32). Apocynin at 300µM and 1000µM inhibited CRP-XL-induced aggregation in PRP (Fig 6.33). Low doses of apocynin (300µM) had no effect on P-selectin expression or fibrinogen binding (Fig 6.34), but did significantly inhibit 12-HETE and $TxB_2$ production from platelets (Fig 6.35), but this dose had an effect on thrombin generation as described previously.

Conversely, DPI had less inhibitory effect on thrombin generation as mediated by platelets or PDMPs (all parameters $p>0.05$; Fig 6.36); it had only a small non-significant

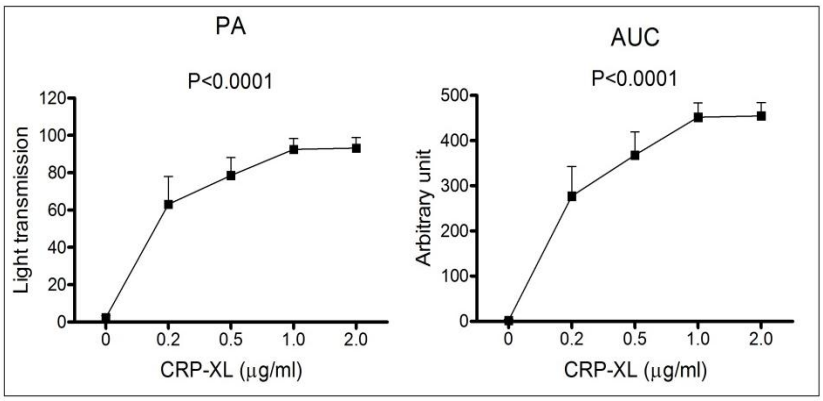



**Fig 6.28: CRP-XL induced platelet aggregation in a dose dependent manner.** Primary aggregation (PA, left) and area under the curve (AUC, right) from 6 healthy donors. Data are the mean±SEM; one-way ANOVA.

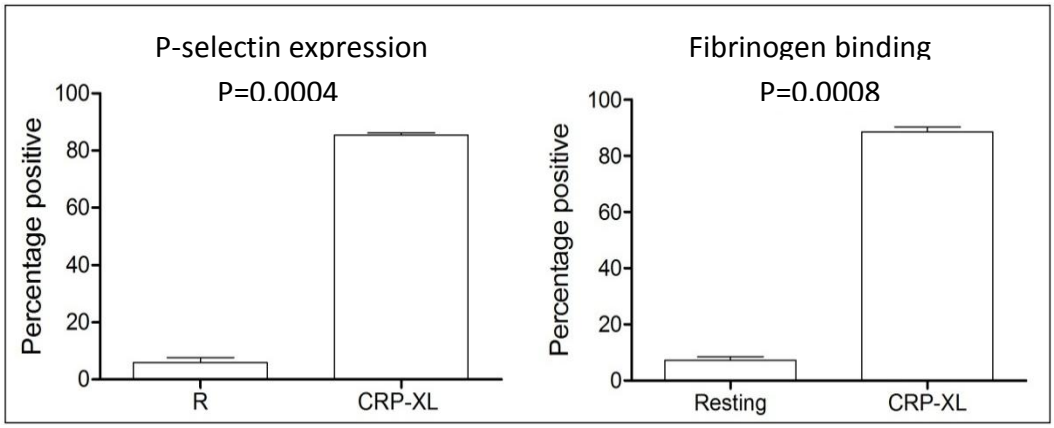



**Fig 6.29: CRP-XL induced P-selectin expression and fibrinogen binding in PRP.** PRP was incubated without CRP-XL for 10 minutes at 37°C. Data are the mean±SEM; n=3; paired student t-test

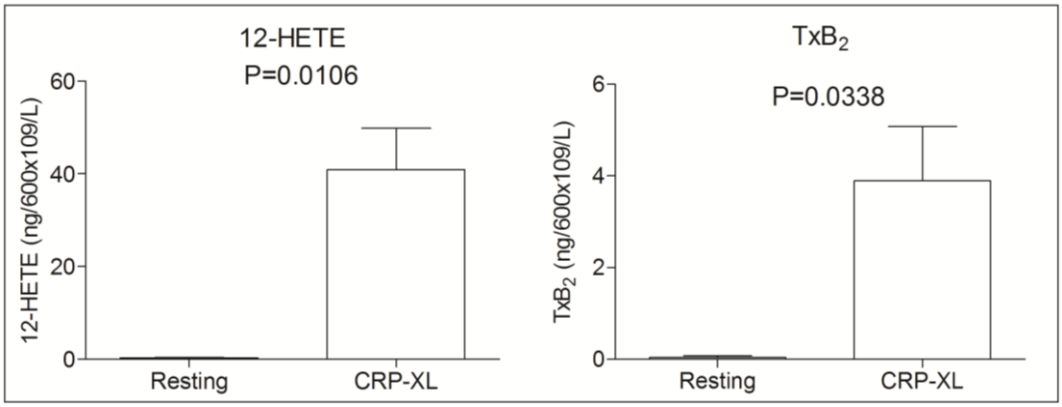



**Fig 6.30: CRP-XL induced 12-HETE and TxB2 production.** Washed platelets were incubated without CRP-XL for 10 minutes at 37°C, then supernatant was obtained by centrifugation of washed platelet for 30 minutes at 1800*xg*. Data are the mean±SEM; n=5; paired student t-test.

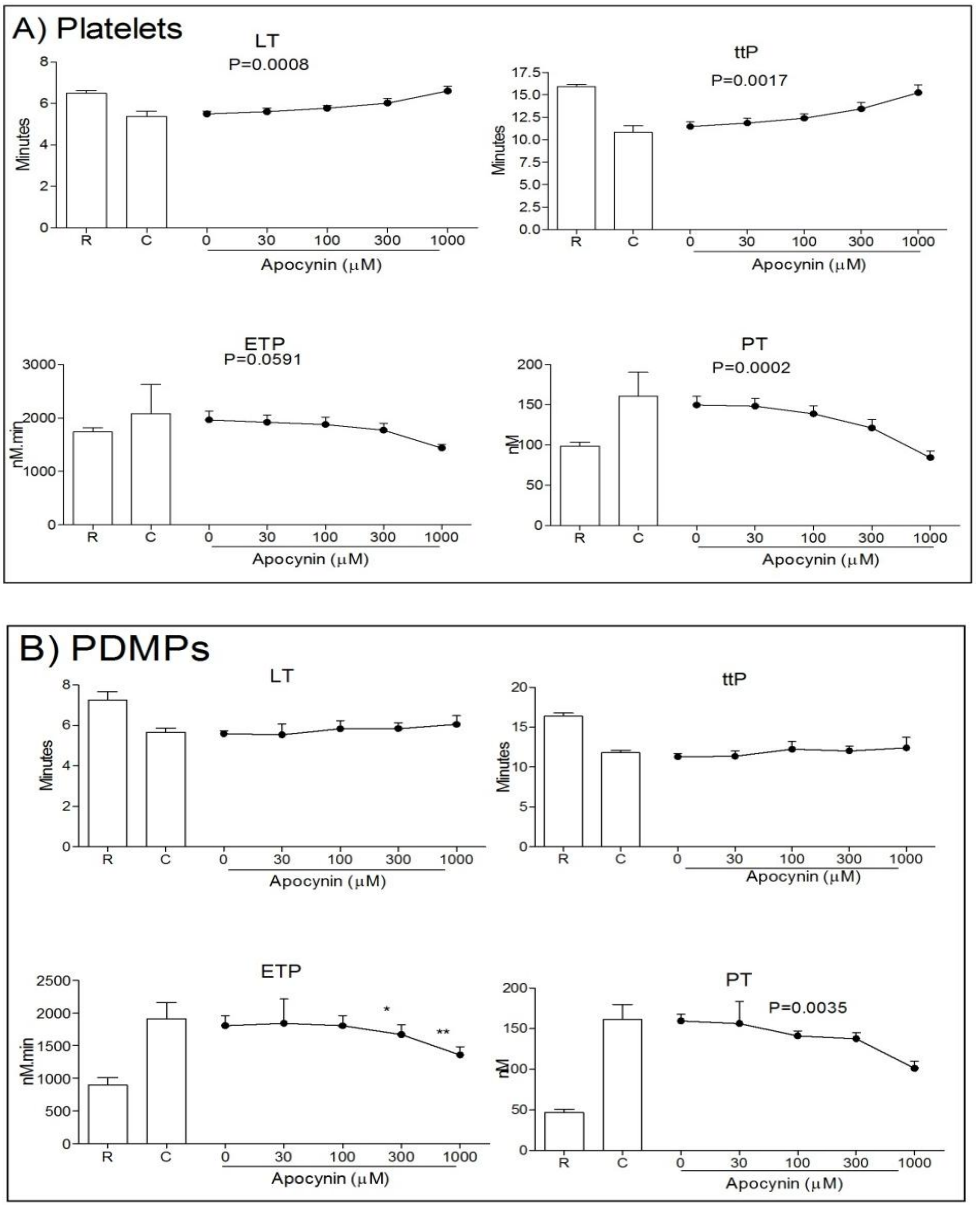



**Fig 6.31: Effect of NAD(P)H oxidase inhibitor, apocynin, on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with apocynin followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml). No agonist (R) or control vehicle (C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800xg for 30 min and analyzed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=4-8); P values are shown by one-way ANOVA analysis. Paired student t-test was used to analyse single concentrations compared with the CRP-XL stimulated sample alone (0); * ($p<0.05$) and ** ($p<0.05$).

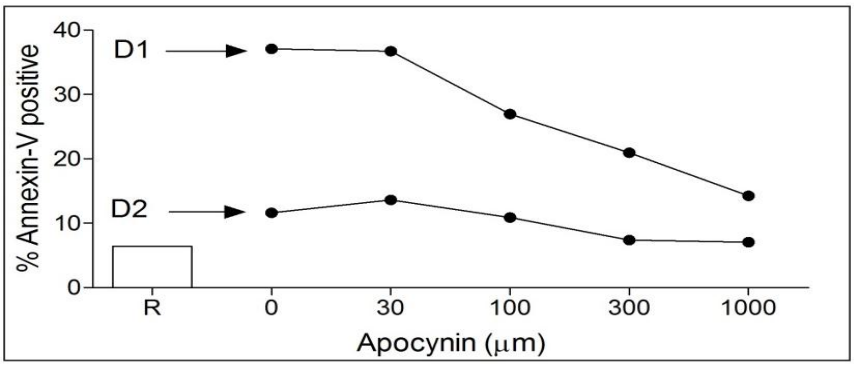



**Fig 6.32: Effect of apocynin on CRP-XL induced PS exposure.** Washed platelets were pre-incubated for 30 minutes at 37°C with apocynin followed by 10 minutes stimulation with CRP-XL (2.0 μg/ml) or no agonist (R=resting). PS exposure was detected by annexin-V using flow cytometry. Data shown are from 2 healthy subjects.

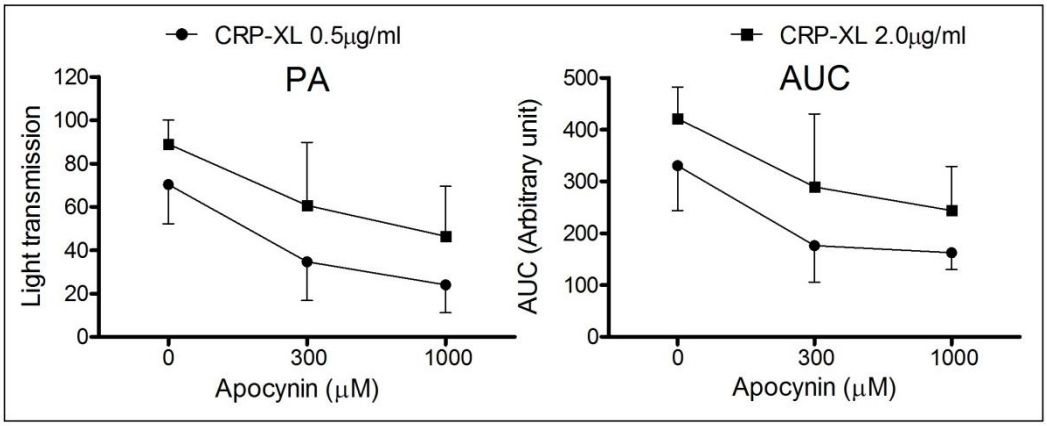



**Fig 6.33: Effect of apocynin on CRP-XL induced platelet aggregation.** Adjusted PRP (150x109/L) was pre-incubated for 30 minutes at 37°C with apocynin followed by 10 minutes stimulation with either 0.5μg/ml (●) or 2.0μg/ml (■)CRP-XL or no agonist (R=resting). Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

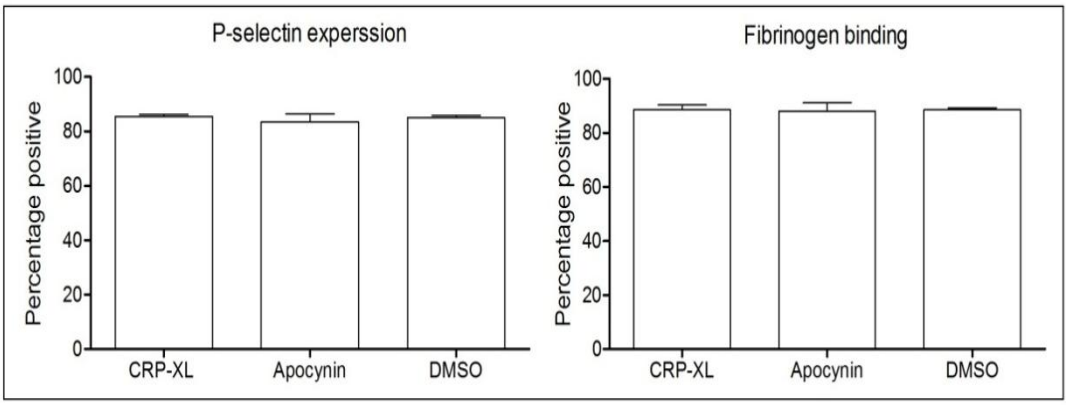



**Fig 6.34. Effect of the apocynin on CRP-XL induced P-selectin expression and fibrinogen binding in PRP.** Either Apocynin (300μM) or control (1%<DMSO) were incubated for 30 minutes at 37°C before CRP-XL stimulation for 10 minutes**.** Data shown are the mean±SEM (n=3).

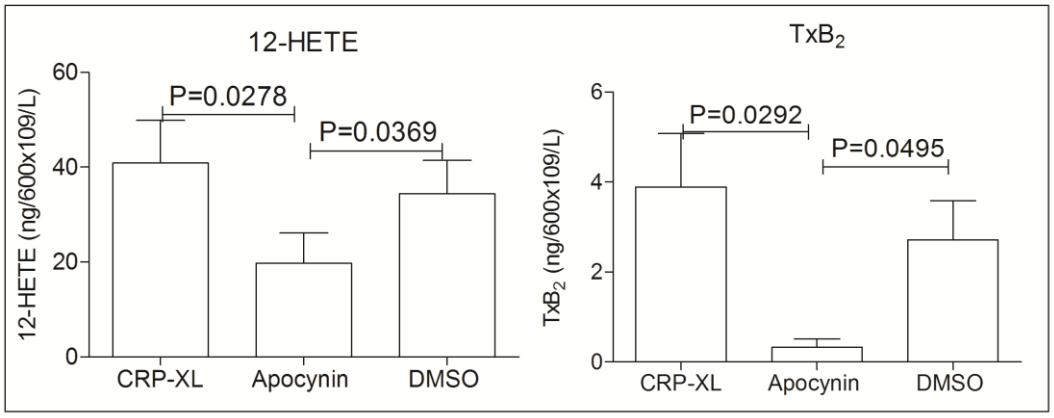



**Fig 6.35. Effect of the apocynin on CRP-XL induced 12-HETE and $TxB_2$ production.** Either Apocynin (300µM) or control (1%<DMSO) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C**,** then supernatant was obtained by centrifugation of washed platelet for 30 minutes at 1800*xg*. Data are the mean±SEM; n=5; paired student t-test.

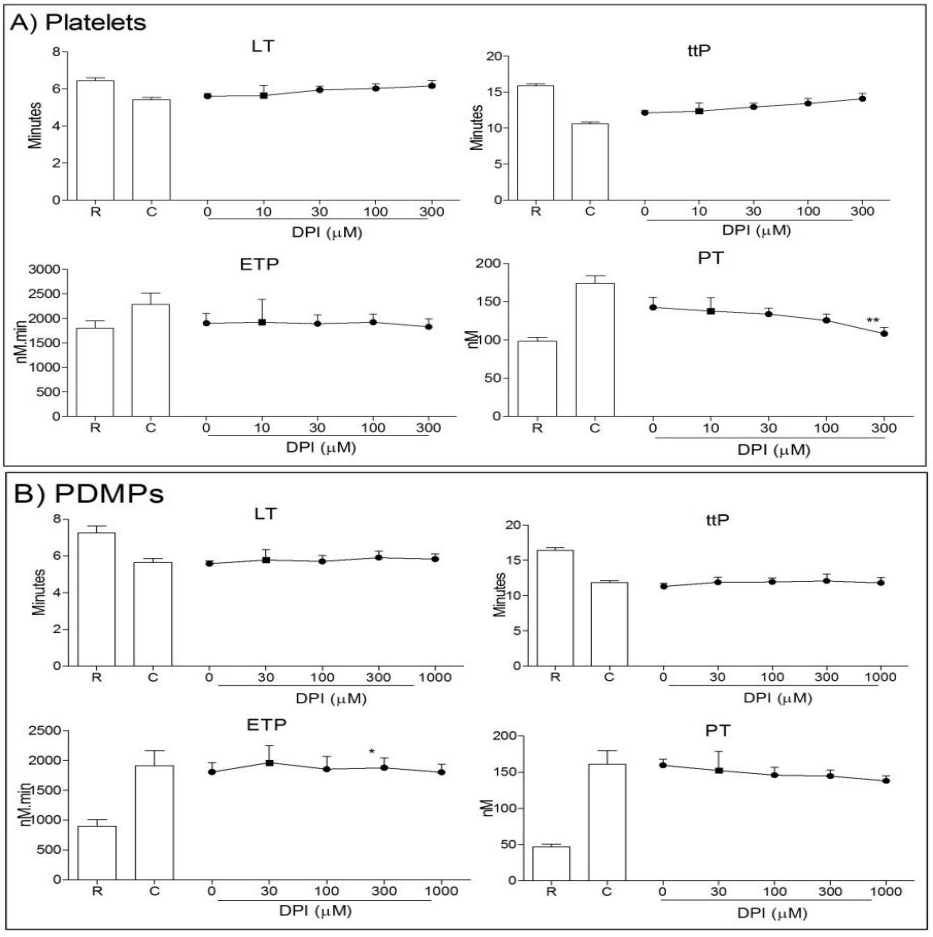



**Fig 6.36: Effect of NAD(P)H oxidase inhibitor, DPI, on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with DPI followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml). No agonist (R) or vehicle (C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800xg for 30 min and analysed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=4-6); P values are for one-way ANOVA analysis. Paired student t-test was used to analyse single concentrations compared with the CRP-XL stimulated sample alone (0); * ($p<0.05$) ** ($p<0.005$).

inhibitory effect at the highest concentrations used (>300μM). The main effect of DPI was seen on the PT only.

### 6.2.6.2. Inhibition of AA pathway

#### 6.2.6.2.1. Effect of 12-LOX pathway inhibitors

Esculetin had only a modest inhibitory effect on thrombin generation by platelets and no effect was observed on thrombin generation by PDMPs (Fig 6.37). Esculetin dose-dependently inhibited the rate of thrombin generation (the LT and ttP; $p<0.05$), but had no effect on the amount of thrombin generated in terms of ETP and PT ($p<0.05$). LT reached a plateau at 50μM but ttP was prolonged significantly with increasing concentrations of esculetin, but it had no inhibitory effect on PMDP-mediated thrombin generation. Esculetin dose-dependently inhibited PS exposure on platelets (n=2; Fig 6.38). Esculetin completely inhibited CRP-XL-induced aggregation by both doses of CRP-XL ($p<0.05$; n=3; Fig 6.39) but had no effect on fibrinogen binding or P-selectin expression in PRP (Fig 6.40). In addition, esculetin attenuated $TxB_2$ and 12-HETE production albeit non-significantly, but to a similar extent (Fig 6.41). Unlike esculetin, baicalein, another 12-LOX inhibitor, had no inhibitory effect on thrombin generation by platelets (Fig 6.42; n=2) so an analysis of the effect of baicalein on the measurement of thrombin generation by PDMPs was not performed.

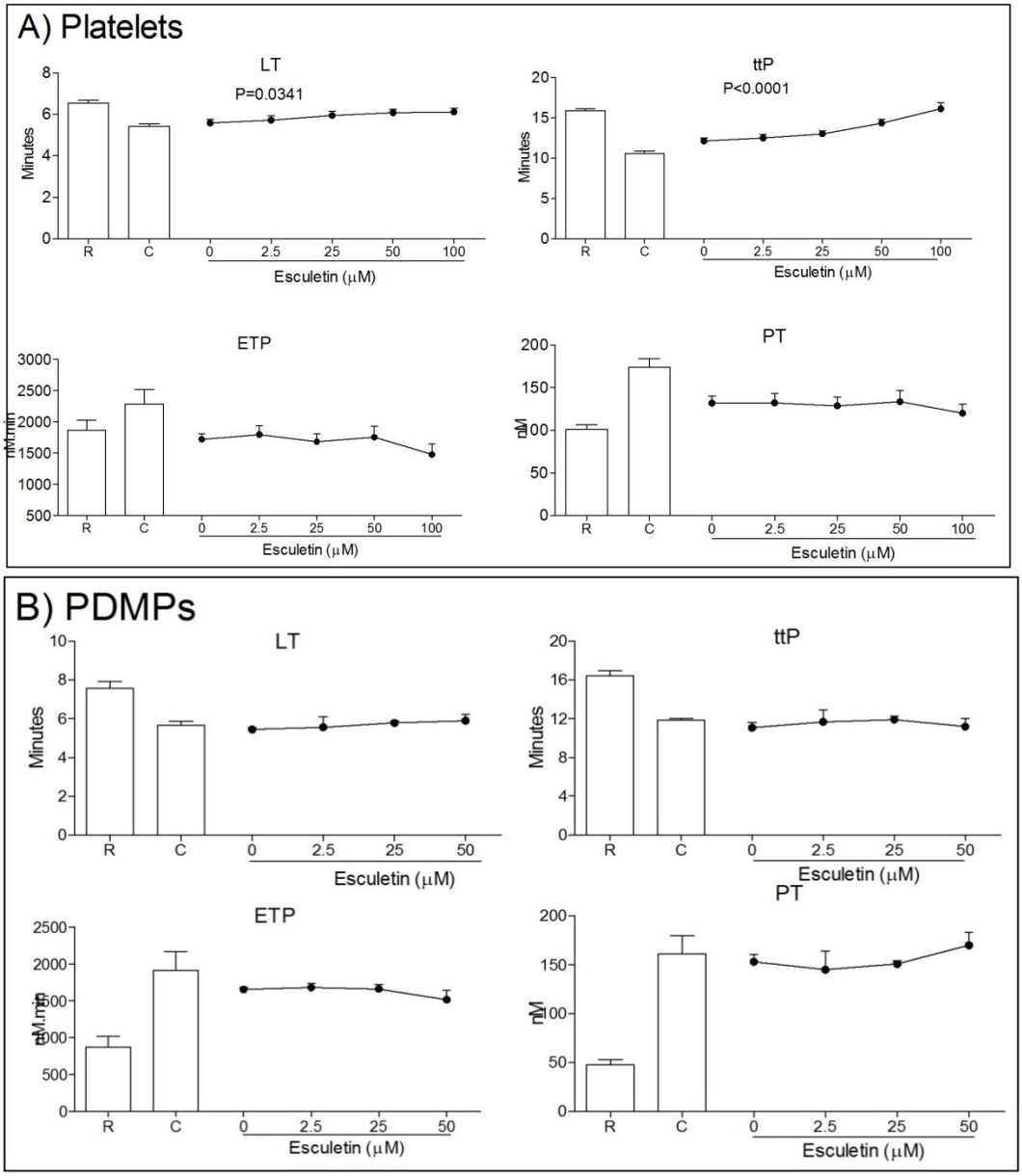



**Fig 6.37: Effect of 12-LOX inhibitor, esculetin, on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with esculetin followed by 10 minutes stimulation with CRP-XL (2.0 μg/ml). No agonist (R) or control ,DMSO, vehicle (C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800*xg* for 30 min and analyzed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=3-5): one-way ANOVA analysis.

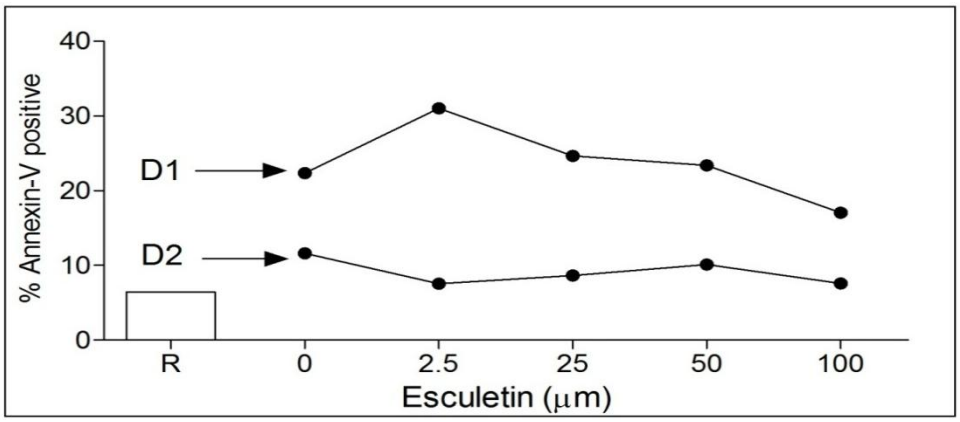



**Fig 6.38: Effect of esculetin on CRP-XL induced PS exposure.** Washed platelets were pre-incubated for 30 minutes at 37°C with esculetin followed by 10 minutes stimulation with CRP-XL (2.0 μg/ml) or no agonist (R=resting). Data shown are from 2 healthy subjects.

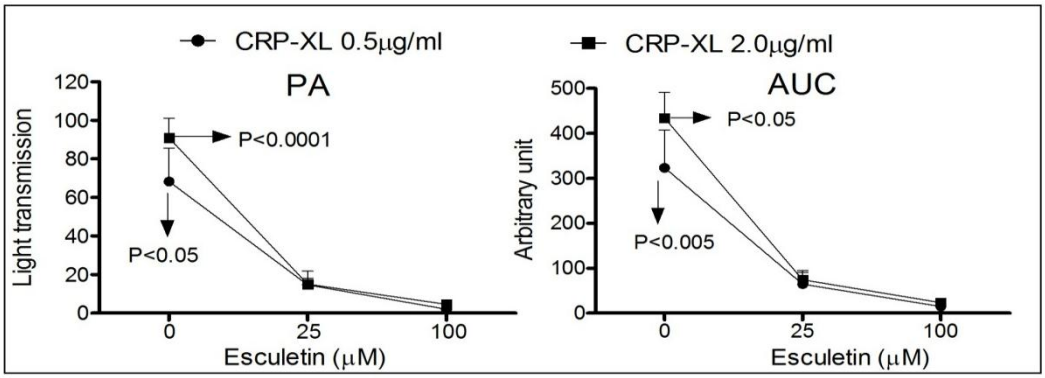



**Fig 6.39: Effect of esculetin on CRP-XL induced platelet aggregation.** Adjusted PRP (150x109/L) was pre-incubated for 30 minutes at 37°C with esculetin followed by 10 minutes stimulation with either 0.5μg/ml or 2.0μg/ml CRP-XL or no agonist (R=resting). Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

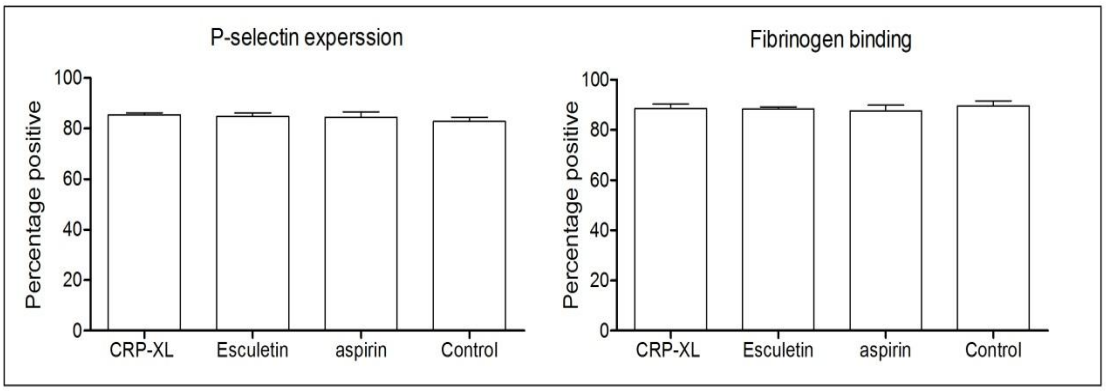



**Fig 6.40: Effect of AA pathway inhibitor (esculetin and aspirin) on CRP-XL induced P-selectin expression and fibrinogen binding in PRP.** Esculetin (25μM), aspirin ($10^{-5}$M) or control (1%<DMSO) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C**.** Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

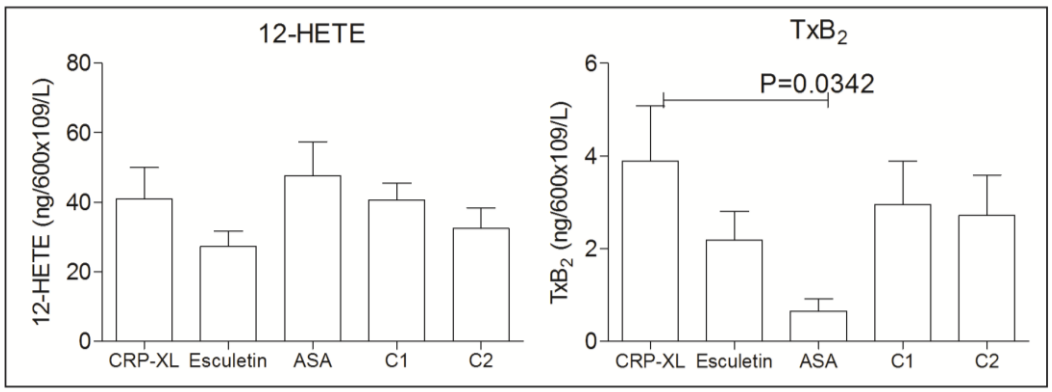



**Fig 6.41: Effect of AA pathway inhibitor (esculetin and aspirin) on CRP-XL induced 12-HETE and $TxB_2$ production.** Esculetin (25µM), aspirin (ASA, $10^{-5}$M) or control (<1%) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C**,** then supernatant was obtained by centrifugation of washed platelet for 30 minutes at 1800*xg*. Data are the mean±SEM; n=5; paired student t-test. C1 is control DMSO for esculetin and C2 is control water for ASA.

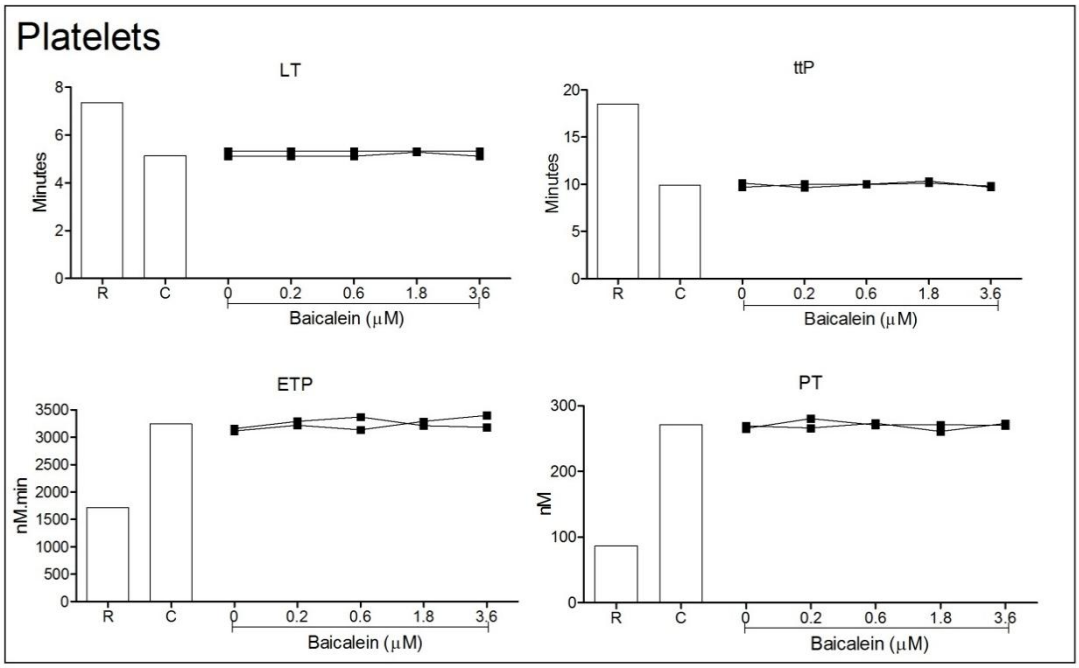



**Fig 6.42: Effect of baicalein on CRP-XL induced thrombin generation in washed platelets.** Washed platelets were pre-incubated for 30 minutes at 37°C with baicalein followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml) or no agonist (R=resting). Vehicle control (C) is DMSO (<1.0%) in CRPLXL stimulated platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the from two runs.

#### 6.2.5.2.2. Effect of COX-1 pathway inhibitor

Aspirin, an inhibitor of COX-1, had no inhibitory effect on thrombin generation by platelets at concentrations up to 1µM (Fig 6.43), however, at the highest concentration of aspirin (10µM), thrombin generation was slightly enhanced reaching statistical significance only for ETP compared to CRP-XL ($p<0.05$; paired t-test). Again, because there was no inhibitory effect on platelets, an analysis of the effect of aspirin on thrombin generation by PDMPs was not performed. At the highest concentration used 10µM) aspirin significantly inhibited $TxB_2$ production and slightly enhanced 12-HETE generation, albeit non-significantly (Fig 6.41).

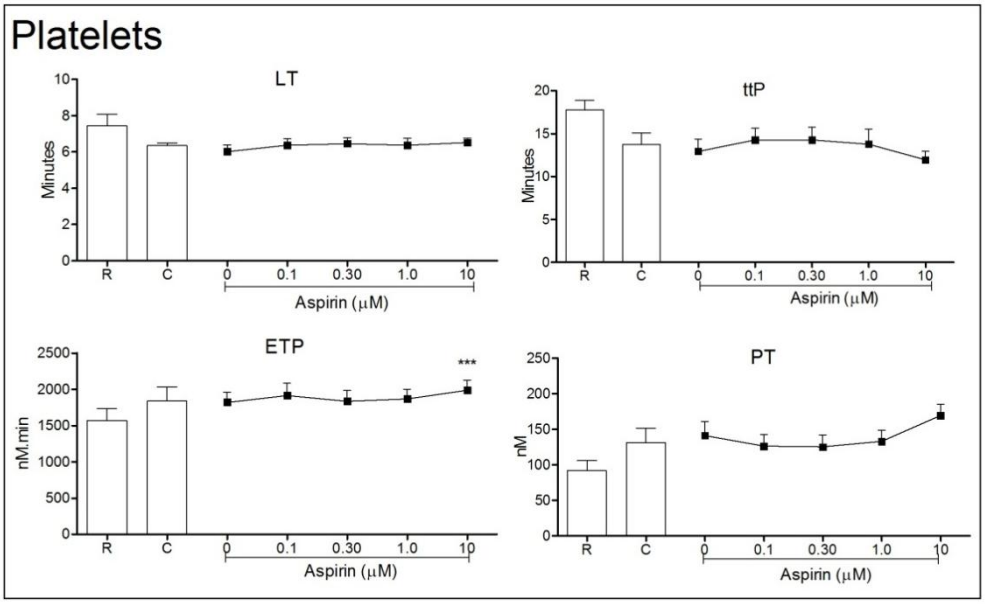



**Fig 6.43. Effect of aspirin on CRP-XL induced thrombin generation in washed platelets.** Washed platelets were pre-incubated for 30 minutes at 37°C with aspirin followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml) or no agonist (R=resting). Vehicle control (C) is water (<1.0%) in CRP-XL stimulated platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=6); one-way ANOVA analysis. Paired student t-test was used to analyse single concentration compared with the CRP-XL stimulated sample alone; *** ($p<0.0001$).

### 6.2.6.3. Effect of ROS scavengers

Tempol inhibited thrombin generation in a dose-dependent manner either by platelet or PDMPs (Fig 6.44). ETP and PT were dose-dependently inhibited by tempol in both platelets and PDMPs ($p<0.05$) although this did not reach statistical significance for ETP induced by PDMPs ($p>0.05$). Tempol had a modest but increasing inhibitory effect on LT and ttP in platelets ($p<0.05$, paired t-test with single doses compared to CRP-XL alone), and on ttP alone with PDMPs. Tempol also inhibited PS exposure (Fig 6.45) and aggregation (Fig 6.46) in a dose-dependent manner, but had no effect on P-selectin expression, or fibrinogen binding (Fig 6.47). It also did not affect 12-HETE or TxB2 production (Fig 6.48).

Tiron had a lesser inhibitory effect compared to tempol (Fig 6.49). At high concentrations, this inhibitor significantly reduced the amount of thrombin measured by ETP in platelets and PDMPs ($p<0.05$; paired t-test) and this was combined with significant inhibition of PT in platelets, ($p<0.0\ 5$, paired t-test), but not PDMPs. Tiron had no effect on LT and ttP in platelets or PDMPs. Unlike, tempol, tiron had no inhibitory effect on aggregation, but in fact it slightly enhanced aggregation at the highest dose ($p>0.05$; Fig 6.50). Nor did tiron affect P-selectin expression, fibrinogen binding (Fig 6.47) or 12-HETE, but it did slightly reduce TxB2 production albeit non-significantly (Fig 6.48).

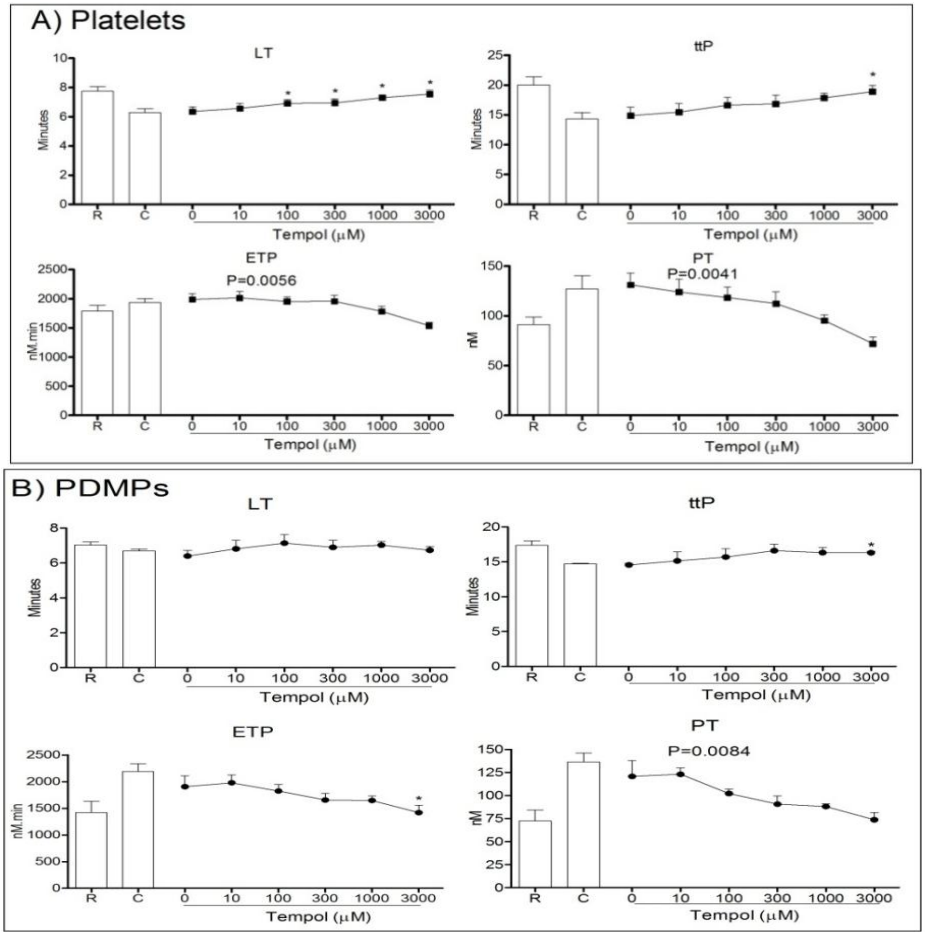



**Fig 6.44. Effect of tempol on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with tempol followed by 10 minutes stimulation with CRP-XL (2.0 μg/ml) or no agonist (R=resting). Vehicle control (C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800 g for 30 min and analyzed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=4-6); one-way ANOVA analysis. Paired student t-test was used to analyse single concentration compared with the CRP-XL stimulated sample alone; * (P<0.05).

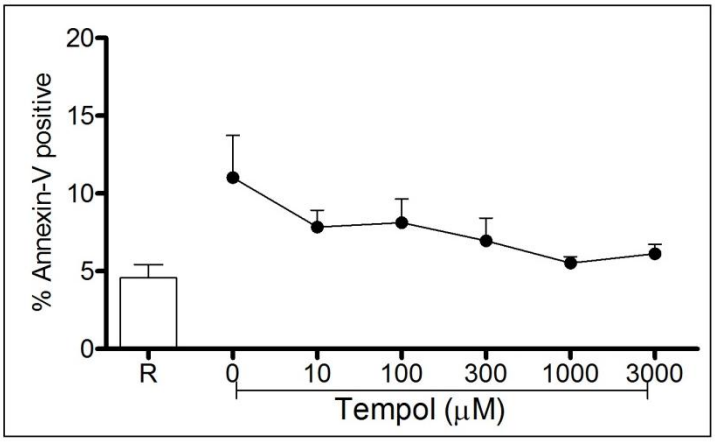



**Fig 6.45 Effect of tempol on CRP-XL induced PS exposure.** Washed platelets were pre-incubated for 30 minutes at 37°C with tempol followed by 10 minutes stimulation with CRP-XL (2.0 μg/ml) or no agonist (R=resting). Data shown as mean±SEM from 3 healthy subjects; One-Way ANOVA.

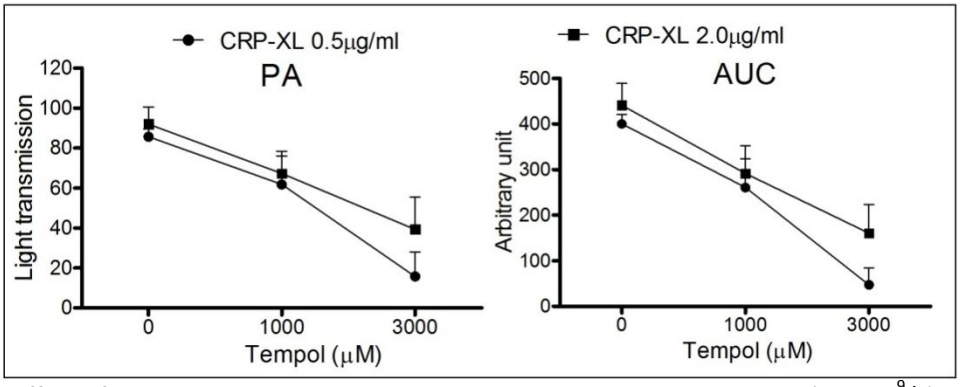



**Fig 6.46. Effect of tempol on CRP-XL induced platelet aggregation.** Adjusted PRP ($150x10^9$/L) was pre-incubated for 30 minutes at 37°C with tempol followed by 10 minutes stimulation with either 0.5μg/ml or 2.0μg/ml CRP-XL or no agonist (R=resting). Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

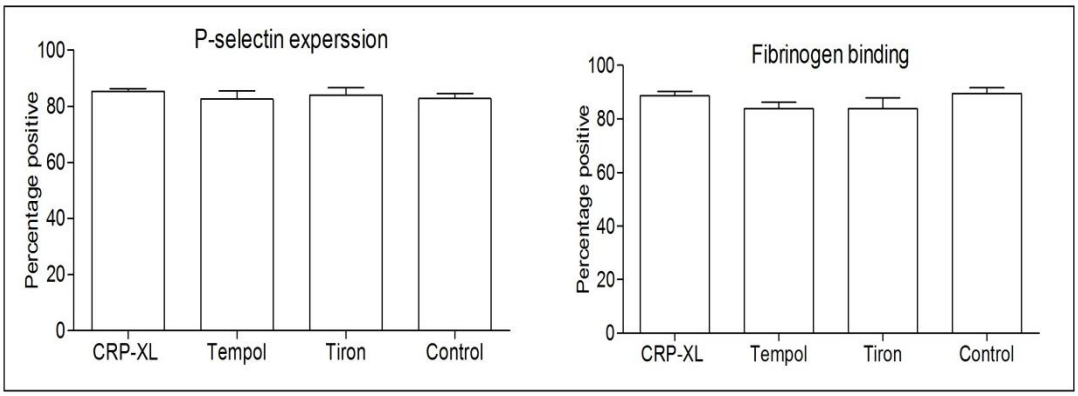



**Fig 6.47. Effect of the ROS scavengers on CRP-XL induced P-selectin expression and fibrinogen binding in PRP.** PPR was pre-incubated with tempol, tiron or control vehicle (DMSO; <1.0%) for 30 minutes at 37°C before CRP-XL stimulation for 10 minutes at 37°C. Data shown are the mean±SEM (n=3); paired t-test.

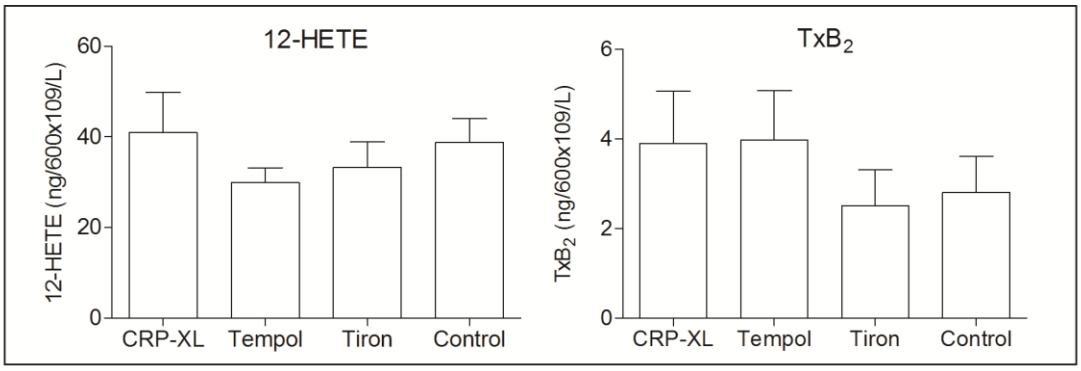



**Fig 6.48. Effect of ROS scavengers on CRP-XL induced 12-HETE and $TxB_2$ production.** Either Apocynin or control (<1%DMSO) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C, then supernatant was obtained by centrifugation of washed platelet for 30 minutes at 1800 xg. Data are the mean±SEM; n=5; paired student t-test.

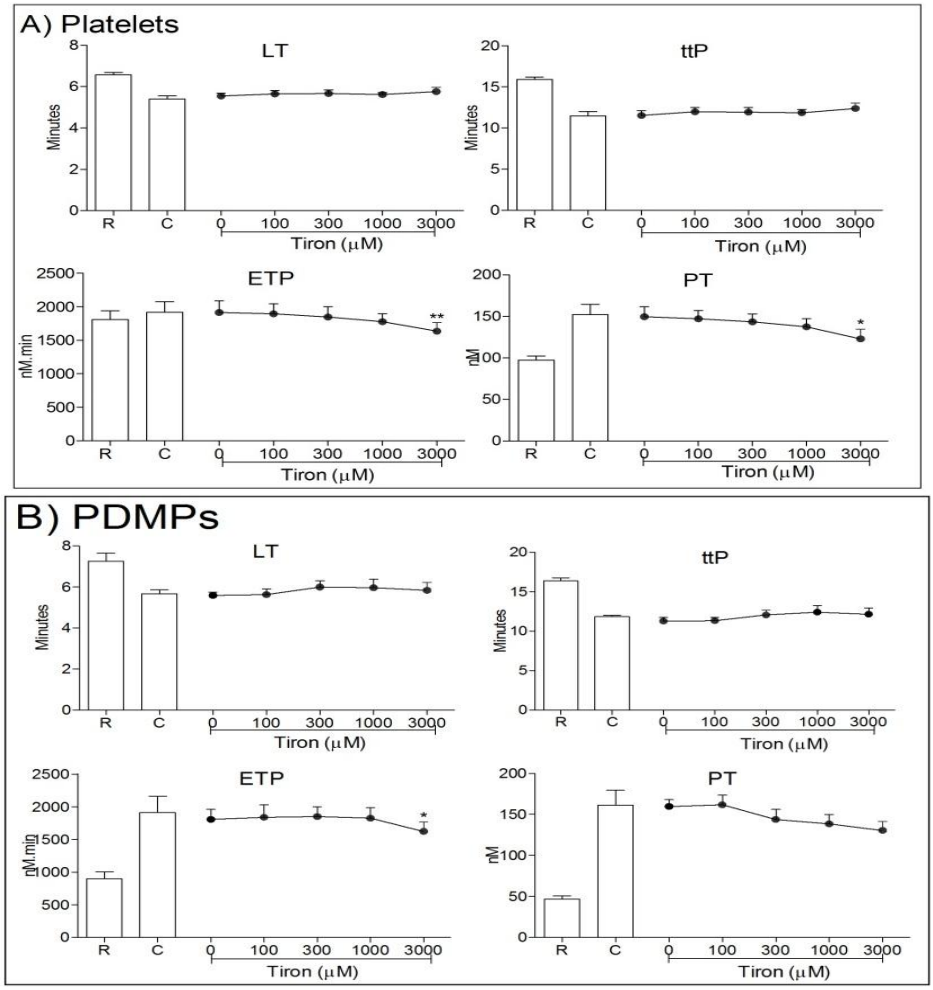



**Fig 6.49. Effect of tiron on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with tiron followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml) or no agonist (R=resting). Vehicle control (C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800 g for 30 min and analysed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=4-6); one-way ANOVA analysis. Paired student t-test was used to analyse single concentration compared with the CRP-XL stimulated sample alone; * (P<0.05).

