`

Development of platelet function analysis for use in haematological and clinical investigations

Mohammad Algahtani,

BSc Medical Technology, MSc Laboratory Medicine

Thesis submitted to the University of Nottingham for the degree of Doctor of Philosophy, January 2016

Dedicated to my parents, father and mother in law, wife and children.

Abstract

Platelets are highly specialised cells that play a pivotal role in the regulation of haemostasis and thrombosis. Accurate measurement of platelet function is important in identifying patients with platelet abnormalities: for example, platelet hyperfunction, which may result in hyperthrombotic risk, or platelet hypofunction, which may lead to enhanced bleeding. Also, accurate measurement is becoming crucial for assessing the adequacy of treatment with antiplatelet therapy. Platelet function testing to assess the efficacy of antiplatelet drugs is becoming widely used and a range of assays has been developed. However, assays such as Light Transmittance Aggregometry (LTA), VerifyNow and Multiplate Electrode Aggregometry (MEA) are cumbersome for regular clinical use and have many limitations. Only a limited number of assays offer the advantage of assessing multiple platelet activation pathways simultaneously. In this thesis, I describe the development of a 96-well plate-based assay carried out in whole blood, where flow cytometry is used concomitantly to assess platelet aggregation (measured as the decrease in number of single platelets in the blood) together with platelet leucocyte conjugate (PLCs) formation, using lysing and non-lysing conditions (fluorescence triggering) – both measurements performed on the same fixed sample of whole blood.

First, this thesis evaluates the effect of blood volume and different fixation approaches, double fixation flow cytometry (DFF) and single fixation Ultra-Flo

100 (SFU), on measuring platelet aggregation in whole blood. The smallest blood volume that was appropriate to study platelet aggregation was 125µl. Both fixation methods were shown to be highly comparable. The preliminary results revealed the suitability of using the 96-well plate format to evaluate the platelet response to a range of different platelet agonists.

The second part of this thesis explores the suitability of the 96-well plate format to study platelet aggregation, and to assess inhibition, using aspirin and the $P2Y_{12}$ antagonist, cangrelor. The 96-well plate format has successfully demonstrated dose-dependent inhibition of adenosine diphosphate (ADP)-induced platelet aggregation with cangrelor and of arachidonic acid (AA)-induced platelet aggregation with aspirin, except when using high concentrations of AA. The apparent failure of aspirin to inhibit AA-induced platelet aggregation at a high concentration could have been due to the fact that endogenous ADP, which may have leaked from red blood cells (RBCs), may have overcome the inhibition. Dual antiplatelet therapy, using aspirin in conjunction with cangrelor, has confirmed this explanation and also demonstrated that more inhibition is obtained when antiplatelet agents are used in pairs. When the glycoprotein (GP) IIb/IIIa antagonist MK-0852 was used to block the final aggregation pathway, it failed to achieve a complete inhibition of platelet aggregation, when measured using the single platelet counting technique. This could be due to the binding of platelets to leucocytes, as demonstrated previously by our group. To investigate whether it was PLCs formation that was responsible for the decrease in the number of single platelets in the presence of MK-852, platelet leucocyte interaction was studied

using lysing and non-lysing approaches, based on a 96-well plate format. The findings demonstrated that PLCs formation in the presence of MK-0852 tends to increase, especially platelet-monocyte conjugate formation. As expected, there was inhibition of PLCs formation with KPL-1, an agent that blocks P-selectin glycoprotein ligand-1 (PSGL-1) on leucocytes, to which P-selectin binds. In this regard, thrombocytopenia, which sometimes occurs after administration of a GPIIb/IIIa antagonist, and the accompanied increase in mortality, could be thereby explained and also implies a potential limitation to the use of GPIIb/IIIa antagonists.

The final part of this thesis focused on the possibility of studying more than one platelet function and the effect of antiplatelet therapy. PLC formation can be measured using fluorescence triggering to capture the leucocyte population, instead of lysing RBCs, to avoid manipulation of the cells. The results have indicated that the use of more than one antiplatelet agent can achieve more inhibition by blocking more activation pathways than a single antiplatelet agent. In conclusion, 96-well plate methods have shown the advantage of assessing multiple platelet activation pathways using a small volume of whole blood. This method has the advantage of using only one sample of fixed whole blood to assess both aggregation and PLCs formation. Moreover, development of this assay can also be useful to testing the effects of new compounds on platelet function.