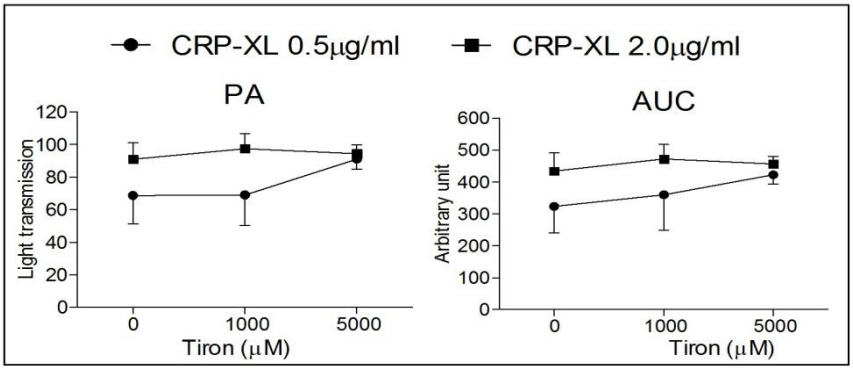



**Fig 6.50: Effect of tiron on CRP-XL induced platelet aggregation.** Adjusted PRP (150x109/L) was pre-incubated for 30 minutes at 37°C with tiron followed by 10 minutes stimulation with either 0.5µg/ml or 2.0µg/ml CRP-XL or no agonist (R=resting). Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

### 6.2.6.4. Effect of Antioxidants

The effect of three antioxidants, BHT, NAC and the vitamin E analogue trolox, on thrombin generation by platelets was also investigated (Fig 6.51), but none of them showed any inhibition for any of the parameters. Surprisingly, BHT increases thrombin generation at high concentrations. Again, since they had no effect on thrombin generation the analysis of the effect of the antioxidants on the measurement of thrombin generation by PDMPs was not performed. One of the antioxidants, (BHT) was tested for its effect on aggregation, which it inhibited in a dose-dependent manner ($p>0.05$) (Fig 6.52), but BHT had no effect on P-selectin expression or fibrinogen binding (Fig 6.53). Conversely, and paradoxically it significantly enhanced 12-HETE production but inhibited (albeit non-significantly) $TxB_2$ production (Fig 6.54).

### 6.2.6.5. Effect of dexamethasone

Dexamethasone has been shown to inhibit ROS and MP formation in A23187-treated platelets (Nardi*, et al* 2007). In Fig 6.55, dexamethasone had no effect on the thrombin generated by CRP-XL-stimulated platelets. However, it slightly inhibited thrombin generation by PDMPs in a dose-dependent manner ($p>0.05$) (Fig 6.55). The maximum inhibitory effect was observed on ETP and PT, but no effect was seen on LT and ttP (all $p>0.05$). Dexamethasone gave a small reduction of PS exposure at low doses, but this effect was not observed with increasing doses of dexamethasone (Fig 6.56).

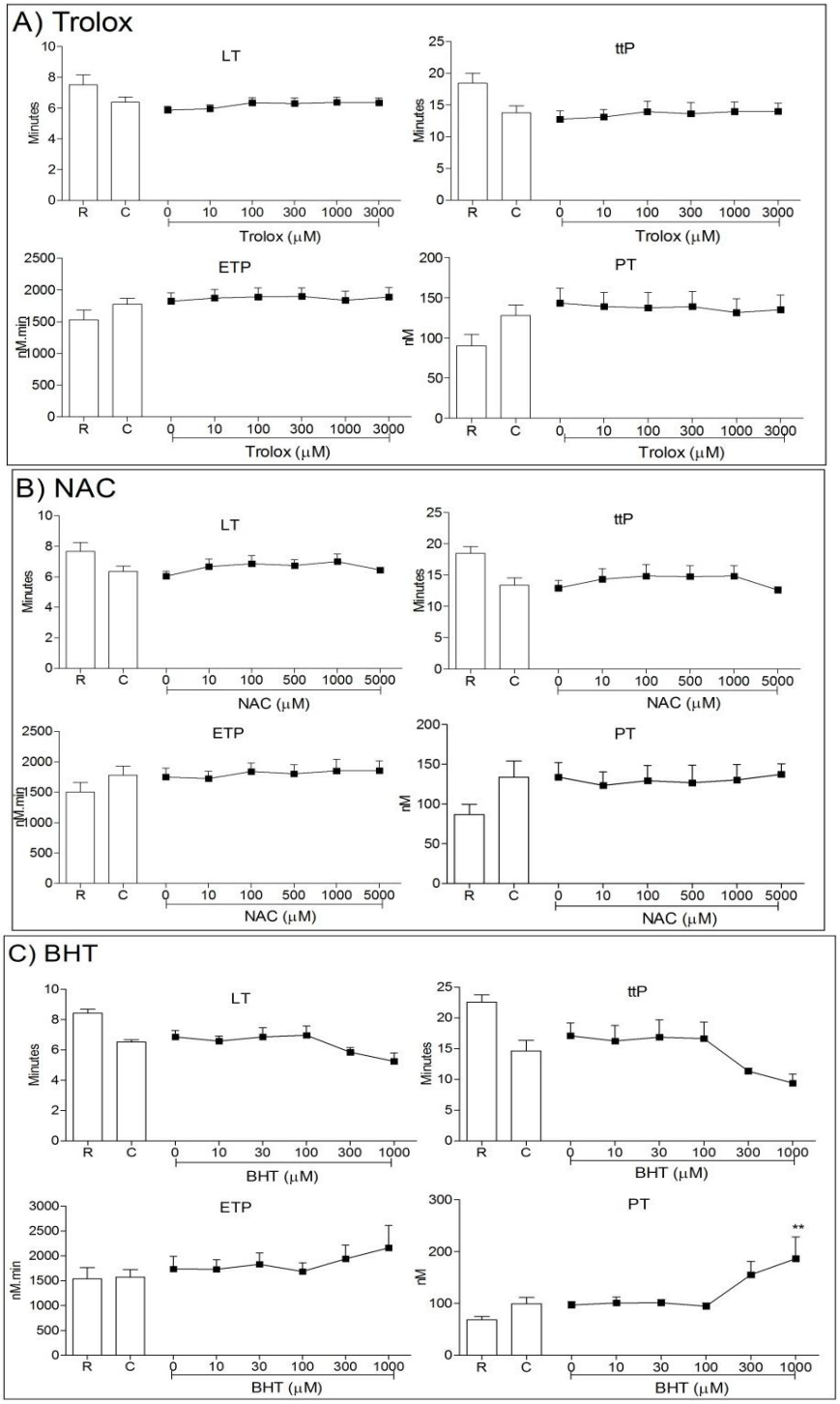



**Fig 6.51: Effect of antioxidants on CRP-XL induced thrombin generation in washed platelets.** Washed platelets were pre-incubated for 30 minutes at 37°C with (A) Trolox (n=6), (B) NAC (n=7) or (C) BHT (n=3C) followed by 10 minutes stimulation with CRP-XL (2.0 μg/ml) or no agonist (R=resting). Vehicle control (C). Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM; one-way ANOVA analysis. Paired student t-test was used to analyse single concentration analysis compared with the CRP-XL stimulated sample alone; ** (P<0.05).

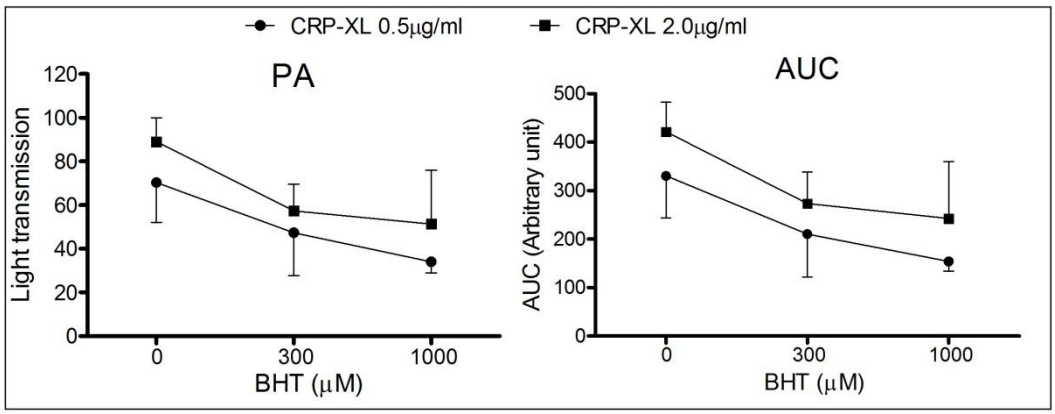



**Fig 6.52: Effect of BHT on CRP-XL induced platelet aggregation.** Adjusted PRP ($150x10^9$/L) was pre-incubated for 30 minutes at 37°C with BHT followed by 10 minutes stimulation with either 0.5μg/ml or 2.0μg/ml CRP-XL or no agonist (R=resting). Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

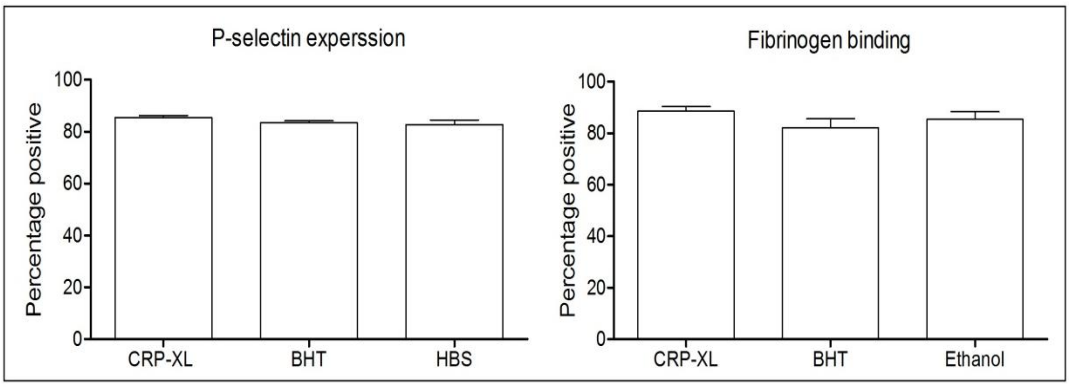



**Fig 6.53: Effect of the BHT on CRP-XL induced P-selectin expression and fibrinogen binding in PRP.** Either BHT or control (1%<ethanol ) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C**.** Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

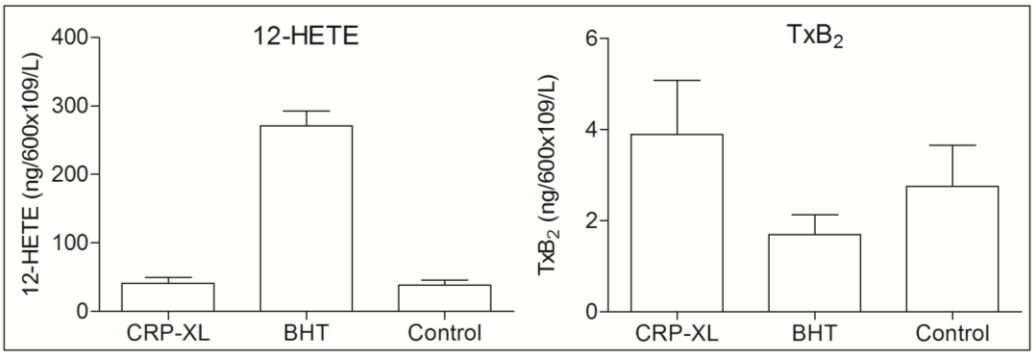



**Fig 6.54: Effect of the BHT on CRP-XL induced 12-HETE and $TxB_2$ production.** Either BHT or control (1%<ethanol) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C**,** then supernatant was obtained by centrifugation of washed platelet for 30 minutes at 1800*xg*. Data are the mean±SEM; n=5; paired student t-test.

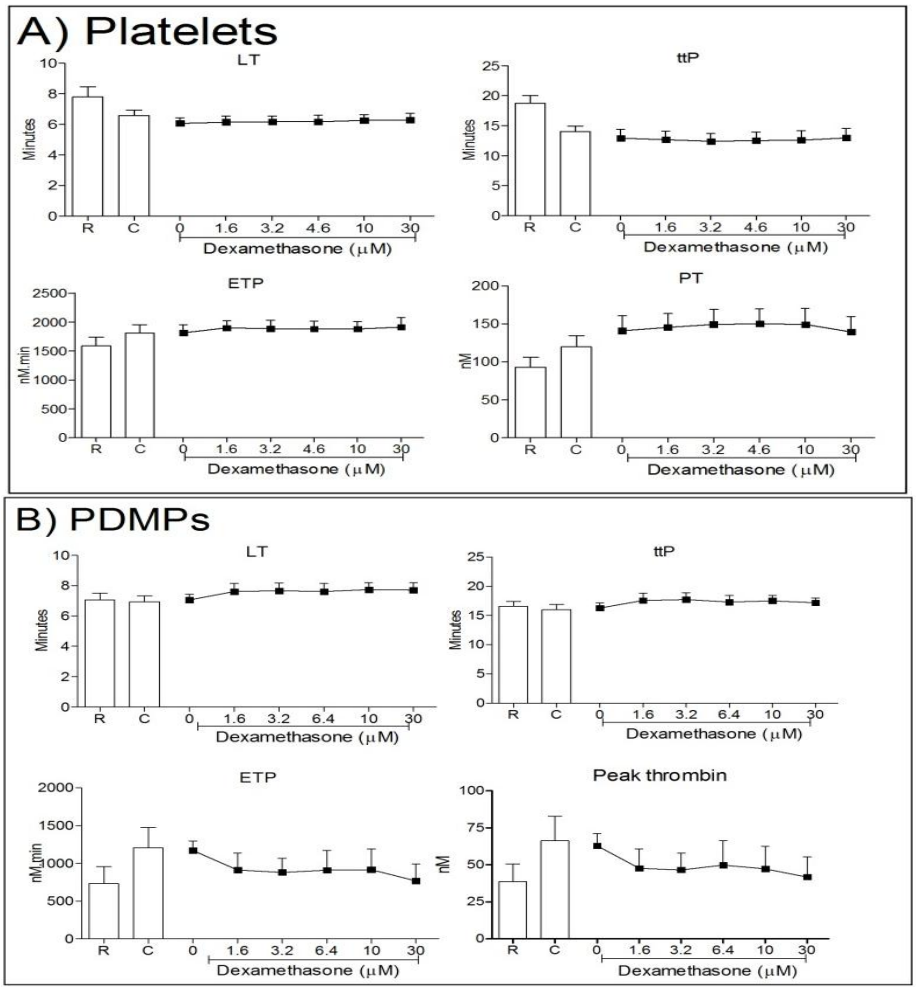



**Fig 6.55.: Effect of dexamethasone on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with dexamethasone followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml) or no agonist (R=resting). Vehicle control (<1% DMSO, C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800 g for 30 min and analyzed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=4-6); one-way ANOVA analysis. Paired student t-test was used to analyse single concentration compared with the CRP-XL stimulated sample alone.

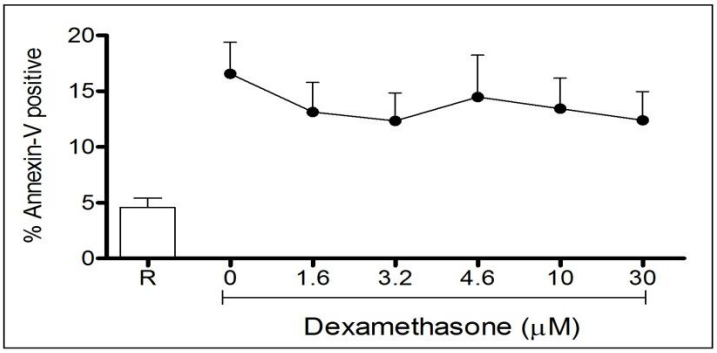



**Fig 6.56: Effect of dexamethasone on CRP-XL induced PS exposure.** Washed platelets were pre-incubated for 30 minutes at 37°C with apocynin followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml) or no agonist (R=resting). Samples were analysed using annexin-V. Data are shown as mean±SEM from 3 healthy subjects; one-way ANOVA analysis.

### 6.2.6.6. Effect of NOS inhibition

Platelets has the ability to produce NO (Chen and Mehta 1996, Freedman*, et al* 1997), which is a free radical that rapidly converts $O^{\bullet}_{2}^{-}$ to ONOO (Chakrabarti*, et al* 2004, Clutton*, et al* 2004). Inhibition of NO production reduces the conversion of $O^{\bullet}_{2}^{-}$ to ONOO and so could reduce the effects on the procoagulant activity. L-NAME is an NOS inhibitor, which inhibits NO production. L-NAME slightly reduced thrombin generation by platelets and PDMPs (Fig 6.57). At 10mM, the LT and TP became significantly prolonged compared to CRP-XL alone ($p<0.05$, paired t-test) and PT and ETP were reduced. This inhibitory effect could only be observed at the highest concentrations of the L-NAME. In PDMPs, although thrombin generation was dose-dependently reduced by L-NAME it did not reach statistical significance ($p>0.05$; one-way ANOVA). When tested for its effect on CRP-XL-induced aggregation. L-NAME dose-dependently attenuated aggregation (Fig 6.58), which is contrary to the NO antiplatelet effect since the inhibition of NO production by L-NAME should enhance platelet activation (Tymvios*, et al* 2009, van Goor*, et al* 2001). L-NAME also inhibited $TxB_2$ production, but had no effect on 12-HETE generation (Fig 6.59), P-selectin expression or fibrinogen binding (data not shown).

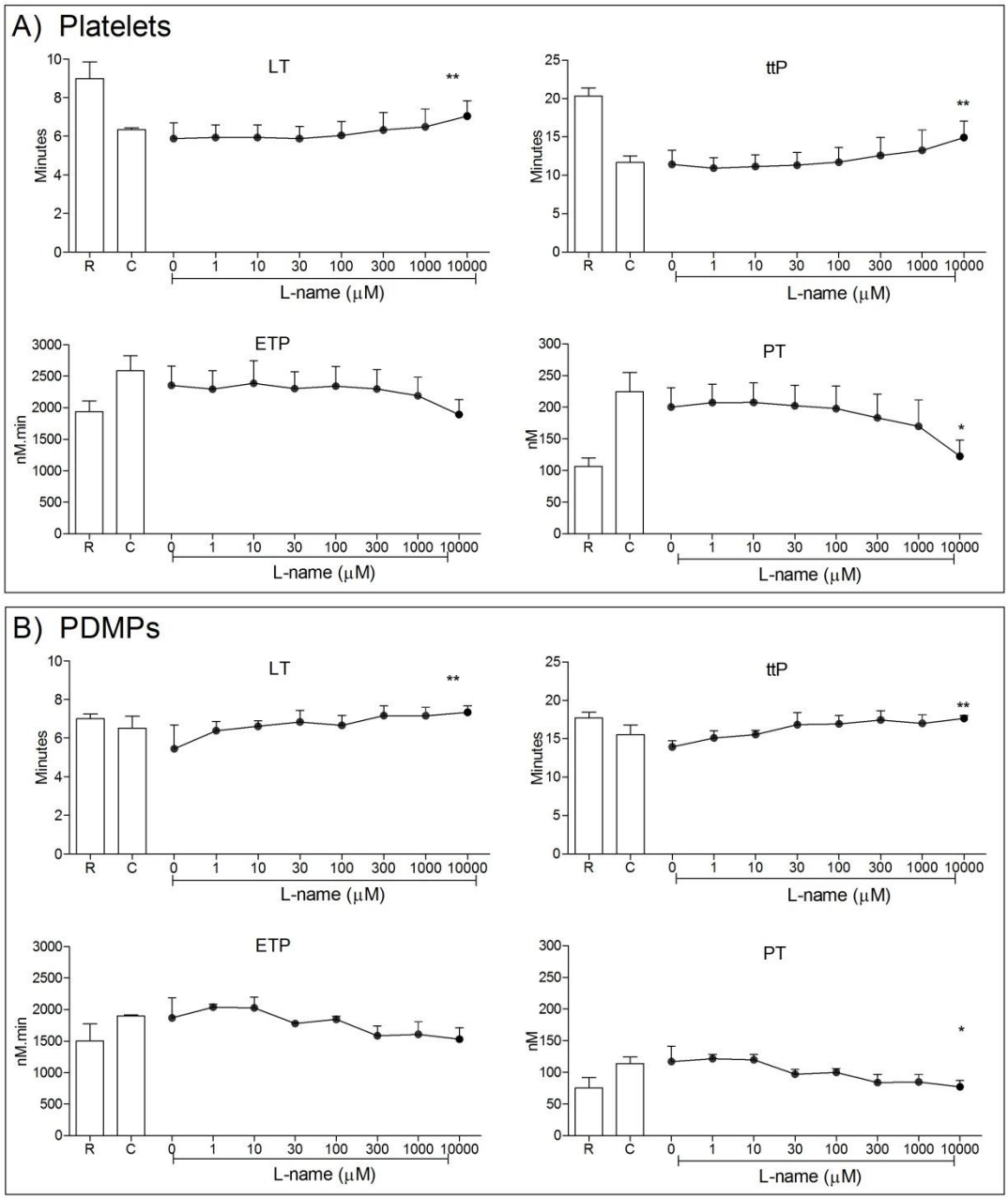



**Fig 6.57: Effect of NOS inhibitor, L-NAME, on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with NOS inhibitor, L-NAME, followed by 10 minutes stimulation with CRP-XL (2.0 μg/ml) or no agonist (R=resting). Vehicle control (C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800 g for 30 min and analysed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=3); one-way ANOVA analysis. Paired student t-test was used to analyse single concentration compared with the CRP-XL stimulated sample alone; * ($p<0.05$).

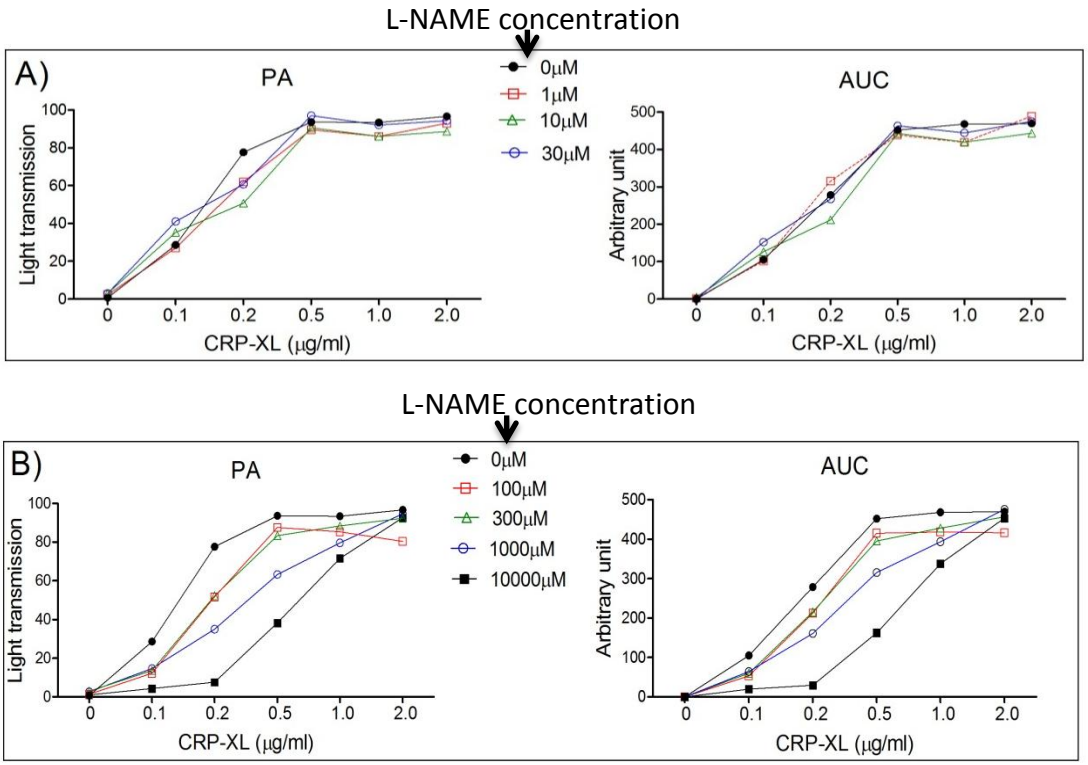



**Fig 6.58. Effect of L-NAME on CRP-XL induced platelet aggregation.** A) Shows L-NAME concentrations (1, 10, 30μM) compared to 0 (CRP-XL alone). B) Shows L-NAME concentrations ( 100, 300, 1000, 10000μM) compared to 0 (CRP-XL alone). Adjusted PRP (150x109/L) was pre-incubated for 30 minutes at 37°C with L-NAME followed by 10 minutes stimulation with dose-response of CRP-XL (0-2.0). Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

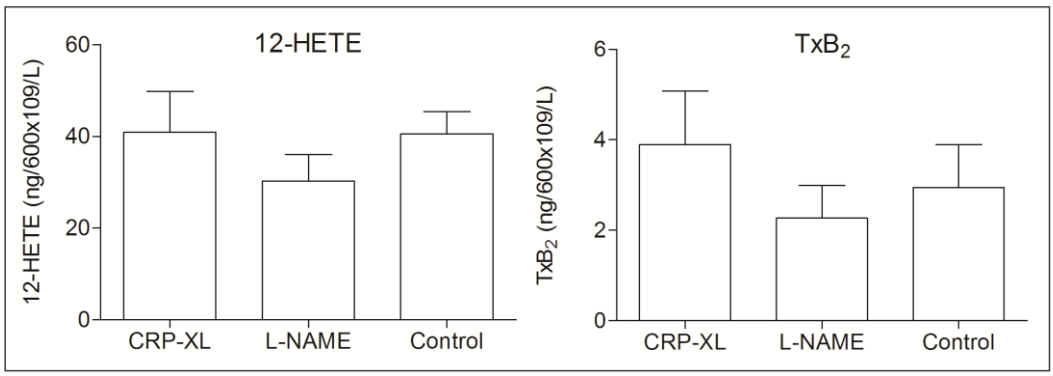



**Fig 6.59. Effect of the L-NAME on CRP-XL induced 12-HETE and $TxB_2$ production.** Either Apocynin or control (1%<water) were incubated 30 minutes before CRP-XL stimulation of washed platelets for 10 minutes at 37°C**,** then supernatant was obtained by centrifugation of washed platelet for 30 minutes at 1800 xg. Data are the mean±SEM n=5; paired student t-test.

### 6.2.6.7. Effect of PI3K inhibition

Since PI3K is an important downstream signalling event in the collagen model (Watson, *et al* 2005), so wortmannin was used to illustrates that all of platelet responses induced by CRP-XL is mediated by GPVI signalling. Wortmannin was found to significantly attenuate both the rate and amount of thrombin generation by both platelets (Fig 6.60a) and PDMPs (Fig 6.60b) in a dose-dependent manner. The inhibitory effect was pronounced for all thrombin generation parameters and greater than any of the other inhibitor tested. In platelets, the LT ($p>0.05$) and ttP ($p<0.005$) were prolonged at the lowest concentrations of the inhibitor (1nM), reaching a plateau at ≥10nM wortmannin. The amount of thrombin generated decreased with increasing concentrations of wortmannin; PT ($p<0.05$) and ETP ($p>0.05$) were markedly reduced. ETP was reduced by wortmannin but did not reach statistical significance ($p>0.05$) by one way ANOVA. Asterisks in Fig 6.60a showed a significant inhibition of ETP compared to CRP-XL stimulation using a paired t-test. With PDMPs, PT ($p=0.0181$) and ETP ($p=0.0087$) were significantly decreased with increasing concentrations of wortmannin. LT and ttP were prolonged and reached a plateau at 1nM and 10nM respectively ($P>0.05$). It is important to mention that wortmannin reduced PT and ETP to a level that was even lower than the amount of PT and ETP obtained in the resting samples for both platelets and PDMPs. Wortmannin (100nM) completely inhibited aggregation with both doses of CRP-XL ($p<0.05$; Fig 6.61). Of all the inhibitors, only wortmannin inhibited P-selectin expression and fibrinogen binding induced by CRP-XL ($p>0.05$; Fig 6.62). Further to this, wortmannin abolished $TxB_2$ and 12-HETE production (Fig 6.63).

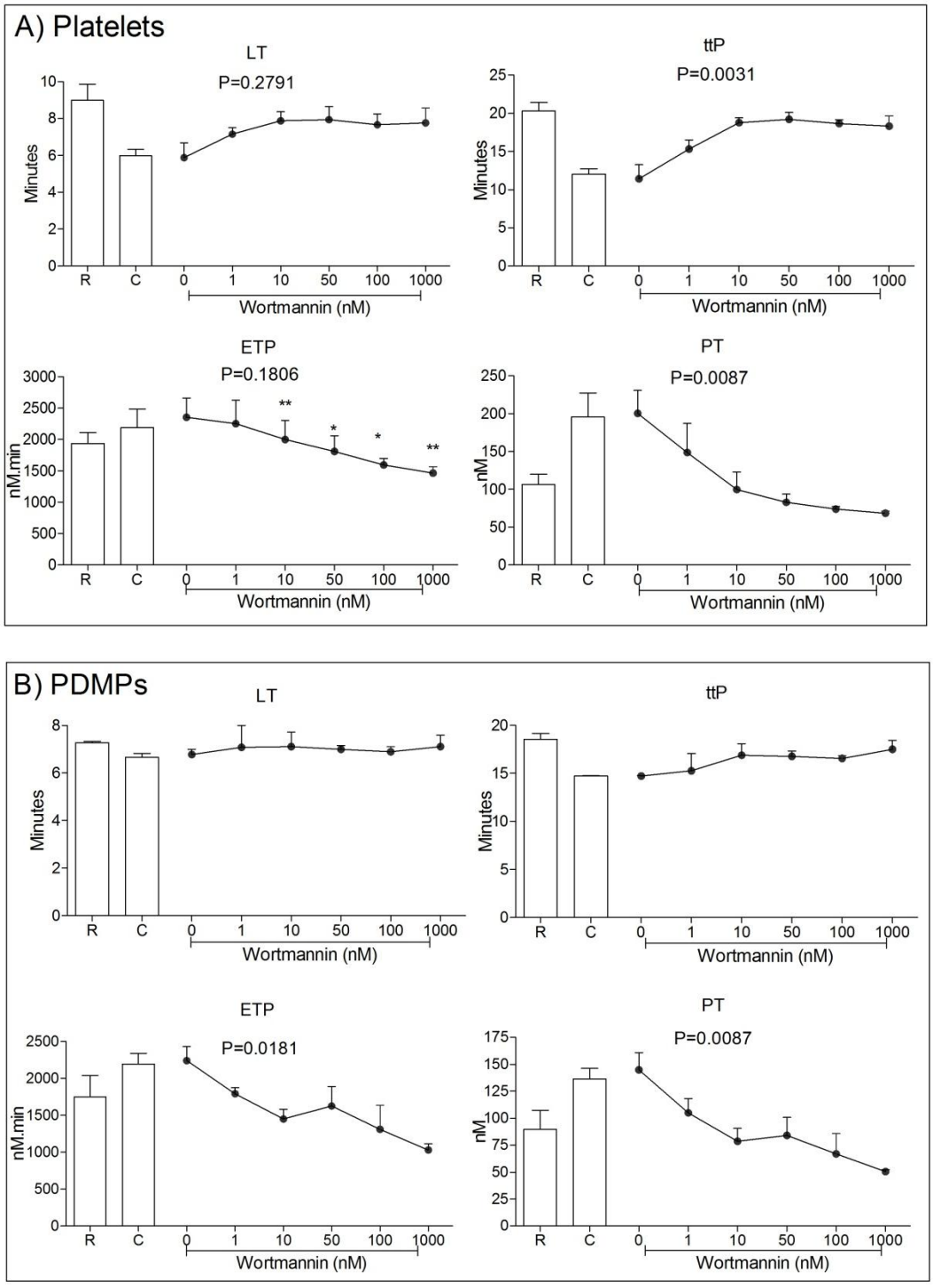



**Fig 6.60: Fig6.24. Effect of PI3K inhibitor, wortmannin, on CRP-XL induced thrombin generation in washed platelets (A) and PDMPs (B).** Washed platelets were pre-incubated for 30 minutes at 37°C with wortmannin followed by 10 minutes stimulation with CRP-XL (2.0 µg/ml) or no agonist (R=resting). Vehicle control (C). PDMP-rich supernatant was obtained from washed platelets by centrifugation at 1800 g for 30 min and analysed in parallel with the washed platelets. Thrombin generation was initiated by the 1pMTF reagent. Data shown are the mean±SEM (n=4-6); one-way ANOVA analysis. Paired student t-test was used to analyse single concentration compared with the CRP-XL stimulated sample alone; * ($p<0.05$).

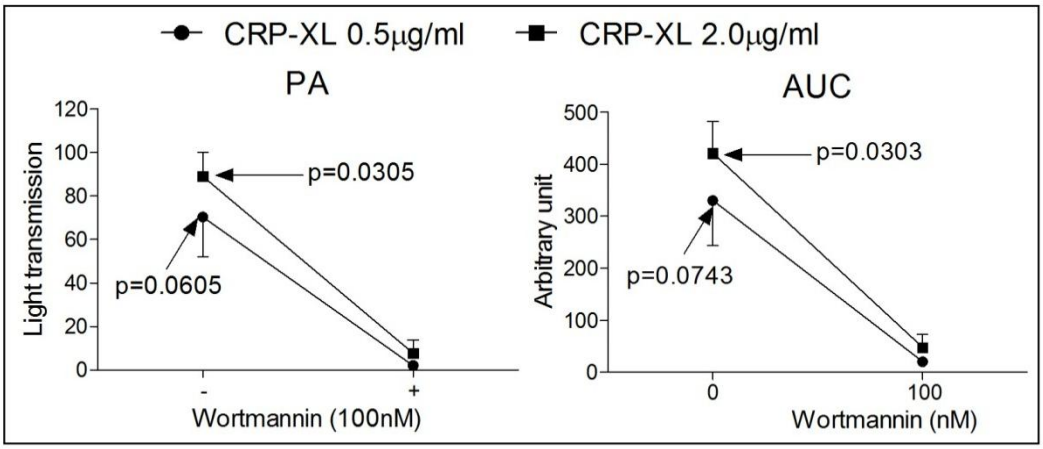



**Fig 6.61. Effect of wortmannin on CRP-XL induced platelet aggregation.** Adjusted PRP ($150x10^9$/L) was pre-incubated for 30 minutes at 37°C with apocynin followed by 10 minutes stimulation with either 0.5μg/ml or 2.0μg/ml CRP-XL or no agonist (R=resting). Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

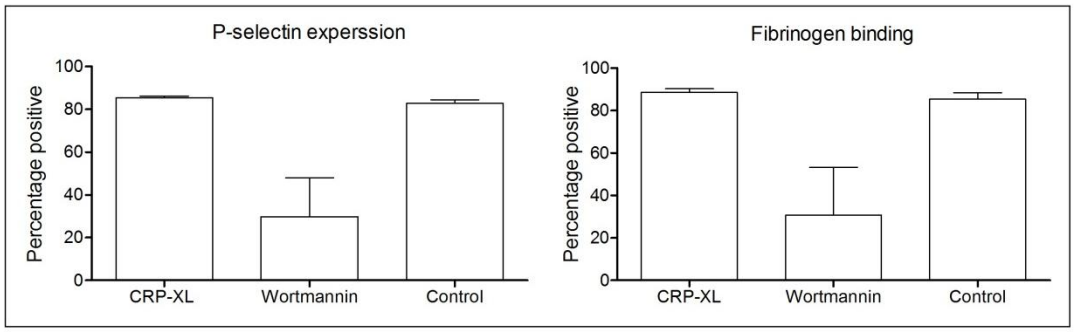



**Fig 6.62: Effect of the wortmannin on CRP-XL induced P-selectin expression and fibrinogen binding in PRP.** Either wortmannin or control (1%<DMSO) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C**.** Data shown are the mean±SEM (n=3); one-way ANOVA analysis.

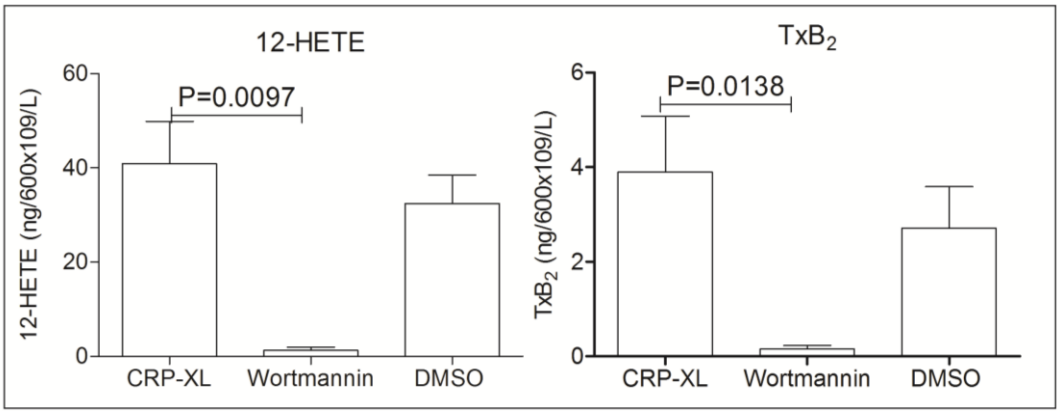



**Fig 6.63: Effect of the wortmannin on CRP-XL induced 12-HETE and $TxB_2$ production.** Either wortmannin or control (1%<DMSO) were incubated 30 minutes before CRP-XL stimulation for 10 minutes at 37°C**,** then supernatant was obtained by centrifugation of washed platelet for 30 minutes at 1800*xg*. Data are the mean±SEM (n=5); paired student t-test.

### 6.2.6.8. Additive effect of the inhibitors

To investigate whether any of the inhibitors had additive effect, apocynin (300µM), tempol (1000µM) and tiron (1000µM) were combined. Consistent with the effects previously observed, apocynin had the significant effect in inhibiting thrombin generation by platelets (Fig 6.64). Neither tiron nor tempol added significantly to the inhibition, as observed with apocynin. Nor did a combination of tiron and tempol induce inhibition, or further inhibit the effect of apocynin. With the PDMPs, apocynin was inhibitory, as previously observed, but the other agents had no effect on their own. However, there was a small additive effect on PT by tiron, but tempol did not increase this effect further.

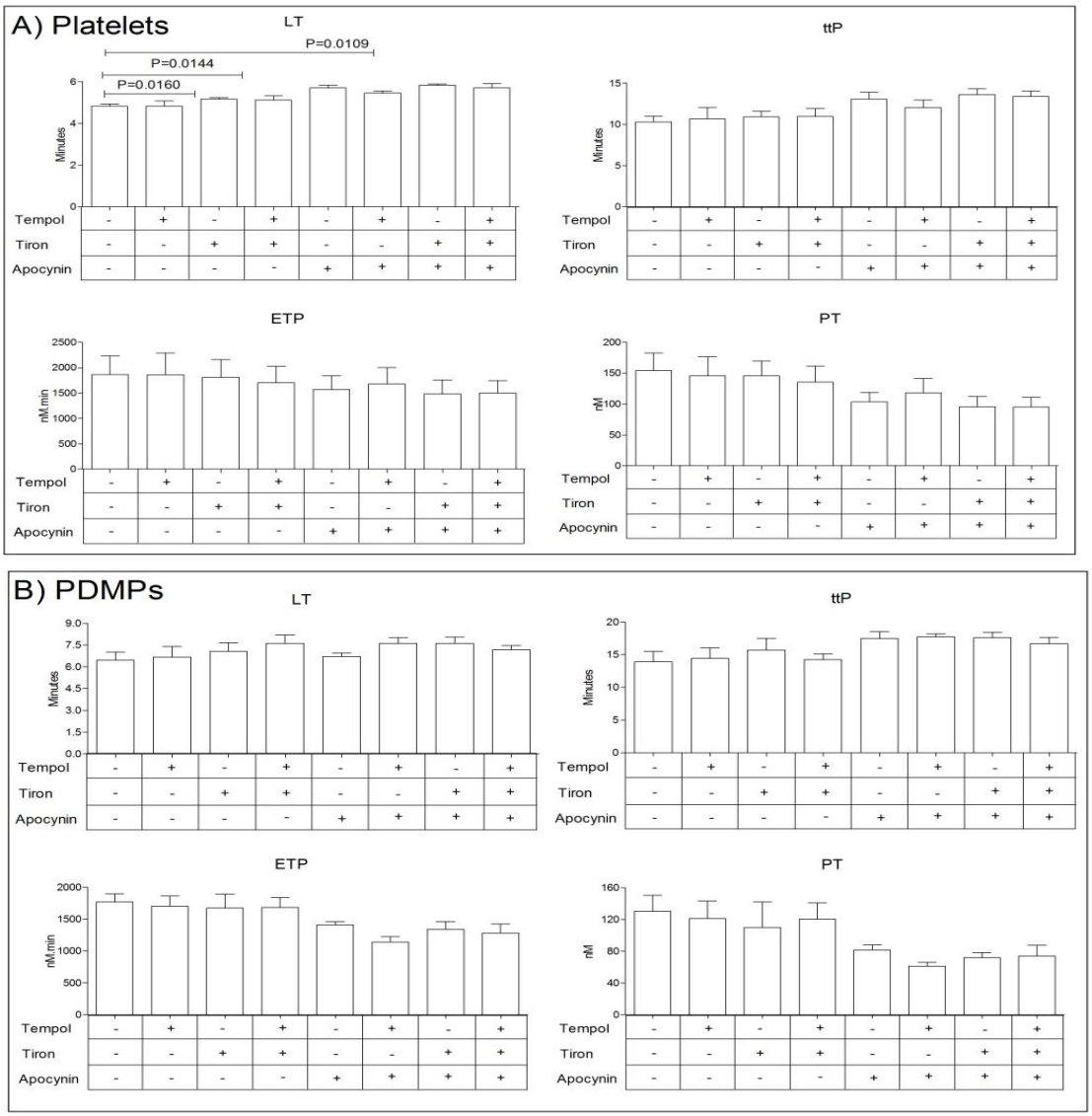



**Fig 6.64: Additive effect of the inhibition of thrombin generation by known ROS inhibitors.** Additive effect of the inhibition of thrombin generation by NAD(P)H oxidase pathway complex in combination with ROS scavengers on thrombin generation by platelets (A, upper panel, n=4) or by PDMPs (B, lower panel, n=3). Data are the mean±SEM; paired t-test.

### 6.2.6.9. Generation of esterified 12-HETE by platelets

There are limited studies on the role of 12-HETE in platelets. Recently, Thomas, *et al* (2009) using LC/MS/MS has shown the incorporation of 12-HETE into membrane PL (mainly PC and PE) following thrombin or collagen stimulation of platelets. Those esterified 12-HETE has been suggested to be thrombogenic (Thomas, *et al* 2009). We, therefore, used the same procedure described by Thomas, *et al* (2009) to analyse CRP-XL activated platelet ($600x10^9$/L) for esterified 12-HETE incorporated into PL. The analysis was carried out in the laboratory of Professor Valerie O'Donnell, (Cardiff University) examining 12-HETE incorporation into PE only. Washed platelets were pelleted by centrifugation at 1,800*xg* for 30 minutes at 37°C and 75% of the supernatant was removed. Pelleted platelets were used for LC/MS/MS analysis. Our preliminary results showed that CRP-XL induced 12-HETE incorporated into PE on the surface of activated platelets at position 16.0p/12-HETE-PE, 18.0p/12-HETE-PE, 18.1p/12-HETE-PE and 18.0a/12-HETE-PE (Fig 6.65). Control vehicles (DMSO) had no effect on esterification of 12-HETE. Apocynin (300µM), wortmannin (100nM) and esculetin (25µM) significantly attenuated the incorporation of 12-HETE into PE (Fig 6.66), which was statistically significant ($p<0.05$ for all) apart from apocynin at 18.1p/12-HETE-PE ($p>0.05$). This is in agreement with effect of these inhibitors on thrombin generation driven by platelets, aggregation and 12-HETE production, as described previously in this chapter. In addition, L-NAME (1mM) attenuated all the forms of 12-HETE incorporation into PE, although this only reached statistical significant with 16.0p/12-HETE-PE (data not shown). Tempol (1mM), tiron (1mM) and aspirin ($10^{-5}$ M) had no effect (data not shown). Conversely BHT (300µM) significantly

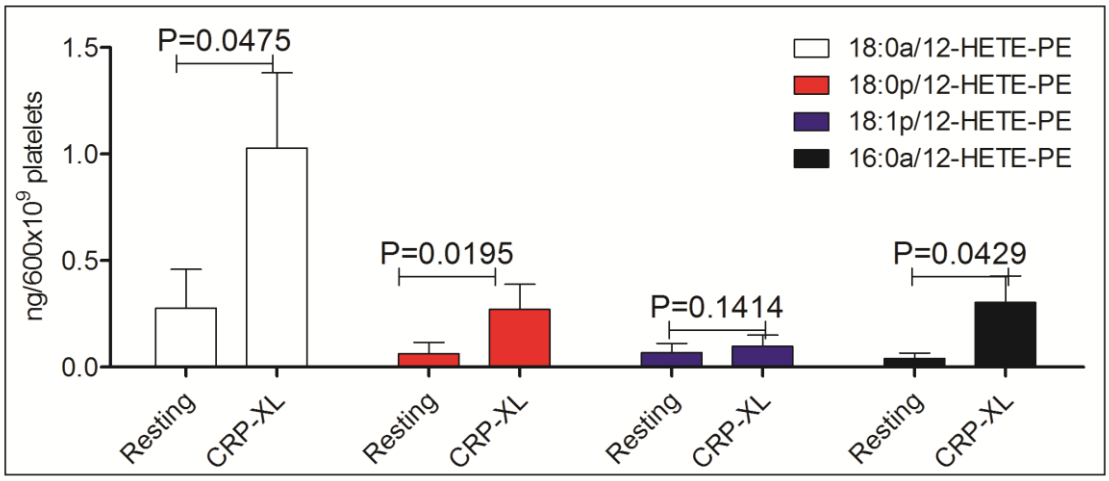



**Fig 6.65: Esterified 12-HETE incorporated into PE upon platelet activation through GPVI.** Washed platelets at ($150x10^9$/L) were stimulated with CRP-XL (2.0µg/ml) for 10 minutes at 37°C. Platelets were pelleted by centrifugation at 1800*xg* for 30 minutes and 75% of the supernatant was removed. Pelleted platelets were used for analysis by LC/MS/MS. Data are shown as mean±SEM (n=3). Paired t-test.

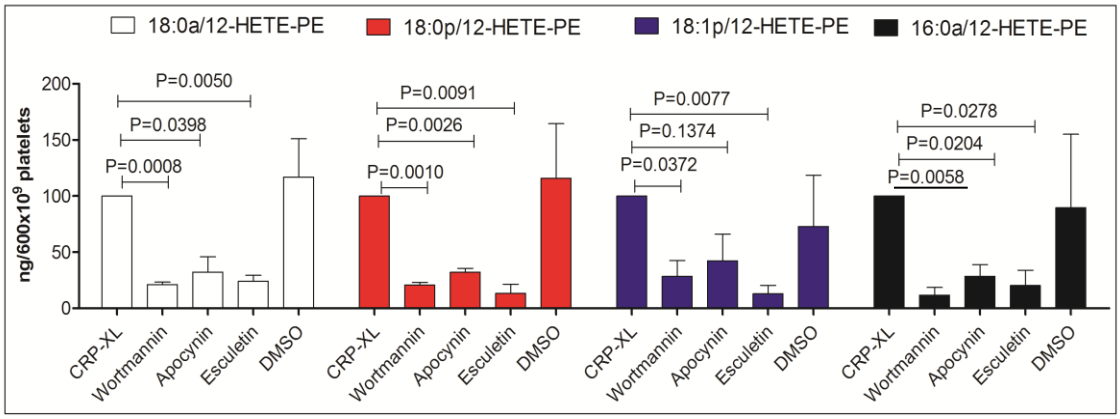



**Fig 6.66: Inhibition of esterified 12-HETE incorporated into PE upon platelet activation through GPVI by (A) wortmannin, (B) apocynin and (C) esculetin.** Washed platelets at ($150x10^9$/L) were incubated with wortmannin (100nM), apocynin (300µM) or esculetin (25µM) and then stimulated with CRP-XL (2.0µg/ml) for 10 minutes at 37°C. Platelets were pelleted by centrifugation at 1800*xg* for 30 minutes and 75% of the supernatant was removed. Pelleted platelets were used for analysis by LC/MS/MS. Data are shown as mean±SEM (n=3), paired t-test.

enhanced the incorporation of 12-HETE into PE in line with its effect on thrombin generation.

### 6.2.6.10. Summary of the results

The current results are summarized in table 6.2, the number of down arrows (↓) indicates the potent of inhibition, while up arrows (↑) indicate enhancement. For example wortmannin showed significant inhibition(↓↓↓) of all platelet responses including PS exposure, thrombin generation driven by platelets and PDMPs, aggregation, fibrinogen binding, P-selectin expression, $TXB_2$, 12-HETE production and 12-HETE incorporation into PE. Significant but less inhibition, compared to wortmannin, was observed by using apocynin (↓↓ ) on PS exposure, thrombin generation driven by platelets and PDMPs, less effect on (↓) on aggregation and no effect (-) on P-selectin expressions and no effect on P-selectin inhibition and fibrinogen binding. Tiron had little or no effect on thrombin generation. On the other hand, BHT enhances (↑) thrombin generation by platelets and 12-HETE production.

| Mode of action | Inhibitor | Platelet TG | PDMP TG | PS exposure | Aggregation | P-selectin expression | Fibrinogen binding | 12-HETE | $TxA_2$ | Esterified 12-HETE |
|---|---|---|---|---|---|---|---|---|---|---|
| NAD(P)H oxidase | Apocynin | ↓↓ | ↓↓ | ↓↓ | ↓ | - | - | ↓↓ | ↓↓ | ↓↓ |
| | DPI | ± | ± | - | NT | NT | NT | NT | NT | NT |
| 12-LOX | Esculetin | ↓ | ± | ↓ | ↓↓↓ | - | - | ↓↓ | ↓↓ | ↓↓↓ |
| COX-1 | Aspirin | - | - | - | NT | - | - | - | ↓↓ | - |
| ROS scavengers | Tempol | ↓↓ | ↓↓ | ↓↓ | ↓↓ | - | - | - | - | - |
| | Tiron | ±↓ | ± | - | - | - | - | - | - | - |
| Antioxidants | BHT | - ↑ | - | - | ↓ | - | - | ↑ | ↓ | ↑ |
| | NAC | - | NT | NT | NT | NT | NT | NT | NT | NT |
| | Trolox | - | NT | NT | NT | NT | NT | NT | NT | NT |
| | Dexa | - | ± | ±↓ | NT | NT | NT | NT | NT | NT |
| NOS | L-NAME | ± | ↓ | NT | ↓ | - | - | - | - | ↓ |
| PI3K | Wortmannin | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ | ↓↓↓ |

**Table 6.2. Summary of the effect of the inhibitors on platelet responses upon GP-VI activation with CRP-XL.** not tested (NT), (↑) indicates enhancement and (↓) indicates inhibition, (–) no effect, (±) a small inhibition. Thrombin generation (TG)

## 6.3. Discussion

The current chapter confirms the participation of ROS in enhancing the procoagulant activity of both platelets and PDMPs in response to CRP-XL stimulation of platelets, and the data suggest an important role for ROS in MP formation, as well as regulating platelet function. In addition, this study suggests that this is mainly mediated by the NAD(P)H oxidase complex pathway and 12-HETE generation with little or no effect of the antioxidants on thrombin generation.

The procoagulant activity of apoptotic cells has been found to be affected by lipid peroxidation linked to ROS (Pickering*, et al* 2008). Furthermore, it was found that A23187 and the Ab against platelet integrin GPIIIa that recognize a.as 49-66 from HIV patients (Nardi*, et al* 2007) involved in MP formation from platelets, which is mediated by ROS generation. So the current study investigated whether lipid peroxidation could be a factor that drives the platelet procoagulant response and if so whether this could be due to the generation of ROS upon physiological agonist, CRP-XL stimulation of platelets. In addition, it investigated the mechanism of driven ROS production in platelets using known ROS inhibitors.

Initially this study compared thrombin generation in PRP and washed platelets. PRP generates more thrombin compared to washed platelets (Fig 6.1) because the washed platelet were diluted 1/2 with filtered autologous plasma (to add back coagulation factors). Therefore, the coagulation factors and platelet count are diluted by half. The effect of platelet count (Chapter 5, Vanschoonbeek *et al* 2004 and Gerotziafas *et al* 2005) and coagulation factors (Duchemin*, et al* 2008) on thrombin generation is well

studied. Duchemin, *et al* (2008) showed that reductions in the levels of prothrombin, FVIII and FIX reduce thrombin generation. However, the data showed that when added back to plasma washed platelets still support thrombin generation, which indicates that thrombin generation can be evaluated in those samples accurately and washed platelets behave like PRP. So the experiments were carried out in washed platelet to avoid the inhibitory effect of plasma antioxidants on the platelets or inhibitors.

The current analysis confirms the generation of ROS upon CRP-XL stimulation compared with A23817 and PMA (Nardi, *et al* 2007). Indeed, collagen (Chlopicki*, et al* 2004), and other agonists including thrombin, TRAP, the $TxA_2$ analog (U46619), convulxin (Begonja*, et al* 2005), 12-HETE (Nardi*, et al* 2007, Seno*, et al* 2001) can also induce ROS generation in platelets. In addition, the current study, although small numbers (n=1-3), confirms the finding that the NADP(H) inhibitors, apocynin and DPI, and dexamethasone, a glucocorticoid drug that has an anti-inflammatory action, attenuated the procoagulant activity of A23187-treated platelets. In line with Karpatkin group findings on the effect of these inhibitors on the MP formation (Nardi, *et al* 2007).

Our data clearly demonstrate that lipid peroxidation induced by exogenous $H_2O_2$ induces significant procoagulant activity in platelets, as shown by enhanced thrombin generation (Fig 6.4), PS exposure (annexin-V binding, Fig 6.5) and lipid peroxidation measured by TBARS (Fig 6.6). This is in agreement with the finding that exogenous ROS induces lipid peroxidation in platelets. Iuliano et al (1994) demonstrated that the stimulation of platelets with exogenous ROS caused lipid peroxidation. Further to this, $H_2O_2$ induces lipid peroxidation in other cells types, such as in cultured endothelial

cells, as measured by C11-BODIPY an oxidative sensitive probe, which was inhibited by vitamin E (van Dam*, et al* 2003). Indeed, $H_2O_2$ has been shown to induce PS oxidation in nucleated cells (Pickering, *et al* 2008), HL-60 cell line (Tyurina*, et al* 2004) and in endothelial cells (van Dam*, et al* 2003), which was inhibited by the use of antioxidants. The procoagulant activity induced by $H_2O_2$ might be mainly dependent on the oxidation of membrane PLs, or may be, due to lysis of the platelets, because peroxidation damages the cell membrane, which would allow access of coagulation factors, or annexin-V to the PS normally present on the inner membrane. The involvement of oxidized PLs in this study was supported by the detection of lipid peroxidation in washed platelets treated with exogenous $H_2O_2$, using the TBARS assay (Fig 6.6), which was completely inhibited by a combination of a ROS scavenger (tiron), 12-LOX inhibitor (esculetin), and NAD(P)H oxidase inhibitors (apocynin and DPI). In addition, numerous studies have shown the effect of exogenous ROS on platelet functions (Pratico*, et al* 1999, Salvemini and Botting 1993) including aggregation (Begonja*, et al* 2005) and $TxB_2$ release, but not on the effect of platelet procoagulant activity. Furthermore, Oxidative stress, ROS, can induce lipid peroxidation in ageing platelets (Okuma*, et al* 1969) and high levels of lipid peroxidation was detected in platelets from haemodialysis patients (Taccone-Gallucci*, et al* 1989), hyperlipidemic patients (Sener*, et al* 2005), and schizophrenic patients (Dietrich-Muszalska*, et al* 2005), compared to normal controls, and in platelets treated with haemoglobin to generate $OH^-$ (Chung*, et al* 2002).

TBARS did not detect lipid peroxidation in CRP-XL-treated platelets (Fig 6.6). The likely explanation for this is that the TBARS assay is insensitive so could not detect the level

of endogenous ROS produced by platelets upon activation CRP-XL, which is likely to be relatively low compared to the high amount of exogenous $H_2O_2$ (0.1 up to 0.33M) used in this study, or compared to that produced by the leukocytes "ROS burst" (Chlopicki*, et al* 2004).

CRP-XL activation of platelets acts in a similar way to collagen through tyrosine phosphorylation (Gibbins*, et al* 1997), and, therefore, the activation of intracellular signalling pathways including PLCγ, PI3K and PLA2 leads to activation of all the responses of platelets, including aggregation, PS exposure and degranulation (Fig 6.67). All of these responses have been observed using CRP-XL in this study, and were associated with ROS production in a dose-dependent manner. PI3K is an early, essential, stage of the GPVI receptor signalling pathway (Gibbins*, et al* 1998), so it was used as a positive control as a known inhibitor of GPVI signalling by CRP-XL. In our study, wortmannin, a potent inhibitor of PI3K, inhibited all aspect of platelet activation including aggregation, P-selectin expression, fibrinogen binding, $TxB_2$ and 12-HETE production, as well as thrombin generation by both platelets and PDMPs (table 6.2). In concordance with previous reports, wortmannin has been shown to inhibit platelet activation by CRP-XL (Coffey*, et al* 2004) and by activation through PAR receptor using TRAP (Clutton*, et al* 2004). Wortmannin also inhibits 12-LOX upon collagen stimulation (Coffey*, et al* 2004) and $O^{-2}$ production in platelets stimulated by thrombin (Zielinski*, et al* 2001).

In the current data, the inhibition of the PI3K using wortmannin on the procoagulant activity measured using the PT in platelets and PDMPs, was even lower than in the unstimulated platelets and their supernatants, which might suggest that platelets at a

basal, resting, level generate ROS. In addition, this study demonstrates that ROS generation from CRP-XL-stimulated platelets was inhibited by apocynin (n=1) to a level that was lower than seen in unstimulated platelets. This supports the observation, that platelets at a basal level do generate ROS (Marcus*, et al* 1977). The main enzymatic pathway responsible for ROS generation in platelets is not fully determined, but most studies suggest the NAD(P)H oxidase pathway upon stimulation with physiological agonists or A23187 (Chlopicki*, et al* 2004, Krotz*, et al* 2002, Nardi*, et al* 2007, Seno*, et al* 2001). The main activator of NAD(P)H oxidase complex pathway is suggested to be PKC, which is a downstream signalling pathway of PI3K. Indeed, the PI3K inhibitor, LY294002, prevents the translocation of the cytosolic $p67^{phox}$ subunit of the NAD(P)H oxidase complex from coupling with the cellular membrane subunits of the NAD(P)H oxidase upon TRAP activation (Clutton*, et al* 2004).

The data in this chapter shows a clear inhibition, by apocynin but not DPI; two structurally-unrelated inhibitors of the NAD(P)H oxidase complex, on the procoagulant activity of platelets and PDMP measured by thrombin generation and PS exposure using annexin-V binding induced by CRP-XL. Apocynin also inhibited aggregation, suppression of 12-HETE generation and $TxB_2$ production, but had no effect on fibrinogen binding and P-selectin expression. These findings are in agreement with the finding of inhibition of collagen-induced aggregation and $TxB_2$ production by apocynin reported by Chlopicki *et al,* (2004). In addition, apocynin and DPI reduced aggregation and thrombus formation on collagen coated surface under high stress (Begonja*, et al* 2005). Indeed, apocynin has been shown to inhibit aggregation, but not the alpha and dense granule secretion induced by thrombin (Begonja*, et al* 2005). While our data

contradict the finding reported by Begonja, *et al* (2005) that apocynin inhibits integrin αIIbβ3 activation assessed by active form of αIIbβ3, PAC-1, as in our study apocynin (300μM) did not affect fibrinogen binding assessed by flow cytometry. This might be due to the differences in apocynin concentration (300μM) used in our study compared to their concentration (600μM) or may be due to the agonist used. Our data suggest that apocynin affect the inside out-signalling induced by aggregation rather than the signalling pathway of integrin αIIbβ3 activation, but why PAC-1 is inhibited in Begonja, *et al* (2005) study may be specific to thrombin.

The mechanism of action of apocynin and DPI is different, so this might explain why apocynin had more effect than DPI. Apocynin is a specific inhibitor of the NAD(P)H oxidase complex, the translocation of the 47phox to the plasma membrane, thereby blocking the assembly of a functional NAD(P)H oxidase complex. On the other hand, DPI is a flavoprotein-dependent inhibitor of the NAD(P)H oxidase complex. It has been shown that DPI inhibits ADP and thrombin- induced platelet aggregation (Salvemini*, et al* 1991) and ROS production mediated by the NAD(P)H oxidase complex in platelets (Seno*, et al* 2001). Furthermore, the data in the present study suggest that in the presence of CRP-XL, the NAD(P)H in platelets might be working in a flavoprotein-independent manner, as it is inhibited by apocynin but not by DPI. However, both inhibitors been shown to inhibit ROS production by platelets induced by collagen (Chlopicki*, et al* 2004). These results also suggest that the NAD(P)H oxidase pathway activation is upstream of PS exposure, PDMP formation and release of AA pathway; this is contrary to the proposal by Nardi *et al* (2001 and 2007) that the NAD(P)H oxidase pathway responsible for ROS generation is downstream of 12-LOX. Lastly the

data might point that NADP(H) is acting in the CRP-XL induced PS exposure via a pathway that is not dependent on ROS. The possible explanation for this is that NAD(P)H is a downstream signalling pathway for GPVI; the main inducer of PS exposure in platelets, which, the NAD(P)H complex is associated with the cell membrane PLs, and this is regulated by the migration of Rac1 and other NAD(P)H subunits in cytoplasm to the cell membrane (Cheng*, et al* 2006), which might be linked to PS exposure.

Nardi et al (2007) have shown that PDMP formation upon platelet stimulation by A23187, PMA or Abs against platelet integrin GPIIIa49-66 is mediated by ROS production via the NAD(P)H oxidase pathway, since it is inhibited by the NAD(P)H oxidase pathway inhibitor DPI, by 12-LOX inhibitors, and by dexamethasone. ROS production in PMA-induced platelets was confirmed by Sanner et al (2002), which inhibited by dexamethasone. The current study used similar doses of dexamethasone as those used by Nardi *et al* (2007), which are within the therapeutic range. However, dexamethasone had no significant effect on platelets upon CRP-XL stimulation, as measured by thrombin, even using similar concentrations of dexamethasone to those that have been shown to inhibit PDMP formation and ROS generation by Nardi *et al* (2007) and Sanner *et al,* (2002); although, dexamethasone did slightly reduce PDMP-mediated thrombin generation and PS exposure ($p>0.05$). This suggests that dexamethasone has an effect on MP formation, but has little or no effect on the procoagulant activity provided by platelets. This finding of the effect of dexamethasone on PDMP to some extent is in line with finding of Nardi, *et al* (2007), but needs more investigation. However, these initial results might suggest a similar

pathway for platelets stimulated with CRP-XL, A23187, PMA and GPIIIa49-66-mediated MP formation and PS exposure. We confirmed the effect of dexamethasone and DPI on PS exposure induced by A23187, in our study, with similar to the findings to these of Nardi *et al* (2007), as discussed previously.

AA is another source of ROS production in platelets and ROS can also activate AA release via PLA2 (Xu*, et al* 2003). The current study showed that CRP-XL activates the AA pathway measured by the release of $TxB_2$ and 12-HETE via COX-1 and 12-LOX respectively. In addition esculetin an inhibitor of 12-LOX but not aspirin, an inhibitor of COX-1, had an effect on platelet procoagulant activity measured by thrombin generation and annexin-V binding. In addition in the current analysis esculetin inhibited 12-HETE production and aggregation, but aspirin inhibited $TxB_2$ only. This was confirmed by the inhibition of 12-HETE incorporation into PE by esculetin but not with aspirin. The analysis showed that COX-1 does not play a role in driving the procoagulant activity upon CRP-XL activation, but does 12-LOX. However, these results must be viewed with caution because of the non-specificity of those 12-LOX inhibitors to 12-LOX. Although, esculetin has been reported to selectively inhibit 12-HETE production (Sekiya and Okuda 1982b, Sekiya*, et al* 1982a), but it has been proposed as antioxidant as well.

Also, it has been suggested that the NAD(P)H oxidase pathway could be activated by the AA pathway in platelets using COX (Caccese*, et al* 2000) and 12-LOX (Nardi*, et al* 2007), while others have suggested that the NAD(P)H oxidase complex could be involved in the activation of PLA2 and COX pathways in other cell types (Lowe*, et al* 1998, Novalija*, et al* 2003, Pricci*, et al* 2003), the latter suggestion is in agreement with

our findings that NADP(H) oxidase pathway is an upstream of AA pathway, because apocynin inhibited 12-HETE and $TxB_2$ production and aggregation, as well as the incorporation of 12-HETE into PE.

Aspirin is an anti-platelet drug that has been used for long time to block $TxA_2$ synthesis via the inhibition of COX-1. Aspirin had no effect on the procoagulant activity of platelet or PDMPs measured by thrombin generation, but did inhibit $TxB_2$ production and slightly enhanced 12-HETE generation. In addition, aspirin had no effect on PS exposure, as assessed by annexin-V binding in PRP (data not shown). Barry *et al* (1997) have also shown that the COX inhibitor indomethacin had no effect on MP formation upon CRP-XL or A23187 stimulation of platelets, but significantly inhibited TxA2 (the end product of COX-1 pathway) production upon collagen stimulation, as reported by Chlopicki *et al* (2004). Although aspirin has no effect on thrombin generation, it has been found to inhibit ROS production upon thrombin stimulation (Begonja*, et al* 2005), which has different signalling pathway from CRP-XL.

The involvement of ROS generation in the GPVI signalling pathway was confirmed by flow cytometry and by the significant attenuation of thrombin generation in platelets and PDMPs using a general ROS scavenger, tempol. It is therefore necessary to determine if $O^{\bullet}_2^-$ is the form specifically responsible for this effect, as $O^{\bullet}_2^-$ is produced from the NAD(P)H oxidase pathway. Tiron, an $O^{\bullet}_2^-$ specific scavenger, had only a small effect at low concentrations (<1000µM), although it significantly inhibit thrombin generation by platelets and PDMPs at high doses — 1000µM and 3000µM. However, tempol nor tiron had any effect on P-selectin expression, fibrinogen binding 12-HETE generation and TxB2 production; this is in agreement with previous observations

(Begonja*, et al* 2005), who found that tiron at 3000µM had no effect on P-selectin expression and serotonin secretion induced by thrombin stimulation, but did inhibit ROS production (Begonja*, et al* 2005). In addition, the current study showed that only tempol inhibited aggregation but not tiron.

$O^{\bullet}_{2}{}^{-}$ is unstable and can be rapidly converted by either superoxide dismutase (SOD) to $H_2O_2$ or by NO into $ONOO^-$ (Chakrabarti*, et al* 2004, Clutton*, et al* 2004) showing more reactivity with NO. It is known that platelets are rich in antioxidant enzymes such as SOD, catalase, glutathione peroxidase (GPx), and also produce NO upon agonist activation. The inhibition of NO synthesis might reduce the conversion of $O^{\bullet}_{2}{}^{-}$into $ONOO^-$, and therefore attenuate the effect on the procoagulant activity of platelets and PDMPs mediated by $ONOO^-$. However, NO also regulates platelet function; indeed NO signalling is a potent inhibitor of all aspects of platelet activation through elevation of cGMP. Therefore, a reduction in the NO level should enhance the platelets responses to agonists inducing their procoagulant activity. NO is mainly produced by endothelial cells (Broekman*, et al* 1991, de Graaf*, et al* 1992), and inhibits platelet adhesion to the vessel wall and aggregation. It has been found that NO inhibits the platelet activation associated with P-selectin expression and the activation of GP IIb-IIIa. Platelets and megakaryocytes have been found to contain constitutive NOS (cNOS) (Sase and Michel 1995). Platelets have the ability to produce NO upon activation (Chen and Mehta 1996, Freedman*, et al* 1997) that is comparable to that observed in endothelial cells (Zhou*, et al* 1995), which can be inhibited by an NOS inhibitor (Chen and Mehta 1996). Treatment of platelets with L-NAME, the inhibitor of NOS, before activation should reduce NO production according to Freedman *et al* (1997), who

observed a significant reduction in NO production by L-NAME in ADP-treated platelets. In the current study L-NAME inhibited thrombin generation induced by CRP-XL by platelets at high doses and had a slight dose-dependent inhibition of thrombin generation by PDMPs. This inhibition was paralleled by a dose-dependent inhibition of CRP-XL-induced aggregation by L-NAME, but there was no effect on P-selectin expression and fibrinogen binding. This conflict with the accepted view that L-NAME enhances ADP, inducing aggregation and release of serotonin, by reducing cyclic GMP in platelets (Freedman*, et al* 1997) and P-selectin expression (Freedman*, et al* 1997, Murohara*, et al* 1995). This anomalous finding cannot be explained. The inhibition at high concentration might be due to a toxic effect of L-NAME on platelets, but if the dose was sufficient to kill platelets, this should increase PS exposure. The highest concentration of L-NAME used in this study has been used previously by others to show an inhibition of NO, so why does inhibition of NO reduce thrombin generation? This might suggest that NO in our CRP-XL treated platelets in our condition might induce $O^{\bullet}_{2}{}^{-}$ production, because if NOS is uncoupled from its essential cofactors, such as tetrahydrobiopterin (BH4) which acts as a source of $O^{\bullet}_{2}{}^{-}$ and has been observed in endothelial cells (Landmesser*, et al* 2003). The effect of L-NAME in this study needs further investigation.

Antioxidant systems exist in all cells to protect cells from the damage of free radicals, which are seen as endogenous antioxidants (for review see (Rahman 2007). These can be enzymatic, such as SOD, catalase and GPx, (Mates*, et al* 1999) and non-enzymatic, such as vitamin E, C and thiol antioxidant systems (McCall and Frei 1999). Furthermore, exogenous antioxidants can enhance antioxidant capacity, and these can

be supplied from the diet, including flavonoids (Rice-Evans 2001). Therefore, the effect of non-enzymatic exogenous antioxidants was investigated on thrombin generation, and BHT was used to investigate the effect of other platelet responses, including degranulation, secretion and fibrinogen binding. In this study, none of the exogenous antioxidants had any effect of inhibiting CRP-XL induced platelet thrombin generation, apart from modest attenuation of PDMP-mediated thrombin generation and inhibition of CRP-XL induced platelet aggregation by BHT. In fact the highest concentration use BHT enhanced thrombin generation by platelets to a high level and significantly increased 12-HETE production, but had no effect on $TxB_2$. A similar effect was reported with 15-LOX. (Schnurr*, et al* 1995) In addition, BHT has been shown to attenuate aggregation induced by collagen in rat platelets, in concordance with our results (Takahashi 1991). Each antioxidant used in this study has a different mechanism of action; for example, vitamin E is the most important antioxidant in the cell membrane, it is the only lipid-soluble chain-breaking antioxidant and it protects cell membranes from lipid peroxidation (Burton*, et al* 1982). The mechanisms of action of BHT and NAC will be discussed in the next chapter; however, the data suggest no involvement of lipid peroxidation with platelet activation upon CRP-XL stimulation.

GPVI activation by collagen is associated with ROS production, which could generate oxidized PLs and has shown to be associated with 12-HETE-PE generation (Maskrey, *et al* 2007). Our preliminary data showed significant production of esterified 12-HETE incorporated into PE upon CRP-XL stimulation, and this generation was significantly attenuated by apocynin, esculetin and wortmannin. Recently, Thomas, *et al* (2009), showed that those esterified 12-HETE-PE incorporated into PE or PC remain in the

membrane. In addition esterified 12-HETE-PE and 12-HETE-PC have been shown to be translocated into the external membrane layer, and both esterified 12-HETE-PE and 12-HETE-PC were able to support thrombin generation. In agreement with our results the 12-LOX inhibitor esculetin has shown to inhibit 12-HETE PE. The current data might illustrate the important of 12-HETE in regulation of platelet procoagulant activity.

The proposed model (Fig 6.67) added to the current knowledge in the GPVI signalling pathways, which shows that there are at least three sources of the anionic PL, (i) PS transduction from the inner leaflet of the membrane by scramblase- driven directly by the increase in $[Ca^{2+}]i$ (Suzuki*, et al* 2010) and cleavage of calpain. (ii) This could be due to esterification by 12-HETE, which might be activated through PLA2 dependent of the NADP(H) oxidase complex pathway activation (iii) ROS causing lipid peroxidation driven by mainly the NADP(H) oxidase complex pathway. In addition, the inhibition of aggregation by 12-LOX inhibitor, NADP(H) oxidase complex inhibitor or ROS scavenger, tempol, NOS inhibitor or BHT is not due to the inhibition of integrin activation but due to the outside-in signalling.

It should be emphasised that the doses of the inhibitors used in this study were selected from the literature, were they have not showed any toxicity unless used in a very high concentrations. In addition, it should be considered that inhibitors are not ideal and sometimes they lack the selectively and not specific and knockout mice should be considered for future work. In conclusion, thrombin generation is enhanced after CRP-XL activation, which acts mainly through GPVI to drive the procoagulant response in platelets and in forming PDMPs. This involves ROS generation through the

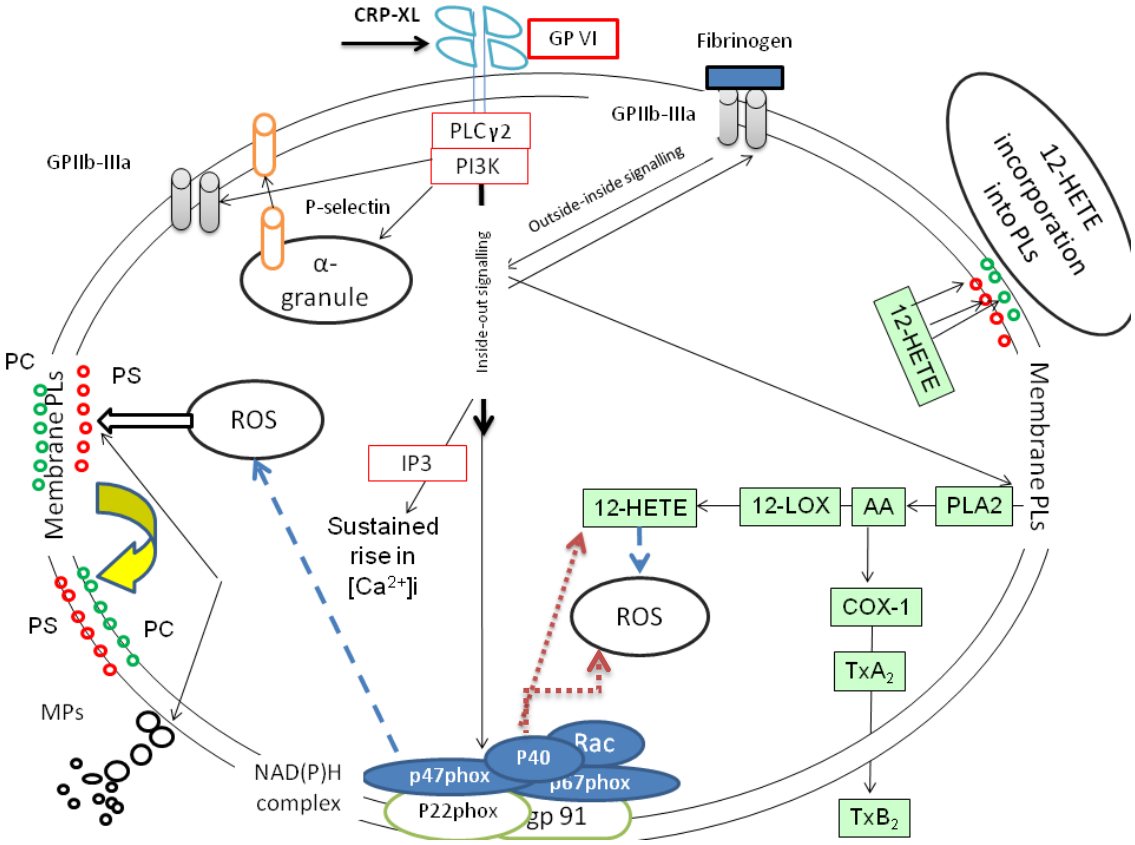



**Fig 6.67: Proposed pathways of ROS generation induced by CRP-XL activation.** Engagement of GPVI with CRP-XL activates inside-out signalling, leading to activation of PI3K which contributes to the activation of all aspects of platelet signalling pathways, including IP3, leading to a sustained increase in $[Ca^{2+}]$; activation of PLA2 leading to 12-HETE and TxA2 production by 12-LOX and COX-1 respectively; activation of the NAD(P)H oxidase, alpha granules, GPIIb-IIIa activation and fibrinogen binding, PS exposure, MP formation and ROS generation; integrin activation leads to the activation of outside-in signalling, leading to an amplification of the activation responses. In this study, the potential sources of ROS are inhibited upon CRP-XL activation.

NAD(P)H oxidase pathway. The addition of a specific NAD(P)H oxidase inhibitor, apocynin or a general ROS scavenger such as tempol markedly reduced the increase in thrombin generation and the percentage of PS exposure after CRP-XL stimulation, but no effect was observed for antioxidants on thrombin generation. These data suggest that oxidized PL, but not lipid peroxidation, enhanced the generation of the platelet procoagulant activity and thrombin formation in platelets upon GPVI stimulation.

## 6.4. Further work

- More work needed on ROS measurement in the platelets using specific probe for $O^{\bullet}_{2}{}^{-}$ such as lucigenin , $H_2O_2$ such as Amplex red, etc. to confirm the exact type of ROS produced upon CRP-XL stimulation and also to confirm the inhibition of ROS using the inhibitors used in this study using plate reader.
- Direct measurement of lipid peroxidation using a more sensitive and specific method than TBARS such as LC/MS/MS.
- Clarify the anomalous finding with L-NAME-use other inhibitors and inducers of NO. Then measurement of aggregation, cGMP level and protein phosphorylation.
- Problem with the inhibitors (specificity) therefore use KO mice, to elucidate the role of NAD(P)H pathway and 12-LOX in driven the procoagulant response.
- Investigate whether the modest, but significant effect of NAD(P)H/12-LOX/ROS on the platelet procoagulant response is a cause of inter-individual variation over and above known effect of the genetic variation and measuring antioxidant capacity using commercial kits

# Chapter 7: Involvement of lipid peroxidation in the procoagulant activity of apoptotic platelets

## 7.1 Introduction

The lifespan of platelets in circulation is approximately 10 days (Harker 1978), after which they undergo programmed cell death through apoptosis. The exact signal for platelet clearance by the RE system is not fully understood, but dying platelets, like other apoptotic cells, express PS on their surfaces (Bevers*, et al* 1998), and this has been identified as being involved in clearance of cells *in vitro* (Fadok*, et al* 1992a, Fadok*, et al* 1992b, Gardai*, et al* 2006, Savill*, et al* 1993) and *in vivo* (Rand*, et al* 2004). Stored platelets undergoing apoptosis have the well-characterized features of nucleated cell apoptosis, including PS exposure (Pereira*, et al* 2002, Rand*, et al* 2004), loss of ΔΨm (Perrotta*, et al* 2003, Verhoeven*, et al* 2005), cytochrome C release, activation of caspases and gelosin cleavage (Li*, et al* 2000). In addition, some of these features of apoptosis have also been reported in platelets undergoing activation using A23187 or thrombin or a combination of thrombin/convulxin including cytochrome C release, activation of caspases and gelosin cleavage (Leytin*, et al* 2006, Remenyi*, et al* 2005, Rosado*, et al* 2006, Shcherbina and Remold-O'Donnell 1999, Tonon*, et al* 2002) and loss of ΔΨm (Leytin*, et al* 2009). From the these contradicting reports of the involvement of cytochrome C, caspase activation and loss of ΔΨm in both processes, the mechanism of both platelet apoptosis and activation in platelets is still controversial and poorly understood (Lopez*, et al* 2008). Recently, it has been found

that PS exposure in both processes is tightly regulated by two distinct pathways (Schoenwaelder*, et al* 2011, Schoenwaelder*, et al* 2009, Vogler*, et al* 2011).

The exact process for triggering platelet apoptosis is unclear, but it has been suggested that it is associated with the deterioration in Bcl-2 family proteins (Pereira*, et al* 2002). Platelets contain and express apoptotic regulators such as caspase proteins and Bcl-2 family proteins, including the pro-apoptotic Bax and Bak and the anti-apoptotic Bcl-xL protein which both play a key role in platelet survival (Zhang*, et al* 2007), indeed, as Bcl-xL diminishes, Bak induces apoptosis. The pro- and anti-apoptotic proteins need to be in balance to maintain platelet viability. Bak activation has been reported to be important in platelet apoptosis, and this process can be inhibited by BCL-xL (Mason*, et al* 2007). Bax and Bak have been shown to play a role in PS exposure in platelets (Bertino*, et al* 2003, Mason*, et al* 2007, Zhang*, et al* 2007). Indeed, *in vivo* experiments show platelets live longer in Bak-deficient mice than in normal mice (Mason*, et al* 2007). A BH3 mimetic inhibitor of Bcl-2 and Bcl-xL, ABT-737 (and its analogue ABT-263) has recently been developed for the treatment of tumour cells (Oltersdorf*, et al* 2005). It is in early clinical trials and the major side effect of this drug is thrombocytopenia (Roberts*, et al* 2008, Zhang*, et al* 2007). ABT-737 (and its analogue ABT-263) provides an excellent model for studying platelet apoptosis derived through the intracellular apoptotic pathway induced by the mitochondrial pathway (Zhang*, et al* 2007).

The well-studied hallmark of both platelet activation and apoptosis is PS exposure, which enhances thrombin generation. It has also been shown previously that lipid peroxidation can occur during apoptosis. Pickering et al (2008) and others (Wang*, et al*

2001) have demonstrated the involvement of lipid peroxidation in enhancing thrombin generation in nucleated cells undergoing apoptosis, which could be completely inhibited by the antioxidant, BHT (Pickering*, et al* 2008). Platelets are also susceptible to lipid peroxidation (Knight*, et al* 1994, Vlachakis and Aledort 1979), which raises the question of whether lipid peroxidation in platelets can enhance thrombin generation. This chapter compares the effect of the BH3 antagonists (ABT-737 and ABT-263) of Bcl-2/BCL-xL on inducing a procoagulant surface and the activation of platelets with physiological agonists (CRP-XL and TRAP) and a non-physiological agonist (A23187), and the involvement of lipid peroxidation in thrombin generation via these different pathways.

## 7.2 Results

In this study, washed platelets ($50x10^9$/L) were pre-incubated with either ABT-737 or ABT-263 for two hours at 37°C, which had been previously shown to be the time required for apoptosis to occur (Vogler*, et al* 2011) unless otherwise stated. They were then either analysed immediately in the test of interest or stimulated with agonists for 10 minutes at 37°C, Washed platelets were mixed with autologous filtered plasma to provide coagulation factors for the thrombin generation assay. Thrombin generation was measured using the 1pMTF reagent because this reagent is sensitive to the level of the procoagulant surface in the sample.

## 7.2.1. Apoptosis enhances thrombin generation in platelets

### 7.2.1.1. ABT-737 triggers thrombin generation is time and dose-dependent

A preliminary experiment investigated the time course and dose response of the effect of ABT-737 and ABT-263 on platelet procoagulant activity as measured by thrombin generation. Washed platelets were pre-incubated with increasing concentrations of either ABT-737 or with ABT-263 for 10, 30, 60 and 120 minutes. The platelets showed time and dose-dependent increases in thrombin generation (Fig 7.1 shows the representative curves and Fig 7.2 shows the numerical data; n=1). At 10 and 30 minutes, no effect was observed on thrombin generation at any concentrations of either ABT-737 or ABT-263. After 60 minutes, ABT-737 and ABT-263 enhanced thrombin generation at the highest concentration (1.0μM), while after 120 minutes thrombin generation was further enhanced by both compounds. The effects of the drugs were seen on all parameters of thrombin generation. On the basis of these findings, two hours was selected as the best time to induce platelet procoagulant activity. The same dose has been reported for annexin-V binding (Vogler*, et al* 2011). These results indicate that the ABT-737 inducing PS exposure is slow, and dose-dependent.

Then a wider range of concentrations of ABT-737 drug in three donors was used to confirm the effect seen in the preliminary data in Fig 7.2. Washed platelets were pre-incubated with increasing concentrations of ABT-737 (0-100μM) for two. Representative curves from one experiment (Fig 7.3a) illustrate that, as the concentration of ABT-737 increased the thrombin generation curves increased in height and shifted to the left, indicating a faster rate and greater amount of thrombin

generation. The numerical data from the three experiments is shown in Figure 7.3b; which shows a significant increase in ETP ($p=0.0017$) and PT ($p<0.0001$) with increasing concentrations of ABT-737, reaching a plateau at 0.1µM. The LT ($p=0.1356$) and ttP ($p=0.0218$) became slightly shorter, also reaching the maximum effect at 0.1µM. It is worth mentioning that PT and ETP were affected more than LT and ttP.

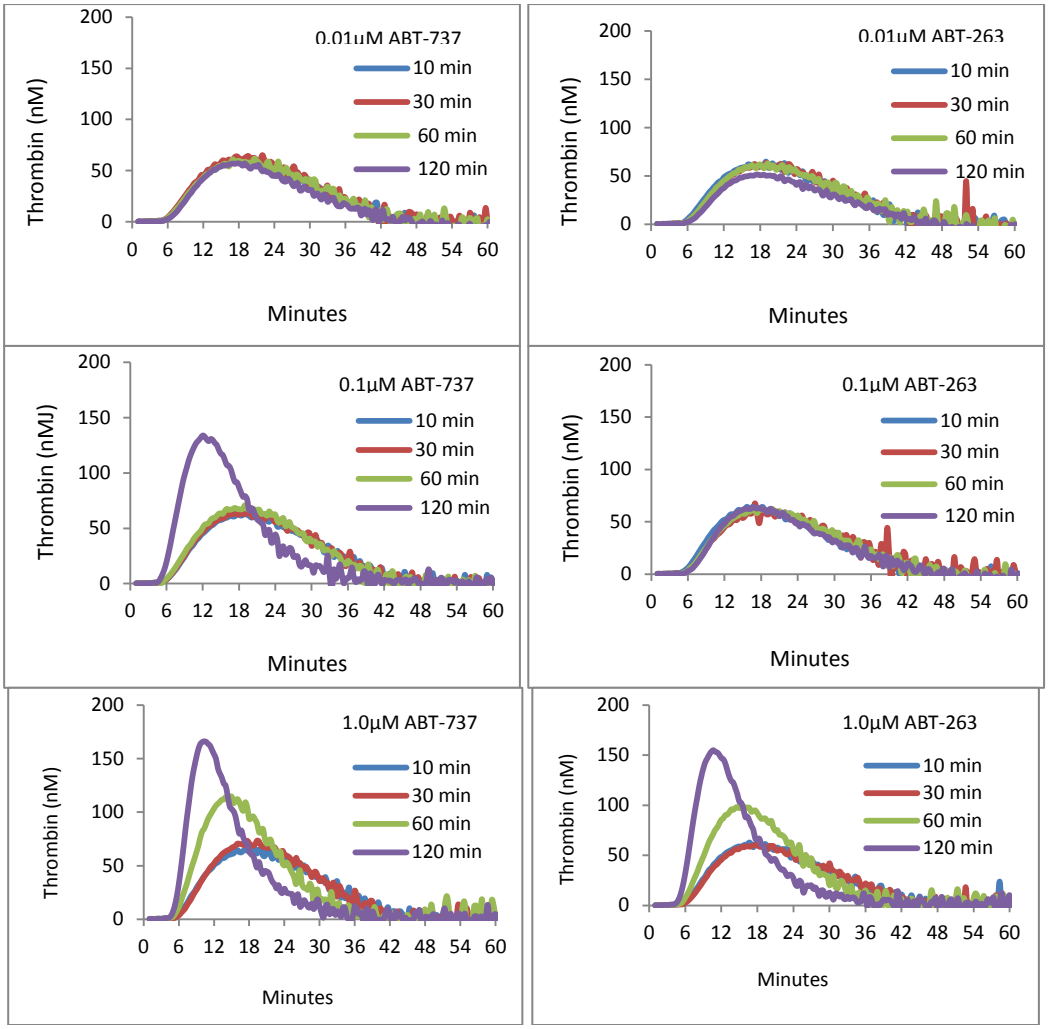



**Fig 7.1: Representative thrombin generation curves of platelets treated with ABT-737 (left figures) or with ABT-263 (right figures) up to 2 hours.** Washed platelets were pre-incubated with increasing concentrations (0.01, 0.1 and 1.0µM) of either ABT-737 or with ABT-263 for 10 minutes (blue line), 30 minutes (red line), 1 hour (green line) and 2 hours at 37°C. Thrombin generation was triggered with 1pM TF. Data shown are from one experiment.

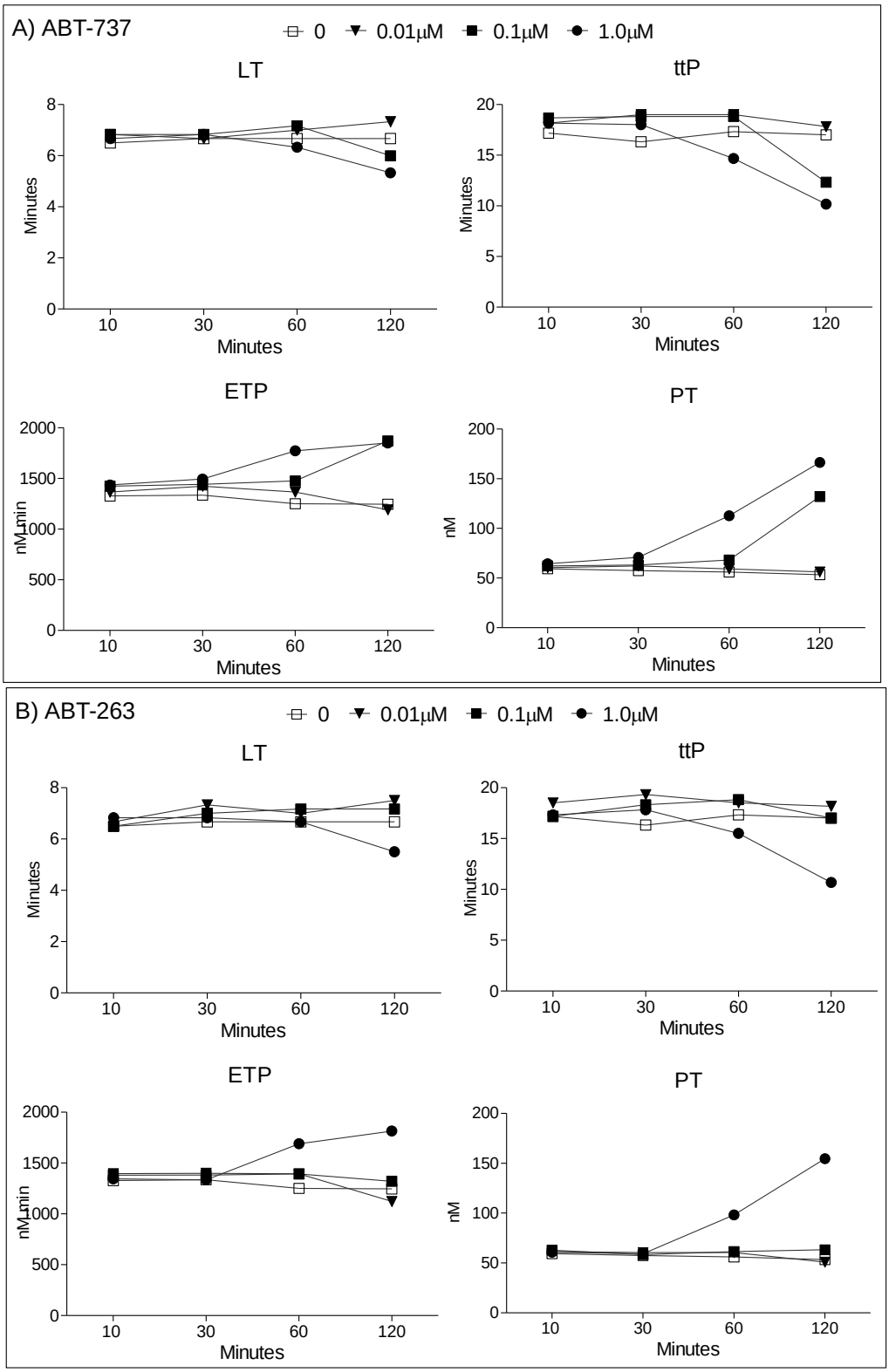
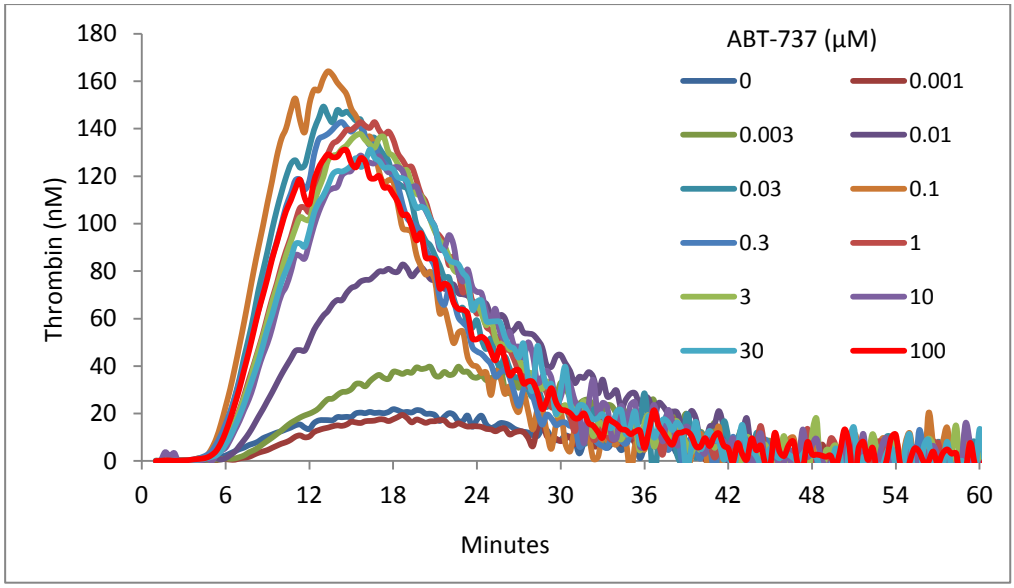



**Fig 7.2: ABT-737 and ABT-263 induce platelet thrombin generation in time and dose-dependent manner.** Washed platelets were incubated with different concentrations of ABT-737 (upper panel) or ABT-263 (lower panel) 0 (□), 0.01μM (▼), 0.1μM (■) and 1.0μM (●) up 2 hours at 37°C. Thrombin generation was triggered with the 1pMTF reagent. Data shown are from one experiment.

A)




B)

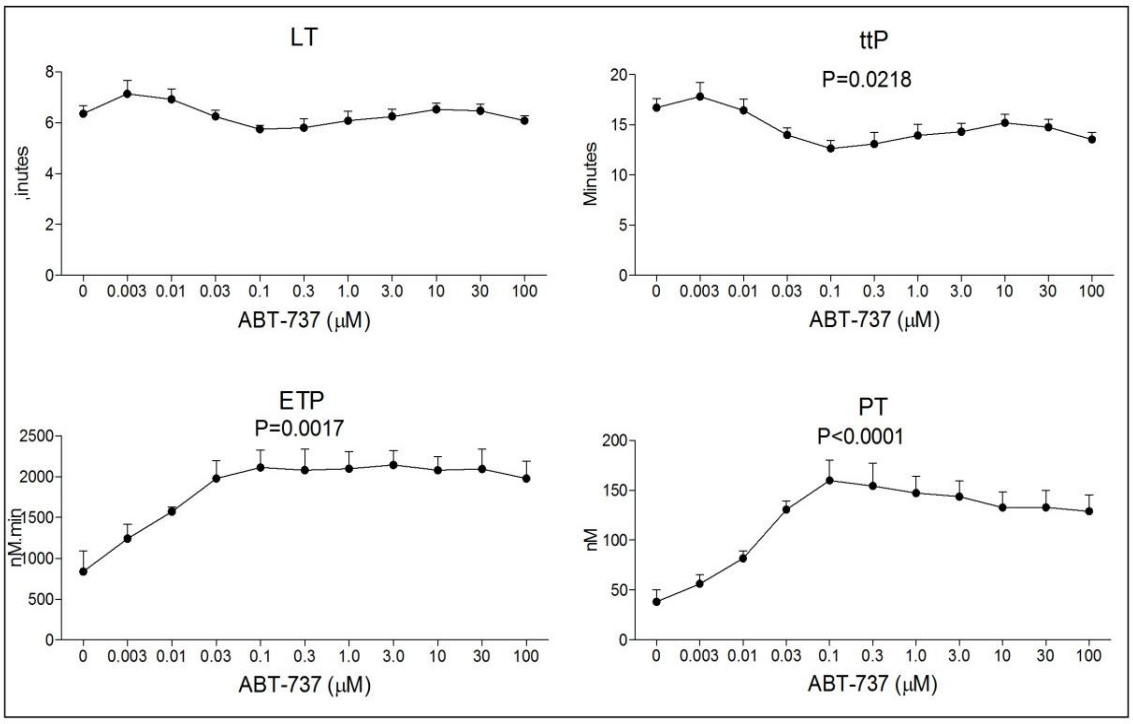
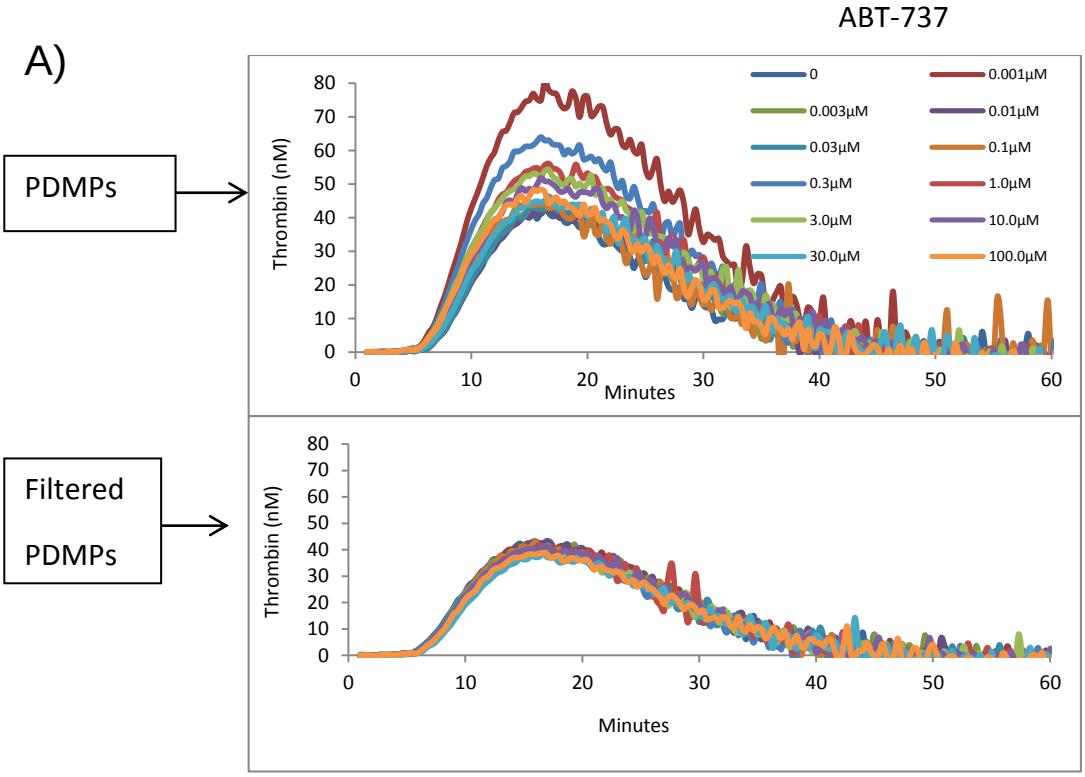



**Fig 7.3: ABT-737 triggers thrombin generation in a dose-dependent manner.** Panel A shows representative curves from one experiment. Panel B shows the numerical data from 3 experiments. Washed platelets were pre- incubated with different concentrations of ABT-737 for 2 hour at 37˚C. Thrombin was triggered with 1pM TF. Data are shown as mean±SEM; One-way ANOVA ($p<0.05$ considered significant).

### 7.2.1.2. Does ABT-737 induce procoagulant MPs?

To confirm if ABT-737 induces the formation of PDMPs, supernatants were obtained from washed platelets pre-treated for two hours with increasing concentrations of ABT-737 (Fig 7.3) by centrifugation at 1,800x*g* for 30 minutes, and the thrombin generation was analysed before and after filtration (to remove PMDPs) (Fig 7.4; n=3). The representative curves for thrombin generation from one experiment (Fig 7.4a) illustrate that no procoagulant PDMPs were released at concentration of ABT-737 above 0.003µM. Filtration had no effect on LT and ttP and reduced PT and ETP ($p>0.05$). These data suggest that no procoagulant MPs were released from platelets undergoing apoptosis incubated for 2 hours. However, the resting sample (0) gave the highest PT, which might suggest that platelets not inhibited by ABT-737 can generate procoagulant MPs.



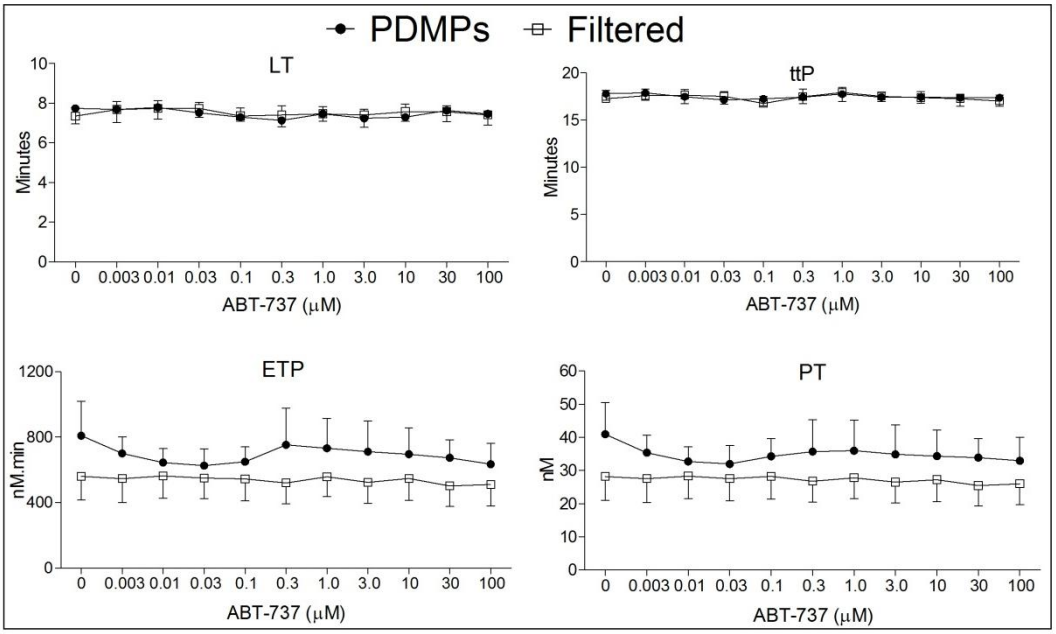




**Fig 7.4: ABT-737 does not release procoagulant PDMPs into the supernatant of treated washed platelets.** Washed platelets were pre-incubated with different concentrations of ABT-737 for two hour at 37˚C. Then the supernatants were obtained by centrifugation for 30 minutes at 1800 g. Thrombin generation was measured with (●) and without (□) filtration (through a 0.2µm filter) using the 1pMTF reagent. Data are shown as mean±SEM from three different experiments.