Acknowledgments

First of all, many thanks to God, who gave me comfort at the difficult times during this study.

Second, I would like to express my sincere gratitude to my research group in the Division of Cardiovascular Medicine (Stan Heptinstall, Susan C. Fox, Natalia Dovlatova, Andrew Johnson, Jane May, Jacqueline R. Glenn, Ann E. White, Gordon May and Linda Pycroft) for their support and guidance. Also, I would like to extend my thanks to Mr Liaque Latif from the School of Life Sciences.

Third, a special thanks to my friend Abdulrahim for his continuous support, encouragement and delicious kabsa (a traditional Saudi dish). Also, thanks to Mr Robert Dawson for sharing his life experience in Nottingham.

Last but not the least, a thank you to the King Abdullah (Allah's mercy be upon him) scholarship program for the unlimited financial support to my PhD study and living expenses during my stay in the United Kingdom.

Contents

List of tables

List of figures

Abbreviations:

% P/M	% platelet positive monocyte
% P/N	% platelet positive neutrophil
5-HT	5-hydroxytryptamine
AA	Arachidonic acid
ACS	Acute coronary syndrome
ADA	Adenosine deaminase
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ASA	Acetylsalicylate
ATP	Adenosine triphosphate
BD	Becton Dickinson
BP	Bandpass
BSS	Bernard-Soulier syndrome
Ca^{2+}	Calcium ion
CADP	Collagen adenosine-5–diphosphate
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CD40-L	CD40 ligand
CEPI	Collagen epinephrine
cGMP	Cyclic guanosine monophosphate
CO_2	Carbon dioxide
COX	Cyclooxygenase
DAG	Diacyl glycerol
DAO	Dot array occupancy
DAPT	Dual antiplatelet therapy

DFF	Double fixation flow cytometry
DMSO	Dimethyl sulphoxide
DTS	Dense tubular system
EC50	Half the maximum effective concentration
EDHF	Endothelial-derived polarizing factor
EDTA	Ethyldiaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ESPS	European Stroke Prevention study
FA	Formaldehyde
FBC	Full blood count
FC	Flow cytometry
FS	Forward scatter
GI	Gastrointestinal
GMP-140	Granule membrane protein-140
GPCRs	G protein-coupled receptors
GRK	G protein-coupled receptor kinase
GT	Glanzmann thrombasthenia
H_2S	Hydrogen sulphide
HCT	Haematocrit
HGB	Haemoglobin
HPR	High platelet reactivity
ICAM	Intracellular adhesion molecule
IP_3	Inositol triphosphate
LFA-1	Leukocytes function associated antigen-1
LTA	Light transmittance aggregometry
MACE	Major adverse cardiovascular events
MAPK	Mitogen activated protein kinase
MEA	Multiplate electrode aggregometry

MMF	Monocyte median fluorescence
MPV	Mean platelet volume
MSA	Multi-sample agitator
NSAID	Non-steroidal anti-inflammatory drug
NMF	Neutrophil median fluorescence
NO	Nitric oxide
PADGEM	Platelet activation-dependent granule external membrane
PAM	Platelet activation marker
PAR	Protease activated receptor
PBS	Phosphate-buffered saline
PCI	Percutaneous coronary intervention
PDE	Phosphodiesterase
PDGF	Platelet derived growth factor
PDW	Platelet distribution width
PF-4	Platelet factor-4
PFA	Paraformaldehyde
PFA-100	Platelet function analyser-100
PFT	Platelet function testing
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PLA_2	Phospholipase A_2
PLATO	Platelet inhibition and patient outcome
PLC	Phospholipase C
PLCs	Platelet-leucocyte conjugates
PMTs	Photomultiplier tubes
POC	Point of care
PP2A	protein phosphatase 2A
PPACK	Phe-Pro-Arg-chloromethyl-ketone

PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PSGL-1	P-selectin Glycoprotein Ligand-1
PT	Prothrombin time
PTCA	Percutaneous transluminal angioplasty
PTT	Partial thromboplastin time
QMC	Queens Medical Centre
QSM	Quorum sensing molecule
RBCs	Red blood cells
RIA	Radioimmunoassay
RPFA	Rapid platelet function analyser
RT	Room temperature
SFU	Single Fixation Ultra-Flo 100
SPC	Single platelet counting
SPCT	Single platelet counting technique
s-P-selectin	soluble-P-selectin
SSC	Side scatter
TEG	Thromboelastography
TGF-β	Transforming growth factor-β
TRAP	Thrombin receptor activated peptide
TREM	Triggering receptor expressed on myeloid cells.
TT	Thrombin time
TXA_2	Thromboxane A_2
VASP	Vasodilator-stimulated phosphoprotein
VCAM	Vascular cell adhesion molecule
VWF	Von Willebrand factor
WBA	Whole blood aggregation
WBC	White blood cell