## 7.2.2. Comparison between apoptosis and activation

The procoagulant surface is a common feature of both apoptosis and activation, so a comparison between activation and apoptosis in promoting thrombin generation and PS exposure was made. Other markers of platelet activation including P-selectin expression, fibrinogen binding and aggregation were also analysed. For activation two hours conditions were used, washed platelets were either stimulated with CRP-XL (2.0µg/ml), TRAP ($10^{-4}$M) or A23187 (10µM) for 10 minutes at 37°C immediately after preparation or pre-incubated for two hours and then stimulated for 10 minutes at 37°C with and without the agonists. For apoptosis, washed platelets were pre-incubated with ABT-737 or ABT-263 for two hours at 37°C, prior to incubation with agonists for 10 minutes

### 7.2.2.1. Apoptosis enhances PT but less ETP than activation

In a comparison of the two conditions of activation, thrombin generation was slightly lower in washed platelets that had been incubated for two hours than in platelets that had been activated immediately (n=2; Figure 7.5). Therefore, 10 minutes of activation was selected to compare activation with apoptosis in promoting thrombin generation (Fig 7.6). Both activation and apoptosis significantly increased thrombin generation compared to resting platelets (Figure 7.6a). Both doses of ABT-737 and ABT-263 induced more thrombin generation in terms of the rate of reaction and PT than the physiological agonists, CRP-XL and TRAP ($p>0.05$; unpaired t-test), but the reaction with A23187 was faster (shorter LT and ttP) and stronger (higher PT and ETP) than ABT drugs ($p<0.05$; unpaired t-test) (Fig 7.6b). ETP was slightly higher in the activation process compared to in apoptosis ($p>0.05$; unpaired t-test). The control vehicle

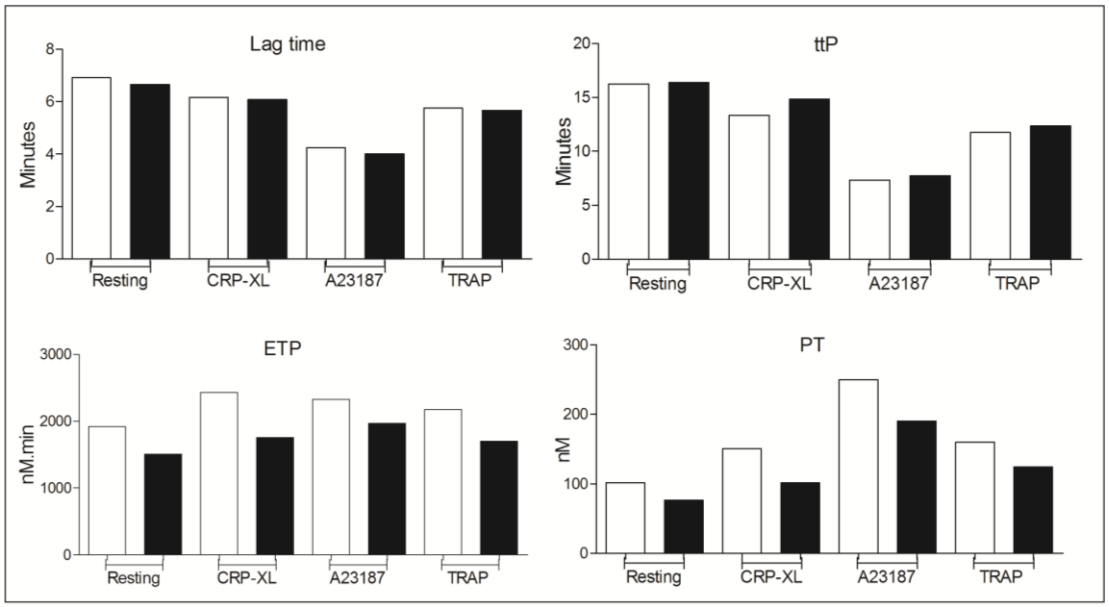
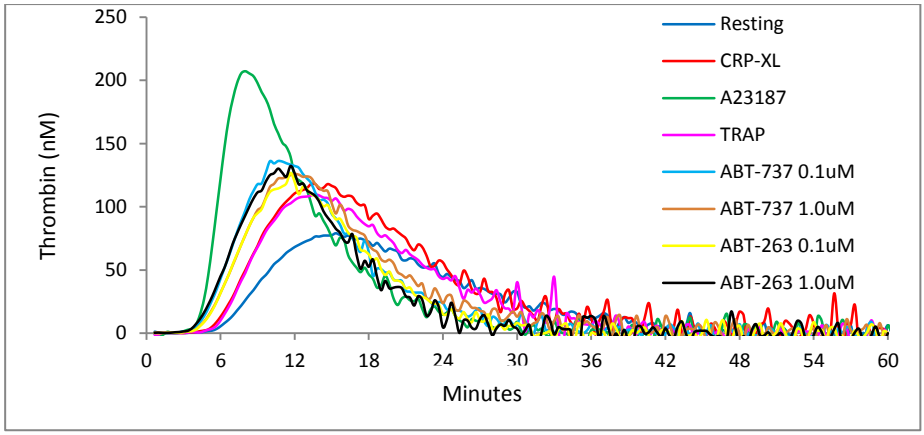



**Fig 7.5: Comparison between the two conditions of washed platelets activation.** Washed platelet either (i) immediately after preparation stimulated with CRP-XL (2.0µg/ml), TRAP ($10^{-4}$M) or A23187 (10µM) for 10 minutes at 37◦C (white bars) or (ii) pre-incubated for two hours and then stimulated for 10 minutes at 37◦C (black bars). Thrombin generation was triggered with 1pM TF. Data are the mean±SEM from n=2.

A)




B)

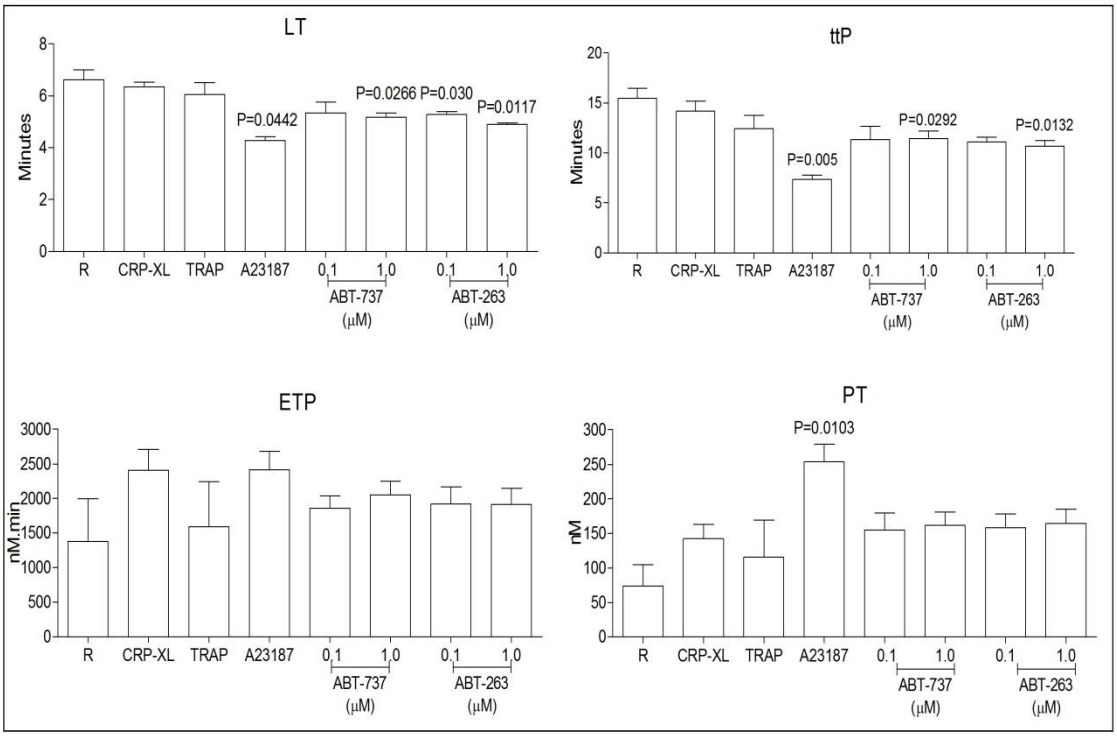



**Fig 7.6: Comparison between thrombin generations induced by activation and apoptosis.** A) Shows a representative curve from one run. B) Shows data from 3 independent experiments. Washed platelets were activated with CRP-XL (2.0µg/ml), TRAP (100µM) or A23187 (10µM) for 10 minutes or incubated with ABT-737 (0.1 and 1.0µM), ABT-263 (0.1 and 1.0µM) for two hours at 37°C. Thrombin generation was triggered with 1pM TF. Data are the mean±SEM. Paired t-test was used for paired data (agonists' comparison with resting platelets) and unpaired t-test for unpaired data (ABT-737 and ABT-263) compared to resting (R) sample.

(DMSO, <0.5% concentration) gave a similar result as the resting sample (data not shown).

### 7.2.2.2. Detection of PS exposure by annexin-V binding

Again, in a comparison of the two conditions of activation, there were no significant differences in PS exposure between whether a sample was incubated for 10 minutes or for 2 hours (n=2; Fig 7.7). There was also no difference between the resting platelets and the control vehicle (DMSO; <0.5% concentration) at the two different time points (data not shown). The percentage of platelets exhibiting PS exposure was significantly increased by CRP-XL, A23187, ABT-737 and ABT-263 compared to resting platelets (Fig 7.8; $p<0.05$; paired t-test for agonists and unpaired t-test for ABT-737 and ABT-263), but not by TRAP. ABT-737 and ABT-263 induced PS was similar to the level observed with A23187 ($p>0.05$; unpaired t-test), and 4.5 times higher than CRP-XL and TRAP, which is significant ($p<0.05$; unpaired t-test). When comparing activation and apoptosis, ABT-737 and ABT-263 (0.1μM and at 1.0μM) increased PS exposure significantly ($p<0.0001$ for both doses; unpaired t-test except ABT-263 0.1μM $p<0.05$ for 0.1μM) compared to CRP-XL and TRAP. No significance differences were observed between A23187 and ABT-737 or ABT-263 ($p>0.5$; unpaired t-test). It is notable that A23187 and ABT give similar results in the annexin-V binding assay, but A23187-treated platelets are more procoagulant measured by thrombin generation (Fig 7.6).

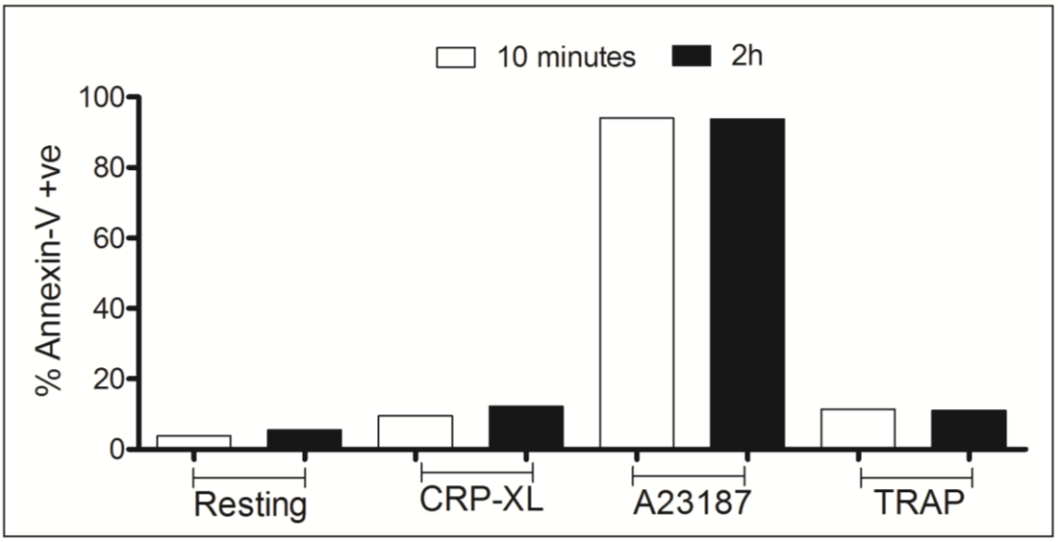



**Fig 7.7: Comparison between two the conditions of washed platelets activation.** Washed platelets were either (i) immediately after preparation stimulated with CRP-XL (2.0µg/ml), TRAP ($10^{-4}$M) or A23187 (10µM) for 10 minutes at 37◦C (red colour) or (ii) incubated for two hours and then stimulated for 10 minutes at 37◦C (blue colour). PS exposure was detected using annexin-V binding assay. Data are the mean±SEM from n=2.

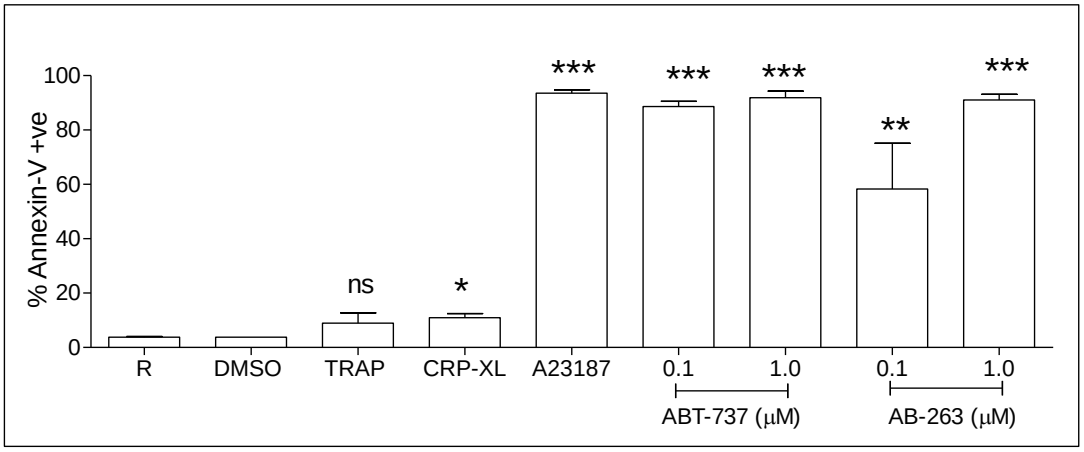



**Fig 7.8. PS exposure is a common feature of activation and apoptosis.** Washed platelets were activated with CRP-XL (2.0µg/ml), TRAP ($10^{-4}$M) or A23187 (10µM) for 10 minutes at 37◦C or with pre-incubated with ABT-737 (0.1 and 1.0µM), ABT-263 (0.1 and 1.0µM) for 2 hours at 37°C. PS exposure was detected in the annexin-V binding assay. Data are the mean±SEM from n=3. Paired t-test was used for paired data (agonists) and unpaired t-test for unpaired data (ABT-737 and ABT-263) compared to resting (R) value. ns ($p>0.05$), * ($p<0.05$), ** ($p<0.005$), *** ($p<0.0001$).

### 7.2.2.3. Platelet activation but not apoptosis induces P-selectin expression

There were no significant differences in P-selectin expression in platelets incubated for 10 minutes or for 2 hours (data not shown). In addition, the control vehicle (DMSO) gave similar results to the resting platelets (data not shown). As expected, CRP-XL, A23187 and TRAP significantly induced P-selectin expression compared to resting platelets ($p<0.005$) (Fig 7.9). On the other hand, ABT-737 or ABT-263 only induced a negligible percentage (<18.0%) of P-selectin expression ($p>0.05$; unpaired t-test) (Fig 7.9).

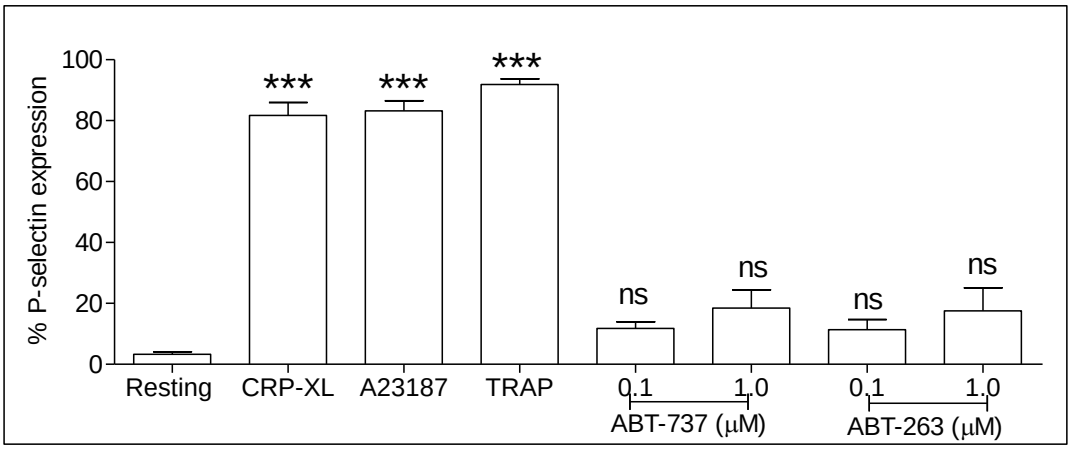



**Fig 7.9. Platelet degranulation measured by P-selectin expression is induced in activation but not apoptosis.** Washed platelets were activated with CRP-XL (2.0μg/ml), TRAP (100μM) or A23187 (10μM) for 10 minutes or pre-incubated with ABT-737 (0.1 and 1.0μM), ABT-263 (0.1 and 1.0μM) for 2 hours at 37°C. P-selectin expression was detected using CD62. Data are the mean±SEM from n=3. Paired t-test was used for paired data (agonists) and unpaired t-test for unpaired data (ABT-737 and ABT-263) compared to resting value. ns ($p>0.05$),*** ($p<0.0001$).

### 7.2.2.4. Platelet activation but not apoptosis induces activation of GP IIb/IIIa

In this experiment, PRP was used instead of washed platelets to analyse the binding of activated GPIIb/IIIa to fibrinogen in the plasma. Whole blood was processed immediately or incubated for 2 hours at 37°C in a slow rotator. Then PRP was prepared and the platelet count in the PRP was adjusted to $150x10^9$/L with autologous plasma and the platelets were stimulated either immediately after preparation or after the two hours at 37°C with CRP-XL (2.0µg/ml) or TRAP ($10^{-4}$M) or A23187 (10µM) for 10 minutes at 37°C. For apoptosis, whole blood was incubated with 1.0 or 10.0µM of either ABT-737 or ABT-263 for two hours at 37°C, and then PRP was prepared and analysed for fibrinogen binding. The results showed that CRP-XL and TRAP significantly induced fibrinogen binding ($p<0.005$; paired t-test) in either fresh samples or after two hours' incubation, with no significant differences between the two time points for either the CRP-XL or TRAP-treated samples (Fig 7.10). A23187 was investigated on the fresh samples and was found to induce significant fibrinogen binding ($p=0.0048$; paired t-test). There were no differences between the resting sample and the control vehicle (DMSO <0.5% concentration; data not shown). Conversely, ABT-737 or ABT-263 (0.1 and 1.0µM) did not induce any fibrinogen binding ($p>0.05$; unpaired t-test) (Fig 7.11).

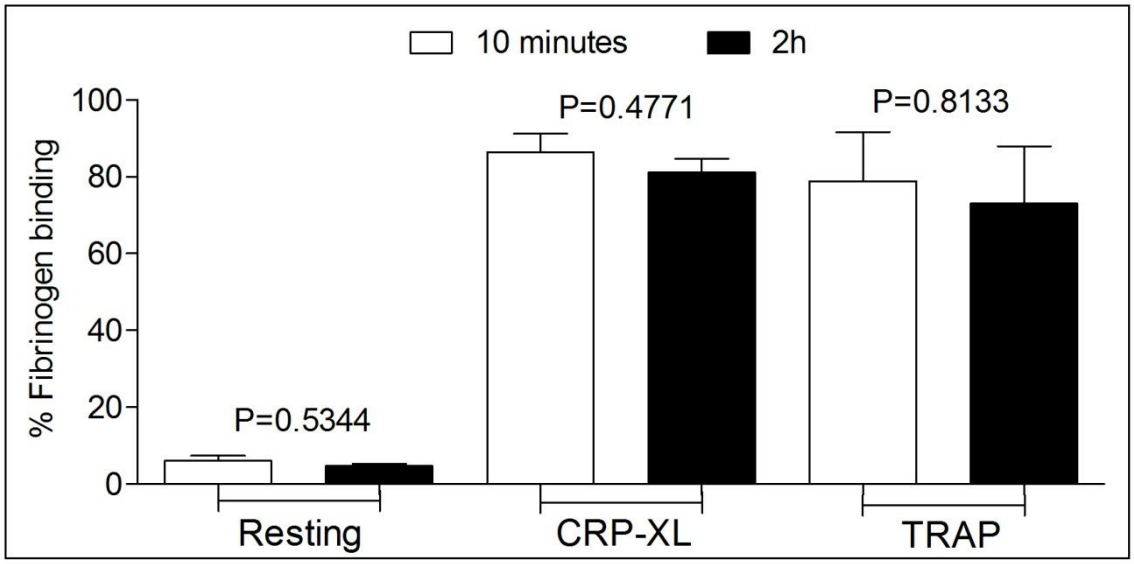



**Fig 7.10. Comparison between two conditions of platelet incubation using fibrinogen binding.** Washed platelets were either (i) immediately after preparation stimulated with CRP-XL (2.0µg/ml) or TRAP ($10^{-4}$M) for 10 minutes at 37◦C (white bars) or (ii) incubated for two hours and then stimulated for 10 minutes at 37◦C (black bars). Data are the mean±SEM from n=3; Paired t-test was used for paired data.

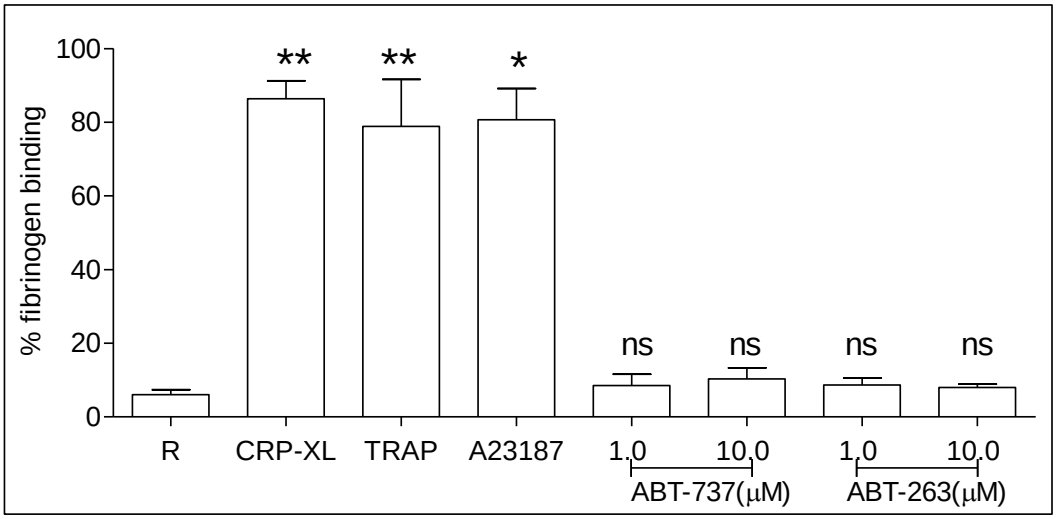


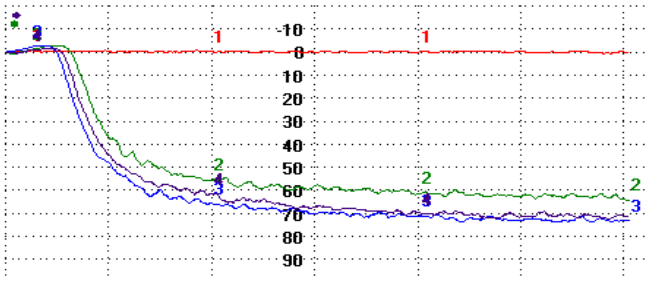

**Fig 7.11. GP IIb/IIIa binds fibrinogen in activation but not apoptosis.** For activation platelets were obtained from whole blood and then activated with (2.0µg/ml), TRAP (100µM) or A23187 (10µM) for 10 minutes. For apoptosis, whole blood was pre-incubated incubated with ABT-737 (0.1 and 1.0µM), ABT-263 (0.1 and 1.0µM) for two hours at 37°C. Data are the mean±SEM from n=3. Paired t-test was used for paired data (agonists) and unpaired t-test for unpaired data for ABT-737 and ABT-263 compared to resting sample. ns ($p>0.05$), * ($p<0.05$), ** ($p<0.005$)

### 7.2.2.5. Platelet aggregation is induced by activation but not apoptosis

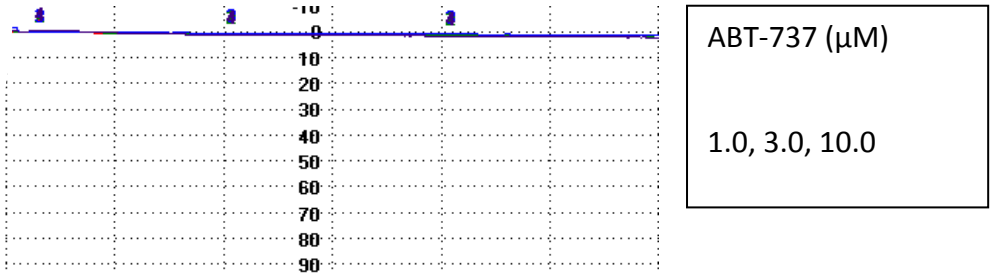
In this experiment, PRP was used instead of washed platelets as described in 7.2.2.4. In all donors (n=3), aggregation was measured using a dose response of either CRP-XL or TRAP in order to select the concentration of agonists that induced the maximum aggregation for each donor. Both agonists induced maximum aggregation at the lowest concentrations of CRP-XL or TRAP used. An example of the aggregation traces with CRP-XL from one run is shown in Fig 7.12a. Aggregation was reported by the PA and AUC, which both increased in a dose-dependent manner with both agonists ($p<0.05$; One-way ANOVA).

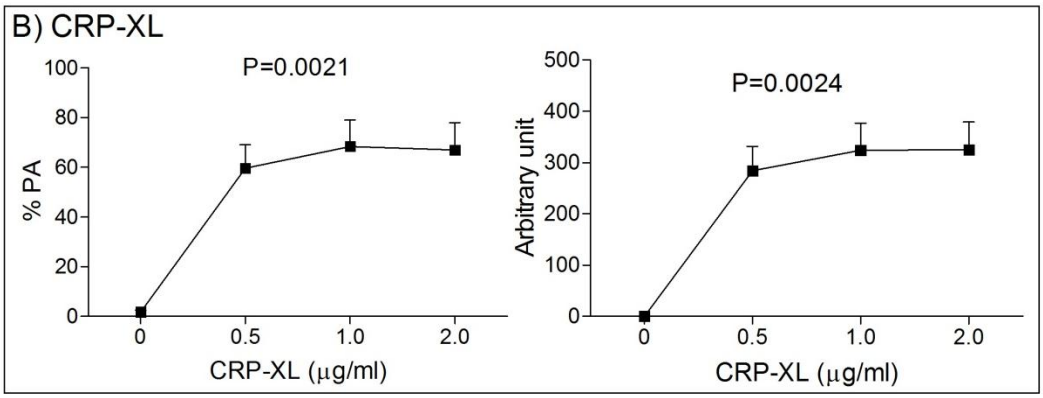
In a comparison of the two conditions of activation (10 minutes vs. 2 hours), CRP-XL- (Fig7.12b) and TRAP- (Fig 7.12c) induced aggregation were similar (data not shown). Incubation of whole blood for two hours with ABT-737 or ABT-263 (1.0, 3.0 and 10.0µM) and then placed in the aggregometer did not induce aggregation at any concentration (all concentration data were from n=3 except at 3.0µM n=2; representative traces are shown in Fig 7.12a).

A upper panel




A lower panel



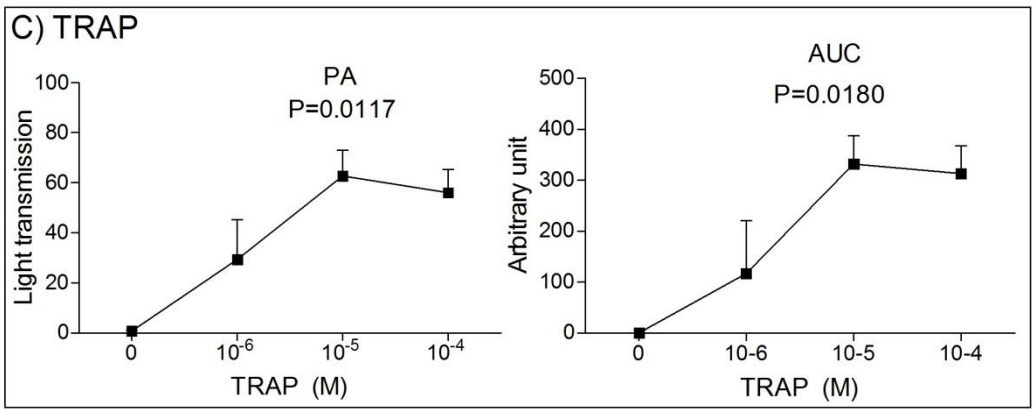




**Fig 7.12. Aggregation is induced by activation but not apoptosis.** Panel A shows representative traces of aggregation induced by CRP-XL (A upper panel) and ABT-737 (A lower panel). Panel B shows the numerical data of CRP-XL (dose response) induced aggregation. Panel C shows the numerical data of TRAP (dose response) induced aggregation. For activation PRP was obtained from whole blood and then activated with (2.0µg/ml), TRAP (100µM) or A23187 (10µM). For apoptosis, whole blood was pre-incubated incubated with ABT-737 (0.1 and 1.0µM), ABT-263 (0.1 and 1.0µM) for two hours at 37°C. Data are the mean±SEM from n=3. Paired t-test was used for paired data (agonists) and unpaired t-test for unpaired data for ABT-737 and ABT-263 compared to resting sample.

## 7.2.3. Apoptotic platelets do not respond to activation

To test whether platelets undergoing apoptosis induced by the Bcl-2/BCL-xL inhibitor subsequently respond to agonists, washed platelets were pre-incubated with either ABT-737 or ABT-263 (0, 0.1 and 1.0μM) for two hours at 37°C, and then were activated for 10 minutes at 37°C with agonists [CRP-XL (2.0μg/ml), A23187 (10μM) or TRAP ($10^{-4}$M)]. To measure aggregation and fibrinogen binding, whole blood was pre-incubated with 1.0, 3.0 or 10.0μM of either ABT-737 or ABT-263 for two hours at 37°C at slow rotation, then the PRP was prepared, the platelet count was adjusted to $150x10^{9}$/L with autologous plasma, then the PRP was either stimulated for 10 minutes at 37°C in the fibrinogen binding assay or used immediately to study aggregation. A23187, CRP-XL and TRAP did not increase thrombin generation over and above that seen with maximum effect of ABT-73 and ABT-263 (Fig 7.13).

Platelets undergoing apoptosis induced by ABT-737 or ABT-263 (all doses) did not respond to subsequent activation with agonists measured by PS exposure using annexin-V binding (Fig 7.14) or P-selectin expression in washed platelet (Fig 7.15) or fibrinogen in PRP (Fig 7.16). A23187 enhanced PS exposure only in platelets treated with a low concentration (0.1μM) of ABT-263 (Fig 7.14b); PS exposure was 58.3%±29.2 with ABT-263 compared to 94.4%±2.3 in ABT-263 platelets treated with A23187.

To measure aggregation, whole blood was pre-incubated with ABT-737 or ABT-263 (1.0, 3.0 and 10.0μM; all n=3 except at 3.0μM n=2) for two hours at 37°C. Representative examples of CRP-XL induced aggregation and ABT-263 inhibited CRP-XL-induced aggregation in a dose-dependent manner are shown in Figure 7.17. Aggregation induced by CRP-XL and TRAP was significantly ($p<0.05$) inhibited in a

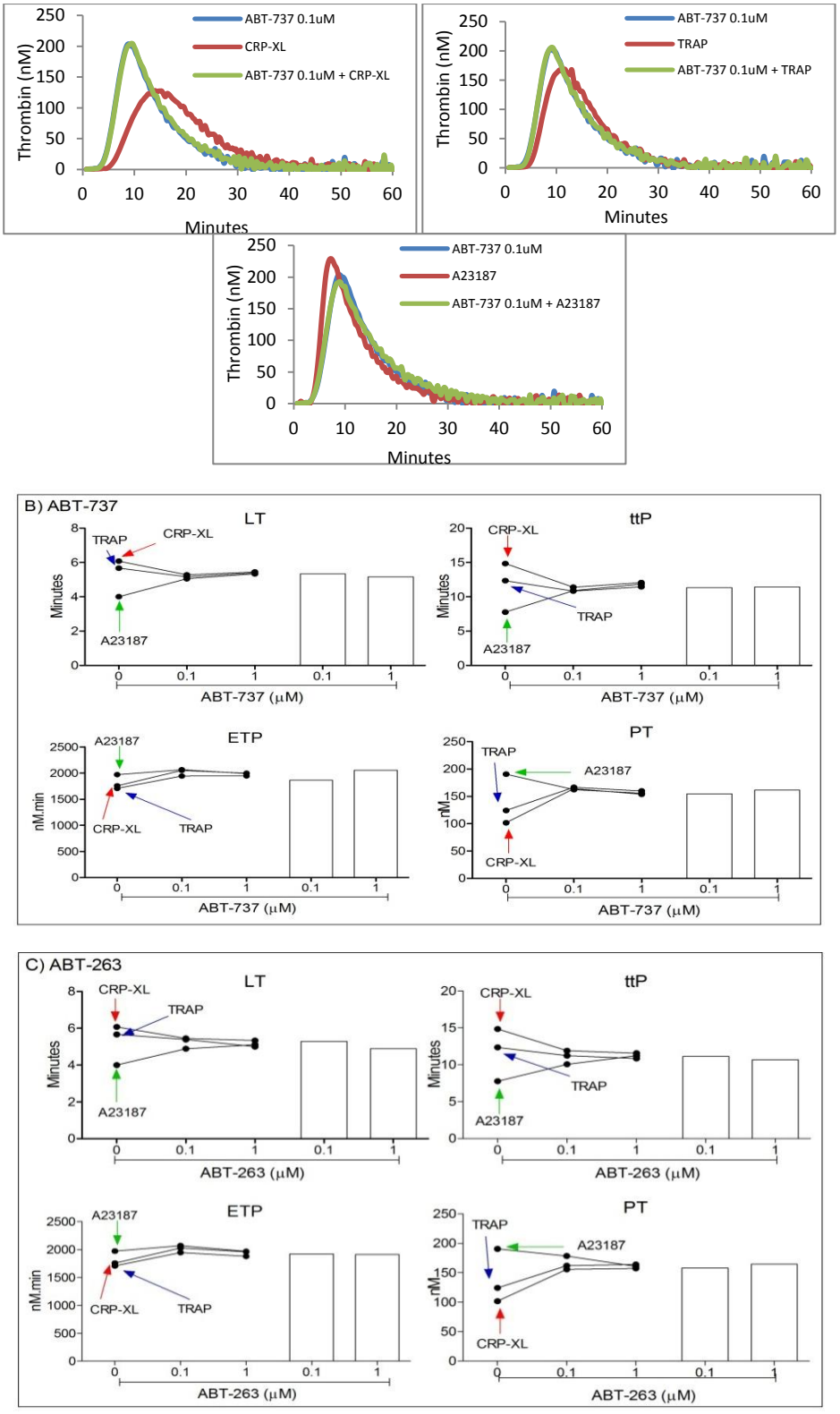



**Fig 7.13. Bcl-2/Bcl-xL antagonists induce maximum thrombin generation in platelets undergoing apoptosis.** Washed platelet were pre-incubated with ABT-737 or ABT-263 for 2 hours at 37°C, then were treated with CRP-XL (2.0μg/ml), A23187 (10μM) or TRAP ($10^{-4}$M) for 10 minutes at 37◦C (line bars) or noting (bars). Thrombin generation was triggered with 1pM TF. Data are the mean from n=3.

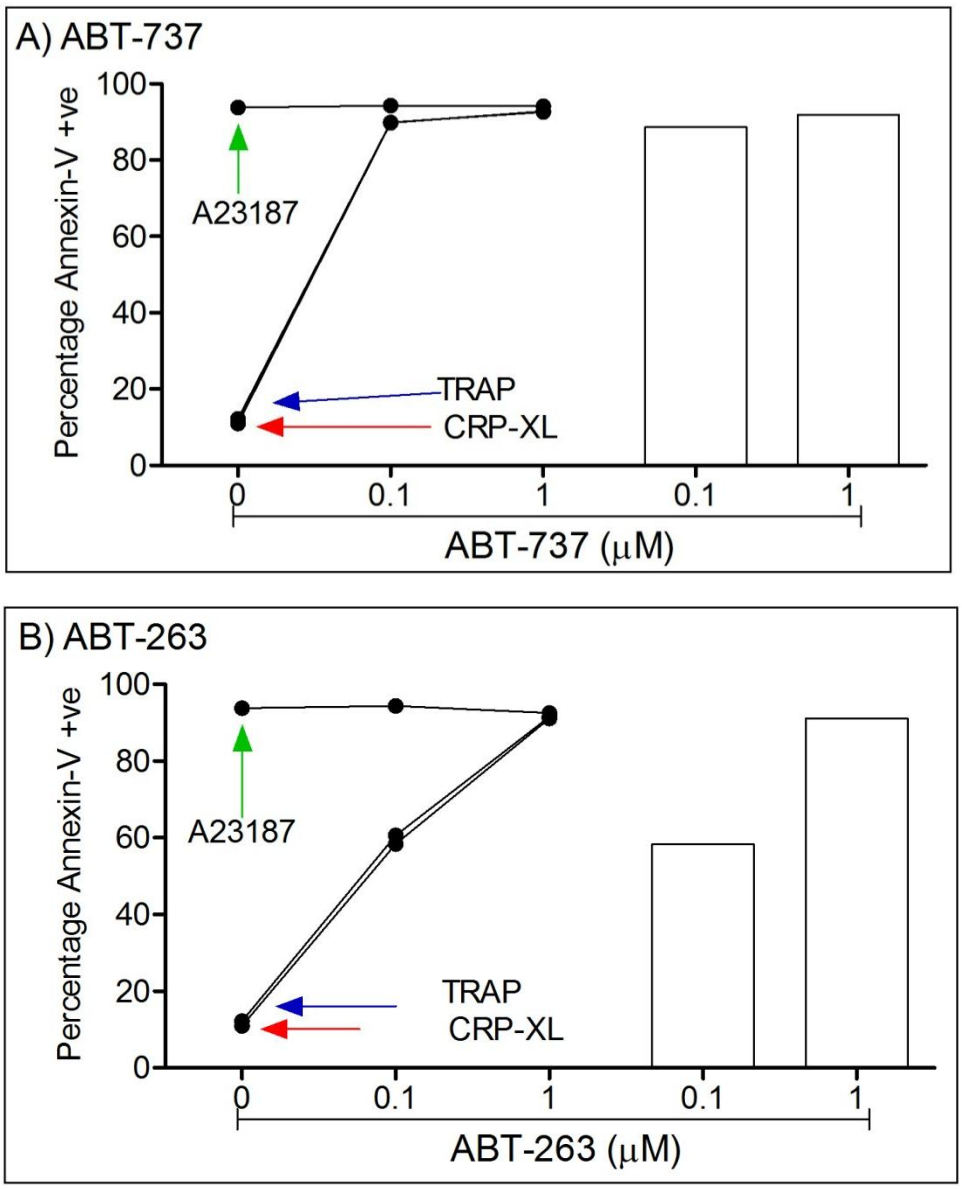



**Fig 7.14. Bcl-2/Bcl-xl antagonists induce maximum PS exposure in platelets undergoing apoptosis.** Washed platelet were pre-incubated with ABT-737 (panel A) or ABT-263 (panel A) for 2 hours at 37°C, then were treated with CRP-XL (2.0μg/ml), A23187 (10μM) or TRAP ($10^{-4}$M) for 10 minutes at 37◦C (line bars) or noting (bars). PS exposure was detected by Annexin-V binding assay. Data are the mean from n=3.

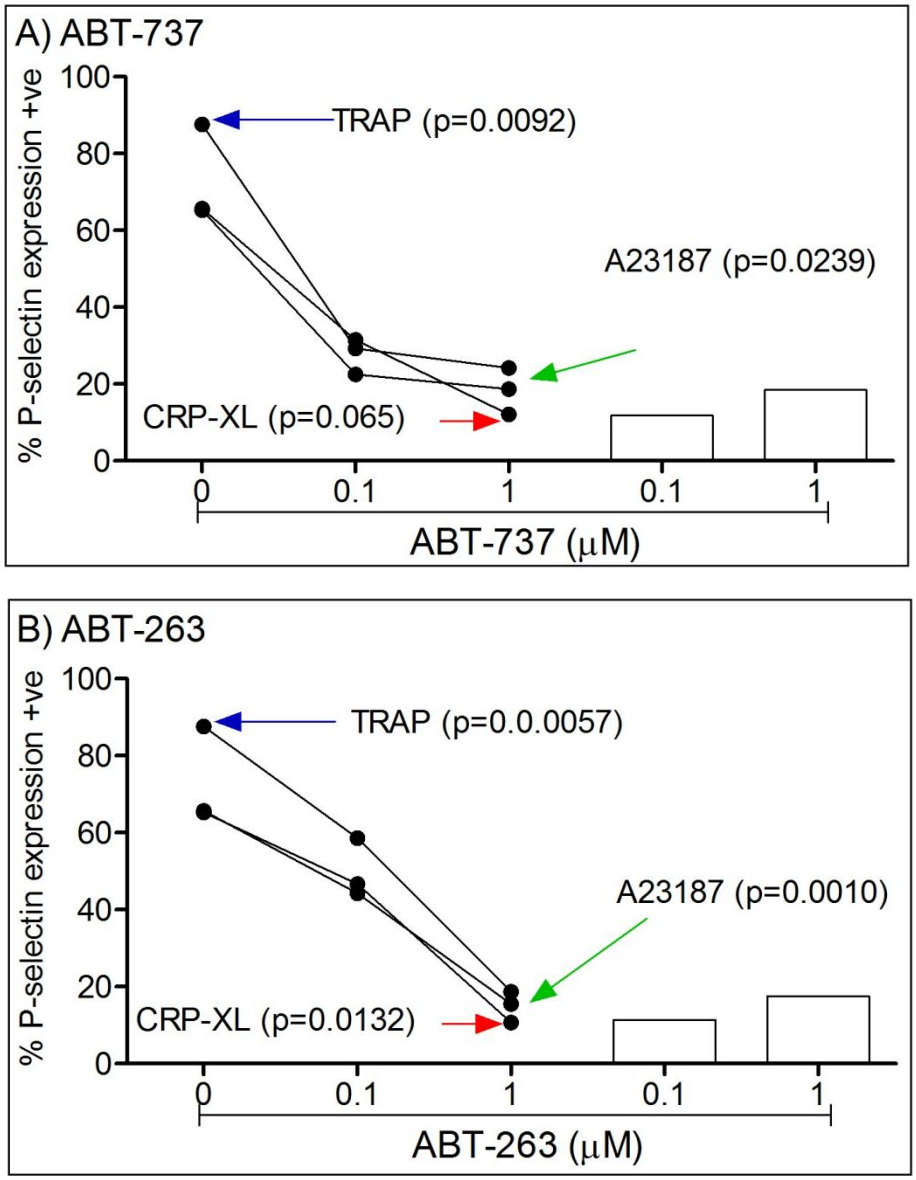



**Fig 7.15. Bcl-2/Bcl-xl antagonists inhibited activation in a dose-dependent manner assessed by degranulation of P-selectin expression.** Washed platelet were pre-incubated with ABT-737 (panel A) or ABT-263 (panel B) for 2 hours at 37°C, then were treated with CRP-XL (2.0μg/ml), A23187 (10μM) or TRAP ($10^{-4}$M) for 10 minutes at 37◦C (line bars) or noting (bars). P-selectin expression was detected by flow cytometry using CD62b. Data are the mean from n=3.

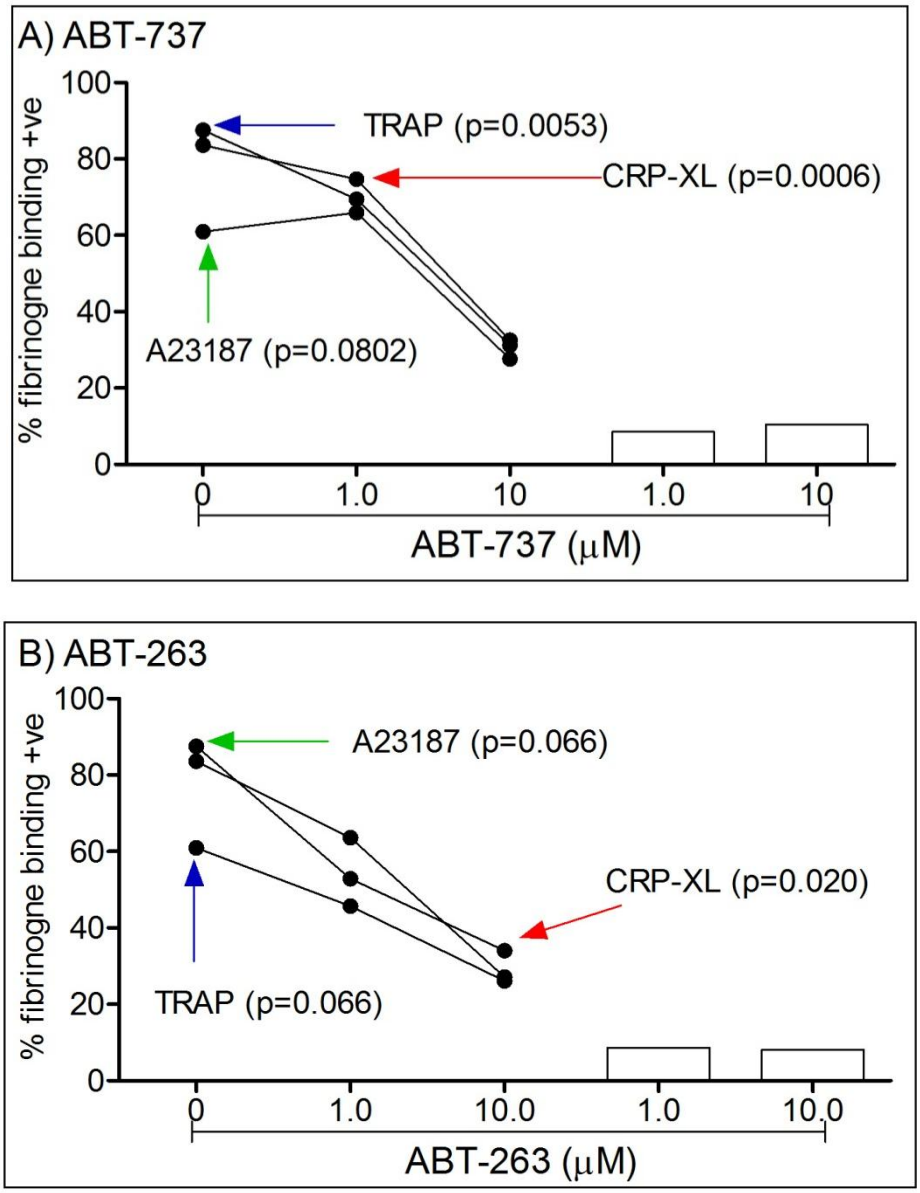
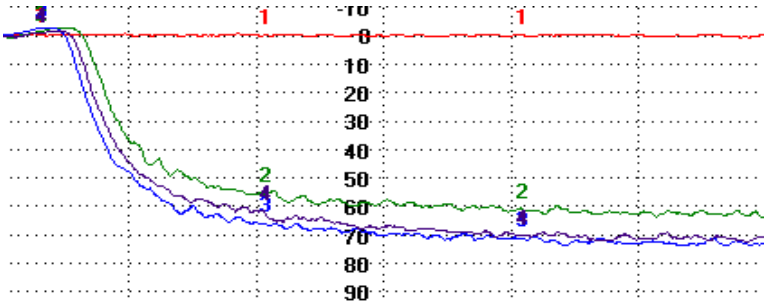


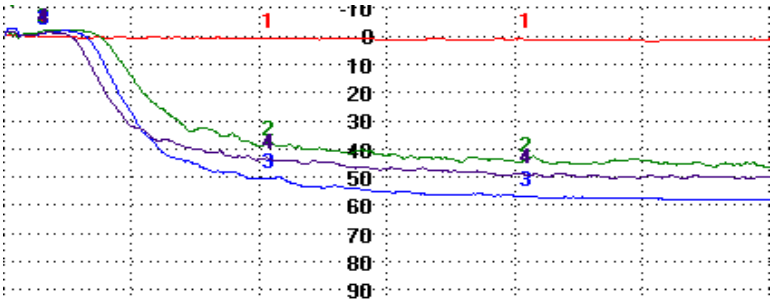

**Fig 7.16: BCL-XL antagonists inhibited activation in a dose-dependent manner assessed by fibrinogen binding.** Washed platelet were pre-incubated with ABT-737 or ABT-263 for 2 hours at 37°C, then were treated with CRP-XL (2.0μg/ml), A23187 (10μM) or TRAP ($10^{-4}$M) for 10 minutes at 37◦C (line bars) or noting (bars). Fibrinogen binding expression was detected by flow cytometry. Data are the mean from n=3.