Poster presentations:

- Mohammad Algahtani, Jane May, Ann White, Natalia Dovlatova, Andrew Johnson, Stan Heptinstall, Sue Fox. Whole blood platelet aggregation measured by single platelet counting using a new double fixation approach to facilitate remote testing, **British Society for Thrombosis and Haemostasis Conference (BSTH), Bath, UK, 18—19 October 2012.**

- Mohammad Algahtani, Natalia Dovlatova, Marie Lordkipanidzé, Jane May, Steve P Watson and Sue Fox. The utility of the 96-well plate based whole blood assay for platelet aggregation to assess the effects of antiplatelet agents, **The 7th Saudi Students' Conference, Edinburgh, UK, 1-2 February 2014.**

- Mohammad Algahtani, Natalia Dovlatova, Jane May, Stan Heptinstall, Sue Fox. Immediate fixation of treated blood samples enables flow cytometric measurement of platelet aggregation and platelet-leucocyte conjugate formation in small volumes of blood, **International Society for Laboratory Haematology (ISLH), The Hague, Netherland, 15-17 May 2014.**
    - **Trainee travel award**

Publications:

- ALGAHTANI, M., DOVLATOVA, N., MAY, J., HEPTINSTALL, S. & FOX, S.2015. A new approach to measuring platelet aggregation and platelet-leucocyte conjugate formation in a small volume of fixed whole blood. Journal of Thrombosis and Haemostasis, 13, 655-656 **(abstract).**

- ALGAHTANI, M., DOVLATOVA, N., MAY, J., HEPTINSTALL, S. & FOX, S.2016. FLOW CYTOMETRIC MEASURMENT OF PLATELET AGGREGATION AND PLATELET-LEUCOCYTE CONJUGATE FORMATION USING SMALL VOLUME OF FIXED WHOLE BLOOD. Platelets, 27, 2-3 **(abstract).**

- ALGAHTANI, M., DOVLATOVA, N., MAY, J., HEPTINSTALL, S. & FOX, S.2015. Immediate fixation allow simultaneous measurment of platelet aggregation (PA) and platelet-leucocyte conjugates (PLCs) formation in a small fixed whole blood **(in preparation).**

- ALGAHTANI, M., DOVLATOVA, N., MAY, J., HEPTINSTALL, S. & FOX, S. 2015. Measurement of platelet aggregation using a 96-well plate methodology involving fixation and platelet counting. The effects of aspirin and a P2Y12 antagonist used in vitro and in patients with acute coronary syndromes **(in preparation).**

1 Introduction

1.1 Platelet

1.1.1 Production

Platelets are the smallest cells in the circulating blood. They are derived from the cytoplasm of megakaryocytes in the bone marrow and typically circulate for 10 days, at a concentration of 150-400 X 10^9/L and a mean volume of a 7-11fl (Pommer et al., 2008). The dimensions of the platelet are approximately 2.0-4.0 by 0.5µm, thus, facilitating their margination toward the vessels wall, where they can frequently check the integrity of the vascular endothelium.

1.1.2 Structure

The platelet consists of three different zones: the peripheral zone, the organelle zone and the sol gel zone, as seen in Figure 1-1. Platelets play a vital role in haemostasis as a response to blood vessel injury, as well as in thrombotic events, which is accomplished by the stimulation of activated platelets toward thrombus formation in response to rupture of an atherosclerotic plaque. Platelets also interact with endothelial cells and leucocytes to promote inflammation, which contributes to atherosclerosis (Jennings, 2009). Human platelets are disc-shaped, anucleated cells as they circulate in the blood in their inactivated (resting) state. When damage occurs to the blood vessel wall, platelets become activated and adhere to the site of injury. This is associated with a change in platelet morphology from disc-shaped to spherical and the formation of pseudopodia, as shown in Figure 1-1. This initiates a cascade of intracellular events (Strauss et al., 2011), resulting in the secretion of the contents of α-and dense granules, which themselves modulate platelet function.