A)



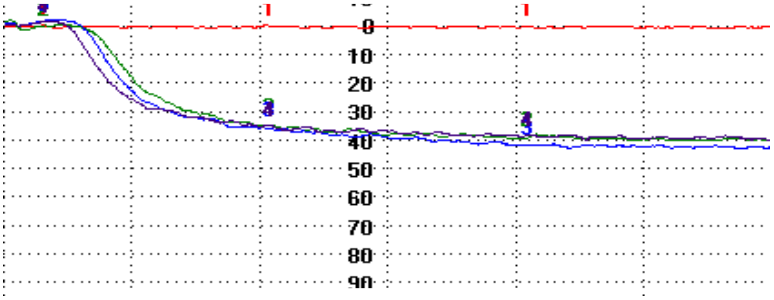
CRP-XL
0.0µg/ml (red line)
0.5µg/ml (green line)
1.0µg/ml (purple line)
2.0µg/ml (blue line)

B) ABT-263 (1.0µM)



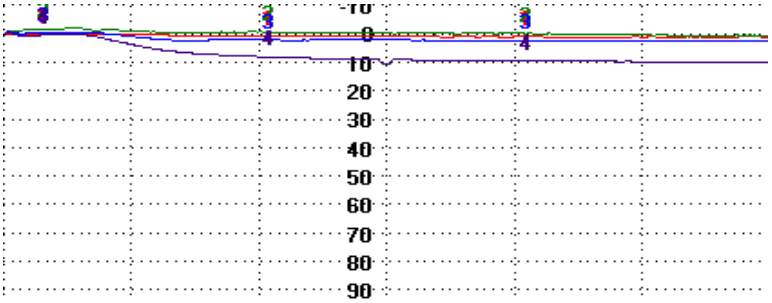
CRP-XL
0.0µg/ml (red line)
0.5µg/ml (green line)
1.0µg/ml (purple line)
2.0µg/ml (blue line)

C) ABT-263 (3.0µM)



CRP-XL
0.0µg/ml (red line)
0.5µg/ml (green line)
1.0µg/ml (purple line)
2.0µg/ml (blue line)

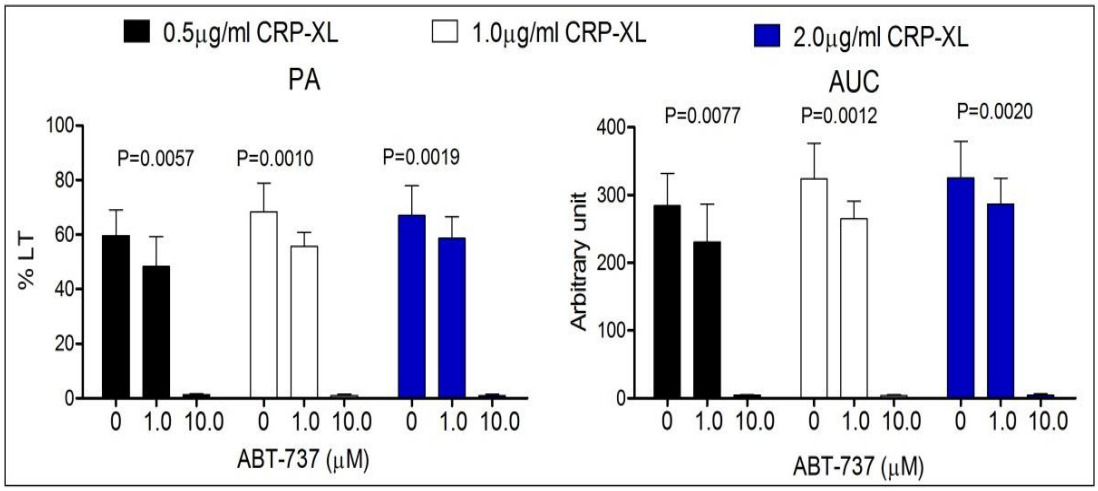
D) ABT-263 (10.0µM)



CRP-XL
0.0µg/ml (red line)
0.5µg/ml (green line)
1.0µg/ml (purple line)
2.0µg/ml (blue line)

**Fig 7.17. Representative traces of ABT-263 inhibited aggregation induced by CRP-XL in a dose-dependent manner.**

A)




B)

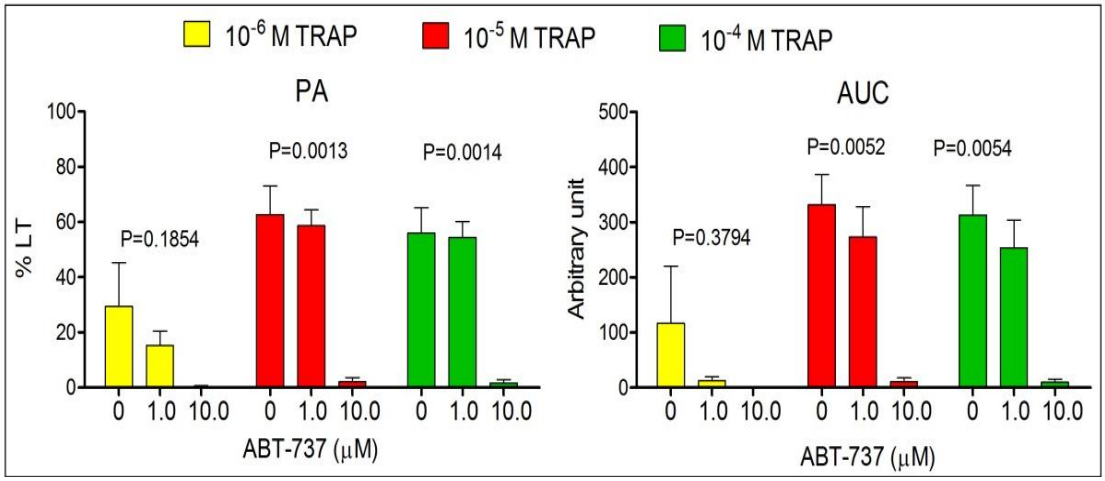
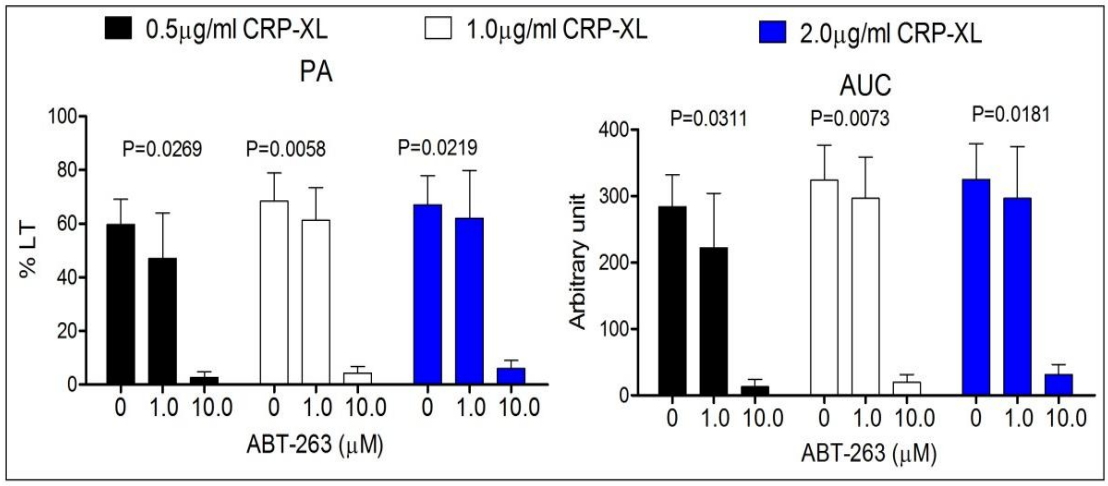



**Fig 7.18: ABT-737 inhibited platelet aggregation induced by physiological agonists in a dose-dependent manner.** Whole blood was pre-incubated with ABT-737 for 2 hours at 37°C, then aggregation was induced by 0.5µg/ml (black bars), 1.0µg/ml (white bars) or 2.0µg/ml (blue bars) of CRP-XL (panel A) or $10^{-6}$M (yellow bars), $10^{-5}$M (red bars) or $10^{-4}$M (green bars) of TRAP (panel B). Data are the mean±SEM from n=3; One-Way ANOVA.

A)




B)

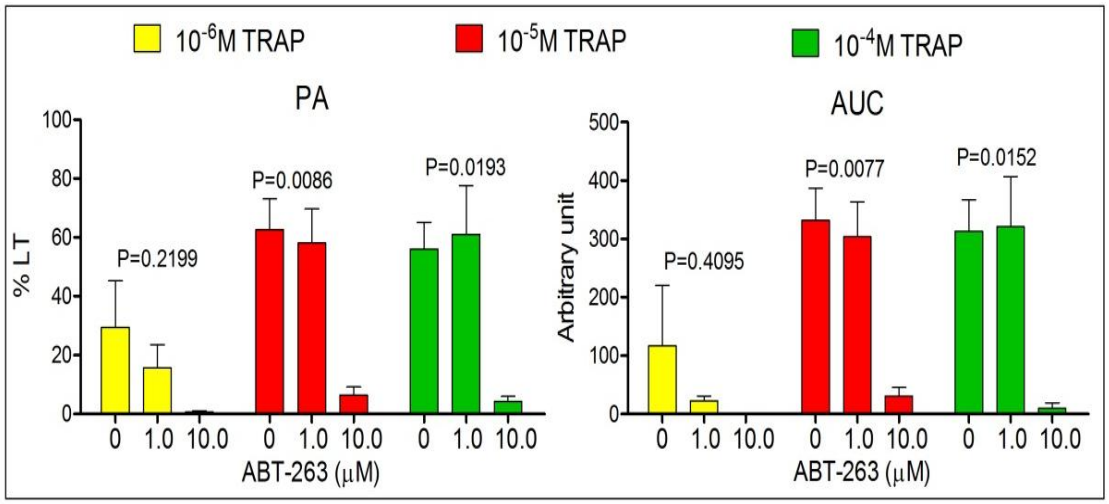



**Fig 7.19. ABT-263 inhibited platelet aggregation induced by physiological agonists in a dose-dependent manner.** Whole blood was pre-incubated with ABT-737 for 2 hours at 37°C, then aggregation was induced by 0.5µg/ml (black bars), 1.0µg/ml (white bars) or 2.0µg/ml (blue bars) of CRP-XL or $10^{-6}$M (yellow bars), $10^{-5}$M (red bars) or $10^{-4}$M (green bars) of TRAP. Data are the mean±SEM from n=3; One-Way ANOVA.

dose-dependent manner by ABT-737 (Fig 7.18) and ABT-263 (Fig 7.19), except for $10^{-6}$M TRAP ($p>0.05$); at this concentration of TRAP, it is a weaker inducer of aggregation. The data for ABT-737 and ABT-263 at 3.0µM are not shown.

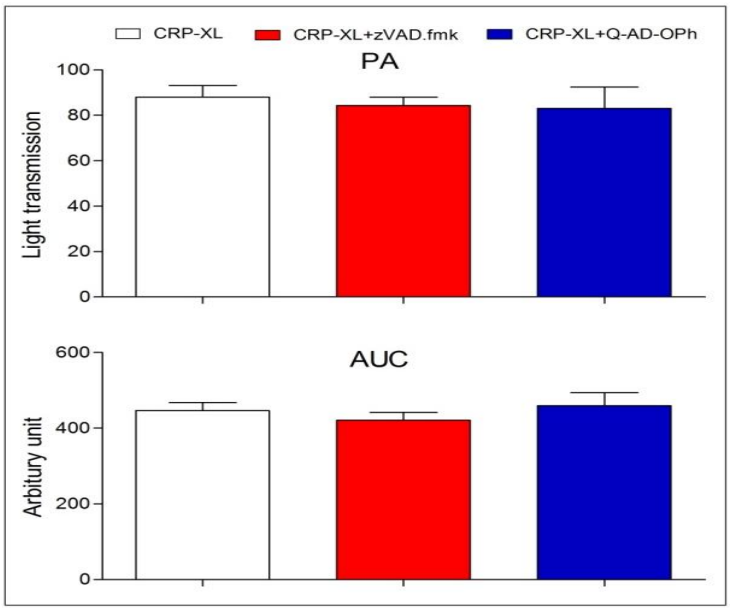
## 7.2.4. Caspase inhibitors did not affect activation

z-VAD.fmk and Q-VD-OPh (50µM; a pan caspase inhibitor) caspase inhibitors were used to investigate if they can reverse the effect of ABT-induced apoptosis or if they can affect activation with CRP-XL or TRAP on aggregation. Neither z-VAD.fmk nor Q-VD-OPh had any effect on CRP-XL (2.0µg/ml) or TRAP ($10^{-4}$M) –induced aggregation (Fig 7.20). CRP-XL and TRAP alone did induce aggregation (data not shown). Then ABT-737 treated platelets in the presence of either z-VAD.fmk (Fig 7.21a) and Q-VD-OPh (Fig 7.21b) did not respond to CRP-XL or TRAP induced aggregation. Again ABT-737 alone did not induce aggregation (data not shown). This mainly because ABT-737 affects $Ca^{2+}$ homeostasis in platelets, as reported by Vogler, *et al* (2011), so abolishes platelets response to agonists inducing activation, because activation is a $Ca^{2+}$ dependent process.

## 7.2.5. Shedding of GP-Ibα

Platelets express GP-Ibα (CD42b) on their surface, with approximately 25 thousand copies. Our data confirmed this with complete shedding of CD42b in platelets treated for 2 hours with ABT-737 at 0.1µM and 1.0µM, ABT-263 at 1.0µM and A23187 ($p<0.05$), but not following activation with physiological agonists. In addition, a reduction of around 60% was observed with the low concentration of ABT-263 (0.1µM) (Fig 7.22).

A) CRP-XL (2.0µg/ml)




B) TRAP ($10^{-4}$M)

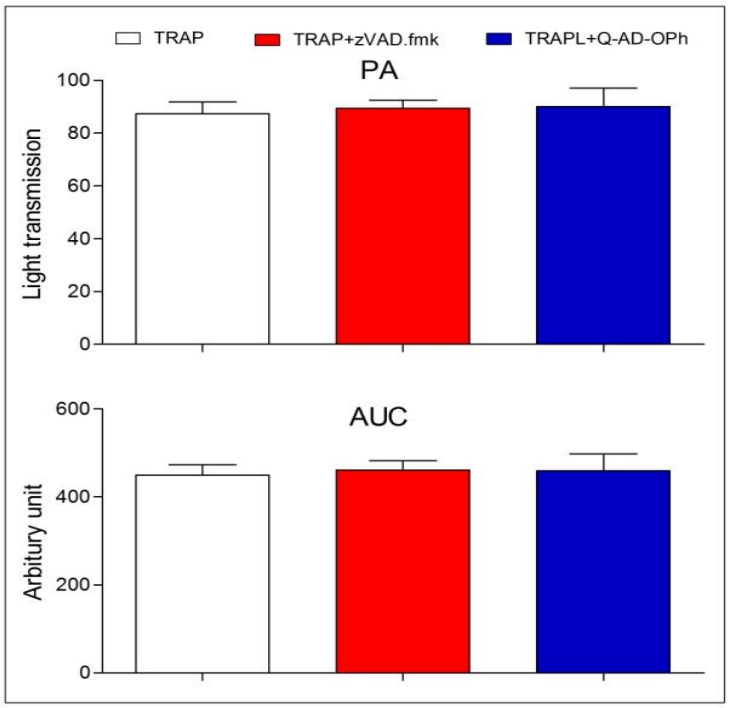
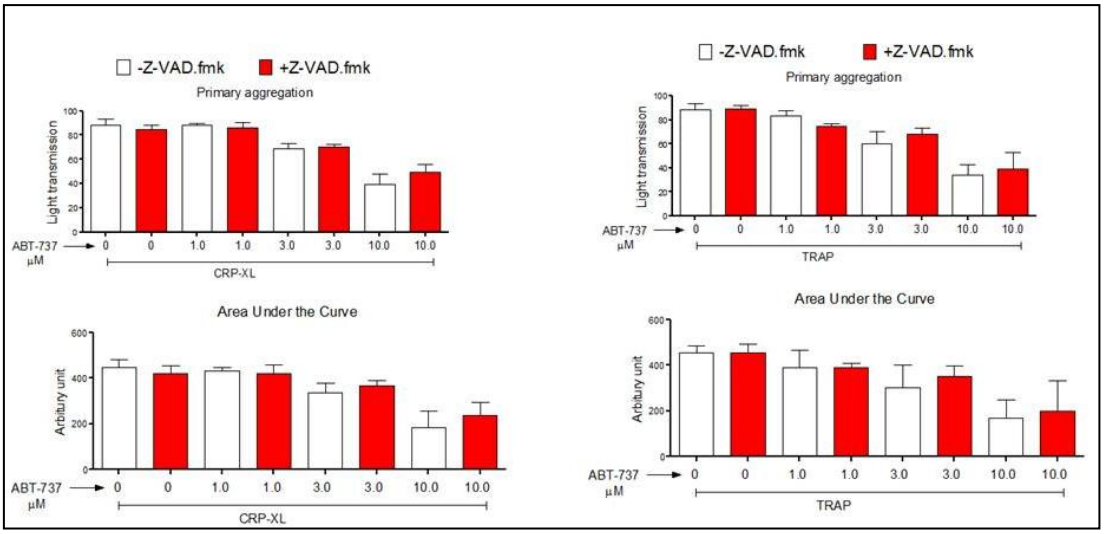



**Fig 7.20: Caspase inhibitor had no effect on agonist induced-aggregation.** PRP was incubated with zVAD-fmk (50µM, red bars), Q-AD-OPh (50µM, blue bars) or nothing for 30 minutes at 37°C under slow rotation. Then aggregation was induced with either (A) CRP-XL (2.0µg/ml) or TRAP ($10^{-4}$M). Data are the mean±SEM from n=3; paired t-test,

A)




B)

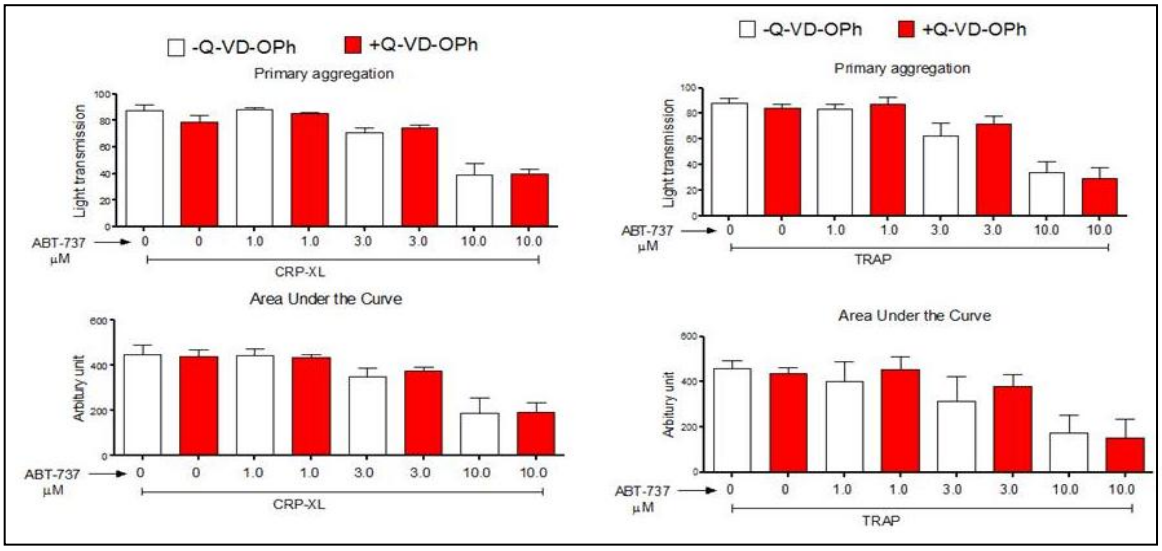



**Fig 7.21: Caspase inhibitor did reverse the effect ABT-263 induced apoptosis assessed by aggregation induced by physiological agonists in a dose-dependent manner.** PRP was pre-incubated with zVAD-fmk (50µM) (red bars panel A), Q-AD-OPh (50µM) (red bars panel B), or nothing (open bars penal A and B) at the same time of adding ABT-737 (0, 1.0, 3.0, 10.0) for 2 hours at 37°C, then aggregation was induced by CRP-XL (2.0µg/ml; left panels) or TRAP ($10^{-4}$M; right panels. Data are the mean±SEM from n=3; paired t-test.

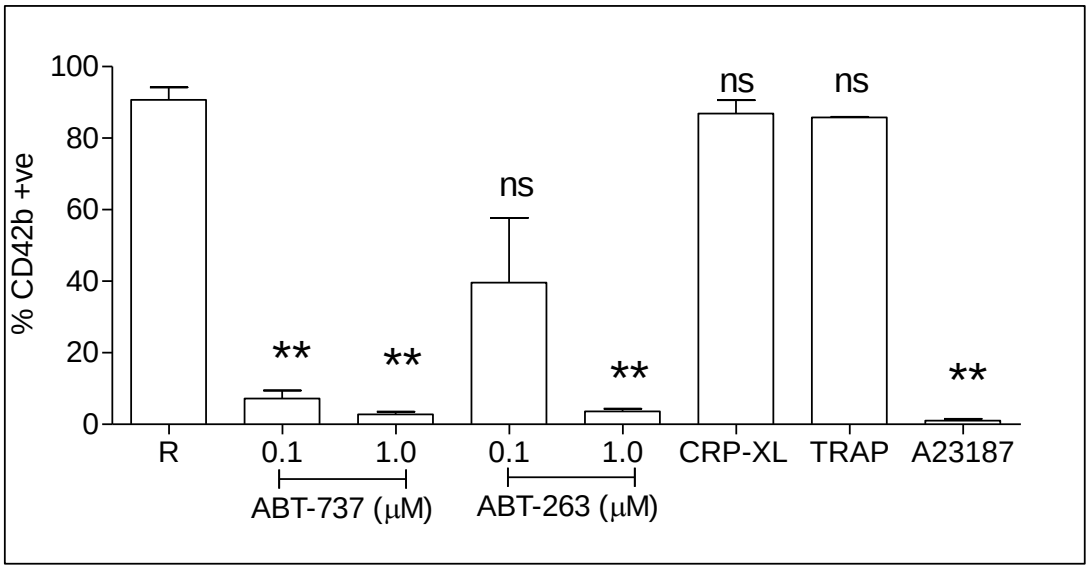



**Fig 7.22. Shedding of GP-Ibα is associated with apoptosis but not activation with physiological agonists.** For activation, washed platelets were pre-treated with CRP-XL (2μg/ml), TRAP $10^{-4}$M) or A23187 (10μM) for 10 minutes at 37°C. For apoptosis, washed platelets were pre-treated with ABT-737 (0.1 or 1.0μM) or ABT-263 (0.1 or 1.0μM) for 2 hours at 37°C. Data are the mean±SEM from 3 healthy donors; paired t-test. ns ($p>0.05$), ** ($p<0.005$)

## 7.2.6. Involvement of lipid peroxidation in apoptosis

To elucidate the involvement of ROS generation and lipid peroxidation in apoptosis through enhancing the procoagulant activity of platelets compared to activation (chapter 6), washed platelets were pre-incubated with the same ROS inhibitors, including the NAD(P)H oxidase complex inhibitor apocynin, the 12-LOX inhibitor esculetin, ROS scavengers tempol and tiron, antioxidants BHT and NAC, dexamethasone, or with cyclosporine A (Cys) at the same time as adding ABT-737 (0.1µM) for two hours at 37°C, and compared with platelets activated with CRP-XL. As has been shown previously, ABT-737 (0.1µM) induced a significant enhancement of thrombin generation (all parameters $p<0.05$ except the LT, $p=0.1796$*;* Fig 7.23).

### 7.2.6.1 Effect of NAD(P)H oxidase inhibitor

Apocynin caused dose-dependent inhibition of thrombin generation induced by ABT-737 (n=6; Fig 7.24; one-way ANOVA). The rate of the reaction (LT and ttP) was slightly affected ($p>0.05$), while the amount of thrombin generated (ETP and PT) was significantly reduced ($p<0.05$). The control vehicles (<1% DMSO) showed similar results to those treated with ABT-737; the control is represented by the letter “C” in Fig 7.24.

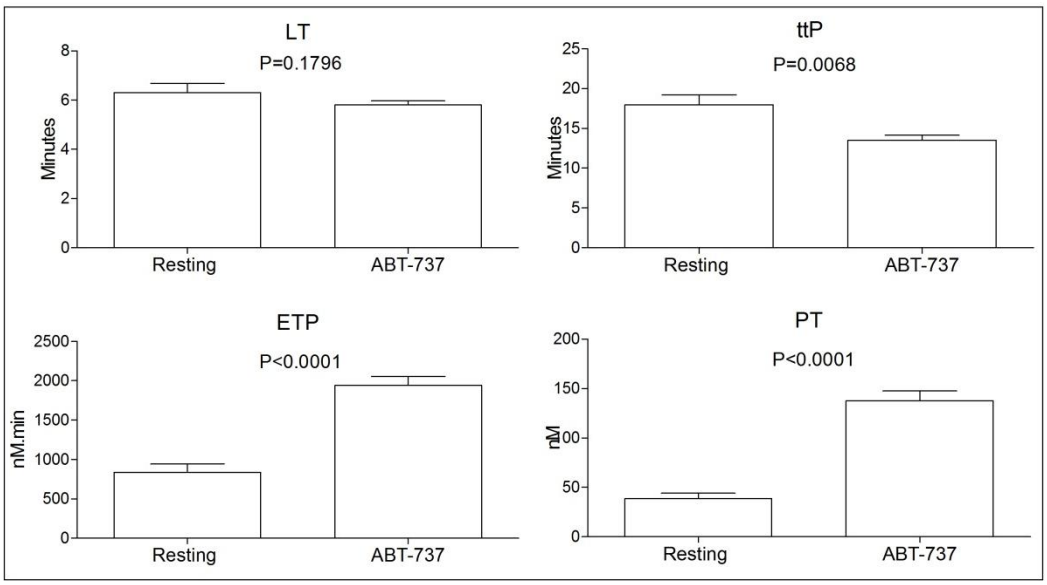



**Fig 7.23: ABT-737 induces thrombin generation**. Washed platelets were incubated with 0.1µM ABT-737 for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with 1pM TF. Data are the mean±SEM from 9 healthy donors; paired t-test.

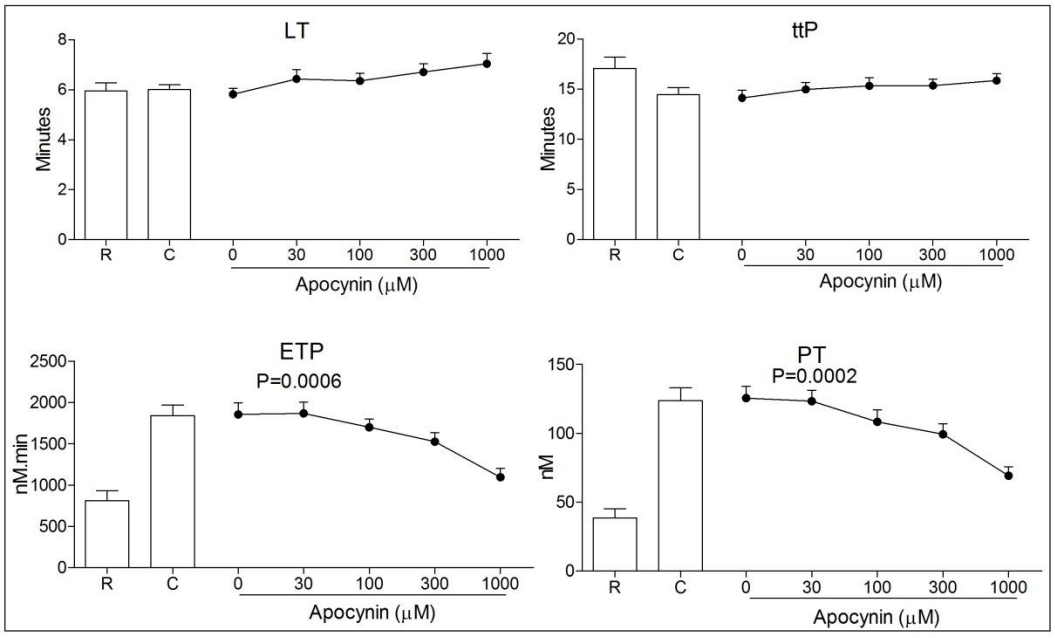



**Fig 7.24: Effect of NAD(P)H oxidase inhibitor, apocynin, on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1µM ABT-737 and apocynin at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with 1pM TF. Untreated sample (R) and control vehicle (C). Data are the mean±SEM from n=6; One-way ANOVA.

### 7.2.6.2 Effect of 12-LOX inhibitor

Esculetin had no effect on ABT-737-induced thrombin generation ($p>0.05$; n=4; one-way ANOVA) (Fig 7.25).

### 7.2.6.3 Effect of ROS scavengers

Unlike the activation with CRP-XL (Chapter 6) where tempol had a significant effect on thrombin generation, tempol had no effect on ABT-737-induced thrombin generation (Fig 7.26, $p>0.05$; n=7; one-way ANOVA). Conversely, tiron inhibited ABT-induced thrombin generation in a dose-dependent manner (Fig 7.27, n=6; one-way ANOVA), having the greatest effect on ETP and PT ($p<0.005$).

### 7.2.6.4 Effect of antioxidants

As shown in Chapter 6, the antioxidants BHT and NAC had no inhibitory effect on thrombin generation with CRP-XL; however, BHT (Fig 7.28) and NAC (Fig 7.29) inhibited ABT-induced thrombin generation in a dose-dependent manner. This is observed as a significant inhibition with NAC on all four parameters ($p<0.05$ except ETP); BHT had a smaller effect, with its greatest effect on PT ($p<0.05$).

### 7.2.6.5 Effect of dexamethasone

As with activation in chapter 6, dexamethasone had no effect on thrombin generation in ABT-treated platelets ($p>0.05$; n=3, Fig 7.30; one-way ANOVA).

### 7.2.6.6 Effect of L-NAME

Unlike activation in chapter 6, L-NAME at 1000µM and 10000µM had no effect on thrombin generation in ABT-treated (data not shown).

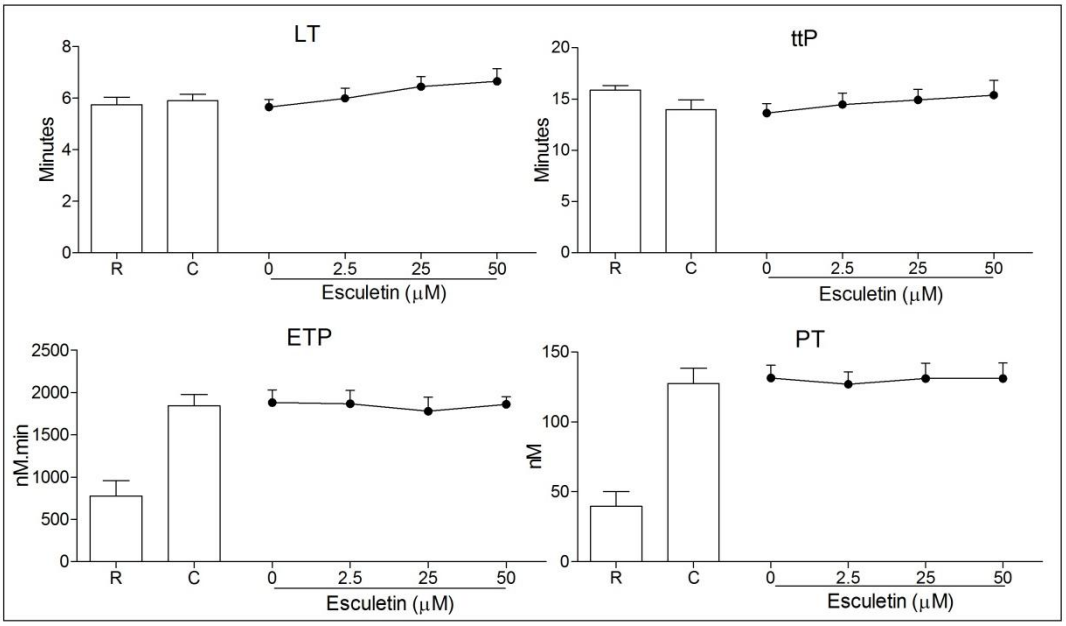



**Fig 7.25: Effect of 12-LOX inhibitor, esculetin, on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1μM ABT-737 and esculetin at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with 1pM TF. Untreated sample (R) and control vehicle (C). Data are the mean±SEM from n=4; One-way ANOVA.

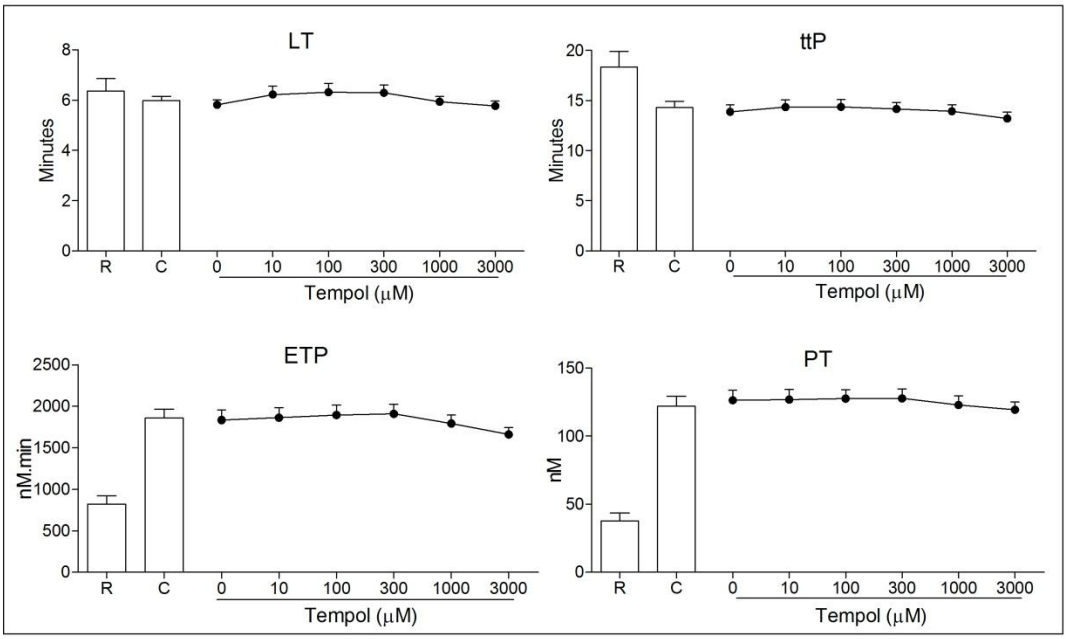



**Fig 7.26: Effect of general ROS scavenger, tempol, on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1μM ABT-737 and tempol at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with 1pM TF. Untreated sample (R) and control vehicle (C). Data are the mean±SEM from n=7; One-way ANOVA.

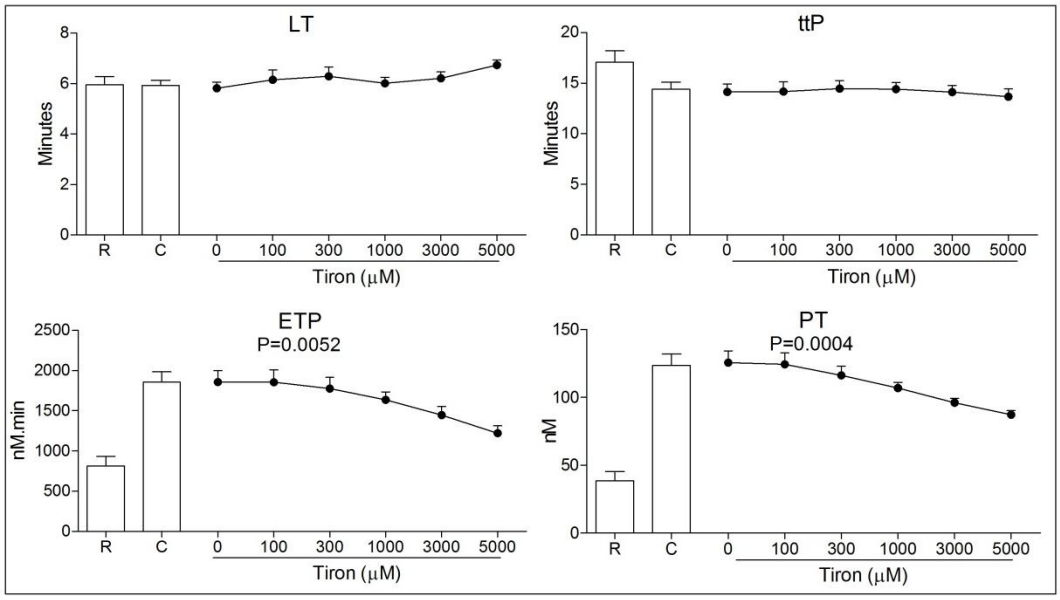



**Fig 7.27: Effect of superoxide scavenger, tiron on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1μM ABT-737 and tiron at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with 1pM TF. Untreated sample (R) and control vehicle (C). Data are the mean±SEM from n=6; One-way ANOVA.

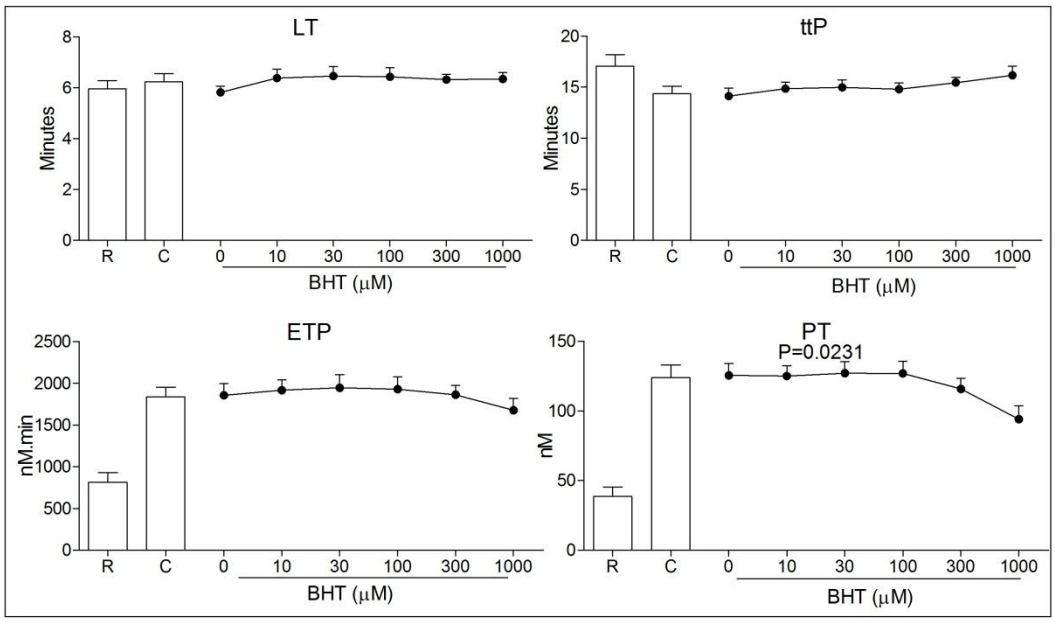



**Fig 7.28: Effect of antioxidant, BHT, on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1μM ABT-737 and BHT at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with 1pM TF. Untreated sample (R) and control vehicle (C). Data are the mean±SEM from n=6; One-way ANOVA.

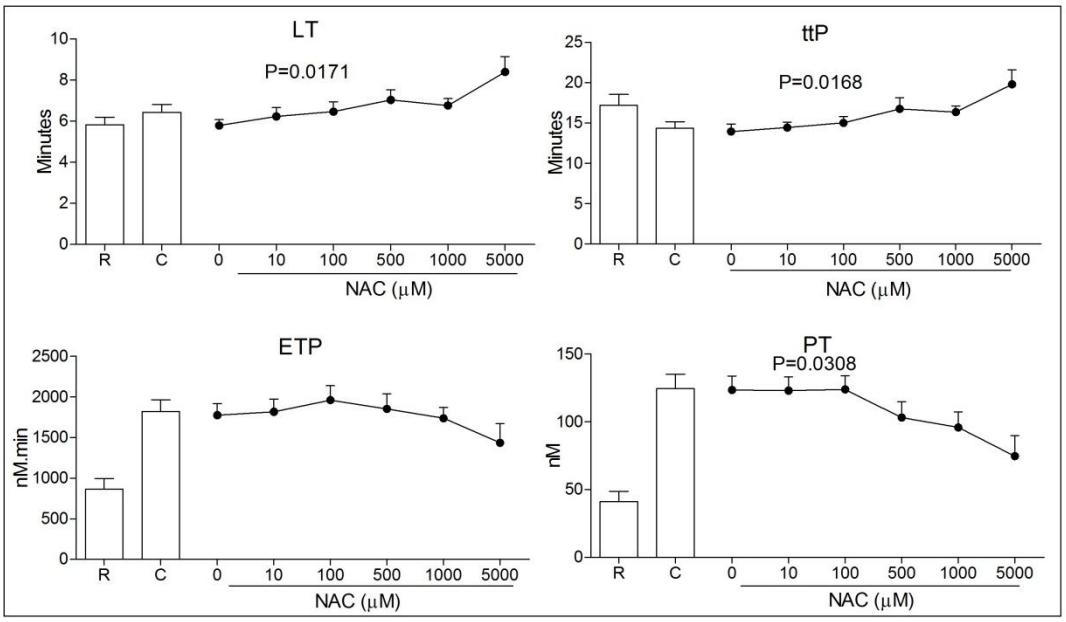



**Fig 7.29: Effect of antioxidant, NAC, on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1μM ABT-737 and NAC at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with the 1pMTF reagent. Untreated sample (R) and control vehicle (C). Data are the mean±SEM from n=5; One-way ANOVA.

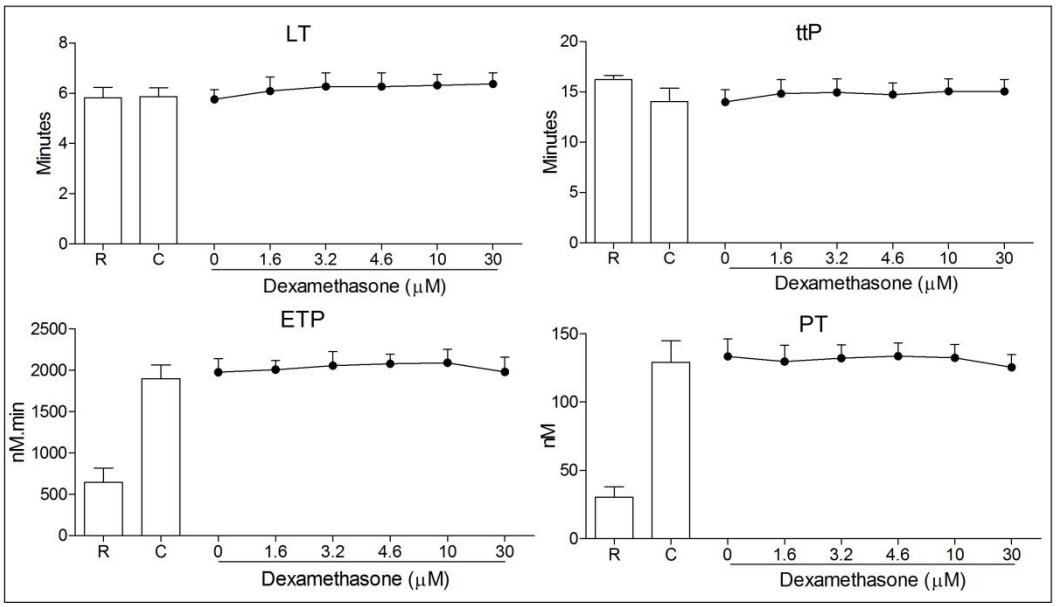



**Fig 7.30: Effect of dexamethasone on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1μM ABT-737 and dexamethasone at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with the 1pMTF reagent. Untreated sample (R) and control vehicle (C). Data are the mean±SEM from n=5; One-way ANOVA.

### 7.2.6.7 Effect of Cyclosporine A (Cys)

Cys is an inhibitor of mitochondrial permeability transition pore (MPTP), which can prevent the loss of ΔΨm induced by apoptosis. Cys inhibited ABT-737-induced thrombin generation ($p>0.05$; Fig 6.31; one-way ANOVA) with the greatest effect observed on PT and ttP. Comparing a single concentration of Cys with resting platelets, the PT induced by ABT-737 was significantly reduced ($p<0.05$: paired t-test) by Cys at 10, 25 and 50 μM.

### 7.2.5.8 Additive effect of the inhibitors

The main effect of the inhibitors on thrombin generation induced by ABT-737 was observed with apocynin (300μM), tiron (1000μM) and NAC (500μM). To investigate whether there is an additive effect of inhibitors there were used at concentrations that were partially inhibitory on their own (Fig 6.32). As in previous experiments, the control vehicle had no effect on thrombin generation (data not shown). Each inhibitor on its own behaved as it did in the previous experiments (inhibiting thrombin generation, apocynin>NAC>tiron). Both tiron and NAC had an additive effect in the inhibition on apocynin, which was greatest in the measurement of PT. Combining both NAC and tiron with apocynin did not increase the inhibition over that seen with either two in combination.

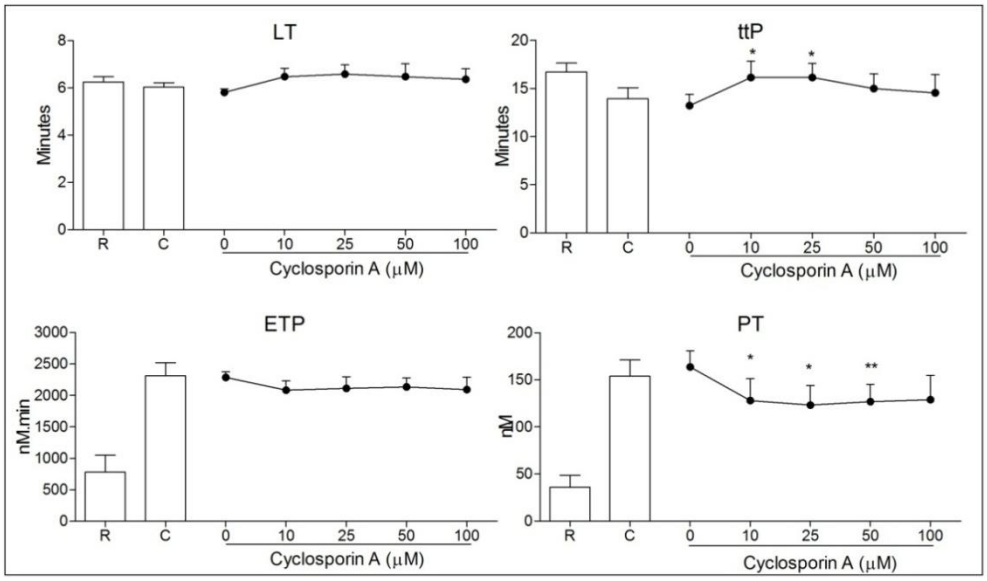



**Fig 7.31: Effect of cyclosporine A on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1µM ABT-737 and cyclosporine A at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with the 1pMTF reagent. Untreated sample (R) and control vehicle (C).Data are the mean±SEM from n=3; One-way ANOVA.

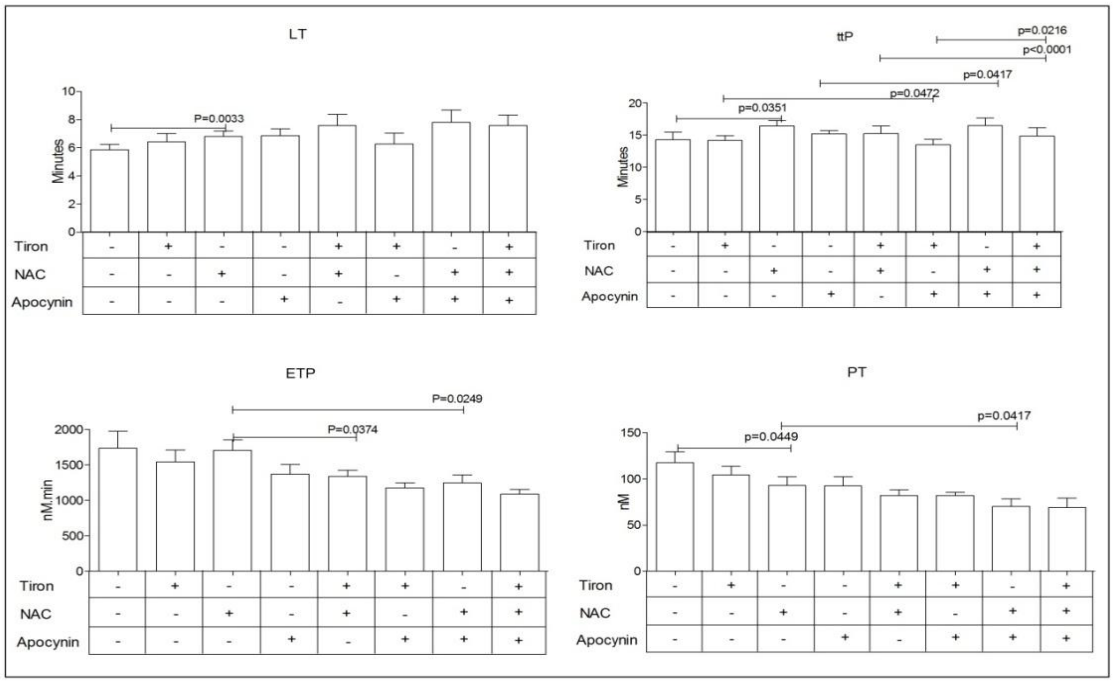



**Fig 7.32: Additive effect of NAC, apocynin and tiron on thrombin generation induced by ABT-737.** Washed platelets were incubated with 0.1µM ABT-737 and /or apocynin (300µM), NAC (500µM) and/or tiron (1000µM) at the same time for 2 hours at 37°C. Washed platelets were mixed with equal volume of autologous filtered PPP and thrombin generation was trigger with the 1pMTF reagent. Data are the mean±SEM from n=3; paired t-test analysis.

## 7.3. Discussion

The current chapter investigated the consequences of platelet apoptosis induced by the Bcl-2/BCL-xL antagonists (ABT-737 and its analogue ABT-263) compared to activation of platelets with CRP-XL, TRAP and A23187 and the involvement of ROS and lipid peroxidation in thrombin generation in platelets undergoing apoptosis. Platelet apoptosis, similar to apoptosis in nucleated cells, is tightly regulated by the Bcl-2 family of both the pro- and anti-apoptotic proteins; with BCL-xL as the sole BCL member in platelets. Once the level of BCL-xL diminishes, Bak induces apoptosis. Indeed, most of the studies of apoptosis in platelets found that the BCL-xL level deteriorates in stored platelets (Bertino*, et al* 2003, Jobe*, et al* 2008), and is linked with increasing PS exposure (Bertino*, et al* 2003, Li*, et al* 2000, Perrotta*, et al* 2003, Wadhawan*, et al* 2004). Therefore, the inhibition of BCL-xL/Bcl-2 proteins in platelets with the BH3 antagonists (ABT-737 and ABT-263) provides an elegant way to study apoptosis in platelets *in vitro*.

Our study (Vogler*, et al* 2011) has clarified the previous confusion about the role of the apoptotic mechanism in platelet activation and shows that the two pathways are distinct, in line with previous observations (Schoenwaelder*, et al* 2009) and recently confirmed (Schoenwaelder*, et al* 2011). Platelet apoptosis comprises caspase activation, cytochrome C release and the loss of $\Delta\Psi m$, but these changes are not seen when platelets are activated with CRP-XL, TRAP and A23187 (Vogler*, et al* 2011). Apoptosis results in more PS exposure than activation, which had been detected by annexin-V binding (Fig 7.8) and thrombin generation (Fig 7.6), which is mainly seen in increased PT. Apoptosis is a slow process within hours, while platelet activation occurs

within minutes as measured by thrombin generation (Fig 7.1) and annexin-V binding (Vogler*, et al* 2011).

The data in this study, showed that apoptosis does not cause activation of integrins, i.e. no functional αIIbβ3 (Fig 7.12) or degranulation (Fig 7.11), in agreement with Zhang, *et al* (2007) and Schoenwaelder, *et al* (2011), who reported no P-selectin expression using CD62b or active αIIbβ3 using the PAC-1 Ab which recognises the active confirmation of αIIbβ3. Our data showed complete shedding of GPIbα in apoptotic platelets (complete reduction in CD42b % events using flow cytometry), similar to A23187 but not with activation with CRP-XL and TRAP in agreement with (Schoenwaelder*, et al* 2011), who also observed shedding of GPVI. In addition, unlike activation, apoptotic platelets were resistant to subsequent activation. Therefore apoptotic platelets are less likely to attach to the vessel wall (no functional GPIbα) (Andrews and Berndt 2004) or to be incorporated into a thrombus/clot (no fibrinogen binding) and do no express P-selectin that can mediate binding of platelets to leukocytes via PSGL-1 (Furie and Furie 2008). They are therefore unlikely to enhance thrombin generation *in vivo* as they would not be become incorporated in a thrombus, but are likely to be cleared from the circulation via scavenger receptors. This has been recently reported that *in vivo* in ABT-737 or ABT-263 treated mice have a defect in platelet aggregation at the site of vascular injury by intravital microscopy and increased tail bleeding time (Schoenwaelder*, et al* 2011), the same group showed under physiological flow condition defect in ABT-treated platelet to adhere to fibrillar collagen and also defect in thrombus growth. Therefore the data suggest that *in vivo*, PS exposure as a result of activation is to promote normal haemostatic process, while

PS exposure on platelets undergoing apoptosis is a trigger for clearance of senescent platelets. Indeed, PS is a sign for macrophage recognition to clear senescent platelet from circulation (Fadok*, et al* 1992a, Fadok*, et al* 1992b, Gardai*, et al* 2006, Savill*, et al* 1993).

The apoptotic mechanism is caspase dependent, but not activation (Vogler*, et al* 2011), we found that the pan caspase inhibitors (z-VAD.fmk and Q-VD-OPH) did not resume platelets ability to activation by CRP-XL and TRAP measured by aggregation (Fig 7.19) and P-selectin (Vogler*, et al* 2011), once apoptosis is commenced by ABT-737 (or ABT-263) induced apoptosis. This mainly because ABT-737 affect $Ca^{2+}$ homeostasis in platelets (Vogler*, et al* 2011). z-VAD.fmk has been shown to inhibit caspase cleavage and cytochrome C release from the mitochondrial upon induced ABT-737 and ABT-263 apoptosis in platelets (Vogler*, et al* 2011).

The current analysis showed that ETP was lower in apoptotic platelets compared to activated platelets (Fig 6.6). This is because apoptotic platelets do not degranulate, so there is no release of procoagulant factor such as FV from the α-granules and also shed GPIbα, which is a receptor for thrombin binding to platelets surface (De Marco*, et al* 1994) and other coagulation factors including FIX (Baglia*, et al* 2002) and FXII (Bradford*, et al* 2000), and HMWK (Joseph*, et al* 1999). Although the level of GPIbα has been reported to internalize with platelet activation this does not lead to a complete absence, and is accompanied by markers of platelet degranulation (Michelson and Barnard 1987). Moreover, apoptotic platelets do not respond to activation (Fig 7.14, Fig 7.15 and Fig 7.16), which would remove the feedback loop of

thrombin generated in the reaction milieu in our experimental condition, which suggests that those apoptotic platelets have limited involvement in clot/thrombus formation.

The current study did not observe the release of procoagulant MPs from apoptotic platelets induced by ABT-737, but cannot rule out the presence of non-procoagulant MPs, because the method used in this study only measure the contribution of procoagulant MPs to thrombin generation. Although it is well-documented that apoptotic platelets in stored platelets release procoagulant MP (Bertino*, et al* 2003, Jobe*, et al* 2008, Li*, et al* 2000, Perrotta*, et al* 2003, Wadhawan*, et al* 2004), this does not contradicts, because the main difference between our study and those reports is timing. The time used for the current study is 2 hours incubation, which is shorter compared to at least more than 8 or 24 hours incubation for stored platelets as (Bertino*, et al* 2003, Jobe*, et al* 2008, Li*, et al* 2000, Perrotta*, et al* 2003, Wadhawan*, et al* 2004). Indeed, recently Schoenwaelder*, et al* (2011) showed membrane budding and MP formation in apoptotic platelet –induced by ABT-737 after 3 hours incubation, using electron microscopy.

PLs oxidation of the cell membrane, mainly PS, occurs during apoptosis (Kagan 2002). Furthermore, oxidised PLs have been shown to increase the activity of the prothrombinase complex, which leads to more thrombin being generated in cells undergoing apoptosis (Pickering*, et al* 2008, Wang*, et al* 2001). Other compounds that induced apoptosis and lipid peroxidation in cells have been also reported to induce PS oxidation in D32 cell (Fabisiak*, et al* 1997). As illustrated in chapter 6, ROS generated

upon platelet activation enhances the procoagulant activity mainly through the NADP(H) oxidase pathway and this has been significantly attenuated by the NAD(P)H inhibitor and ROS scavengers. This study explores the participation of ROS in the procoagulant activity of apoptotic platelets and the subsequent involvement of the lipid peroxidation.

In this study, two different antioxidants, BHT and NAC, significantly attenuated the increase of thrombin generation induced by ABT-737, mainly affecting the PT ($p<0.05$), which is a parameter sensitive to the procoagulant PLs (Chapter 3) (Bidot*, et al* 2008, Hron*, et al* 2007, Kaufmann*, et al* 2007). BHT is a phenolic antioxidant that acts by terminating the chain reaction of the free radicals, while NAC is a cysteine derivative antioxidant known as an amino-thiol precursor of intracellular cysteine and glutathione. The data might suggest that the increase in the procoagulant activity of apoptotic platelets is, to some extent, mediated by the deterioration of platelet antioxidant capacity, mainly the depletion of the intracellular thiols. In addition, the $O^{\bullet}_2^-$ scavenger tiron, but not the $H_2O_2$ inhibitor tempol, was shown to significantly inhibit thrombin generation, similar to that seen with the antioxidants. This might suggest that the generation of $O^{\bullet}_2^-$ levels in apoptosis is high compared to activation. Or it could be due the antioxidant capacity within platelets that has been deteriorated with the two hours incubation in this study; therefore $O^{\bullet}_2^-$ cannot be converted by SOD to $H_2O_2$. This is in reverse of the observation in activated platelets

Unlike the antioxidants and the superoxide scavenger, the NAD(P)H oxidase complex inhibitor, apocynin, reduced all parameters of thrombin generation in apoptotic

platelets to a level nearly similar to that observed in resting platelets. Our observation confirms the fact that ABT-737–induced thrombin generation *in vitro* is mediated by ROS generation. The combination of NAD(P)H oxidase inhibitor (apocynin) with a superoxide scavenger (tiron), or with an antioxidant (NAC), markedly reduced the procoagulant activity of the PLs measured by PT by 30.2% and 40.3%, respectively, compared to their independent effects. The results appear to support an additive effect of an ROS scavenger or antioxidant on top of apocynin. This might suggest the generation of ROS from different sources such as mitochondrial respiration, xanthine oxidase, and NOS on top of the NAD(P)H oxidase pathway. This also indicates a role of the NAD(P)H oxidase complex mediated by ROS on PS exposure, but whether this is via a direct or indirect effect is unclear and still needs to be elucidated compare to activated platelets.

We (Vogler*, et al* 2011) and others (Schoenwaelder*, et al* 2011, Schoenwaelder*, et al* 2009) have shown the loss of ΔΨm in apoptosis induced by the Bcl-2 inhibitors in platelets, which is normally controlled by the MPTM. The loss of ΔΨm is important in apoptosis because it leads to subsequent release of cytochrome C to cytosol and consequent activation of caspase (Danial and Korsmeyer 2004). Cys is an inhibitor of MPTM, which can prevent the loss of ΔΨm induced by apoptosis in nucleated cells (Crompton 1999), which has been used clinically and showed to reduce the infarct size in the heart and brain (Mattson and Kroemer 2003). It is an immunosuppressive drug to prevent organ rejection after transplantation (Kahan 1989). Indeed, 10μM Cys reverses the effect of A23187 induced platelet apoptosis by preventing the loss of ΔΨm, caspase 3 activation, cell shrinkage, and MP formation but has modest or no

effect on PS exposure (Leytin*, et al* 2009). In the current study, Cys (10-100µM) attenuated thrombin generation (Fig 7.28), by ABT-737, in agreement with the finding of Arachiche, *et al* (2009) who showed inhibition of PS exposure by Cys in A23187- and thrombin/convulxin treated platelets, but contradicts the finding of Tomasiak *et al.* (2007) that Cys potentiates PS is induced by collagen in platelets. Other groups have also seen enhancement of platelet aggregation in response to agonists after Cys treatment (Cohen*, et al* 1988). However, the doses of Cys above 10µM used in this study are considered high compared to other studies (Leytin*, et al* 2009, Remenyi*, et al* 2005), and the effect of lower doses (<10µM) should be investigated.

The current study showed that dexamethasone, which had a small inhibitory effect on activation-induced PS exposure (Chapter 6) at therapeutic doses, had no effect on ABT-737 induced thrombin generation by platelets, suggesting that dexamethasone only play a role in A23187- and PMA- induced MPs formation by inhibiting NAD(P)H oxidase, due to the participation of ROS generation in MP formation that had been suggested by Karpatkin group (Nardi*, et al* 2004, Nardi*, et al* 2007). The rational of using dexamethasone is that ROS generation is high in apoptosis.

The main limitations of this study can be observed by the lack of direct measurement of lipid peroxidation and ROS generation. In addition, those inhibitors, especially apocynin, can they prevent the loss of ΔΨm, cytochrome C release or caspase activation in ABT-737 treated platelets, which should be investigated.

In conclusion, the BH3 mimetic peptide inhibitors of BCL-2 (ABT-737 or ABT263) induced platelet apoptosis in a slower process compared to activation, and trigger

thrombin generation by the exposure of PS on the platelet surface but not MP formation. Platelets in which apoptosis are induced by ABT-737 or ABT-263 are no longer susceptible to activation by physiological agonists or by the calcium ionophore A23187 and lose their ability to participate in the thrombotic response. Lipid peroxidation is associated with the procoagulant activity of apoptotic platelets, which is mediated by the NAD(P)H oxidase complex pathway and by $O^{\bullet}_{2}{}^{-}$. Apocynin, the NAD(P)H oxidase complex inhibitor, a $O^{\bullet}_{2}{}^{-}$ scavenger and antioxidants dose-dependently inhibited the increase in thrombin generation by the ABT-737, which was more inhibited by the addition of the antioxidant NAC or the $O^{\bullet}_{2}{}^{-}$ scavenger, tiron, indicating the involvement of lipid peroxidation. The data also suggest the level of ROS is relatively high in apoptosis compared to activation, which lead to deterioration in the antioxidants defences within platelets.

## 7.4. Further work

- The lack of procoagulant MP formation from apoptotic platelets reported in the current study may be due to the short time frame of the study. Whether using a longer incubation time (more than 2 hours) would lead to procoagulant MP formation should be explored.
- To explore the mechanism of ROS generation in apoptotic platelets compared to activation and the use of enzymatic antioxidants to investigate if they have similar effect as BHT and NAC.
- To demonstrate lipid peroxidation and 12-HETE incorporation into PLs in apoptotic platelets compared to activation using a specific methods such as LC/MS/MS.

# Chapter 8: General discussion and conclusions

The current study investigates the regulation of the procoagulant surface on activated platelets and PDMPs. It has been known for almost three decades that exposure of negatively charged procoagulant PLs on the platelet surface, in particular in the form of PS, increases the binding and catalytic activity of the tenase and prothrombinase complexes to enhance thrombin generation. Platelets that express a negatively-charged, $PS^{+ve}$ surface also readily bud off (MPs), which are also negatively charged and support thrombin generation. PDMPs form the majority of the MPs found in the normal circulation and can be elevated in a number of disease states. MPs in the circulation are increasingly recognized as playing a role in normal haemostasis, pathological and inflammatory conditions and may prove to be useful biomarkers for clinical studies (Morel*, et al* 2006, VanWijk*, et al* 2003).

The procoagulant activity of MPs can be accurately evaluated by the CAT, which is a measure of thrombin generation. Several reagents are available for the analysis of thrombin generation by CAT that differ in their levels of TF and PL. In the study described in Chapter 3 we have demonstrated that the most sensitive reagent for detecting the contribution of negatively charged PLs uses the PRP (1pM TF) reagent, which was initially designed to measure the procoagulant activity of platelets (Hemker, *et al* 2003). Under this condition, the assay is most sensitive to the level of endogenous PL in the form of MPs, with PT –which is a measure of the maximum amount of thrombin generated at any one time. This is in agreement with other recently-published studies (Bidot*, et al* 2008, Kaufmann*, et al* 2007), while ETP

represents the prothrombotic potential of the plasma and only needs a small amount of the procoagulant surface to generate the maximum amount of thrombin (Gerotziafas*, et al* 2005, Hemker*, et al* 2003, Vanschoonbeek*, et al* 2004). Here we showed that other CAT reagents containing PL, or higher concentrations of TF, were insensitive to the procoagulant effect of the PDMPs (or platelets), because these reagent failed to discriminate between the plasma that low or rich in endogenous PL as they already contain sufficient amount of exogenous PL. The present study was focused on MPs derived from platelets but it can be assumed that the 1pM reagent can be used for any type of procoagulant MPs derived from other cell types, such as endothelial cells and monocytes.

The procoagulant activity is not only associated with PLs on the membrane surface of MPs, but also with TF. Our data suggest the presence of TF in normal individuals and this level is enhanced after CRP-XL stimulation of platelets. Indeed, TF can generated by platelets after activation (Panes*, et al* 2007, Weyrich*, et al* 2007) or can be taken up the platelets from other cells, including monocytes (Rauch*, et al* 2000), or from "blood borne TF" in the form of MPs or soluble TF (Giesen*, et al* 1999). TF is the main initiator of coagulation, and can be found in either encrypted (dormant, inactive) or decrypted (active) forms (Bach*, et al* 1986, Bach and Rifkin 1990). High levels of TF have been found to be strongly correlated with thrombin generation (Wang*, et al* 2001), and might lead to thrombosis. Elevated levels of TF in plasma have been associated with the morbidity and mortality associated with various thrombotic diseases. The decryption of TF can be achieved by using agents such as A23187 and oxidants, and

has been associated with a step for oxidation of the disulphide bonds in TF by PDI (Ahamed*, et al* 2006, Chen*, et al* 2006, Cho*, et al* 2008).

Different methods exist to analyse MPs; the most commonly-used methods are flow cytometry and the functional assays (Jy*, et al* 2004). However, each method looks at different characteristics of MPs. Flow cytometry is widely used among laboratories using the binding of fluorescently labelled annexin-V to PS exposure and lineage specific antibodies to characterize the cellular origin of the MPs, but this approach fails to examine the functional activity and cannot detect MPs smaller than the wavelength of the exciting laser (normally 0.5µm). Functional assays, including thrombin generation, provide a measurement of the contribution of MPs, including smaller MPs (<0.5µm), but cannot provide information on the count or cell origin of MPs. This study suggests that these methods are complementary to each other and so it is recommended to use more than one method to study the phenotype of MPs. In this study, the generic marker for negatively charged PLs, annexin-V was used, but other markers have been used specifically to detect PS, such as lactadherin (Albanyan*, et al* 2009) or oxidized PLs, such as the EO6 (T15) antibody (Friedman*, et al* 2002), which might provide increased discrimination between the procoagulant effect of PS and oxidized PLs. Alternative methods for MP detection exist, including FXa generation assays, which is widely used to detect the procoagulant activity and the contribution of TF to the reaction. FXa generation assays normally rely on the addition of known amount of exogenous coagulation factors, which eliminates the variability and the levels of coagulation factors in individuals plasma on the procoagulant activity; however, thrombin generation as measured by CAT is more physiological relevant.

There are contrasting reports on the effect of sample preparation and storage of plasma on the MP integrity and level (Dey-Hazra*, et al* 2010, Trummer*, et al* 2009), and the subsequent effects on thrombin generation (Keuren*, et al* 2006), and there is no consensus on the best methods of MP preparation and storage (Jy*, et al* 2004). Although, it is best to run and analyse fresh samples each time, this is not always possible. Also, running fresh samples increases sample-to-sample variation and the cost of the assay, and limits collaboration between laboratories. In addition, freezing has been shown to break down residual platelets (if they exist in the plasma) and to break down large MPs into smaller MPs (Chantarangkul*, et al* 2004, Xiao*, et al* 2002). Most of the previous studies have used flow cytometry to detect the effects of freezing and thawing, but did not use an assay that can measure the procoagulant activity. The current study showed that multiple repeat freeze-thaw cycles have a deleterious effect (albeit small) on thrombin generation driven by MPs. Our results indicate that freeze–thaw cycle enhances thrombin generation, possibly due to disruption of the MP membrane, and also has an effect on coagulation factors, which is in agreement with the recently-published observations that a single freeze-thaw cycle at 80°C, increases MPs, including PDMPs, as measured by flow cytometry using annexin-V (Ayers*, et al* 2011, Dey-Hazra*, et al* 2010). Not only this condition but also snap freezing of plasma in liquid nitrogen followed storage at -70-80°C increases MP count measured by flow cytometry (van Ierssel*, et al* 2010) and increases thrombin generation measured using 1pM TF with three concentrations of PL (0, 0.5, 1µM) (Chantarangkul, *et al*2004). In addition, prolonged storage of the samples at 80°C has been shown to reduce the level of MPs in the plasma (Ayers*, et al* 2011).

Our data demonstrate that the plasma in stable post-MI patients has no difference in its ability to generate thrombin-driven by procoagulant MPs compared to the matched healthy controls. The analysis was only performed in a 20 of patients, which might lead to the procoagulant activity of their plasma being underestimated; therefore a larger study should be carried out, and a comparison could be made by studying patients with an acute coronary event with those who are in a stable condition. By contrast the procoagulant activity in the plasma of chronic renal patients was showed to be enhanced by the presence of MPs compared to matched healthy individuals. High level of circulatory MPs have been reported in patients with CAD (Bernal-Mizrachi*, et al* 2003) and CKD disease (Amabile*, et al* 2005) compared to normal individuals, but other studies have reported similar level of MPs in patients with stable angina compared to healthy controls (Matsumoto*, et al* 2004, Nomura*, et al* 2003). MPs have also been associated with prothrombotic conditions (Aras*, et al* 2004, Biro*, et al* 2003) and with single and recurrent venous thrombosis (Bidot*, et al* 2008, Hron*, et al* 2007, van Hylckama Vlieg*, et al* 2007) and especially PDMPs and EDMPs have been significant predictors of future CAD (Morel*, et al* 2004). Therefore, from the following results, it is suggested that the elevation of procoagulant MPs may be used as an independent risk factor of thrombosis. Although the pathophysiology between CAD and CKD is different, with the emphasis that at early ages the thrombotic risk in CAD patients with premature MI is not usually due to atherosclerotic plaque burden, which is relatively low in the young population, but may be due to other factors affecting the haemostatic system which are different to those in older patients.

The current study confirms that MPs are more procoagulant than platelets measured using annexin-V binding. The analysis of platelets activated with CRP-XL shows the generation of procoagulant MPs which are smaller in size and can bind to annexin-V more strongly than larger-sized platelets, which is in agreement with recently reported observation that MPs have 50-100-fold more procoagulant activity than platelets (Sinauridze*, et al* 2007). By contrast, the CAT assay (a functional assay) showed that platelets generate and support more thrombin generation compared to pre-formed procoagulant MPs. This is mainly because platelets in the reaction milieu are continuously activated by the feedback loop of thrombin generated, while MPs are pre-formed, so their procoagulant activity does not increase with increasing thrombin generation. Also the current data illustrate a direct correlation relationship between MP count and size and the procoagulant activity measured by thrombin generation. The size of MPs and their count are both important in driving this procoagulant activity because the current study indicates that particles smaller than 0.2µm (removed by filtration) are not able to support thrombin generation (Lawrie*, et al* 2008), in agreement with finding that larger PDMPs have a strong correlation with thrombin generation (Michelsen*, et al* 2008). In addition, the number of MPs, as counted using the NTA technology, was strongly correlated with ETP and PT of thrombin generation. In addition, we have shown that dilution of PDMP-rich plasma reduced thrombin generation, which illustrates the significant effect of MPs count on thrombin generation.

Of the physiological platelet agonists, collagen, signalling through GPVI, is the most potent at inducing PS exposure (flip-flop mechanism), and MP budding. It has also

been shown that production of ROS participate in the generation of PDMP from activated platelets, and previous studies have shown the role of lipid peroxidation in enhancing thrombin generation in nucleated cells undergoing apoptosis. Platelets are known to generate ROS in response to stimulation including with CRP-XL via the GPVI receptor. Therefore, does ROS play a role in PL on platelet and MPs formation? This was investigated by measuring thrombin generation by platelets and PDMPs in the presence of known ROS inhibitors, and compared to the procoagulant activity of platelets undergoing apoptosis. Platelet activation via GPVI has been associated with significant inter-individual variability in PS exposure as well as other platelet responses. Around 30-35% of this variation can be accounted for by the effect of a haplotype in GPVI (Jones*, et al* 2007, Joutsi-Korhonen*, et al* 2003). While additional 10-20% of variability can be attributed to other genetic polymorphism (Jones*, et al* 2009), the remainder could be partially or completely due to the level of ROS generated by the platelets, lipid peroxidation or antioxidant capacity (GPx and SOD levels). Emerging data suggest that GPVI signalling is regulated by ROS generation and antioxidant capacity level upon activation, and it is known that this pathway is the major component of the 'flip-flop' of PS in platelets.

Inhibitors of ROS from different sources were tested for their effect on GPVI activation of platelets' these included inhibitors of the 12-LOX pathway (esculetin), the COX-1 pathway (aspirin), the NAD(P)H oxidase complex pathways (apocynin and DPI), ROS scavengers (tempol and tiron), NOS (L-NAME) and antioxidants (NAC, BHT and vitamin E). Apocynin and tempol attenuate thrombin generation in platelets and PDMP procoagulant activity. None of these inhibitors had any effect on platelet responses

including degranulation (P-selectin expression) and the activation of αIIbβ3 (fibrinogen binding). All inhibitors (apocynin, esculetin, tempol, BHT, L-NAME), but not tiron, attenuate CRP-XL-induced aggregation, but these inhibitors had no effect on αIIbβ3 activation (fibrinogen binding) or P-selectin expression because aggregation is associated with the activation of outside-in signalling, which might suggest that degranulation is not dependent on ROS production. An upstream inhibitor of the GPVI signalling pathway, the PI3K, was used as +ve control to show that if the pathway is blocked, all aspects of platelet activation are diminished. As anticipated, the PI3K inhibitor wortmannin significantly down-regulates all aspects of platelet responses, including thrombin generation by platelets and PDMPs, aggregation and degranulation.

Platelet-platelet interaction (aggregation) induces PS exposure, which illustrates the important role of outside-in signalling, therefore enhancing thrombin generation. This platelet-platelet interaction is abolished by blocking the αIIbβ3 bridge that facilitates this interaction, because the use of anti- αIIbβ3 (RFGP56) has been shown to inhibit platelet-induced thrombin generation under flow conditions and under in conditions (Thomas*, et al* 2003).

In particular, the NAD(P)H oxidase pathway seems to be involved in PS exposure, $TxB_2$ production and 12-HETE generation, as well as the incorporation of esterified 12-HETE into PE, but not degranulation, as apocynin attenuates the procoagulant activity, $TxB_2$ production, 12-HETE generation, and the incorporation of esterified 12-HETE into PE, which might be an upstream of 12-LOX pathway, but a downstream of degranulation

(P-selectin expression), as the data in the current study suggest a key role of the NADP(H) oxidase and 12-LOX pathways. The data indicate that the effect on thrombin generation is not due to $TxB_2$, but may be due to 12-HETE, because aspirin did inhibit $TxB_2$ (but not 12-HETE) but had no effect on thrombin generation. Furthermore, esculetin, a non-specific inhibitor of the 12-LOX pathway had a modest effect on thrombin generation and PS exposure as well as on both $TxB_2$ and 12-HETE generation, and significantly attenuated the incorporation of esterified 12-HETE into PE. The importance of NAD(P)H oxidase and 12-LOX was initially highlighted by the study of MP formation from platelets by the Karpatkin group; Nardi et al (2007) have shown that PDMP formation upon stimulation by A23187, PMA or Abs against platelet integrin GPIIIa49-66 is mediated by ROS production via the NAD(P)H oxidase pathway. These data are in agreement with this study's observation that ROS was involved in the formation of the procoagulant activity of PDMPs, as using known ROS inhibitors including apocynin and tempol attenuated thrombin generation and PS exposure. In addition, it was also found that the procoagulant activity on the surface of activated platelets via GPVI receptor is also mediated by the NAD(P)H oxidase pathway, which might be an upstream of 12-LOX pathway.

Finally, we (Vogler, *et al* 2011) clarify the conflicting data in the literature on the involvement of apoptotic features in platelet activation including caspase activation and the loss of $\Delta\Psi m$. In this study, apoptosis in platelets was induced by the intrinsic pathway using a Bcl-xL/Bcl2 inhibitor, which shows that PS is a feature of platelet apoptosis (Fig 8.1), similar to activation, but does not induce procoagulant MP formation, which was indirectly measured by the thrombin generation assay. This is

unlike activation which was significantly associated with MP formation. Platelets undergoing apoptosis, unlike activated platelets, lose GPIbα (adhesion receptor which bind to vWF), are not able to degranulate (no P-selection expression) and do not express activated αIIbβ3 (GPIIb-IIIa, that binds fibrinogen to facilitate aggregation) (Fig 8.1), which is in agreement with other recently-published observations (Schoenwaelder*, et al* 2011, Schoenwaelder*, et al* 2009, Vogler*, et al* 2011). It also loses the collagen receptor, GPVI (Schoenwaelder*, et al* 2011), which suggests that platelets in which the apoptotic machinery has commenced lose their ability to take part in a thrombotic response. Although they do express a procoagulant surface PS this probably serves as a trigger for their clearance by macrophages and RE cells from circulation. In addition, unlike activated platelets, these apoptotic platelets have less ability to generate massive amounts of thrombin because they are less able to amplify thrombin generation.

The negatively-charged surface, mainly PS, is important in regulating thrombin production, which can be exposed by activated platelets and apoptotic platelets. There are compelling observations indicate the significant effect of oxidative lipid "Lipid peroxidation" on thrombin generation. Oxidation has an effect on the membrane architecture and chemical compositions, so it affects the membrane PLs' bilayers, and those oxidized PLs in form of esterified 12-HETE-PE or esterified 12-HETE-PC induced by thrombin have shown to be thrombogenic (Thomas*, et al* 2010). ROS can be released from activated platelets and apoptotic platelets, which can oxidize the membrane PLS and have been shown to induce the oxidation of lipoproteins (Gorog and Kovacs 1995). Oxidized lipoproteins have shown to accelerate prothrombinase

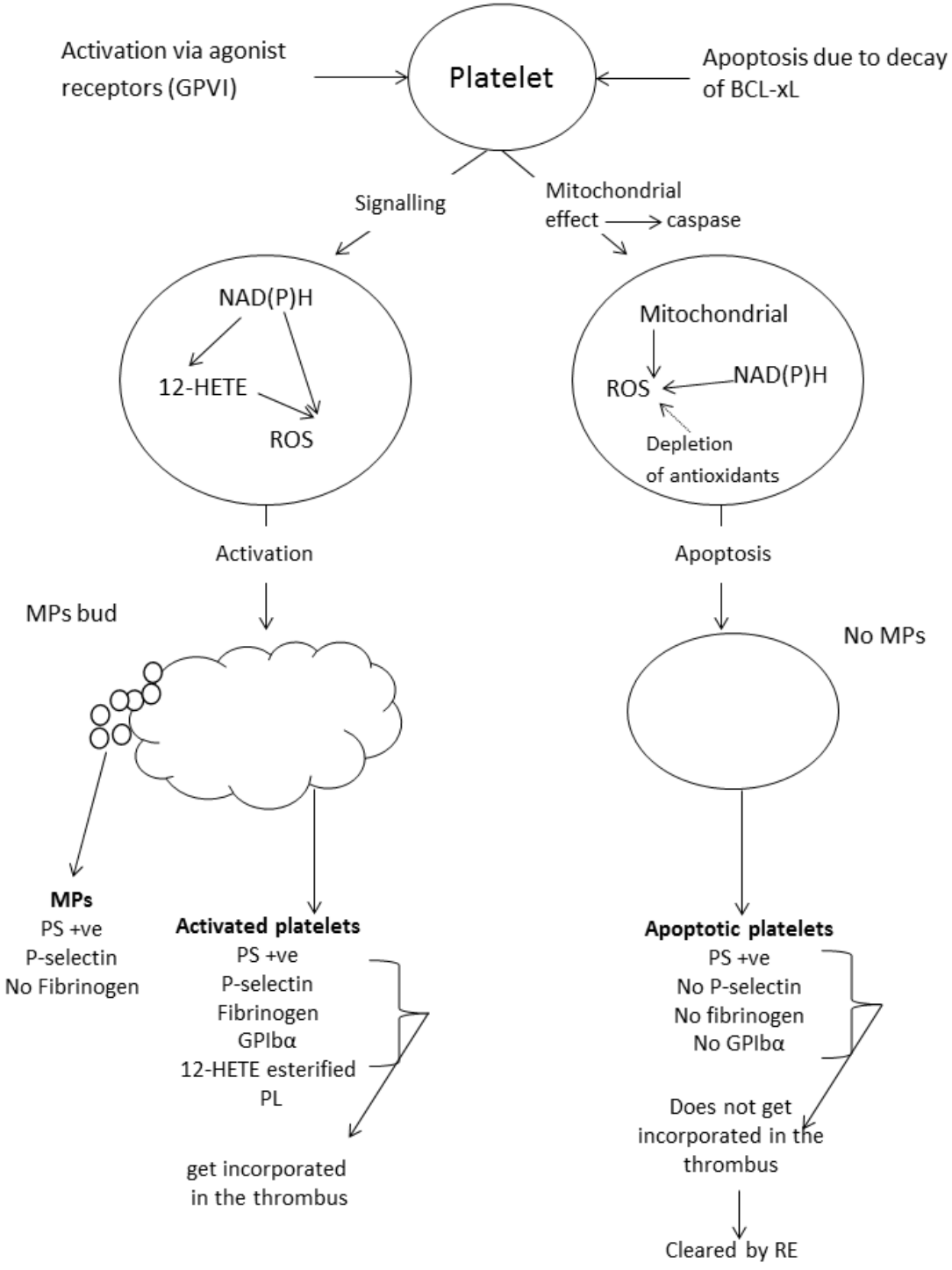


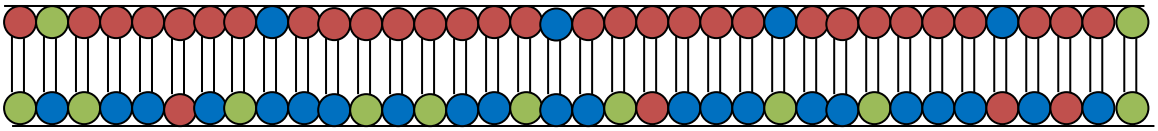

**Fig 8.1. Suggested mechanisms of platelet apoptosis and activation.**

activity and generate more thrombin (Rota*, et al* 1998a). Also, lipid peroxidation of membrane PLs from nucleated cells undergoing apoptosis has been shown to enhance thrombin generation (Pickering*, et al* 2008, Wang*, et al* 2001).

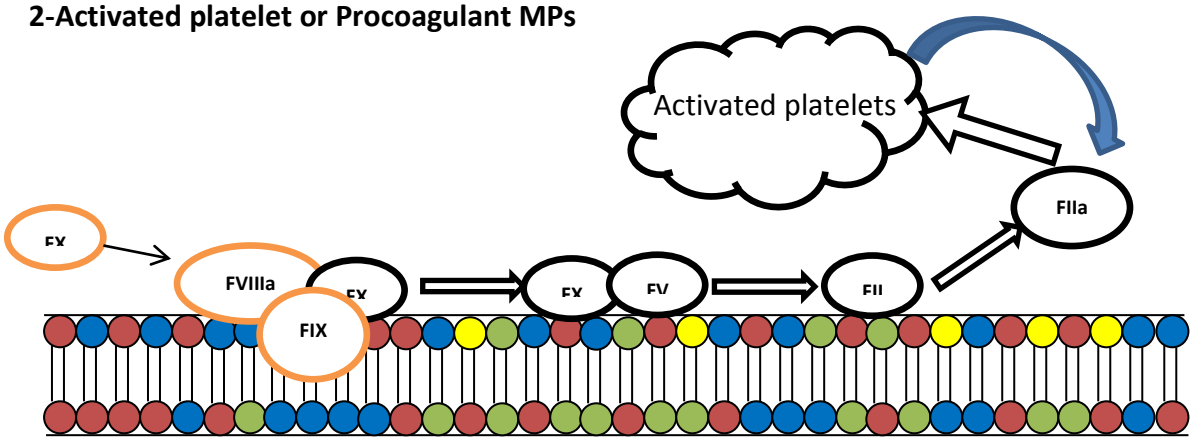
Thrombin generation was significantly attenuated in apoptosis but not in activation by two different non-enzymatic antioxidants, BHT and NAC, whether this is the same for enzymatic antioxidants should be investigated. A more significant inhibition of thrombin generation in apoptotic platelets was associated with the inhibition of NAD(P)H oxidase complex pathway using apocynin, similar to the inhibition in activation. The data proposed a PS exposure mechanism dependent on/mediated by the NAD(P)H oxidase complex pathway, the main producer of ROS in platelets, as well as other cells. Also, the data in this study illustrates that more than one type of ROS is produced upon activation, and that it is different from apoptosis in both level and type. In addition, EO6 (T15) has been suggested to bind apoptotic cells but not viable cells (Chang*, et al* 1999), which can be used in further study to differentiate between apoptotic and activated platelets.

In the current study, we have shown that activated platelets and platelets undergoing apoptosis supported more thrombin generation than normal unstimulated platelets, mainly driven by the procoagulant surface (Fig 8.2). The PT showed that the procoagulant activity (rate of thrombin generation) is faster in apoptotic platelets than in activated platelets, mainly because PS is highly expressed on apoptotic platelets compared to activated platelets as shown by annexin-V binding, but the total amount

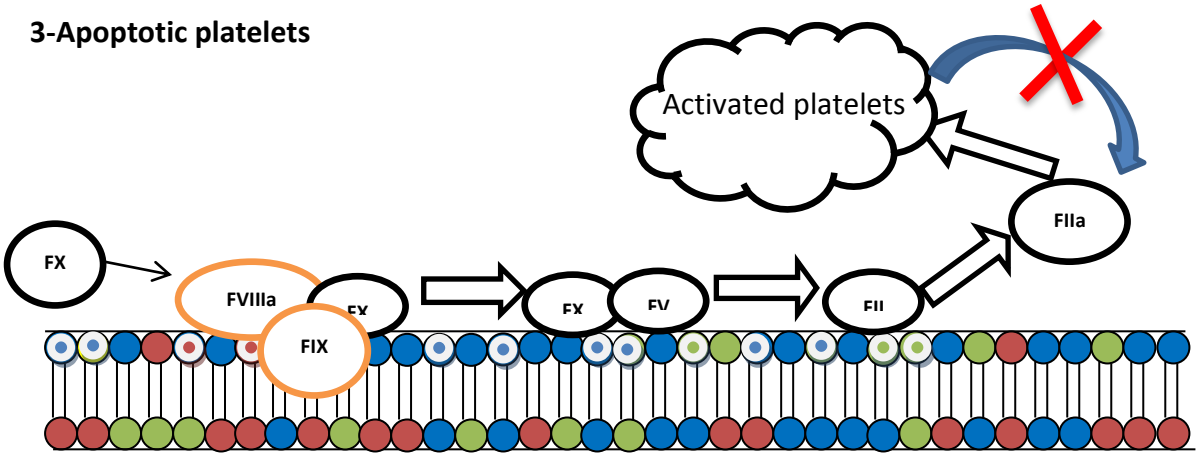
**1-Resting platelet**



**2-Activated platelet or Procoagulant MPs**




**3-Apoptotic platelets**




Phosphatidylcholine (PC) Phosphotidylserine (PS) Phosphatidylethanolamine (PE)

Oxidized PC Oxidized PS Oxidized PE 12-HETE esterified PL.

low medium high rate of binding and activation

**Fig 8.2: Potential types of membrane PLs in (A) resting platelets (B) activated platelets and (C) apoptotic platelets.**

of thrombin generated over one hour (ETP) was lower in apoptotic cells than in activated, because those cells cannot get activated by the feedback loop of thrombin, no able to degranulate, there is no expression of GPIbα to binds thrombin and coagulation factors, and no functional integrin αIIbβ3 to facilitate.

**Conclusion**:

- GPVI signalling induces the generation of a procoagulant surface in platelets and generates procoagulant PDMPs.
- Procoagulant PDMPs enhance thrombin generation, which can be accurately, evaluated using the 1pM TF reagent in both healthy subjects and patients with thrombotic disease states, which can be used as an independent risk factor.
- Platelets are more able to generate thrombin than PDMPs because they can still respond to the feedback from generating thrombin in combination with collagen, which drives the initial steps of platelet activation, but PDMPs are procoagulant than activated platelets because they bind more annexin-V.
- ROS has shown to contribute partially to the production of the procoagulant surface on platelets and maybe to the production of PDMPs.
- Platelet activation is distinct from platelet apoptosis, with a common feature between the two processes being PS exposure and enhancement of thrombin generation.
- Oxidized PL in apoptosis, unlike activation, enhances the rate of the reaction of thrombin generation, but not the total amount of thrombin generated, which are mainly affected by antioxidants.

# 9. References

Abid Hussein, M.N., Boing, A.N., Sturk, A., Hau, C.M. & Nieuwland, R. (2007) Inhibition of microparticle release triggers endothelial cell apoptosis and detachment. *Thromb Haemost,* **98,** 1096-1107.

Abrams, C.S., Ellison, N., Budzynski, A.Z. & Shattil, S.J. (1990) Direct detection of activated platelets and platelet-derived microparticles in humans. *Blood,* **75,** 128-138.

Ahamed, J., Versteeg, H.H., Kerver, M., Chen, V.M., Mueller, B.M., Hogg, P.J. & Ruf, W. (2006) Disulfide isomerization switches tissue factor from coagulation to cell signaling. *Proc Natl Acad Sci U S A,* **103,** 13932-13937.

Albanyan, A.M., Murphy, M.F., Rasmussen, J.T., Heegaard, C.W. & Harrison, P. (2009) Measurement of phosphatidylserine exposure during storage of platelet concentrates using the novel probe lactadherin: a comparison study with annexin V. *Transfusion,* **49,** 99-107.

Alberio, L., Safa, O., Clemetson, K.J., Esmon, C.T. & Dale, G.L. (2000) Surface expression and functional characterization of alpha-granule factor V in human platelets: effects of ionophore A23187, thrombin, collagen, and convulxin. *Blood,* **95,** 1694-1702.

Altman, R., Scazziota, A., Herrera, L. & Gonzalez, C. (2007) Relationship between thrombin generation and international normalized ratio in patients receiving oral vitamin K antagonist therapy. *Journal of Thrombosis and Haemostasis,* **5,** 1552-1554.

Amabile, N., Guerin, A.P., Leroyer, A., Mallat, Z., Nguyen, C., Boddaert, J., London, G.M., Tedgui, A. & Boulanger, C.M. (2005) Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure. *J Am Soc Nephrol,* **16,** 3381-3388.

Ando, M., Iwata, A., Ozeki, Y., Tsuchiya, K., Akiba, T. & Nihei, H. (2002) Circulating platelet-derived microparticles with procoagulant activity may be a potential cause of thrombosis in uremic patients. *Kidney International,* **62,** 1757-1763.

Andree, H.A., Reutelingsperger, C.P., Hauptmann, R., Hemker, H.C., Hermens, W.T. & Willems, G.M. (1990) Binding of vascular anticoagulant alpha (VAC alpha) to planar phospholipid bilayers. *J Biol Chem,* **265,** 4923-4928.

Andrews, R.K. & Berndt, M.C. (2004) Platelet physiology and thrombosis. *Thromb Res,* **114,** 447-453.

Andrews, R.K., Suzuki-Inoue, K., Shen, Y., Tulasne, D., Watson, S.P. & Berndt, M.C. (2002) Interaction of calmodulin with the cytoplasmic domain of platelet glycoprotein VI. *Blood,* **99,** 4219-4221.

Appleby, J.A., Appleby, J.A. & Goodall, A.H. (2006) Role of platelets in the procoagulant environment in blood. *PhD thesis*.

Arachiche, A., Kerbiriou-Nabias, D., Garcin, I., Letellier, T. & Dachary-Prigent, J. (2009) Rapid procoagulant phosphatidylserine exposure relies on high cytosolic calcium rather than on mitochondrial depolarization. *Arterioscler Thromb Vasc Biol,* **29,** 1883-1889.

Aras, O., Shet, A., Bach, R.R., Hysjulien, J.L., Slungaard, A., Hebbel, R.P., Escolar, G., Jilma, B. & Key, N.S. (2004) Induction of microparticle- and cell-associated intravascular tissue factor in human endotoxemia. *Blood,* **103,** 4545-4553.

Arthur, J.F., Dunkley, S. & Andrews, R.K. (2007) Platelet glycoprotein VI-related clinical defects. *Br J Haematol,* **139,** 363-372.

Arthur, J.F., Gardiner, E.E., Kenny, D., Andrews, R.K. & Berndt, M.C. (2008) Platelet receptor redox regulation. *Platelets,* **19,** 1-8.

Arthur, J.F., Shen, Y., Mu, F.T., Leon, C., Gachet, C., Berndt, M.C. & Andrews, R.K. (2006) Calmodulin interacts with the platelet ADP receptor P2Y1. *Biochem J,* **398,** 339-343.

Asazuma, N., Ozaki, Y., Satoh, K., Yatomi, Y., Handa, M., Fujimura, Y., Miura, S. & Kume, S. (1997) Glycoprotein Ib-von Willebrand factor interactions activate tyrosine kinases in human platelets. *Blood,* **90,** 4789-4798.

Ashkenazi, A. & Dixit, V.M. (1998) Death receptors: signaling and modulation. *Science,* **281,** 1305-1308.

Ay, C., Freyssinet, J.M., Sailer, T., Vormittag, R. & Pabinger, I. (2009) Circulating procoagulant microparticles in patients with venous thromboembolism. *Thromb Res,* **123,** 724-726.

Ayers, L., Kohler, M., Harrison, P., Sargent, I., Dragovic, R., Schaap, M., Nieuwland, R., Brooks, S.A. & Ferry, B. (2011) Measurement of circulating cell-derived microparticles by flow cytometry: sources of variability within the assay. *Thromb Res,* **127,** 370-377.

Bach, R., Gentry, R. & Nemerson, Y. (1986) Factor VII binding to tissue factor in reconstituted phospholipid vesicles: induction of cooperativity by phosphatidylserine. *Biochemistry,* **25,** 4007-4020.

Bach, R. & Rifkin, D.B. (1990) Expression of tissue factor procoagulant activity: regulation by cytosolic calcium. *Proc Natl Acad Sci U S A,* **87,** 6995-6999.

Baglia, F.A., Badellino, K.O., Li, C.Q., Lopez, J.A. & Walsh, P.N. (2002) Factor XI binding to the platelet glycoprotein Ib-IX-V complex promotes factor XI activation by thrombin. *J Biol Chem,* **277,** 1662-1668.

Bajaj, M.S., Birktoft, J.J., Steer, S.A. & Bajaj, S.P. (2001) Structure and biology of tissue factor pathway inhibitor. *Thromb Haemost,* **86,** 959-972.

Bal, L., Ederhy, S., Di Angelantonio, E., Toti, F., Zobairi, F., Dufaitre, G., Meuleman, C., Mallat, Z., Boccara, F., Tedgui, A., Freyssinet, J.M. & Cohen, A. (2009) Circulating procoagulant microparticles in acute pulmonary embolism: a case-control study. *Int J Cardiol,* **145,** 321-322.

Bandi, A., Mosialos, L., Katsiki, N., Zarifis, N., Vessara, J., Kaprinis, I., Kiskinis, D., Elalamy, I. & Gerotziafas, G.T. (2007) Treatment with aspirin and clopidogrel inhibit thrombin generation in patinetss with stable coronory artery disease. *Joirnal of thrombosis and haemostasis,* **7**.

Barry, O.P., Pratico, D., Lawson, J.A. & FitzGerald, G.A. (1997) Transcellular activation of platelets and endothelial cells by bioactive lipids in platelet microparticles. *The Journal of Clinical Investigation,* **99,** 2118-2127.

Begonja, A.J., Gambaryan, S., Geiger, J., Aktas, B., Pozgajova, M., Nieswandt, B. & Walter, U. (2005) Platelet NAD(P)H-oxidase-generated ROS production regulates {alpha}IIb{beta}3-integrin activation independent of the NO/cGMP pathway. *Blood,* **106,** 2757-2760.

Beltran-Miranda, C.P., Khan, A., Jaloma-Cruz, A.R. & Laffan, M.A. (2005) Thrombin generation and phenotypic correlation in haemophilia A. *Haemophilia,* **11,** 326-334.

Bennett, M.R., Gibson, D.F., Schwartz, S.M. & Tait, J.F. (1995) Binding and phagocytosis of apoptotic vascular smooth muscle cells is mediated in part by exposure of phosphatidylserine. *Circ Res,* **77,** 1136-1142.

Berckmans, R.J., Nieuwland, R., Boing, A.N., Romijn, F.P., Hack, C.E. & Sturk, A. (2001) Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. *Thromb Haemost,* **85,** 639-646.

Bernal-Mizrachi, L., Jy, W., Fierro, C., Macdonough, R., Velazques, H.A., Purow, J., Jimenez, J.J., Horstman, L.L., Ferreira, A., de Marchena, E. & Ahn, Y.S. (2004) Endothelial microparticles correlate with high-risk angiographic lesions in acute coronary syndromes. *International Journal of Cardiology,* **97,** 439-446.

Bernal-Mizrachi, L., Jy, W., Jimenez, J.J., Pastor, J., Mauro, L.M., Horstman, L.L., de Marchena, E. & Ahn, Y.S. (2003) High levels of circulating endothelial microparticles in patients with acute coronary syndromes. *Am Heart J,* **145,** 962-970.

Berndt, M.C., Arthur, J.F., Shen, Y., Gardiner, E.E., Coleman, L., Kenny, D. & Andrews, R.K. (2010) TNF-receptor associated factor 4 (TRAF4) is a novel binding partner of glycoprotein IB and glycoproetin VI - a potential link to redox-dependent signalling in human platelets. *Platelets,* **21,** 393-419.

Bertino, A.M., Qi, X.Q., Li, J., Xia, Y. & Kuter, D.J. (2003) Apoptotic markers are increased in platelets stored at 37°C. *Transfusion,* **43,** 857-866.

Bevers, E.M., Comfurius, P., Dekkers, D.W., Harmsma, M. & Zwaal, R.F. (1998) Transmembrane phospholipid distribution in blood cells: control mechanisms and pathophysiological significance. *Biol Chem,* **379,** 973-986.

Bevers, E.M., Comfurius, P., Dekkers, D.W. & Zwaal, R.F. (1999) Lipid translocation across the plasma membrane of mammalian cells. *Biochim Biophys Acta,* **1439,** 317-330.

Bevers, E.M., Comfurius, P., van Rijn, J.L., Hemker, H.C. & Zwaal, R.F. (1982) Generation of prothrombin-converting activity and the exposure of phosphatidylserine at the outer surface of platelets. *Eur J Biochem,* **122,** 429-436.

Bevers, E.M., Comfurius, P. & Zwaal, R.F. (1983) Changes in membrane phospholipid distribution during platelet activation. *Biochim Biophys Acta,* **736,** 57-66.

Bevers, E.M., Comfurius, P. & Zwaal, R.F. (1991) Platelet procoagulant activity: physiological significance and mechanisms of exposure. *Blood Rev,* **5,** 146-154.

Bidot, L., Jy, W., Bidot, C., Jimenez, J.J., Fontana, V., Horstman, L.L. & Ahn, Y.S. (2008) Microparticle-mediated thrombin generation assay: increased activity in patients with recurrent thrombosis. *Journal of Thrombosis and Haemostasis,* **6,** 913-919.

Bigalke, B., Lindemann, S., Ehlers, R., Seizer, P., Daub, K., Langer, H., Schonberger, T., Kremmer, E., Siegel-Axel, D., May, A.E. & Gawaz, M. (2006) Expression of platelet collagen receptor glycoprotein VI is associated with acute coronary syndrome. *Eur Heart J,* **27,** 2165-2169.

Biro, E., Sturk-Maquelin, K.N., Vogel, G.M., Meuleman, D.G., Smit, M.J., Hack, C.E., Sturk, A. & Nieuwland, R. (2003) Human cell-derived microparticles promote thrombus formation in vivo in a tissue factor-dependent manner. *J Thromb Haemost,* **1,** 2561-2568.

Bode, A.P., Sandberg, H., Dombrose, F.A. & Lentz, B.R. (1985) Association of factor V activity with membranous vesicles released from human platelets: requirement for platelet stimulation. *Thromb Res,* **39,** 49-61.

Boersma, E., Harrington, R.A., Moliterno, D.J., White, H., Théroux, P., Van de Werf, F., de Torbal, A., Armstrong, P.W., Wallentin, L.C., Wilcox, R.G., Simes, J., Califf, R.M., Topol, E.J. & Simoons, M.L. (2002) Platelet glycoprotein IIb/IIIa inhibitors in acute coronary syndromes: a meta-analysis of all major randomised clinical trials. *The Lancet,* **359,** 189-198.

Boesze-Battaglia, K. & Schimmel, R. (1997) Cell membrane lipid composition and distribution: implications for cell function and lessons learned from photoreceptors and platelets. *J Exp Biol,* **200,** 2927-2936.

Bogdanov, V.Y., Balasubramanian, V., Hathcock, J., Vele, O., Lieb, M. & Nemerson, Y. (2003) Alternatively spliced human tissue factor: a circulating, soluble, thrombogenic protein. *Nat Med,* **9,** 458-462.

Bom, V.J. & Bertina, R.M. (1990) The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII. *Biochem J,* **265,** 327-336.

Bonderman, D., Teml, A., Jakowitsch, J., Adlbrecht, C., Gyongyosi, M., Sperker, W., Lass, H., Mosgoeller, W., Glogar, D.H., Probst, P., Maurer, G., Nemerson, Y. & Lang, I.M. (2002) Coronary no-reflow is caused by

shedding of active tissue factor from dissected atherosclerotic plaque. *Blood,* **99,** 2794-2800.

Born, G.V. & Cross, M.J. (1963) The Aggregation of Blood Platelets. *J Physiol,* **168,** 178-195.

Boskovic, D.S., Bajzar, L.S. & Nesheim, M.E. (2001) Channeling during prothrombin activation. *J Biol Chem,* **276,** 28686-28693.

Bradford, H.N., Pixley, R.A. & Colman, R.W. (2000) Human factor XII binding to the glycoprotein Ib-IX-V complex inhibits thrombin-induced platelet aggregation. *J Biol Chem,* **275,** 22756-22763.

Bretscher, M.S. (1972) Asymmetrical lipid bilayer structure for biological membranes. *Nat New Biol,* **236,** 11-12.

Brodin, E., Seljeflot, I., Arnesen, H., Hurlen, M., Appelbom, H. & Hansen, J.B. (2009) Endogenous thrombin potential (ETP) in plasma from patients with AMI during antithrombotic treatment. *Thrombosis Research,* **123,** 573-579.

Broekman, M.J., Eiroa, A.M. & Marcus, A.J. (1991) Inhibition of human platelet reactivity by endothelium-derived relaxing factor from human umbilical vein endothelial cells in suspension: blockade of aggregation and secretion by an aspirin-insensitive mechanism. *Blood,* **78,** 1033-1040.

Brooks, M.B., Catalfamo, J.L., Brown, H.A., Ivanova, P. & Lovaglio, J. (2002) A hereditary bleeding disorder of dogs caused by a lack of platelet procoagulant activity. *Blood,* **99,** 2434-2441.

Burnier, L., Fontana, P., Kwak, B.R. & Angelillo-Scherrer, A. (2009) Cell-derived microparticles in haemostasis and vascular medicine. *Thromb Haemost,* **101,** 439-451.

Burton, G.W., Joyce, A. & Ingold, K.U. (1982) First proof that vitamin E is major lipid-soluble, chain-breaking antioxidant in human blood plasma. *Lancet,* **2,** 327.