There is also following exposure of adhesive protein on the platelet surface GPIb/V/IX and consequent binding of aggregating protein GPIIb/IIIa to fibrinogen. This leads to platelet aggregation and adherence to the site of injury, ultimately resulting in the formation of a platelet plug. Accurate measurement of platelet function is critical for the identification and diagnosis of patients with diseases featuring platelet dysfunction or hyperfunction. In addition, platelet function testing has become increasingly useful as an effective platelet monitoring tool in modern anti platelet therapy.

As mentioned earlier, platelets possess two types of granules that play a role in the secretion process and the subsequent aggregation phase:

1) Dense granules, which store ADP, adenosine triphosphate (ATP), 5-hydroxytryptamine (5-HT) and calcium ions (Ca^{2+}).

2) Alpha (α)-granules, which contain numerous platelet modulators such as P-selectin, platelet factor-4 (PF-4), von Willebrand factor (VWF), fibrinogen, platelet derived growth factor (PDGF), factor V and β-thromboglobulin (Kaplan et al., 1979).

The surface of platelets contains numerous glycoprotein receptors, such as GPIb/IX/V, which are important for platelet adhesion and GPIIb/IIIa binding during platelet aggregation. Various other receptors, such as $P2Y_{12}$ and protease activated receptors (PAR-1 and PAR-4) for ADP thrombin ligation, respectively, also play an important role in the activation process. Figure 1-1

describes the ultrastructure of the platelet in its resting and activated states (George, 2000a).

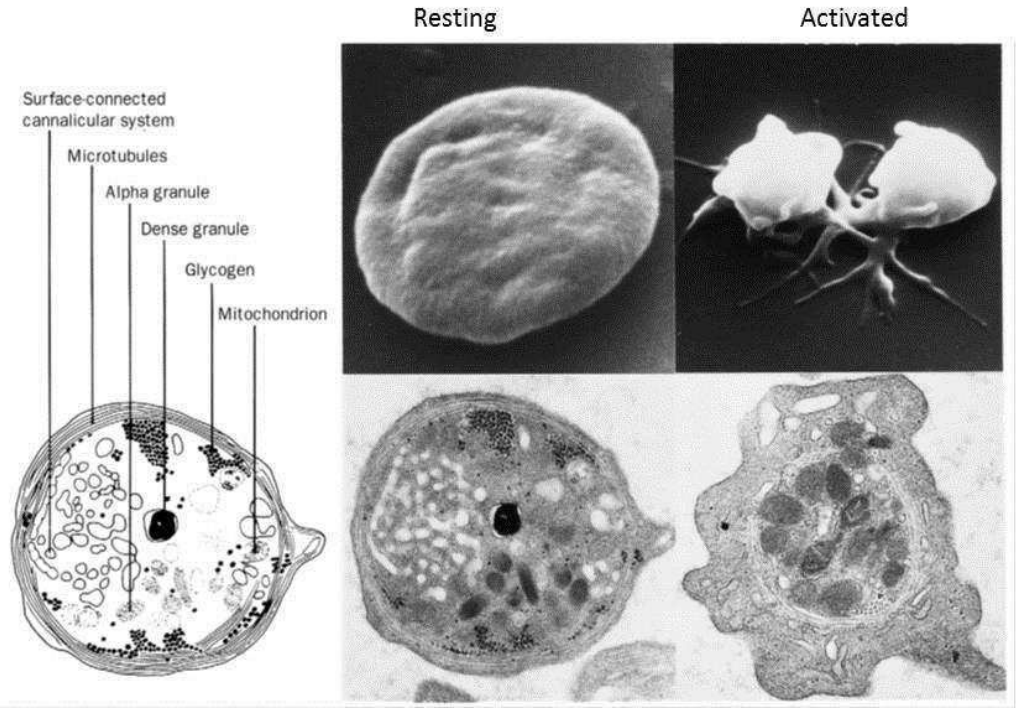

Figure 1-1: Electron microscopy (EM) of platelet ultrastructure.