Burton, J., Hamali, H., Stansfield, R., Patel, A., Goodall, A. & Brunskill, N. (Submitted) Elevated levels of procoagulant plasma microparticles in dialysis patients. *Kidney international*.

Butenas, S. & Mann, K.G. (2004) Active tissue factor in blood? *Nat Med,* **10,** 1155-1156; author reply 1156.

Butenas, S., van 't Veer, C. & Mann, K.G. (1997) Evaluation of the initiation phase of blood coagulation using ultrasensitive assays for serine proteases. *J Biol Chem,* **272,** 21527-21533.

Byzova, T.V. & Plow, E.F. (1997) Networking in the hemostatic system. Integrin alphaiibbeta3 binds prothrombin and influences its activation. *J Biol Chem,* **272,** 27183-27188.

Cabeza, N., Li, Z., Schulz, C., Kremmer, E., Massberg, S., BÃ¼ltmann, A. & Gawaz, M. (2004) Surface Expression of Collagen Receptor Fc Receptor-Î³/Glycoprotein VI Is Enhanced on Platelets in Type 2 Diabetes and

Mediates Release of CD40 Ligand and Activation of Endothelial Cells. *Diabetes,* **53,** 2117-2121.

Caccese, D., Pratico, D., Ghiselli, A., Natoli, S., Pignatelli, P., Sanguigni, V., Iuliano, L. & Violi, F. (2000) Superoxide anion and hydroxyl radical release by collagen-induced platelet aggregation--role of arachidonic acid metabolism. *Thromb Haemost,* **83,** 485-490.

Carson, S.D. & Konigsberg, W.H. (1981) Coagulation factor III (tissue factor) interaction with phospholipid vesicles induced by cadmium: characterization of the reconstituted protein-membrane complex. *Biosci Rep,* **1,** 197-205.

Casciola-Rosen, L., Rosen, A., Petri, M. & Schlissel, M. (1996) Surface blebs on apoptotic cells are sites of enhanced procoagulant activity: implications for coagulation events and antigenic spread in systemic lupus erythematosus. *Proc Natl Acad Sci U S A,* **93,** 1624-1629.

Chakrabarti, S., Clutton, P., Varghese, S., Cox, D., Mascelli, M.A. & Freedman, J.E. (2004) Glycoprotein IIb/IIIa inhibition enhances platelet nitric oxide release. *Thrombosis Research,* **113,** 225-233.

Chang, M.-K., Bergmark, C., Laurila, A., H¡¶rkk¡¶, S., Han, K.-H., Friedman, P., Dennis, E.A. & Witztum, J.L. (1999) Monoclonal antibodies against oxidized low-density lipoprotein bind to apoptotic cells and inhibit their phagocytosis by elicited macrophages: Evidence that oxidation-specific epitopes mediate macrophage recognition. *Proceedings of the National Academy of Sciences,* **96,** 6353-6358.

Chantarangkul, V., Clerici, M., Bressi, C., Giesen, P.L.A. & Tripodi, A. (2003) Thrombin generation assessed as endogenous thrombin potential in patients with hyper- or hypo-coagulability. *Haematologica,* **88,** 547-554.

Chantarangkul, V., Clerici, M., Bressi, C. & Tripodi, A. (2004) Standardization of the endogenous thrombin potential measurement: how to minimize the effect of residual platelets in stored plasma. *Br J Haematol,* **124,** 355-357.

Chen, L.Y. & Mehta, J.L. (1996) Variable effects of L-arginine analogs on L-arginine-nitric oxide pathway in human neutrophils and platelets may relate to different nitric oxide synthase isoforms. *J Pharmacol Exp Ther,* **276,** 253-257.

Chen, V.M., Ahamed, J., Versteeg, H.H., Berndt, M.C., Ruf, W. & Hogg, P.J. (2006) Evidence for activation of tissue factor by an allosteric disulfide bond. *Biochemistry,* **45,** 12020-12028.

Cheng, G., Diebold, B.A., Hughes, Y. & Lambeth, J.D. (2006) Nox1-dependent Reactive Oxygen Generation Is Regulated by Rac1. *Journal of Biological Chemistry,* **281,** 17718-17726.

Chlopicki, S., Olszanecki, R., Janiszewski, M., Laurindo, F.R., Panz, T. & Miedzobrodzki, J. (2004) Functional role of NADPH oxidase in activation of platelets. *Antioxid Redox Signal,* **6,** 691-698.

Cho, J., Furie, B.C., Coughlin, S.R. & Furie, B. (2008) A critical role for extracellular protein disulfide isomerase during thrombus formation in mice. *J Clin Invest,* **118,** 1123-1131.

Chung, T.W., Tyan, Y.C., Hsieh, J.H., Wang, S.S. & Chu, S.H. (2002) Shear stress-induced aggregation of oxidized platelets. *Thromb Res,* **105,** 325-329.

Ciferri, S., Emiliani, C., Guglielmini, G., Orlacchio, A., Nenci, G.G. & Gresele, P. (2000) Platelets release their lysosomal content in vivo in humans upon activation. *Thromb Haemost,* **83,** 157-164.

Clemetson, J.M., Polgar, J.s., Magnenat, E., Wells, T.N.C. & Clemetson, K.J. (1999) The Platelet Collagen Receptor Glycoprotein VI Is a Member of the Immunoglobulin Superfamily Closely Related to FcÎ±R and the Natural Killer Receptors. *Journal of Biological Chemistry,* **274,** 29019-29024.

Clemetson, K.J. (1995) Platelet activation: signal transduction via membrane receptors. *Thromb Haemost,* **74,** 111-116.

Clutton, P., Miermont, A. & Freedman, J.E. (2004) Regulation of endogenous reactive oxygen species in platelets can reverse aggregation. *Arterioscler Thromb Vasc Biol,* **24,** 187-192.

Coffey, M.J., Jarvis, G.E., Gibbins, J.M., Coles, B., Barrett, N.E., Wylie, O.R. & O'Donnell, V.B. (2004) Platelet 12-lipoxygenase activation via glycoprotein VI: involvement of multiple signaling pathways in agonist control of H(P)ETE synthesis. *Circ Res,* **94,** 1598-1605.

Cohen, H., Neild, G.H., Patel, R., Mackie, I.J. & Machin, S.J. (1988) Evidence for chronic platelet hyperaggregability and in vivo activation in cyclosporin-treated renal allograft recipients. *Thromb Res,* **49,** 91-101.

Coller, B.S. (1997) Platelet GPIIb/IIIa antagonists: the first anti-integrin receptor therapeutics. *J Clin Invest,* **100,** S57-60.

Coller, B.S., Peerschke, E.I., Scudder, L.E. & Sullivan, C.A. (1983) Studies with a murine monoclonal antibody that abolishes ristocetin-induced binding of von Willebrand factor to platelets: additional evidence in support of GPIb as a platelet receptor for von Willebrand factor. *Blood,* **61,** 99-110.

Comfurius, P., Senden, J.M., Tilly, R.H., Schroit, A.J., Bevers, E.M. & Zwaal, R.F. (1990) Loss of membrane phospholipid asymmetry in platelets and red cells may be associated with calcium-induced shedding of plasma membrane and inhibition of aminophospholipid translocase. *Biochim Biophys Acta,* **1026,** 153-160.

Croft, S.A., Samani, N.J., Teare, M.D., Hampton, K.K., Steeds, R.P., Channer, K.S. & Daly, M.E. (2001) Novel platelet membrane glycoprotein VI dimorphism is a risk factor for myocardial infarction. *Circulation,* **104,** 1459-1463.

Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem J,* **341 ( Pt 2),** 233-249.

Dale, G.L., Friese, P., Batar, P., Hamilton, S.F., Reed, G.L., Jackson, K.W., Clemetson, K.J. & Alberio, L. (2002) Stimulated platelets use serotonin to enhance their retention of procoagulant proteins on the cell surface. *Nature,* **415,** 175-179.

Dale, G.L., Remenyi, G. & Friese, P. (2005) Quantitation of microparticles released from coated-platelets. *J Thromb Haemost,* **3,** 2081-2088.

Danial, N.N. & Korsmeyer, S.J. (2004) Cell death: critical control points. *Cell,* **116,** 205-219.

Dargaud, Y., Beguin, S., Lienhart, A., Al Dieri, R., Trzeciak, C., Bordet, J.C., Hemker, H.C. & Negrier, C. (2005) Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. *Thromb Haemost,* **93,** 475-480.

Dargaud, Y., Luddington, R., Gray, E., Negrier, C., Lecompte, T., Petros, S., Hogwood, J., Bordet, J.-C., Regnault, V., Siegemund, A. & Baglin, T. (2007) Effect of standardization and normalization on imprecision of calibrated automated thrombography: an international multicentre study. *British Journal of Haematology,* **139,** 303-309.

Davie, E.W., Fujikawa, K. & Kisiel, W. (1991) The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry,* **30,** 10363-10370.

Davie, E.W. & Ratnoff, O.D. (1964) Waterfall sequence for intrinsic blood clotting. *Science,* **145,** 1310-1312.

de Graaf, J.C., Banga, J.D., Moncada, S., Palmer, R.M., de Groot, P.G. & Sixma, J.J. (1992) Nitric oxide functions as an inhibitor of platelet adhesion under flow conditions. *Circulation,* **85,** 2284-2290.

De Marco, L., Mazzucato, M., Masotti, A. & Ruggeri, Z.M. (1994) Localization and characterization of an alpha-thrombin-binding site on platelet glycoprotein Ib alpha. *J Biol Chem,* **269,** 6478-6484.

Delaney, M.K., liu, J., Zheng, Y. & Du, X. (2010) An Important Role for Rac1 and RhoA in Regulating Platelet Microparticle Formation and Phosphatidylserine Exposure *Circulation,* **122**.

Denis, M.M., Tolley, N.D., Bunting, M., Schwertz, H., Jiang, H., Lindemann, S., Yost, C.C., Rubner, F.J., Albertine, K.H., Swoboda, K.J., Fratto, C.M., Tolley, E., Kraiss, L.W., McIntyre, T.M., Zimmerman, G.A. & Weyrich, A.S. (2005) Escaping the nuclear confines: signal-dependent pre-mRNA splicing in anucleate platelets. *Cell,* **122,** 379-391.

Dey-Hazra, E., Hertel, B., Kirsch, T., Woywodt, A., Lovric, S., Haller, H., Haubitz, M. & Erdbruegger, U. (2010) Detection of circulating microparticles by flow cytometry: influence of centrifugation, filtration of buffer, and freezing. *Vasc Health Risk Manag,* **6,** 1125-1133.

Diaz-Ricart, M., Estebanell, E., Lozano, M., Aznar-Salatti, J., White, J.G., Ordinas, A. & Escolar, G. (2000) Thrombin facilitates primary platelet adhesion

onto vascular surfaces in the absence of plasma adhesive proteins: studies under flow conditions. *Haematologica,* **85,** 280-288.

Dietrich-Muszalska, A., Olas, B. & Rabe-Jablonska, J. (2005) Oxidative stress in blood platelets from schizophrenic patients. *Platelets,* **16,** 386-391.

Drueke, T.B. (2000) Aspects of cardiovascular burden in pre-dialysis patients. *Nephron,* **85,** 9-14.

Dubois, C., Panicot-Dubois, L., Merrill-Skoloff, G., Furie, B. & Furie, B.C. (2006) Glycoprotein VI-dependent and -independent pathways of thrombus formation in vivo. *Blood,* **107,** 3902-3906.

Duchemin, J., Pan-Petesch, B., Arnaud, B., Blouch, M.T. & Abgrall, J.F. (2008) Influence of coagulation factors and tissue factor concentration on the thrombin generation test in plasma. *Thromb Haemost,* **99,** 767-773.

Dursun, I., Poyrazoglu, H.M., Gunduz, Z., Ulger, H., Yykylmaz, A., Dusunsel, R., Patyroglu, T. & Gurgoze, M. (2009) The relationship between circulating endothelial microparticles and arterial stiffness and atherosclerosis in children with chronic kidney disease. *Nephrol Dial Transplant,* **24,** 2511-2518.

Dusting, G.J., Selemidis, S. & Jiang, F. (2005) Mechanisms for suppressing NADPH oxidase in the vascular wall. *Mem Inst Oswaldo Cruz,* **100 Suppl 1,** 97-103.

Eichinger, S., Mannucci, P.M., Tradati, F., Arbini, A.A., Rosenberg, R.D. & Bauer, K.A. (1995) Determinants of plasma factor VIIa levels in humans. *Blood,* **86,** 3021-3025.

Eilertsen, K.E. & Osterud, B. (2004) Tissue factor: (patho)physiology and cellular biology. *Blood Coagul Fibrinolysis,* **15,** 521-538.

Emsley, J., Knight, C.G., Farndale, R.W., Barnes, M.J. & Liddington, R.C. (2000) Structural basis of collagen recognition by integrin alpha2beta1. *Cell,* **101,** 47-56.

Escolar, G., Cases, A., Bastida, E., Garrido, M., Lopez, J., Revert, L., Castillo, R. & Ordinas, A. (1990) Uremic platelets have a functional defect affecting the interaction of von Willebrand factor with glycoprotein IIb-IIIa. *Blood,* **76,** 1336-1340.

Esmon, C. (2000a) The protein C pathway. *Crit Care Med,* **28,** S44-48.

Esmon, C.T. (2000b) The endothelial cell protein C receptor. *Thromb Haemost,* **83,** 639-643.

Essayagh, S., Xuereb, J.M., Terrisse, A.D., Tellier-Cirioni, L., Pipy, B. & Sie, P. (2007) Microparticles from apoptotic monocytes induce transient platelet recruitment and tissue factor expression by cultured human vascular endothelial cells via a redox-sensitive mechanism. *Thromb Haemost,* **98,** 831-837.

Essex, D.W. & Li, M. (2003) Redox control of platelet aggregation. *Biochemistry,* **42,** 129-136.

Ezumi, Y., Shindoh, K., Tsuji, M. & Takayama, H. (1998) Physical and functional association of the Src family kinases Fyn and Lyn with the collagen receptor glycoprotein VI-Fc receptor gamma chain complex on human platelets. *J Exp Med,* **188,** 267-276.

Fabisiak, J.P., Kagan, V.E., Ritov, V.B., Johnson, D.E. & Lazo, J.S. (1997) Bcl-2 inhibits selective oxidation and externalization of phosphatidylserine during paraquat-induced apoptosis. *American Journal of Physiology - Cell Physiology,* **272,** C675-C684.

Fadok, V.A., Savill, J.S., Haslett, C., Bratton, D.L., Doherty, D.E., Campbell, P.A. & Henson, P.M. (1992a) Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J Immunol,* **149,** 4029-4035.

Fadok, V.A., Voelker, D.R., Campbell, P.A., Cohen, J.J., Bratton, D.L. & Henson, P.M. (1992b) Exposure of phosphatidylserine on the surface of apoptotic lymphocytes triggers specific recognition and removal by macrophages. *J Immunol,* **148,** 2207-2216.

Faure, V., Dou, L., Sabatier, F., Cerini, C., Sampol, J., Berland, Y., Brunet, P. & Dignat-George, F. (2006) Elevation of circulating endothelial microparticles in patients with chronic renal failure. *J Thromb Haemost,* **4,** 566-573.

Favaloro, E.J., Soltani, S. & McDonald, J. (2004) Potential laboratory misdiagnosis of hemophilia and von Willebrand disorder owing to cold activation of blood samples for testing. *Am J Clin Pathol,* **122,** 686-692.

Fitzgerald, D.J., Roy, L., Catella, F. & FitzGerald, G.A. (1986) Platelet activation in unstable coronary disease. *N Engl J Med,* **315,** 983-989.

Fleck, R.A., Rao, L.V., Rapaport, S.I. & Varki, N. (1990) Localization of human tissue factor antigen by immunostaining with monospecific, polyclonal anti-human tissue factor antibody. *Thromb Res,* **59,** 421-437.

Freedman, J.E. (2008) Oxidative stress and platelets. *Arterioscler Thromb Vasc Biol,* **28,** s11-16.

Freedman, J.E., Loscalzo, J., Barnard, M.R., Alpert, C., Keaney, J.F. & Michelson, A.D. (1997) Nitric oxide released from activated platelets inhibits platelet recruitment. *J Clin Invest,* **100,** 350-356.

Friedman, P., H¡¶rkk¡¶, S., Steinberg, D., Witztum, J.L. & Dennis, E.A. (2002) Correlation of Antiphospholipid Antibody Recognition with the Structure of Synthetic Oxidized Phospholipids. *Journal of Biological Chemistry,* **277,** 7010-7020.

Furie, B. & Furie, B.C. (2008) Mechanisms of Thrombous Formation. *NEJM,* **359,** 938-949.

Gailani, D. & Broze, G.J., Jr. (1991) Factor XI activation in a revised model of blood coagulation. *Science,* **253,** 909-912.

Gailani, D., Ho, D., Sun, M.F., Cheng, Q. & Walsh, P.N. (2001) Model for a factor IX activation complex on blood platelets: dimeric conformation of factor XIa is essential. *Blood,* **97,** 3117-3122.

Garcia, B.A., Smalley, D.M., Cho, H., Shabanowitz, J., Ley, K. & Hunt, D.F. (2005) The platelet microparticle proteome. *J Proteome Res,* **4,** 1516-1521.

Gardai, S.J., Bratton, D.L., Ogden, C.A. & Henson, P.M. (2006) Recognition ligands on apoptotic cells: a perspective. *J Leukoc Biol,* **79,** 896-903.

Gatt, A., van Veen, J.J., Woolley, A.M., Kitchen, S., Cooper, P. & Makris, M. (2008) Thrombin generation assays are superior to traditional tests in assessing anticoagulation reversal in vitro. *Thromb Haemost,* **100,** 350-355.

Gawaz, M., Langer, H. & May, A.E. (2005) Platelets in inflammation and atherogenesis. *J Clin Invest,* **115,** 3378-3384.

Gawaz, M.P., Dobos, G., Spath, M., Schollmeyer, P., Gurland, H.J. & Mujais, S.K. (1994) IMPAIRED FUNCTION OF PLATELET MEMBRANE GLYCOPROTEIN-IIB-IIIA IN END-STAGE RENAL-DISEASE. *Journal of the American Society of Nephrology,* **5,** 36-46.

Gemmell, C.H., Sefton, M.V. & Yeo, E.L. (1993) Platelet-derived microparticle formation involves glycoprotein IIb-IIIa. Inhibition by RGDS and a Glanzmann's thrombasthenia defect. *J Biol Chem,* **268,** 14586-14589.

Gerotziafas, G.T., Depasse, F., Busson, J., Leflem, L., Elalamy, I. & Samama, M.M. (2005) Towards a standardization of thrombin generation assessment: the influence of tissue factor, platelets and phospholipids concentration on the normal values of Thrombogram-Thrombinoscope assay. *Thromb J,* **3,** 16.

Gerotziafas, G.T., Depasse, F., Chakroun, T., Van Dreden, P., Samama, M.M. & Elalamy, I. (2004) Comparison of the effect of fondaparinux and enoxaparin on thrombin generation during in-vitro clotting of whole blood and platelet-rich plasma. *Blood Coagul Fibrinolysis,* **15,** 149-156.

Gibbins, J.M., Briddon, S., Shutes, A., van Vugt, M.J., van de Winkel, J.G., Saito, T. & Watson, S.P. (1998) The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin. *J Biol Chem,* **273,** 34437-34443.

Gibbins, J.M., Okuma, M., Farndale, R., Barnes, M. & Watson, S.P. (1997) Glycoprotein VI is the collagen receptor in platelets which underlies tyrosine phosphorylation of the Fc receptor gamma-chain. *FEBS Lett,* **413,** 255-259.

Giesen, P.L., Rauch, U., Bohrmann, B., Kling, D., Roque, M., Fallon, J.T., Badimon, J.J., Himber, J., Riederer, M.A. & Nemerson, Y. (1999) Blood-borne tissue factor: another view of thrombosis. *Proc Natl Acad Sci U S A,* **96,** 2311-2315.

Go, A.S., Chertow, G.M., Fan, D.J., McCulloch, C.E. & Hsu, C.Y. (2004) Chronic kidney disease and the risks of death, cardiovascular events, and hospitalization. *New England Journal of Medicine,* **351,** 1296-1305.

Go, Y.M. & Jones, D.P. (2005) Intracellular proatherogenic events and cell adhesion modulated by extracellular thiol/disulfide redox state. *Circulation,* **111,** 2973-2980.

Goodall, A.H. & Appleby, J. (2004) Flow-cytometric analysis of platelet-membrane glycoprotein expression and platelet activation. *Methods Mol Biol,* **272,** 225-253.

Gorog, P. & Kovacs, I.B. (1995) Lipid peroxidation by activated platelets: a possible link between thrombosis and atherogenesis. *Atherosclerosis,* **115,** 121-128.

Green, D.R. & Reed, J.C. (1998) Mitochondria and apoptosis. *Science,* **281,** 1309-1312.

Greenberg, M.E., Li, X.M., Gugiu, B.G., Gu, X., Qin, J., Salomon, R.G. & Hazen, S.L. (2008) The lipid whisker model of the structure of oxidized cell membranes. *J Biol Chem,* **283,** 2385-2396.

Gregory, S.A., Morrissey, J.H. & Edgington, T.S. (1989) Regulation of tissue factor gene expression in the monocyte procoagulant response to endotoxin. *Mol Cell Biol,* **9,** 2752-2755.

Greinacher, A. & Selleng, K. (2010) Thrombocytopenia in the intensive care unit patient. *Hematology Am Soc Hematol Educ Program,* **2010,** 135-143.

Gross, A., McDonnell, J.M. & Korsmeyer, S.J. (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev,* **13,** 1899-1911.

Hamer, R.J., Koedam, J.A., Beeser-Visser, N.H. & Sixma, J.J. (1987) The effect of thrombin on the complex between factor VIII and von Willebrand factor. *Eur J Biochem,* **167,** 253-259.

Handagama, P., Scarborough, R.M., Shuman, M.A. & Bainton, D.F. (1993) Endocytosis of fibrinogen into megakaryocyte and platelet alpha-granules is mediated by alpha IIb beta 3 (glycoprotein IIb-IIIa). *Blood,* **82,** 135-138.

Harker, L.A. (1978) Platelet survival time: its measurement and use. *Prog Hemost Thromb,* **4,** 321-347.

Harrison, P., Dragovic, R., Albanyan, A., Lawrie, A.S., Murphy, M. & Sargent, I. (2010) Application of dynamic light scattering to the measurement of microparticles. *Journal of Thrombosis and Haemostasis,* **7**.

Harrison, P., Wilbourn, B., Debili, N., Vainchenker, W., Breton-Gorius, J., Lawrie, A.S., Masse, J.M., Savidge, G.F. & Cramer, E.M. (1989) Uptake of plasma fibrinogen into the alpha granules of human megakaryocytes and platelets. *J Clin Invest,* **84,** 1320-1324.

Hartwig, J.H. (1992) Mechanisms of actin rearrangements mediating platelet activation. *J Cell Biol,* **118,** 1421-1442.

Haubold, K., Rink, M., Spath, B., Friedrich, M., Chun, F.K., Marx, G., Amirkhosravi, A., Francis, J.L., Bokemeyer, C., Eifrig, B. & Langer, F. (2009) Tissue factor procoagulant activity of plasma microparticles is increased in patients with early-stage prostate cancer. *Thromb Haemost,* **101,** 1147-1155.

Hechler, B., Zhang, Y., Eckly, A., Cazenave, J.P., Gachet, C. & Ravid, K. (2003) Lineage-specific overexpression of the P2Y1 receptor induces platelet hyper-reactivity in transgenic mice. *J Thromb Haemost,* **1,** 155-163.

Heemskerk, J.W., Bevers, E.M. & Lindhout, T. (2002) Platelet activation and blood coagulation. *Thromb Haemost,* **88,** 186-193.

Heemskerk, J.W., Siljander, P., Vuist, W.M., Breikers, G., Reutelingsperger, C.P., Barnes, M.J., Knight, C.G., Lassila, R. & Farndale, R.W. (1999) Function of glycoprotein VI and integrin alpha2beta1 in the procoagulant response of single, collagen-adherent platelets. *Thromb Haemost,* **81,** 782-792.

Heijnen, H.F., Schiel, A.E., Fijnheer, R., Geuze, H.J. & Sixma, J.J. (1999) Activated platelets release two types of membrane vesicles: microvesicles by surface shedding and exosomes derived from exocytosis of multivesicular bodies and alpha-granules. *Blood,* **94,** 3791-3799.

Hemker, H.C. & Beguin, S. (2000) Phenotyping the clotting system. *Thromb Haemost,* **84,** 747-751.

Hemker, H.C., Giesen, P., Al Dieri, R., Regnault, V., de Smedt, E., Wagenvoord, R., Lecompte, T. & Beguin, S. (2003) Calibrated automated thrombin generation measurement in clotting plasma. *Pathophysiol Haemost Thromb,* **33,** 4-15.

Hoffman, M. & Monroe, D.M. (2001) A cell-based model of hemostasis. *Thrombosis and Haemostasis,* **85,** 958-965.

Hoffman, R.A., Johnson, T.S. & Britt, W.B. (1981) Flow cytometric electronic direct current volume and radiofrequency impedance measurements of single cells and particles. *Cytometry,* **1,** 377-384.

Hollopeter, G., Jantzen, H.M., Vincent, D., Li, G., England, L., Ramakrishnan, V., Yang, R.B., Nurden, P., Nurden, A., Julius, D. & Conley, P.B. (2001) Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature,* **409,** 202-207.

Holmsen, H. & Weiss, H.J. (1979) Secretable storage pools in platelets. *Annu Rev Med,* **30,** 119-134.

Horii, K., Kahn, M.L. & Herr, A.B. (2006) Structural basis for platelet collagen responses by the immune-type receptor glycoprotein VI. *Blood,* **108,** 936-942.

Horstman, L.L., Jy, W., Jimenez, J.J., Bidot, C. & Ahn, Y.S. (2004) New horizons in the analysis of circulating cell-derived microparticles. *Keio J Med,* **53,** 210-230.

Hron, G., Kollars, M., Weber, H., Sagaster, V., Quehenberger, P., Eichinger, S., Kyrle, P.A. & Weltermann, A. (2007) Tissue factor-positive microparticles: cellular origin and association with coagulation activation in patients with colorectal cancer. *Thromb Haemost,* **97,** 119-123.

Hubbard, G.P., Stevens, J.M., Cicmil, M., Sage, T., Jordan, P.A., Williams, C.M., Lovegrove, J.A. & Gibbins, J.M. (2003) Quercetin inhibits collagen-stimulated platelet activation through inhibition of multiple components of the glycoprotein VI signaling pathway. *J Thromb Haemost,* **1,** 1079-1088.

Hubbard, G.P., Wolffram, S., de Vos, R., Bovy, A., Gibbins, J.M. & Lovegrove, J.A. (2006) Ingestion of onion soup high in quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in man: a pilot study. *Br J Nutr,* **96,** 482-488.

Hubbard, G.P., Wolffram, S., Lovegrove, J.A. & Gibbins, J.M. (2004) Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *J Thromb Haemost,* **2,** 2138-2145.

Hughan, S.C., Hughes, C.E., McCarty, O.J., Schweighoffer, E., Soultanova, I., Ware, J., Tybulewicz, V.L. & Watson, S.P. (2007) GPVI potentiation of platelet activation by thrombin and adhesion molecules independent of Src kinases and Syk. *Arterioscler Thromb Vasc Biol,* **27,** 422-429.

Hultin, M.B. (1985) Modulation of thrombin-mediated activation of factor VIII:C by calcium ions, phospholipid, and platelets. *Blood,* **66,** 53-58.

Hung, D.T., Vu, T.K., Wheaton, V.I., Ishii, K. & Coughlin, S.R. (1992) Cloned platelet thrombin receptor is necessary for thrombin-induced platelet activation. *J Clin Invest,* **89,** 1350-1353.

Ilveskero, S., Siljander, P. & Lassila, R. (2001) Procoagulant activity on platelets adhered to collagen or plasma clot. *Arterioscler Thromb Vasc Biol,* **21,** 628-635.

Iuliano, L., Pedersen, J.Z., PraticÒ, D., Rotilio, G. & Violi, F. (1994) Role of hydroxyl radicals in the activation of human platelets. *European Journal of Biochemistry,* **221,** 695-704.

Jackson, S.P., Schoenwaelder, S.M., Yuan, Y., Rabinowitz, I., Salem, H.H. & Mitchell, C.A. (1994) Adhesion receptor activation of phosphatidylinositol 3-kinase. von Willebrand factor stimulates the cytoskeletal association and activation of phosphatidylinositol 3-kinase and pp60c-src in human platelets. *J Biol Chem,* **269,** 27093-27099.

Janes, S.L., Wilson, D.J., Cox, A.D., Chronos, N.A. & Goodall, A.H. (1994) ADP causes partial degranulation of platelets in the absence of aggregation. *Br J Haematol,* **86,** 568-573.

Jarvis, G.E., Atkinson, B.T., Frampton, J. & Watson, S.P. (2003) Thrombin-induced conversion of fibrinogen to fibrin results in rapid platelet

trapping which is not dependent on platelet activation or GPIb. *Br J Pharmacol,* **138,** 574-583.

Jesty, J. & Nemerson, Y. (1976) The activation of bovine coagulation factor X. *Methods Enzymol,* **45,** 95-107.

Jimenez, J.J., Jy, W., Mauro, L.M., Soderland, C., Horstman, L.L. & Ahn, Y.S. (2003) Endothelial cells release phenotypically and quantitatively distinct microparticles in activation and apoptosis. *Thromb Res,* **109,** 175-180.

Jobe, S.M., Wilson, K.M., Leo, L., Raimondi, A., Molkentin, J.D., Lentz, S.R. & Di Paola, J. (2008) Critical role for the mitochondrial permeability transition pore and cyclophilin D in platelet activation and thrombosis. *Blood,* **111,** 1257-1265.

Jones, C.I., Bray, S., Garner, S.F., Stephens, J., de Bono, B., Angenent, W.G., Bentley, D., Burns, P., Coffey, A., Deloukas, P., Earthrowl, M., Farndale, R.W., Hoylaerts, M.F., Koch, K., Rankin, A., Rice, C.M., Rogers, J., Samani, N.J., Steward, M., Walker, A., Watkins, N.A., Akkerman, J.W., Dudbridge, F., Goodall, A.H. & Ouwehand, W.H. (2009) A functional genomics approach reveals novel quantitative trait loci associated with platelet signaling pathways. *Blood,* **114,** 1405-1416.

Jones, C.I., Garner, S.F., Angenent, W., Bernard, A., Berzuini, C., Burns, P., Farndale, R.W., Hogwood, J., Rankin, A., Stephens, J.C., Tom, B.D., Walton, J., Dudbridge, F., Ouwehand, W.H., Goodall, A.H. & Bloodomics, C. (2007) Mapping the platelet profile for functional genomic studies and demonstration of the effect size of the GP6 locus. *Journal of Thrombosis and Haemostasis,* **5,** 1756-1765.

Jones, D.P., Go, Y.M., Anderson, C.L., Ziegler, T.R., Kinkade, J.M., Jr. & Kirlin, W.G. (2004) Cysteine/cystine couple is a newly recognized node in the circuitry for biologic redox signaling and control. *FASEB J,* **18,** 1246-1248.

Jones, M.E., Lentz, B.R., Dombrose, F.A. & Sandberg, H. (1985) Comparison of the abilities of synthetic and platelet-derived membranes to enhance thrombin formation. *Thromb Res,* **39,** 711-724.

Joop, K., Berckmans, R.J., Nieuwland, R., Berkhout, J., Romijn, F.P., Hack, C.E. & Sturk, A. (2001) Microparticles from patients with multiple organ dysfunction syndrome and sepsis support coagulation through multiple mechanisms. *Thromb Haemost,* **85,** 810-820.

Joseph, K., Nakazawa, Y., Bahou, W.F., Ghebrehiwet, B. & Kaplan, A.P. (1999) Platelet glycoprotein Ib: a zinc-dependent binding protein for the heavy chain of high-molecular-weight kininogen. *Mol Med,* **5,** 555-563.

Joutsi-Korhonen, L., Smethurst, P.A., Rankin, A., Gray, E., M, I.J., Onley, C.M., Watkins, N.A., Williamson, L.M., Goodall, A.H., de Groot, P.G., Farndale, R.W. & Ouwehand, W.H. (2003) The low-frequency allele of the platelet collagen signaling receptor glycoprotein VI is associated with reduced functional responses and expression. *Blood,* **101,** 4372-4379.

Jy, W., Horstman, L.L., Jimenez, J.J., Ahn, Y.S., Biro, E., Nieuwland, R., Sturk, A., Dignat-George, F., Sabatier, F., Camoin-Jau, L., Sampol, J., Hugel, B., Zobairi, F., Freyssinet, J.M., Nomura, S., Shet, A.S., Key, N.S. & Hebbel, R.P. (2004) Measuring circulating cell-derived microparticles. *J Thromb Haemost,* **2,** 1842-1851.

Jy, W., Mao, W.W., Horstman, L., Tao, J. & Ahn, Y.S. (1995) Platelet microparticles bind, activate and aggregate neutrophils in vitro. *Blood Cells Mol Dis,* **21,** 217-231; discussion 231a.

Kagan, V.E., Fabisiak, J.P., Shvedova, A.A., Tyurina, Y.Y., Tyurin, V.A., Schor, N.F. & Kawai, K. (2000) Oxidative signaling pathway for externalization of plasma membrane phosphatidylserine during apoptosis. *FEBS letters,* **477,** 1-7.

Kahan, B.D. (1989) Cyclosporine. *N Engl J Med,* **321,** 1725-1738.

Kahn, M.L., Zheng, Y.W., Huang, W., Bigornia, V., Zeng, D., Moff, S., Farese, R.V., Jr., Tam, C. & Coughlin, S.R. (1998) A dual thrombin receptor system for platelet activation. *Nature,* **394,** 690-694.

Kasthuri, R.S., Taubman, M.B. & Mackman, N. (2009) Role of tissue factor in cancer. *J Clin Oncol,* **27,** 4834-4838.

Kato, K., Kanaji, T., Russell, S., Kunicki, T.J., Furihata, K., Kanaji, S., Marchese, P., Reininger, A., Ruggeri, Z.M. & Ware, J. (2003) The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. *Blood,* **102,** 1701-1707.

Kaufmann, V., Eichinger, S. & Binder, B.R. (2007) Microparticles in thrombin generation: A signifcant parameter influencing peak thrombin levels. *J Thromb Haemost,* **5,** Abstract number: P-M-432.

Kehrel, B., Wierwille, S., Clemetson, K.J., Anders, O., Steiner, M., Knight, C.G., Farndale, R.W., Okuma, M. & Barnes, M.J. (1998) Glycoprotein VI is a major collagen receptor for platelet activation: it recognizes the platelet-activating quaternary structure of collagen, whereas CD36, glycoprotein IIb/IIIa, and von Willebrand factor do not. *Blood,* **91,** 491-499.

Kereiakes, D.J., Kleiman, N., Ferguson, J.J., Runyon, J.P., Broderick, T.M., Higby, N.A., Martin, L.H., Hantsbarger, G., McDonald, S. & Anders, R.J. (1997) Sustained Platelet Glycoprotein IIb/IIIa Blockade With Oral Xemilofiban in 170 Patients After Coronary Stent Deployment. *Circulation,* **96,** 1117-1121.

Kerr, J.F., Wyllie, A.H. & Currie, A.R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer,* **26,** 239-257.

Keuren, J.F., Magdeleyns, E.J., Govers-Riemslag, J.W., Lindhout, T. & Curvers, J. (2006) Effects of storage-induced platelet microparticles on the initiation and propagation phase of blood coagulation. *Br J Haematol,* **134,** 307-313.

Kieffer, N., Guichard, J., Farcet, J.P., Vainchenker, W. & Breton-Gorius, J. (1987) Biosynthesis of major platelet proteins in human blood platelets. *Eur J Biochem,* **164,** 189-195.

Kim, H.K., Song, K.S., Lee, E.S., Lee, Y.J., Park, Y.S., Lee, K.R. & Lee, S.N. (2002) Optimized flow cytometric assay for the measurement of platelet microparticles in plasma: pre-analytic and analytic considerations. *Blood Coagul Fibrinolysis,* **13,** 393-397.

Kjalke, M., Monroe, D.M., Hoffman, M., Oliver, J.A., Ezban, M. & Roberts, H.R. (1998) Active site-inactivated factors VIIa, Xa, and IXa inhibit individual steps in a cell-based model of tissue factor-initiated coagulation. *Thromb Haemost,* **80,** 578-584.

Knight, J.A., Searles, D.A. & Blaylock, R.C. (1994) Lipid peroxidation in platelet concentrates: effects of leukocyte removal by filtration. *Ann Clin Lab Sci,* **24,** 69-75.

Krishnaswamy, S., Russell, G.D. & Mann, K.G. (1989) The reassociation of factor Va from its isolated subunits. *J Biol Chem,* **264,** 3160-3168.

Kroll, M.H., Harris, T.S., Moake, J.L., Handin, R.I. & Schafer, A.I. (1991) von Willebrand factor binding to platelet GpIb initiates signals for platelet activation. *J Clin Invest,* **88,** 1568-1573.

Kroll, M.H., Hellums, J.D., McIntire, L.V., Schafer, A.I. & Moake, J.L. (1996) Platelets and shear stress. *Blood,* **88,** 1525-1541.

Krotz, F., Sohn, H.Y., Gloe, T., Zahler, S., Riexinger, T., Schiele, T.M., Becker, B.F., Theisen, K., Klauss, V. & Pohl, U. (2002) NAD(P)H oxidase-dependent platelet superoxide anion release increases platelet recruitment. *Blood,* **100,** 917-924.

Krotz, F., Sohn, H.Y. & Pohl, U. (2004) Reactive oxygen species: players in the platelet game. *Arterioscler Thromb Vasc Biol,* **24,** 1988-1996.

Kumar, R., Beguin, S. & Hemker, H.C. (1994) The influence of fibrinogen and fibrin on thrombin generation--evidence for feedback activation of the clotting system by clot bound thrombin. *Thromb Haemost,* **72,** 713-721.

Lacroix, R. (2010) Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. . *Journal of Thrombosis and Haemostasis,* **8,** 2571-2574.

Lacroix, R., Robert, S., Poncelet, P. & Dignat-George, F. (2010a) Overcoming limitations of microparticle measurement by flow cytometry. *Semin Thromb Hemost,* **36,** 807-818.

Lacroix, R., Robert, S., Poncelet, P., Kasthuri, R.S., Key, N.S. & Dignat-George, F. (2010b) Standardization of platelet-derived microparticle enumeration by flow cytometry with calibrated beads: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *J Thromb Haemost,* **8,** 2571-2574.

Lamba, O.P., Borchman, D. & Garner, W.H. (1994) Spectral characterization of lipid peroxidation in rabbit lens membranes induced by hydrogen peroxide in the presence of Fe2+/Fe3+ cations: a site-specific catalyzed oxidation. *Free Radic Biol Med,* **16,** 591-601.

Lamba, O.P., Lal, S., Yappert, M.C., Lou, M.F. & Borchman, D. (1991) Spectroscopic detection of lipid peroxidation products and structural changes in a sphingomyelin model system. *Biochim Biophys Acta,* **1081,** 181-187.

Lambeth, J.D. (2004) NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol,* **4,** 181-189.

Landmesser, U., Dikalov, S., Price, S.R., McCann, L., Fukai, T., Holland, S.M., Mitch, W.E. & Harrison, D.G. (2003) Oxidation of tetrahydrobiopterin leads to uncoupling of endothelial cell nitric oxide synthase in hypertension. *J Clin Invest,* **111,** 1201-1209.

Larson, P.J., Camire, R.M., Wong, D., Fasano, N.C., Monroe, D.M., Tracy, P.B. & High, K.A. (1998) Structure/function analyses of recombinant variants of human factor Xa: factor Xa incorporation into prothrombinase on the thrombin-activated platelet surface is not mimicked by synthetic phospholipid vesicles. *Biochemistry,* **37,** 5029-5038.

Lawrie, A.S., Albanyan, A., Cardigan, R.A., Mackie, I.J. & Harrison, P. (2009) Microparticle sizing by dynamic light scattering in fresh-frozen plasma. *Vox Sang,* **96,** 206-212.

Lawrie, A.S., Harrison, P., Cardigan, R.A. & Mackie, I.J. (2008) The characterization and impact of microparticles on haemostasis within fresh-frozen plasma. *Vox Sang,* **95,** 197-204.

Lawson, J.H. & Mann, K.G. (1991) Cooperative activation of human factor IX by the human extrinsic pathway of blood coagulation. *J Biol Chem,* **266,** 11317-11327.

Lee, Y.J., Jy, W., Horstman, L.L., Janania, J., Reyes, Y., Kelley, R.E. & Ahn, Y.S. (1993) Elevated platelet microparticles in transient ischemic attacks, lacunar infarcts, and multiinfarct dementias. *Thromb Res,* **72,** 295-304.

Lefkovits, J., Plow, E.F. & Topol, E.J. (1995) Platelet glycoprotein IIb/IIIa receptors in cardiovascular medicine. *N Engl J Med,* **332,** 1553-1559.

Lenain, N., Freund, M., Leon, C., Cazenave, J.P. & Gachet, C. (2003) Inhibition of localized thrombosis in P2Y1-deficient mice and rodents treated with MRS2179, a P2Y1 receptor antagonist. *J Thromb Haemost,* **1,** 1144-1149.

Leon, C., Hechler, B., Freund, M., Eckly, A., Vial, C., Ohlmann, P., Dierich, A., LeMeur, M., Cazenave, J.P. & Gachet, C. (1999) Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. *J Clin Invest,* **104,** 1731-1737.

Leroyer, A.S., Isobe, H., Leseche, G., Castier, Y., Wassef, M., Mallat, Z., Binder, B.R., Tedgui, A. & Boulanger, C.M. (2007) Cellular origins and

thrombogenic activity of microparticles isolated from human atherosclerotic plaques. *J Am Coll Cardiol,* **49,** 772-777.

Leroyer, A.S., Tedgui, A. & Boulanger, C.M. (2008) Role of microparticles in atherothrombosis. *J Intern Med,* **263,** 528-537.

Lewis, S.J., Stephens, E., Florou, G., Macartney, N.J., Hathaway, L.S., Knipping, J. & Collins, P.W. (2007) Measurement of global haemostasis in severe haemophilia A following factor VIII infusion. *Br J Haematol,* **138,** 775-782.

Leytin, V., Allen, D.J., Mutlu, A., Gyulkhandanyan, A.V., Mykhaylov, S. & Freedman, J. (2009) Mitochondrial control of platelet apoptosis: effect of cyclosporin A, an inhibitor of the mitochondrial permeability transition pore. *Lab Invest,* **89,** 374-384.

Leytin, V., Allen, D.J., Mykhaylov, S., Lyubimov, E. & Freedman, J. (2006) Thrombin-triggered platelet apoptosis. *J Thromb Haemost,* **4,** 2656-2663.

Li, J., Xia, Y., Bertino, A.M., Coburn, J.P. & Kuter, D.J. (2000) The mechanism of apoptosis in human platelets during storage. *Transfusion,* **40,** 1320-1329.

Li, Y.H., Teng, J.K., Tsai, W.C., Tsai, L.M., Lin, L.J., Guo, H.R. & Chen, J.H. (1999) Prognostic significance of elevated hemostatic markers in patients with acute myocardial infarction. *J Am Coll Cardiol,* **33,** 1543-1548.

Li, Z., Nardi, M.A., Wu, J., Pan, R., Zhang, W. & Karpatkin, S. (2008) Platelet fragmentation requires a specific structural conformation of human monoclonal antibody against beta3 integrin. *J Biol Chem,* **283,** 3224-3230.

Lindemann, S., Tolley, N.D., Dixon, D.A., McIntyre, T.M., Prescott, S.M., Zimmerman, G.A. & Weyrich, A.S. (2001) Activated platelets mediate inflammatory signaling by regulated interleukin 1beta synthesis. *J Cell Biol,* **154,** 485-490.

Livnat, T., Zivelin, A., Martinowitz, U., Salomon, O. & Seligsohn, U. (2006) Prerequisites for recombinant factor VIIa-induced thrombin generation in plasmas deficient in factors VIII, IX or XI. *J Thromb Haemost,* **4,** 192-200.

Locke, D., Liu, C., Peng, X., Chen, H. & Kahn, M.L. (2003) Fc Rgamma - independent signaling by the platelet collagen receptor glycoprotein VI. *J Biol Chem,* **278,** 15441-15448.

London, F., Ahmad, S.S. & Walsh, P.N. (1996) Annexin V inhibition of factor IXa-catalyzed factor X activation on human platelets and on negatively-charged phospholipid vesicles. *Biochemistry,* **35,** 16886-16897.

Lopez, J.J., Salido, G.M., Pariente, J.A. & Rosado, J.A. (2008) Thrombin induces activation and translocation of Bid, Bax and Bak to the mitochondria in human platelets. *J Thromb Haemost,* **6,** 1780-1788.

Lorant, D.E., Patel, K.D., McIntyre, T.M., McEver, R.P., Prescott, S.M. & Zimmerman, G.A. (1991) Coexpression of GMP-140 and PAF by endothelium stimulated by histamine or thrombin: a juxtacrine system for adhesion and activation of neutrophils. *J Cell Biol,* **115,** 223-234.

Lowe, G.M., Hulley, C.E., Rhodes, E.S., Young, A.J. & Bilton, R.F. (1998) Free radical stimulation of tyrosine kinase and phosphatase activity in human peripheral blood mononuclear cells. *Biochem Biophys Res Commun,* **245,** 17-22.

Luddington, R. & Baglin, T. (2004) Clinical measurement of thrombin generation by calibrated automated thrombography requires contact factor inhibition. *Journal of Thrombosis and Haemostasis,* **2,** 1954-1959.

Macey, M.G., Enniks, N. & Bevan, S. (2011) Flow cytometric analysis of microparticle phenotype and their role in thrombin generation. *Cytometry B Clin Cytom,* **80,** 57-63.

Macfarlane, R.G. (1964) An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier. *Nature,* **202,** 498-499.

Maekawa, M., Ishizaki, T., Boku, S., Watanabe, N., Fujita, A., Iwamatsu, A., Obinata, T., Ohashi, K., Mizuno, K. & Narumiya, S. (1999) Signaling from Rho to the actin cytoskeleton through protein kinases ROCK and LIM-kinase. *Science,* **285,** 895-898.

Mallat, Z., Benamer, H., Hugel, B., Benessiano, J., Steg, P.G., Freyssinet, J.M. & Tedgui, A. (2000) Elevated levels of shed membrane microparticles with procoagulant potential in the peripheral circulating blood of patients with acute coronary syndromes. *Circulation,* **101,** 841-843.

Mallat, Z., Hugel, B., Ohan, J., Leseche, G., Freyssinet, J.M. & Tedgui, A. (1999) Shed membrane microparticles with procoagulant potential in human atherosclerotic plaques: a role for apoptosis in plaque thrombogenicity. *Circulation,* **99,** 348-353.

Mann, K.G. (1999) Biochemistry and physiology of blood coagulation. *Thromb Haemost,* **82,** 165-174.

Mann, K.G., Nesheim, M.E., Church, W.R., Haley, P. & Krishnaswamy, S. (1990) Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood,* **76,** 1-16.

Marcus, A.J., Silk, S.T., Safier, L.B. & Ullman, H.L. (1977) Superoxide production and reducing activity in human platelets. *J Clin Invest,* **59,** 149-158.

Martin, S.J., Reutelingsperger, C.P., McGahon, A.J., Rader, J.A., van Schie, R.C., LaFace, D.M. & Green, D.R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med,* **182,** 1545-1556.

Mason, K.D., Carpinelli, M.R., Fletcher, J.I., Collinge, J.E., Hilton, A.A., Ellis, S., Kelly, P.N., Ekert, P.G., Metcalf, D., Roberts, A.W., Huang, D.C.S. & Kile, B.T. (2007) Programmed Anuclear Cell Death Delimits Platelet Life Span. *Cell,* **128,** 1173-1186.

Massberg, S., Gawaz, M., Gruner, S., Schulte, V., Konrad, I., Zohlnhofer, D., Heinzmann, U. & Nieswandt, B. (2003) A crucial role of glycoprotein VI

for platelet recruitment to the injured arterial wall in vivo. *J Exp Med,* **197,** 41-49.

Mates, J.M., Perez-Gomez, C. & Nunez de Castro, I. (1999) Antioxidant enzymes and human diseases. *Clin Biochem,* **32,** 595-603.

Matsumoto, N., Nomura, S., Kamihata, H., Kimura, Y. & Iwasaka, T. (2004) Increased level of oxidized LDL-dependent monocyte-derived microparticles in acute coronary syndrome. *Thromb Haemost,* **91,** 146-154.

Mattson, M.P. & Kroemer, G. (2003) Mitochondria in cell death: novel targets for neuroprotection and cardioprotection. *Trends Mol Med,* **9,** 196-205.

Maxwell, M.J., Westein, E., Nesbitt, W.S., Giuliano, S., Dopheide, S.M. & Jackson, S.P. (2007) Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation. *Blood,* **109,** 566-576.

McCall, M.R. & Frei, B. (1999) Can antioxidant vitamins materially reduce oxidative damage in humans? *Free Radic Biol Med,* **26,** 1034-1053.

McEver, R.P., Beckstead, J.H., Moore, K.L., Marshall-Carlson, L. & Bainton, D.F. (1989) GMP-140, a platelet alpha-granule membrane protein, is also synthesized by vascular endothelial cells and is localized in Weibel-Palade bodies. *J Clin Invest,* **84,** 92-99.

Mertens, K. & Bertina, R.M. (1984) The contribution of Ca2+ and phospholipids to the activation of human blood-coagulation Factor X by activated Factor IX. *Biochem J,* **223,** 607-615.

Michelsen, A.E., Brodin, E., Brosstad, F. & Hansen, J.B. (2008) Increased level of platelet microparticles in survivors of myocardial infarction. *Scand J Clin Lab Invest,* **68,** 386-392.

Michelson, A.D. (1994) Platelet activation by thrombin can be measured in whole blood through the use of the peptide GPRP and flow cytometry methdos and clinical applications. *Blood Coagulation & Fibrinolysis,* **5,** 121-131.

Michelson, A.D. (2007) Platelets. *Elsevier/Academic press, San Diego*.

Michelson, A.D. (2010) Antiplatelet therapies for the treatment of cardiovascular disease. *Nat Rev Drug Discov,* **9,** 154-169.

Michelson, A.D. & Barnard, M.R. (1987) Thrombin-induced changes in platelet membrane glycoproteins Ib, IX, and IIb-IIIa complex. *Blood,* **70,** 1673-1678.

Miyoshi, H., Umeshita, K., Sakon, M., Imajoh-Ohmi, S., Fujitani, K., Gotoh, M., Oiki, E., Kambayashi, J. & Monden, M. (1996) Calpain activation in plasma membrane bleb formation during tert-butyl hydroperoxide-induced rat hepatocyte injury. *Gastroenterology,* **110,** 1897-1904.

Mobarrez, F., Antovic, J., Egberg, N., Hansson, M., Jorneskog, G., Hultenby, K. & Wallen, H. (2010) A multicolor flow cytometric assay for measurement of platelet-derived microparticles. *Thromb Res,* **125,** e110-116.

Monkovic, D.D. & Tracy, P.B. (1990) Activation of human factor V by factor Xa and thrombin. *Biochemistry,* **29,** 1118-1128.

Monnet, E., Sizaret, P., Arbeille, B. & Fauvel-Lafeve, F. (2000) Different role of platelet glycoprotein GP Ia/IIa in platelet contact and activation induced by type I and type III collagens. *Thromb Res,* **98,** 423-433.

Monroe, D.M., Hoffman, M. & Roberts, H.R. (1996) Transmission of a procoagulant signal from tissue factor-bearing cell to platelets. *Blood Coagul Fibrinolysis,* **7,** 459-464.

Monroe, D.M., Hoffman, M. & Roberts, H.R. (2002) Platelets and thrombin generation. *Arterioscler Thromb Vasc Biol,* **22,** 1381-1389.

Morel, O., Hugel, B., Jesel, L., Lanza, F., Douchet, M.P., Zupan, M., Chauvin, M., Cazenave, J.P., Freyssinet, J.M. & Toti, F. (2004) Sustained elevated amounts of circulating procoagulant membrane microparticles and soluble GPV after acute myocardial infarction in diabetes mellitus. *Thromb Haemost,* **91,** 345-353.

Morel, O., Toti, F., Hugel, B., Bakouboula, B., Camoin-Jau, L., Dignat-George, F. & Freyssinet, J.M. (2006) Procoagulant microparticles: disrupting the vascular homeostasis equation? *Arterioscler Thromb Vasc Biol,* **26,** 2594-2604.

Moroi, M., Jung, S.M., Okuma, M. & Shinmyozu, K. (1989) A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. *J Clin Invest,* **84,** 1440-1445.

Moroi, M., Jung, S.M., Shinmyozu, K., Tomiyama, Y., Ordinas, A. & Diaz-Ricart, M. (1996) Analysis of platelet adhesion to a collagen-coated surface under flow conditions: the involvement of glycoprotein VI in the platelet adhesion. *Blood,* **88,** 2081-2092.

Morrissey, J.H., Macik, B.G., Neuenschwander, P.F. & Comp, P.C. (1993) Quantitation of activated factor VII levels in plasma using a tissue factor mutant selectively deficient in promoting factor VII activation. *Blood,* **81,** 734-744.

Morton, L.F., Hargreaves, P.G., Farndale, R.W., Young, R.D. & Barnes, M.J. (1995) Integrin alpha 2 beta 1-independent activation of platelets by simple collagen-like peptides: collagen tertiary (triple-helical) and quaternary (polymeric) structures are sufficient alone for alpha 2 beta 1-independent platelet reactivity. *Biochem J,* **306 ( Pt 2),** 337-344.

Mosesson, M.W. (1992) The roles of fibrinogen and fibrin in hemostasis and thrombosis. *Semin Hematol,* **29,** 177-188.

Mower, D.A., Jr., Peckham, D.W., Illera, V.A., Fishbaugh, J.K., Stunz, L.L. & Ashman, R.F. (1994) Decreased membrane phospholipid packing and

decreased cell size precede DNA cleavage in mature mouse B cell apoptosis. *J Immunol,* **152,** 4832-4842.

Murohara, T., Parkinson, S.J., Waldman, S.A. & Lefer, A.M. (1995) Inhibition of nitric oxide biosynthesis promotes P-selectin expression in platelets. Role of protein kinase C. *Arterioscler Thromb Vasc Biol,* **15,** 2068-2075.

Murphy, D., Reddy, E., Forster, R. & O'neill, S. (2010) Platelet reactivity to collagen is diminished in an external reducing environment. *Platelets,* **21,** 393-419.

Nakanishi, T., Ishigami, Y., Otaki, Y., Izumi, M., Hiraoka, K., Inoue, T. & Takamitsu, Y. (2002) Impairment of vascular responses to reactive hyperemia and nitric oxide in chronic renal failure. *Nephron,* **92,** 529-535.

Nardi, M., Feinmark, S.J., Hu, L., Li, Z. & Karpatkin, S. (2004) Complement-independent Ab-induced peroxide lysis of platelets requires 12-lipoxygenase and a platelet NADPH oxidase pathway. *J Clin Invest,* **113,** 973-980.

Nardi, M., Tomlinson, S., Greco, M.A. & Karpatkin, S. (2001) Complement-independent, peroxide-induced antibody lysis of platelets in HIV-1-related immune thrombocytopenia. *Cell,* **106,** 551-561.

Nardi, M.A., Gor, Y., Feinmark, S.J., Xu, F. & Karpatkin, S. (2007) Platelet particle formation by anti GPIIIa49-66 Ab, Ca2+ ionophore A23187, and phorbol myristate acetate is induced by reactive oxygen species and inhibited by dexamethasone blockade of platelet phospholipase A2, 12-lipoxygenase, and NADPH oxidase. *Blood,* **110,** 1989-1996.

Nesbitt, W.S., Kulkarni, S., Giuliano, S., Goncalves, I., Dopheide, S.M., Yap, C.L., Harper, I.S., Salem, H.H. & Jackson, S.P. (2002) Distinct glycoprotein Ib/V/IX and integrin alpha IIbbeta 3-dependent calcium signals cooperatively regulate platelet adhesion under flow. *J Biol Chem,* **277,** 2965-2972.

Nesheim, M.E., Taswell, J.B. & Mann, K.G. (1979) The contribution of bovine Factor V and Factor Va to the activity of prothrombinase. *J Biol Chem,* **254,** 10952-10962.

Nieswandt, B., Brakebusch, C., Bergmeier, W., Schulte, V., Bouvard, D., Mokhtari-Nejad, R., Lindhout, T., Heemskerk, J.W., Zirngibl, H. & Fassler, R. (2001a) Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen. *EMBO J,* **20,** 2120-2130.

Nieswandt, B., Schulte, V., Bergmeier, W., Mokhtari-Nejad, R., Rackebrandt, K., Cazenave, J.P., Ohlmann, P., Gachet, C. & Zirngibl, H. (2001b) Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. *J Exp Med,* **193,** 459-469.

Nieswandt, B. & Watson, S.P. (2003) Platelet-collagen interaction: is GPVI the central receptor? *Blood,* **102,** 449-461.

Nieuwland, R., Berckmans, R.J., Rotteveel-Eijkman, R.C., Maquelin, K.N., Roozendaal, K.J., Jansen, P.G., ten Have, K., Eijsman, L., Hack, C.E. & Sturk, A. (1997) Cell-derived microparticles generated in patients during cardiopulmonary bypass are highly procoagulant. *Circulation,* **96,** 3534-3541.

Noble, M., Smith, J., Power, J. & Mayer-Proschel, M. (2003) Redox state as a central modulator of precursor cell function. *Ann N Y Acad Sci,* **991,** 251-271.

Nomura, S., Shouzu, A., Taomoto, K., Togane, Y., Goto, S., Ozaki, Y., Uchiyama, S. & Ikeda, Y. (2009) Assessment of an ELISA kit for platelet-derived microparticles by joint research at many institutes in Japan. *J Atheroscler Thromb,* **16,** 878-887.

Nomura, S., Uehata, S., Saito, S., Osumi, K., Ozeki, Y. & Kimura, Y. (2003) Enzyme immunoassay detection of platelet-derived microparticles and RANTES in acute coronary syndrome. *Thromb Haemost,* **89,** 506-512.

Novalija, E., Kevin, L.G., Camara, A.K., Bosnjak, Z.J., Kampine, J.P. & Stowe, D.F. (2003) Reactive oxygen species precede the epsilon isoform of protein kinase C in the anesthetic preconditioning signaling cascade. *Anesthesiology,* **99,** 421-428.

Nowak, P., Olas, B., Bald, E., Glowacki, R. & Wachowicz, B. (2003) Peroxynitrite-induced changes of thiol groups in human blood platelets. *Platelets,* **14,** 375-379.

Nurden, A.T. & Nurden, P. (2003) Advantages of fast-acting ADP receptor blockade in ischemic heart disease. *Arterioscler Thromb Vasc Biol,* **23,** 158-159.

O'Donnell, V.B., Maskrey, B. & Taylor, G.W. (2009) Eicosanoids: generation and detection in mammalian cells. *Methods Mol Biol,* **462,** 5-23.

Okuma, M., Steiner, M. & Baldini, M. (1969) Lipid peroxidation in aging platelets. *Blood,* **34,** 712-716.

Oliver, J.A., Monroe, D.M., Roberts, H.R. & Hoffman, M. (1999) Thrombin activates factor XI on activated platelets in the absence of factor XII. *Arterioscler Thromb Vasc Biol,* **19,** 170-177.

Ollivier, V., Wang, J., Manly, D., Machlus, K.R., Wolberg, A.S., Jandrot-Perrus, M. & Mackman, N. Detection of endogenous tissue factor levels in plasma using the calibrated automated thrombogram assay. *Thrombosis Research,* **125,** 90-96.

Ollivier, V., Wang, J., Manly, D., Machlus, K.R., Wolberg, A.S., Jandrot-Perrus, M. & Mackman, N. (2010) Detection of endogenous tissue factor levels in plasma using the calibrated automated thrombogram assay. *Thromb Res,* **125,** 90-96.

Oltersdorf, T., Elmore, S.W., Shoemaker, A.R., Armstrong, R.C., Augeri, D.J., Belli, B.A., Bruncko, M., Deckwerth, T.L., Dinges, J., Hajduk, P.J., Joseph, M.K.,

Kitada, S., Korsmeyer, S.J., Kunzer, A.R., Letai, A., Li, C., Mitten, M.J., Nettesheim, D.G., Ng, S., Nimmer, P.M., O'Connor, J.M., Oleksijew, A., Petros, A.M., Reed, J.C., Shen, W., Tahir, S.K., Thompson, C.B., Tomaselli, K.J., Wang, B., Wendt, M.D., Zhang, H., Fesik, S.W. & Rosenberg, S.H. (2005) An inhibitor of Bcl-2 family proteins induces regression of solid tumours. *Nature,* **435,** 677-681.

Ott, I., Andrassy, M., Zieglgansberger, D., Geith, S., Schomig, A. & Neumann, F.J. (2001) Regulation of monocyte procoagulant activity in acute myocardial infarction: role of tissue factor and tissue factor pathway inhibitor-1. *Blood,* **97,** 3721-3726.

Owen, J.S., Hutton, R.A., Day, R.C., Bruckdorfer, K.R. & McIntyre, N. (1981) Platelet lipid composition and platelet aggregation in human liver disease. *J Lipid Res,* **22,** 423-430.

Panes, O., Matus, V., Saez, C.G., Quiroga, T., Pereira, J. & Mezzano, D. (2007) Human platelets synthesize and express functional tissue factor. *Blood,* **109,** 5242-5250.

Pasquet, J.M., Bobe, R., Gross, B., Gratacap, M.P., Tomlinson, M.G., Payrastre, B. & Watson, S.P. (1999) A collagen-related peptide regulates phospholipase Cgamma2 via phosphatidylinositol 3-kinase in human platelets. *Biochem J,* **342 ( Pt 1),** 171-177.

Pasquet, J.M., Dachary-Prigent, J. & Nurden, A.T. (1998) Microvesicle release is associated with extensive protein tyrosine dephosphorylation in platelets stimulated by A23187 or a mixture of thrombin and collagen. *Biochem J,* **333 ( Pt 3),** 591-599.

Pearce, A.C., Wilde, J.I., Doody, G.M., Best, D., Inoue, O., Vigorito, E., Tybulewicz, V.L.J., Turner, M. & Watson, S.P. (2002) Vav1, but not Vav2, contributes to platelet aggregation by CRP and thrombin, but neither is required for regulation of phospholipase C. *Blood,* **100,** 3561-3569.

Penz, S., Reininger, A.J., Brandl, R., Goyal, P., Rabie, T., Bernlochner, I., Rother, E., Goetz, C., Engelmann, B., Smethurst, P.A., Ouwehand, W.H., Farndale, R., Nieswandt, B. & Siess, W. (2005) Human atheromatous plaques stimulate thrombus formation by activating platelet glycoprotein VI. *FASEB J,* **19,** 898-909.

Pereira, J., Alfaro, G., Goycoolea, M., Quiroga, T., Ocqueteau, M., Massardo, L., Perez, C., Saez, C., Panes, O., Matus, V. & Mezzano, D. (2006a) Circulating platelet-derived microparticles in systemic lupus erythematosus - Association with increased thrombin generation and procoagulant state. *Thrombosis and Haemostasis,* **95,** 94-99.

Pereira, J., Alfaro, G., Goycoolea, M., Quiroga, T., Ocqueteau, M., Massardo, L., Perez, C., Saez, C., Panes, O., Matus, V. & Mezzano, D. (2006b) Circulating platelet-derived microparticles in systemic lupus erythematosus.

Association with increased thrombin generation and procoagulant state. *Thromb Haemost,* **95,** 94-99.

Pereira, J., Soto, M., Palomo, I., Ocqueteau, M., Coetzee, L.M., Astudillo, S., Aranda, E. & Mezzano, D. (2002) Platelet aging in vivo is associated with activation of apoptotic pathways: studies in a model of suppressed thrombopoiesis in dogs. *Thromb Haemost,* **87,** 905-909.

Perrotta, P.L., Perrotta, C.L. & Snyder, E.L. (2003) Apoptotic activity in stored human platelets. *Transfusion,* **43,** 526-535.

Pickering, W., Gray, E., Goodall, A.H. & Barrowcliffe, T.W. (2004) Characterisation and studies of T-lymphoblastoid procogulant actvity. *PhD thesis*.

Pickering, W., Gray, E., Goodall, A.H. & Barrowcliffe, T.W. (2008) Effects of apoptosis and lipid peroxidation on T-lymphoblastoid phospholipid-dependent procoagulant activity. *Journal of Thrombosis and Haemostasis,* **6,** 1122-1130.

Picklo, M.J., Montine, T.J., Amarnath, V. & Neely, M.D. (2002) Carbonyl toxicology and Alzheimer's disease. *Toxicol Appl Pharmacol,* **184,** 187-197.

Pieters, J., Lindhout, T. & Hemker, H.C. (1989) In situ-generated thrombin is the only enzyme that effectively activates factor VIII and factor V in thromboplastin-activated plasma. *Blood,* **74,** 1021-1024.

Plow, E.F., McEver, R.P., Coller, B.S., Woods, V.L., Jr., Marguerie, G.A. & Ginsberg, M.H. (1985) Related binding mechanisms for fibrinogen, fibronectin, von Willebrand factor, and thrombospondin on thrombin-stimulated human platelets. *Blood,* **66,** 724-727.

Plow, E.F., Srouji, A.H., Meyer, D., Marguerie, G. & Ginsberg, M.H. (1984) Evidence that three adhesive proteins interact with a common recognition site on activated platelets. *J Biol Chem,* **259,** 5388-5391.

Poole, A., Gibbins, J.M., Turner, M., van Vugt, M.J., van de Winkel, J.G., Saito, T., Tybulewicz, V.L. & Watson, S.P. (1997) The Fc receptor gamma-chain and the tyrosine kinase Syk are essential for activation of mouse platelets by collagen. *EMBO J,* **16,** 2333-2341.

Pratico, D., Pasin, M., Barry, O.P., Ghiselli, A., Sabatino, G., Iuliano, L., FitzGerald, G.A. & Violi, F. (1999) Iron-dependent human platelet activation and hydroxyl radical formation: involvement of protein kinase C. *Circulation,* **99,** 3118-3124.

Pricci, F., Leto, G., Amadio, L., Iacobini, C., Cordone, S., Catalano, S., Zicari, A., Sorcini, M., Di Mario, U. & Pugliese, G. (2003) Oxidative stress in diabetes-induced endothelial dysfunction involvement of nitric oxide and protein kinase C. *Free Radic Biol Med,* **35,** 683-694.

Quinn, M.J., Plow, E.F. & Topol, E.J. (2002) Platelet Glycoprotein IIb/IIIa Inhibitors: Recognition of a Two-Edged Sword? *Circulation,* **106,** 379-385.

Raff, M.C. (1992) Social controls on cell survival and cell death. *Nature,* **356,** 397-400.

Rahman, K. (2007) Studies on free radicals, antioxidants, and co-factors. *Clin Interv Aging,* **2,** 219-236.

Ramstrom, S., O'Neill, S., Dunne, E. & Kenny, D. (2010) Annexin V binding to platelets is agonist, time and temperature dependent. *Platelets,* **21,** 289-296.

Rand, M.D., Lock, J.B., van't Veer, C., Gaffney, D.P. & Mann, K.G. (1996) Blood clotting in minimally altered whole blood. *Blood,* **88,** 3432-3445.

Rand, M.L., Wang, H., Bang, K.W., Poon, K.S., Packham, M.A. & Freedman, J. (2004) Procoagulant surface exposure and apoptosis in rabbit platelets: association with shortened survival and steady-state senescence. *J Thromb Haemost,* **2,** 651-659.

Rau, J.C., Beaulieu, L.M., Huntington, J.A. & Church, F.C. (2007) Serpins in thrombosis, hemostasis and fibrinolysis. *J Thromb Haemost,* **5 Suppl 1,** 102-115.

Rauch, U., Bonderman, D., Bohrmann, B., Badimon, J.J., Himber, J., Riederer, M.A. & Nemerson, Y. (2000) Transfer of tissue factor from leukocytes to platelets is mediated by CD15 and tissue factor. *Blood,* **96,** 170-175.

Rauch, U. & Nemerson, Y. (2000) Circulating tissue factor and thrombosis. *Curr Opin Hematol,* **7,** 273-277.

Ravanat, C., Archipoff, G., Beretz, A., Freund, G., Cazenave, J.P. & Freyssinet, J.M. (1992) Use of annexin-V to demonstrate the role of phosphatidylserine exposure in the maintenance of haemostatic balance by endothelial cells. *Biochem J,* **282 ( Pt 1),** 7-13.

Razdan, K., Hellums, J.D. & Kroll, M.H. (1994) Shear-stress-induced von Willebrand factor binding to platelets causes the activation of tyrosine kinase(s). *Biochem J,* **302 ( Pt 3),** 681-686.

Rebulla, P. (2001) Platelet transfusion trigger in difficult patients. *Transfus Clin Biol,* **8,** 249-254.

Reininger, A.J., Heijnen, H.F., Schumann, H., Specht, H.M., Schramm, W. & Ruggeri, Z.M. (2006) Mechanism of platelet adhesion to von Willebrand factor and microparticle formation under high shear stress. *Blood,* **107,** 3537-3545.

Remenyi, G., Szasz, R., Friese, P. & Dale, G.L. (2005) Role of mitochondrial permeability transition pore in coated-platelet formation. *Arterioscler Thromb Vasc Biol,* **25,** 467-471.

Rice-Evans, C. (2001) Flavonoid antioxidants. *Curr Med Chem,* **8,** 797-807.

Rickles, F.R., Patierno, S. & Fernandez, P.M. (2003) Tissue factor, thrombin, and cancer. *Chest,* **124,** 58S-68S.

Robbie, L. & Libby, P. (2001) Inflammation and atherothrombosis. *Ann N Y Acad Sci,* **947,** 167-179; discussion 179-180.

Roberts, A.W., Gandhi, L., O'Connor, O.A., Rudin, C.M., Khaira, D., Xiong, H., Chiu, Y., Greco, R., Krivoshik, A.P. & Wilson, W.H. (2008) Reduction in platelet counts as a mechanistic biomarker and guide for adaptive dose-escalation in phase I studies of the Bcl-2 family inhibitor ABT-263. *J Clin Oncol,* **26,** abstr 3542.

Roberts, E., Lamb, D. & Goodall, A.H. (1999) Standardisation and optimisation of experimental conditions to allow analysis and measurement of intracellular oxidative stress in platelets, monocytes and platelet-monocyte aggregates. *MSc thesis, Leicester University*.

Roberts, H.R., Hoffman, M. & Monroe, D.M. (2006) A cell-based model of thrombin generation. *Semin Thromb Hemost,* **32 Suppl 1,** 32-38.

Roffi, M., Chew, D.P., Mukherjee, D., Bhatt, D.L., White, J.A., Heeschen, C., Hamm, C.W., Moliterno, D.J., Califf, R.M., White, H.D., Kleiman, N.S., Theroux, P. & Topol, E.J. (2001) Platelet Glycoprotein IIb/IIIa Inhibitors Reduce Mortality in Diabetic Patients With Non-ST-Segment-Elevation Acute Coronary Syndromes. *Circulation,* **104,** 2767-2771.

Rosado, J.A., Lopez, J.J., Gomez-Arteta, E., Redondo, P.C., Salido, G.M. & Pariente, J.A. (2006) Early caspase-3 activation independent of apoptosis is required for cellular function. *Journal of Cellular Physiology,* **209,** 142-152.

Rota, S., McWilliam, N.A., Baglin, T.P. & Byrne, C.D. (1998a) Atherogenic lipoproteins support assembly of the prothrombinase complex and thrombin generation: modulation by oxidation and vitamin E. *Blood,* **91,** 508-515.

Rota, S., McWilliam, N.A., Baglin, T.P. & Byrne, C.D. (1998b) Atherogenic Lipoproteins Support Assembly of the Prothrombinase Complex and Thrombin Generation: Modulation by Oxidation and Vitamin E. *Blood,* **91,** 508-515.

Ruggeri, Z.M. (1997) Mechanisms initiating platelet thrombus formation. *Thromb Haemost,* **78,** 611-616.

Ruggeri, Z.M. (2002) Platelets in atherothrombosis. *Nat Med,* **8,** 1227-1234.

Ruggeri, Z.M. (2003) Von Willebrand factor, platelets and endothelial cell interactions. *J Thromb Haemost,* **1,** 1335-1342.

Rysz, J., Luciak, M., Kedziora, J., Blaszczyk, J. & Sibinska, E. (1997) Nitric oxide release in the peripheral blood during hemodialysis. *Kidney International,* **51,** 294-300.

Salvemini, D. & Botting, R. (1993) Modulation of platelet function by free radicals and free-radical scavengers. *Trends Pharmacol Sci,* **14,** 36-42.

Salvemini, D., Radziszewski, W., Mollace, V., Moore, A., Willoughby, D. & Vane, J. (1991) Diphenylene iodonium, an inhibitor of free radical formation, inhibits platelet aggregation. *European Journal of Pharmacology,* **199,** 15-18.

Sandberg, H., Bode, A.P., Dombrose, F.A., Hoechli, M. & Lentz, B.R. (1985) Expression of coagulant activity in human platelets: release of membranous vesicles providing platelet factor 1 and platelet factor 3. *Thromb Res,* **39,** 63-79.

Sanner, B.M., Meder, U., Zidek, W. & Tepel, M. (2002) Effects of glucocorticoids on generation of reactive oxygen species in platelets. *Steroids,* **67,** 715-719.

Sase, K. & Michel, T. (1995) Expression of constitutive endothelial nitric oxide synthase in human blood platelets. *Life Sci,* **57,** 2049-2055.

Savill, J., Fadok, V., Henson, P. & Haslett, C. (1993) Phagocyte recognition of cells undergoing apoptosis. *Immunol Today,* **14,** 131-136.

Schnurr, K., Kuhn, H., Rapoport, S.M. & Schewe, T. (1995) 3,5-Di-t-butyl-4-hydroxytoluene (BHT) and probucol stimulate selectively the reaction of mammalian 15-lipoxygenase with biomembranes. *Biochim Biophys Acta,* **1254,** 66-72.

Schoenwaelder, S.M., Jarman, K.E., Gardiner, E., Hua, M., Qiao, J., White, M.J., Josefsson, E.C., Alwis, I., Ono, A., Willcox, A., Andrews, R.K., Mason, K.D., Salem, H.H., Huang, D.C., Kile, B.T., Roberts, A.W. & Jackson, S.P. (2011) Bcl-xL inhibitory BH3 mimetics can induce a transient thrombocytopathy that undermines the hemostatic function of platelets. *Blood*.

Schoenwaelder, S.M., Yuan, Y., Josefsson, E.C., White, M.J., Yao, Y., Mason, K.D., O'Reilly, L.A., Henley, K.J., Ono, A., Hsiao, S., Willcox, A., Roberts, A.W., Huang, D.C.S., Salem, H.H., Kile, B.T. & Jackson, S.P. (2009) Two distinct pathways regulate platelet phosphatidylserine exposure and procoagulant function. *Blood,* **114,** 663-666.

Scholz, T., Temmler, U., Krause, S., Heptinstall, S. & Losche, W. (2002) Transfer of tissue factor from platelets to monocytes: role of platelet-derived microvesicles and CD62P. *Thromb Haemost,* **88,** 1033-1038.

Segal, A.W. (2005) How neutrophils kill microbes. *Annu Rev Immunol,* **23,** 197-223.

Seigneuret, M. & Devaux, P.F. (1984) ATP-dependent asymmetric distribution of spin-labeled phospholipids in the erythrocyte membrane: relation to shape changes. *Proc Natl Acad Sci U S A,* **81,** 3751-3755.

Sekiya, K. & Okuda, H. (1982a) Selective inhibition of platelet lipoxygenase by baicalein. *Biochemical and Biophysical Research Communications,* **105,** 1090-1095.

Sekiya, K. & Okuda, H. (1982b) Selective inhibition of platelet lipoxygenase by baicalein. *Biochem Biophys Res Commun,* **105,** 1090-1095.

Sekiya, K., Okuda, H. & Arichi, S. (1982a) Selective inhibition of platelet lipoxygenase by esculetin. *Biochim Biophys Acta,* **713,** 68-72.

Sekiya, K., Okuda, H. & Arichi, S. (1982b) Selective inhibition of platelet lipoxygenase by esculetin. *Biochimica et Biophysica Acta (BBA) - Lipids and Lipid Metabolism,* **713,** 68-72.

Sener, A., Ozsavci, D., Oba, R., Demirel, G.Y., Uras, F. & Yardimci, K.T. (2005) Do platelet apoptosis, activation, aggregation, lipid peroxidation and platelet-leukocyte aggregate formation occur simultaneously in hyperlipidemia? *Clin Biochem,* **38,** 1081-1087.

Seno, T., Inoue, N., Gao, D., Okuda, M., Sumi, Y., Matsui, K., Yamada, S., Hirata, K.-i., Kawashima, S., Tawa, R., Imajoh-Ohmi, S., Sakurai, H. & Yokoyama, M. (2001) Involvement of NADH/NADPH Oxidase in Human Platelet ROS Production. *Thrombosis Research,* **103,** 399-409.

Shah, M.D., Bergeron, A.L., Dong, J.F. & Lopez, J.A. (2008) Flow cytometric measurement of microparticles: pitfalls and protocol modifications. *Platelets,* **19,** 365-372.

Shcherbina, A. & Remold-O'Donnell, E. (1999) Role of Caspase in a Subset of Human Platelet Activation Responses. *Blood,* **93,** 4222-4231.

Siljander, P., Farndale, R.W., Feijge, M.A., Comfurius, P., Kos, S., Bevers, E.M. & Heemskerk, J.W. (2001) Platelet adhesion enhances the glycoprotein VI-dependent procoagulant response: Involvement of p38 MAP kinase and calpain. *Arterioscler Thromb Vasc Biol,* **21,** 618-627.

Sims, P.J., Faioni, E.M., Wiedmer, T. & Shattil, S.J. (1988) Complement proteins C5b-9 cause release of membrane vesicles from the platelet surface that are enriched in the membrane receptor for coagulation factor Va and express prothrombinase activity. *J Biol Chem,* **263,** 18205-18212.

Sims, P.J., Wiedmer, T., Esmon, C.T., Weiss, H.J. & Shattil, S.J. (1989) Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. *J Biol Chem,* **264,** 17049-17057.

Sinauridze, E.I., Kireev, D.A., Popenko, N.Y., Pichugin, A.V., Panteleev, M.A., Krymskaya, O.V. & Ataullakhanov, F.I. (2007) Platelet microparticle membranes have 50- to 100-fold higher specific procoagulant activity than activated platelets. *Thromb Haemost,* **97,** 425-434.

Sinning, J.M., Losch, J., Walenta, K., Bohm, M., Nickenig, G. & Werner, N. (2010) Circulating CD31+/Annexin V+ microparticles correlate with cardiovascular outcomes. *Eur Heart J*.

Smalley, D.M., Root, K.E., Cho, H., Ross, M.M. & Ley, K. (2007) Proteomic discovery of 21 proteins expressed in human plasma-derived but not platelet-derived microparticles. *Thromb Haemost,* **97,** 67-80.

Smith, S.A. (2009) The cell-based model of coagulation. *J Vet Emerg Crit Care (San Antonio),* **19,** 3-10.

Soslau, G., Class, R., Morgan, D.A., Foster, C., Lord, S.T., Marchese, P. & Ruggeri, Z.M. (2001) Unique pathway of thrombin-induced platelet aggregation mediated by glycoprotein Ib. *J Biol Chem,* **276,** 21173-21183.

Sossdorf, M., Konig, V., Gummert, J., Marx, G. & Losche, W. (2008) Correlations between platelet-derived microvesicles and thrombin generation in patients with coronary artery disease. *Platelets,* **19,** 476-477.

Spronk, H., Dielis, A., Panova-Noeva, M., van Oerle, R., Govers-Riemslag, J., Hamulyák, K., Falanga, A. & Cate, H. (2009) Monitoring thrombin generation: Is addition of corn trypsin inhibitor needed? *Thrombosis and Haemostasis,* **101,** 1156-1162.

Stegner, D. & Nieswandt, B. (2010) Platelet receptor signaling in thrombus formation. *J Mol Med,* **89,** 109-121.

Steppich, B.A., Braun, S.L., Stein, A., Demetz, G., Groha, P., Schomig, A., von Beckerath, N., Kastrati, A. & Ott, I. (2009) Plasma TF activity predicts cardiovascular mortality in patients with acute myocardial infarction. *Thromb J,* **7,** 11.

Sugiyama, T., Okuma, M., Ushikubi, F., Sensaki, S., Kanaji, K. & Uchino, H. (1987) A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. *Blood,* **69,** 1712-1720.

Sumner, W.T., Monroe, D.M. & Hoffman, M. (1996) Variability in platelet procogaulant activity in healthy volunteer. *Thrombosis Research,* **81,** 533-543.

Suzuki-Inoue, K., Tulasne, D., Shen, Y., Bori-Sanz, T., Inoue, O., Jung, S.M., Moroi, M., Andrews, R.K., Berndt, M.C. & Watson, S.P. (2002) Association of Fyn and Lyn with the proline-rich domain of glycoprotein VI regulates intracellular signaling. *J Biol Chem,* **277,** 21561-21566.

Suzuki, J., Umeda, M., Sims, P.J. & Nagata, S. (2010) Calcium-dependent phospholipid scrambling by TMEM16F. *Nature,* **468,** 834-838.

Suzuki, K., Dahlback, B. & Stenflo, J. (1982) Thrombin-catalyzed activation of human coagulation factor V. *J Biol Chem,* **257,** 6556-6564.

Swedenborg, J. (1998) The mechanisms of action of alpha- and beta-isoforms of antithrombin. *Blood Coagul Fibrinolysis,* **9 Suppl 3,** S7-10.

Taccone-Gallucci, M., Lubrano, R., Del Principe, D., Menichelli, A., Giordani, M., Citti, G., Morosetti, M., Meloni, C., Mazzarella, V., Meschini, L. & et al. (1989) Platelet lipid peroxidation in haemodialysis patients: effects of vitamin E supplementation. *Nephrol Dial Transplant,* **4,** 975-978.

Tait, J.F., Gibson, D. & Fujikawa, K. (1989) Phospholipid binding properties of human placental anticoagulant prtoein-1; a member of the lipocortin family. *Journal of Biological Chemistry,* **264,** 7944-7949.

Takahashi, H. & Moroi, M. (2001) Antibody against platelet membrane glycoprotein VI in a patient with systemic lupus erythematosus. *Am J Hematol,* **67,** 262-267.

Takahashi, O. (1991) Some properties of rat platelet aggregation and effects of butylated hydroxytoluene, warfarin and aspirin. *Food Chem Toxicol,* **29,** 173-183.

Tedgui, A. & Mallat, Z. (2001) Apoptosis as a determinant of atherothrombosis. *Thromb Haemost,* **86,** 420-426.

Tesselaar, M.E., Romijn, F.P., Van Der Linden, I.K., Prins, F.A., Bertina, R.M. & Osanto, S. (2007) Microparticle-associated tissue factor activity: a link between cancer and thrombosis? *J Thromb Haemost,* **5,** 520-527.

Thaulow, E., Erikssen, J., Sandvik, L., Stormorken, H. & Cohn, P.F. (1991) Blood platelet count and function are related to total and cardiovascular death in apparently healthy men. *Circulation,* **84,** 613-617.

Thiagarajan, P. & Tait, J.F. (1990) Binding of annexin-V placntal anticoagulant protein to platelets evidenace for phosphotidylserine exposure in the procoagulant respone of activated platelets. *Journal of Biological Chemistry,* **265,** 17420-17423.

Thim, T., Hagensen, M.K., Bentzon, J.F. & Falk, E. (2008) From vulnerable plaque to atherothrombosis. *J Intern Med,* **263,** 506-516.

Thomas, C.P., Morgan, L.T., Maskrey, B.H., Murphy, R.C., Kuhn, H., Hazen, S.L., Goodall, A.H., Hamali, H.A., Collins, P.W. & O'Donnell, V.B. (2010) Phospholipid-esterified eicosanoids are generated in agonist-activated human platelets and enhance tissue factor-dependent thrombin generation. *J Biol Chem,* **285,** 6891-6903.

Thomas, D.W., Mannon, R.B., Mannon, P.J., Latour, A., Oliver, J.A., Hoffman, M., Smithies, O., Koller, B.H. & Coffman, T.M. (1998) Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. *J Clin Invest,* **102,** 1994-2001.

Thomas, S., Rigsby, P., Goodall, A. & Gray, E. (2003) Influence of flow on inhibiton of platelet dependent thrombin generatiomn by an anti-GPIIb/IIIa. *J Thromb Haemost,* **1,** Abstract # P0215.

Thompson, A.R. (1986) Structure, function, and molecular defects of factor IX. *Blood,* **67,** 565-572.

Thon, J.N. & Italiano, J.E. (2010) Platelet formation. *Semin Hematol,* **47,** 220-226.

Thornberry, N.A. & Lazebnik, Y. (1998) Caspases: enemies within. *Science,* **281,** 1312-1316.

Tomasiak, M., Rusak, T., Gacko, M. & Stelmach, H. (2007) Cyclosporine enhances platelet procoagulant activity. *Nephrol Dial Transplant,* **22,** 1750-1756.

Tonon, G., Luo, X., Greco, N.J., Chen, W., Shi, Y. & Jamieson, G.A. (2002) Weak platelet agonists and U46619 induce apoptosis-like events in platelets, in the absence of phosphatidylserine exposure. *Thromb Res,* **107,** 345-350.

Topol, E.J., Moliterno, D.J., Herrmann, H.C., Powers, E.R., Grines, C.L., Cohen, D.J., Cohen, E.A., Bertrand, M., Neumann, F.-J., Stone, G.W., DiBattiste, P.M., Yakubov, S.J., DeLucca, P.T. & Demopoulos, L. (2001) Comparison of Two Platelet Glycoprotein IIb/IIIa Inhibitors, Tirofiban and Abciximab, for the Prevention of Ischemic Events with Percutaneous Coronary Revascularization. *New England Journal of Medicine,* **344,** 1888-1894.

Trip, M.D., Cats, V.M., van Capelle, F.J. & Vreeken, J. (1990) Platelet hyperreactivity and prognosis in survivors of myocardial infarction. *N Engl J Med,* **322,** 1549-1554.

Trumel, C., Payrastre, B., Plantavid, M., Hechler, B., Viala, C., Presek, P., Martinson, E.A., Cazenave, J.P., Chap, H. & Gachet, C. (1999) A key role of adenosine diphosphate in the irreversible platelet aggregation induced by the PAR1-activating peptide through the late activation of phosphoinositide 3-kinase. *Blood,* **94,** 4156-4165.

Trummer, A., De Rop, C., Tiede, A., Ganser, A. & Eisert, R. (2009) Recovery and composition of microparticles after snap-freezing depends on thawing temperature. *Blood Coagul Fibrinolysis,* **20,** 52-56.

Tschoepe, D., Schultheiss, H.P., Kolarov, P., Schwippert, B., Dannehl, K., Nieuwenhuis, H.K., Kehrel, B., Strauer, B. & Gries, F.A. (1993) Platelet membrane activation markers are predictive for increased risk of acute ischemic events after PTCA. *Circulation,* **88,** 37-42.

Tuma, D.J. (2002) Role of malondialdehyde-acetaldehyde adducts in liver injury. *Free Radic Biol Med,* **32,** 303-308.

Tymvios, C., Moore, C., Jones, S., Solomon, A., Sanz-Rosa, D. & Emerson, M. (2009) Platelet aggregation responses are critically regulated in vivo by endogenous nitric oxide but not by endothelial nitric oxide synthase. *Br J Pharmacol,* **158,** 1735-1742.

Tyurina, Y.Y., Serinkan, F.B., Tyurin, V.A., Kini, V., Yalowich, J.C., Schroit, A.J., Fadeel, B. & Kagan, V.E. (2004) Lipid antioxidant, etoposide, inhibits phosphatidylserine externalization and macrophage clearance of apoptotic cells by preventing phosphatidylserine oxidation. *J Biol Chem,* **279,** 6056-6064.

Tyurina, Y.Y., Shvedova, A.A., Kawai, K., Tyurin, V.A., Kommineni, C., Quinn, P.J., Schor, N.F., Fabisiak, J.P. & Kagan, V.E. (2000) Phospholipid signaling in apoptosis: peroxidation and externalization of phosphatidylserine. *Toxicology,* **148,** 93-101.

Ueba, T., Haze, T., Sugiyama, M., Higuchi, M., Asayama, H., Karitani, Y., Nishikawa, T., Yamashita, K., Nagami, S., Nakayama, T., Kanatani, K. & Nomura, S. (2008) Level, distribution and correlates of platelet-derived

microparticles in healthy individuals with special reference to the metabolic syndrome. *Thromb Haemost,* **100,** 280-285.

Utsugi, T., Schroit, A.J., Connor, J., Bucana, C.D. & Fidler, I.J. (1991) Elevated expression of phosphatidylserine in the outer membrane leaflet of human tumor cells and recognition by activated human blood monocytes. *Cancer Res,* **51,** 3062-3066.

van Dam, B., van Hinsbergh, V.W., Stehouwer, C.D., Versteilen, A., Dekker, H., Buytenhek, R., Princen, H.M. & Schalkwijk, C.G. (2003) Vitamin E inhibits lipid peroxidation-induced adhesion molecule expression in endothelial cells and decreases soluble cell adhesion molecules in healthy subjects. *Cardiovasc Res,* **57,** 563-571.

van Goor, H., Albrecht, E.W., Heeringa, P., Klok, P.A., van der Horst, M.L., de Jager-Krikken, A., Bakker, W.W. & Moshage, H. (2001) Nitric oxide inhibition enhances platelet aggregation in experimental anti-Thy-1 nephritis. *Nitric Oxide,* **5,** 525-533.

van Guldener, C., Janssen, M., Lambert, J., Steyn, M., Donker, A.J.M. & Stehouwer, D.A. (1998) Endothelium-dependent vasodilatation is impaired in peritoneal dialysis patients. *Nephrology Dialysis Transplantation,* **13,** 1782-1786.

van Hylckama Vlieg, A., Christiansen, S.C., Luddington, R., Cannegieter, S.C., Rosendaal, F.R. & Baglin, T.P. (2007) Elevated endogenous thrombin potential is associated with an increased risk of a first deep venous thrombosis but not with the risk of recurrence. *Br J Haematol,* **138,** 769-774.

van Ierssel, S.H., Van Craenenbroeck, E.M., Conraads, V.M., Van Tendeloo, V.F., Vrints, C.J., Jorens, P.G. & Hoymans, V.Y. (2010) Flow cytometric detection of endothelial microparticles (EMP): effects of centrifugation and storage alter with the phenotype studied. *Thromb Res,* **125,** 332-339.

van Veen, J.J., Gatt, A., Bowyer, A.E., Cooper, P.C., Kitchen, S. & Makris, M. (2009) Calibrated automated thrombin generation and modified thromboelastometry in haemophilia A. *Thromb Res,* **123,** 895-901.

Van Veen, J.J., Gatt, A., Cooper, P.C., Kitchen, S., Bowyer, A.E. & Makris, M. (2008) Corn trypsin inhibitor in fluorogenic thrombin-generation measurements is only necessary at low tissue factor concentrations and influences the relationship between factor VIII coagulant activity and thromblogram parameters. *Blood Coagulation & Fibrinolysis,* **19,** 183-189.

Vane, J.R. & Botting, R.M. (1998) Anti-inflammatory drugs and their mechanism of action. *Inflamm Res,* **47 Suppl 2,** S78-87.

Vanschoonbeek, K., Feijge, M.A., Van Kampen, R.J., Kenis, H., Hemker, H.C., Giesen, P.L. & Heemskerk, J.W. (2004) Initiating and potentiating role of platelets in tissue factor-induced thrombin generation in the presence of

plasma: subject-dependent variation in thrombogram characteristics. *J Thromb Haemost,* **2,** 476-484.

VanWijk, M.J., VanBavel, E., Sturk, A. & Nieuwland, R. (2003) Microparticles in cardiovascular diseases. *Cardiovasc Res,* **59,** 277-287.

Varga-Szabo, D., Braun, A. & Nieswandt, B. (2009) Calcium signaling in platelets. *J Thromb Haemost,* **7,** 1057-1066.

Vaux, D.L. & Strasser, A. (1996) The molecular biology of apoptosis. *Proc Natl Acad Sci U S A,* **93,** 2239-2244.

Verhoeven, A.J., Verhaar, R., Gouwerok, E.G. & de Korte, D. (2005) The mitochondrial membrane potential in human platelets: a sensitive parameter for platelet quality. *Transfusion,* **45,** 82-89.

Vlachakis, N.D. & Aledort, L. (1979) Platelet lipid peroxides as determinant of platelet survival and sympathetic stimulation in patients with hypertension. *Biochemical Medicine,* **21,** 94-103.

Vogler, M., Hamali, H., Sun, X., Bampton, E., Dinsdale, D., Snowden, R., Dyer, M., Goodall, A. & Cohen, G. (2011) Pathways involved in platelet apoptosis and activation following BCL2-inhibition. *Submitted to Blood*.

Wadhawan, V., Karim, Z.A., Mukhopadhyay, S., Gupta, R., Dikshit, M. & Dash, D. (2004) Platelet storage under in vitro condition is associated with calcium-dependent apoptosis-like lesions and novel reorganization in platelet cytoskeleton. *Archives of Biochemistry and Biophysics,* **422,** 183-190.

Wagner, C.L., Mascelli, M.A., Neblock, D.S., Weisman, H.F., Coller, B.S. & Jordan, R.E. (1996) Analysis of GPIIb/IIIa receptor number by quantification of 7E3 binding to human platelets. *Blood,* **88,** 907-914.

Wang, J., Weiss, I., Svoboda, K. & Kwaan, H.C. (2001) Thrombogenic role of cells undergoing apoptosis. *Br J Haematol,* **115,** 382-391.

Warshaw, A.L., Laster, L. & Shulman, N.R. (1967) Protein synthesis by human platelets. *J Biol Chem,* **242,** 2094-2097.

Watson, S.P., Asazuma, N., Atkinson, B., Berlanga, O., Best, D., Bobe, R., Jarvis, G., Marshall, S., Snell, D., Stafford, M., Tulasne, D., Wilde, J., Wonerow, P. & Frampton, J. (2001) The role of ITAM- and ITIM-coupled receptors in platelet activation by collagen. *Thromb Haemost,* **86,** 276-288.

Watson, S.P., Auger, J.M., McCarty, O.J. & Pearce, A.C. (2005) GPVI and integrin alphaIIb beta3 signaling in platelets. *J Thromb Haemost,* **3,** 1752-1762.

Weinstein, E.A., Li, H., Lawson, J.A., Rokach, J., FitzGerald, G.A. & Axelsen, P.H. (2000) Prothrombinase acceleration by oxidatively damaged phospholipids. *J Biol Chem,* **275,** 22925-22930.

Weiss, H.J. (1995) Flow-related platelet deposition on subendothelium. *Thromb Haemost,* **74,** 117-122.

Weiss, H.J., Vicic, W.J., Lages, B.A. & Rogers, J. (1979) Isolated deficiency of platelet procoagulant activity. *Am J Med,* **67,** 206-213.

Weyrich, A.S., Dixon, D.A., Pabla, R., Elstad, M.R., McIntyre, T.M., Prescott, S.M. & Zimmerman, G.A. (1998) Signal-dependent translation of a regulatory protein, Bcl-3, in activated human platelets. *Proc Natl Acad Sci U S A,* **95,** 5556-5561.

Weyrich, A.S., Lindemann, S. & Zimmerman, G.A. (2003) The evolving role of platelets in inflammation. *J Thromb Haemost,* **1,** 1897-1905.

Weyrich, A.S., Schwertz, H.r. & Mackman, N. (2007) Platelet tissue factor comes of age. *Blood,* **109,** 5069-5070.

White, J.G. (1969) The dense bodies of human platelets: inherent electron opacity of the serotonin storage particles. *Blood,* **33,** 598-606.

White, J.G. (2007) *Platelets Structure.* Academic Press.

Wielders, S., Mukherjee, M., Michiels, J., Rijkers, D.T.S., Cambus, J.P., Knebel, R.W.C., Kakkar, V., Hemker, H.C. & Beguin, S. (1997) The routine determination of the endogenous thrombin potential, first results in different forms of hyper- and hypocoagulability. *Thrombosis and Haemostasis,* **77,** 629-636.

Wilcox, J.N., Smith, K.M., Schwartz, S.M. & Gordon, D. (1989) Localization of tissue factor in the normal vessel wall and in the atherosclerotic plaque. *Proc Natl Acad Sci U S A,* **86,** 2839-2843.

Wolf, B.B. & Green, D.R. (1999) Suicidal tendencies: apoptotic cell death by caspase family proteinases. *J Biol Chem,* **274,** 20049-20052.

Wright, J.R., Thompson, J.A., Singh, R.K., Brouilette, S., Pasi, K.J., Samani, N.J. & Goodall, A.H. (2005) Increased levels of procoagulant factors associated with increased potential for thrombin generation in patients with premature myocardial infarction. *Joirnal of thrombosis and haemostasis,* **3**.

Wu, C.C., Wu, S.Y., Liao, C.Y., Teng, C.M., Wu, Y.C. & Kuo, S.C. (2010) The roles and mechanisms of PAR4 and P2Y12/phosphatidylinositol 3-kinase pathway in maintaining thrombin-induced platelet aggregation. *Br J Pharmacol,* **161,** 643-658.

Wymann, M., Bulgarelli-Leva, G., Zvelebil, M., Pirola, L., Vanhaesebroeck, B., Waterfield, M. & Panayotou, G. (1996) Wortmannin inactivates phosphoinositide 3-kinase by covalent modification of Lys-802, a residue involved in the phosphate transfer reaction. *Mol. Cell. Biol.,* **16,** 1722-1733.

Xiao, H., Jepkorir, C.J., Harvey, K. & Remick, D.G. (2002) Thrombin-induced platelet microparticles improved the aggregability of cryopreserved platelets. *Cryobiology,* **44,** 179-188.

Xu, J., Yu, S., Sun, A.Y. & Sun, G.Y. (2003) Oxidant-mediated AA release from astrocytes involves cPLA(2) and iPLA(2). *Free Radic Biol Med,* **34,** 1531-1543.

Yu, Q., Purwaha, P., Ni, K., Sun, C., Mallik, S. & Qian, S.Y. (2009) Characterization of novel radicals from COX-catalyzed arachidonic acid peroxidation. *Free Radic Biol Med,* **47,** 568-576.

Yuan, Y., Kulkarni, S., Ulsemer, P., Cranmer, S.L., Yap, C.L., Nesbitt, W.S., Harper, I., Mistry, N., Dopheide, S.M., Hughan, S.C., Williamson, D., de la Salle, C., Salem, H.H., Lanza, F. & Jackson, S.P. (1999) The von Willebrand factor-glycoprotein Ib/V/IX interaction induces actin polymerization and cytoskeletal reorganization in rolling platelets and glycoprotein Ib/V/IX-transfected cells. *J Biol Chem,* **274,** 36241-36251.

Yuana, Y., Bertina, R.M. & Osanto, S. (2010) Pre-analytical and analytical issues in the analysis of blood microparticles. *Thromb Haemost,* **105,** 396-408.

Yuana, Y., Bertina, R.M. & Osanto, S. (2011) Pre-analytical and analytical issues in the analysis of blood microparticles. *Thromb Haemost,* **105,** 396-408.

Yuana, Y., Oosterkamp, T.H., Bahatyrova, S., Ashcroft, B., Garcia Rodriguez, P., Bertina, R.M. & Osanto, S. (2009) Atomic force microscopy: a novel approach to the detection of nanosized blood microparticles. *J Thromb Haemost,* **8,** 315-323.

Zaffran, Y., Meyer, S.C., Negrescu, E., Reddy, K.B. & Fox, J.E. (2000) Signaling across the platelet adhesion receptor glycoprotein Ib-IX induces alpha IIbbeta 3 activation both in platelets and a transfected Chinese hamster ovary cell system. *J Biol Chem,* **275,** 16779-16787.

Zahra, S., Anderson, J.A., Stirling, D. & Ludlam, C.A. (2011) Microparticles, malignancy and thrombosis. *Br J Haematol,* **152,** 688-700.

Zhang, H., Nimmer, P.M., Tahir, S.K., Chen, J., Fryer, R.M., Hahn, K.R., Iciek, L.A., Morgan, S.J., Nasarre, M.C., Nelson, R., Preusser, L.C., Reinhart, G.A., Smith, M.L., Rosenberg, S.H., Elmore, S.W. & Tse, C. (2007) Bcl-2 family proteins are essential for platelet survival. *Cell Death Differ,* **14,** 943-951.

Zhou, Q., Hellermann, G.R. & Solomonson, L.P. (1995) Nitric oxide release from resting human platelets. *Thromb Res,* **77,** 87-96.

Zielinski, T., Wachowicz, B., Saluk-Juszczak, J. & Kaca, W. (2001) The generation of superoxide anion in blood platelets in response to different forms of Proteus mirabilis lipopolysaccharide: effects of staurosporin, wortmannin, and indomethacin. *Thromb Res,* **103,** 149-155.

Zwaal, R.F. (1978) Membrane and lipid involvement in blood coagulation. *Biochim Biophys Acta,* **515,** 163-205.

Zwaal, R.F., Comfurius, P. & van Deenen, L.L. (1977) Membrane asymmetry and blood coagulation. *Nature,* **268,** 358-360.

Zwaal, R.F. & Schroit, A.J. (1997) Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. *Blood,* **89,** 1121-1132.

# 10. Appendices

## *Ethical Approval*

- Healthy donors used throughout the study

Blood samples were collected from healthy subjects (staff and students of Leicester University) under either an ethical approval granted by the Leicestershire Local Research Ethics Committee (reference # 5021) entitled ‘Studies of normal platelet and leukocyte function', granted in 1998. The principle investigator for this study was Professor Alison H Goodall.

From September 2009 samples were collected under ethical approval granted by the University of Leicester Research Ethics Committee for a study entitled ‘Studies of haemostasis in healthy human subjects’, granted in September 2009. The principle investigator for this study was Professor Alison H Goodall.

- For patients with MI and matched controls

Blood samples were collected from patients who had suffered an MI, and from matched controls under either an ethical approval granted by the Derbyshire Research Ethics Committee reference (reference 06/Q2401/134) entitled 'Identification of novel monocyte and platelet factors involved in coronary atherothrombosis by comprehensive blood cell transcriptome profiling', granted on 31$^{st}$ October 2006. The principle investigator for this study was Professor Nilesh Samani.

- For patients with chronic kidney disease

Blood samples were collected from renal patients and controls under either an ethical approval granted by the Leicestershire Local Research Ethics Committee (reference 05/Q2502/80) entitled 'Analysis of protein expression patterns in human kidney disease'. The principle investigator for this study was Dr Peter Topham.