EM can be used to show the ultrastructure of platelets and differentiate between the resting (disc-shaped) and activated state (pseudopodia; (George, 2000b). The far left schematic shows the different zones of the platelet: the peripheral zone, which contains the glycocalyx, plasma membrane and dense tubular system (DTS); the second zone, the Sol-Gel zone, which forms the cytoskeleton of the platelet and consists of microtubules and microfilaments; and the third zone, the organelle zone, which contains mitochondria and granules. The right electron micrograph shows morphologies of the resting (left) and activated (right) platelet (George, 2000a).

1.1.3 Function

The main function of the platelet is to mediate haemostasis. This is accomplished, in part, by formation of a haemostatic plug via the following events: platelet adhesion, shape change, activation, aggregation and procoagulant ability.

1.1.3.1 Adhesion

The first step of platelet activation is adhesion; this is an important step to maintain the integrity of blood vessels and is also a potential target for certain antithrombotic drugs. Upon vascular injury, the subendothelium layer is exposed and causes collagen exposure. This enhances the binding of the subendothelial layer with VWF, through GPIb/V/IX, which acts as a bridge between the circulating platelets and the site of injury, as well as protecting factor VIII from proteolytic enzymes. GPVI on the platelet surface facilitates platelet binding to collagen, thereby inducing thromboxane A_2 (TXA_2) formation and release. Finally, α2β1 mediates further platelet adhesion and activation (Jenne et al., 2013).

1.1.3.2 Activation and amplification

Following the adhesion phase, the release reaction takes place and includes the secretion of platelet agonists, such as ADP from dense granules and factor V, fibrinogen and P-selectin from α-granules. This induces further platelet activation and also initiates the coagulation response, as a result of the release

of factor V, and an inflammatory response in response to the exposure of P-selectin on the platelet surface. Moreover, activated platelets generate lipid mediators from the plasma membrane, such as TXA_2. TXA_2 interacts with the thromboxane prostanoid (TP) receptor on the platelet surface while ADP interacts with the $P2Y_1$ and $P2Y_{12}$ receptors.

The binding of these chemicals to their receptors on the platelet plasma membrane leads to induction of different intraplatelet signalling pathways. These signalling events alter the intracellular concentrations of Ca^{+2} and cyclic monophosphates – two ubiquitous second messengers that orchestrate main platelet functions. Changes in concentrations of these messengers mediate both activation and inhibition pathways in platelets.

The elevation of cytosolic Ca^{+2} represents the greatest signalling event in platelets. This is due to the ion's capacity to trigger a variety of regulatory enzymes responsible for different functional responses, including granule release and shape change (Siess, 1989). The majority of the increase in cytosolic Ca^{+2} is attributed to Ca^{+2} mobilization from endoplasmic reticulum (ER) intracellular stores (the platelet dense tubular system) and, to a smaller degree, entry of Ca^{+2} through selective plasma membrane ion channels.

Platelets are activated, in part, by the increase in intraplatelet Ca^{+2} through binding of physiological platelet agonists, TXA_2, ADP, thrombin, serotonin and platelet activating factor (PAF). The interaction of these agonists with their receptors via Gq proteins promotes the activation of phospholipase C (PLC),

which is followed by the formation of secondary messengers, inositol 1, 4, 5-triphosphate (IP_3) and diacyl glycerol (DAG) from membrane phosphatidylinositol (PI) (Litosch, 2002).

Finally, binding of IP3 with its receptors (IP3R) on the ER leads to prompt liberation of Ca^{+2} from ER stores. These eventually leads to a platelet shape change from disc-shaped to spherical, with formation of filopodial projections that appear as a meshwork of platelets in plug formation.

1.1.3.3 Aggregation

This process leads to a conformational change in GPIIb/IIIa receptors on the platelet surface, from a low affinity to a high affinity state, and triggers the binding together of platelets to form aggregates that accumulate and arrest bleeding at the site of injury.

GPIIb/IIIa is the main receptor involved in platelet aggregation; an activated GPIIb/IIIa complex allows binding to soluble plasma fibrinogen, leading to platelet aggregation and promotion of spreading of the stimulated platelets along the site of injury. Therefore, aggregation is considerably dependent on G protein-coupled receptors (GPCRs) and is mediated by the bridge between fibrinogen/VWF (under high shear) and activated GPIIb/IIIa complexes on nearby stimulated platelets (Abbate et al., 2012).

As well as the exposure of anionic phospholipids, exposure of phosphatidylserine (PS) primarily provides a surface upon which platelets can

support thrombin generation. Thrombin, the most powerful agonist and the fundamental enzyme of the coagulation cascade, acts by cleaving the protease activated receptor (PAR)-1. This causes further activation and local recruitment of platelets into the area and inclusion of leucocytes, via the interaction of their P-selectin glycoprotein ligand (PSGL)-1 receptor with P-selectin on platelets.

However, pathophysiological states, such as atherosclerotic plaque rupture, can lead to abnormal platelet activation and consequent arterial thrombosis, which can cause myocardial infarction and ischaemic stroke. Figure 1-2 describes the overall haemostatic process and the signalling events involved (Broos et al., 2011, Dorsam and Kunapuli, 2004).

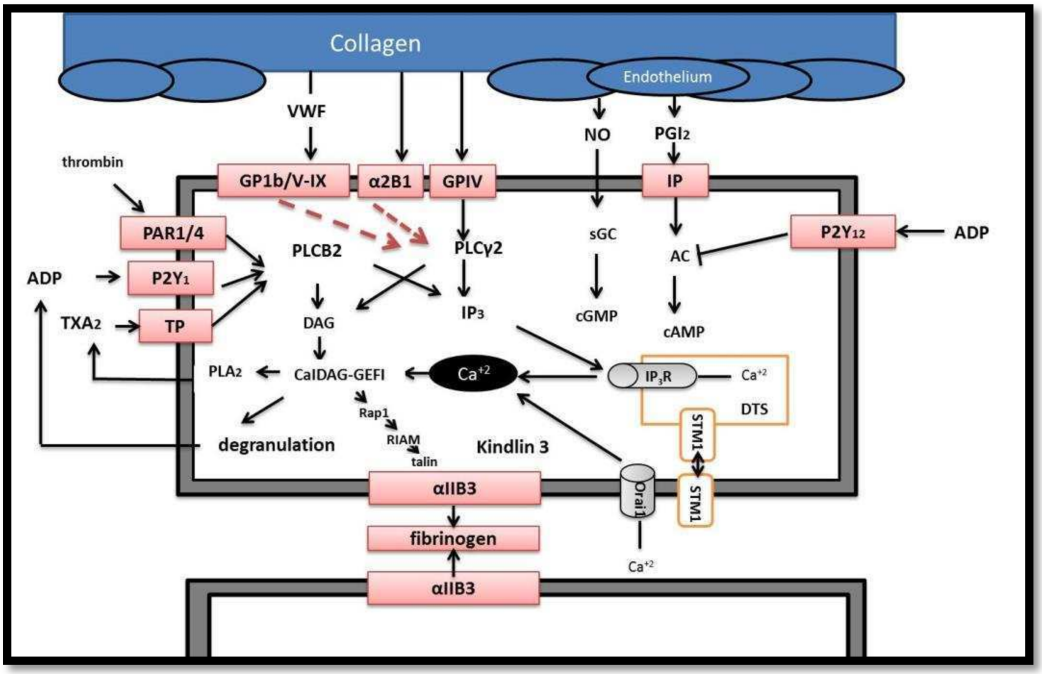

Figure 1-2: The main platelet receptors and signalling pathways (Broos et al., 2011, Dorsam and Kunapuli, 2004).

Overview of the main platelet receptors and effectors involved in platelet signalling: Upon vascular injury, platelets become attached to the vessel wall by interaction with von Willebrand factor (VWF) and collagen (noted as black strands). These cause platelet shape change and the release of platelet mediators such as adenosine diphosphate (ADP), which is secreted from dense granules, and thromboxane A_2 (TXA_2). Both ADP and TXA_2 are agonists that cause further platelet activation and accumulation at the site of injury. Vascular injury also causes the exposure of tissue factor, which initiates the coagulation response and leads to formation of thrombin. Thrombin further activates the platelets, resulting in fibrin aggregation at the site of injury to form a stable haemostatic plug that arrests bleeding. Figure adapted from (Broos et al., 2011).

1.1.3.4 Platelet-leucocyte interaction

Stimulated circulating platelets promptly bind to other platelets in close vicinity to form platelet aggregates. Activated platelets can also bind to leucocytes and this is mediated by the binding of P-selectin and CD40-ligand (CD40L) to PSGL-1 and CD40, respectively, on the leucocyte surface, thereby representing the important role that platelets play in the inflammatory process. This binding has a fast dissociation rate (Kd), for which reason stable adhesion is required and is mediated by β2-integrin Mac-1 (CD11b/CD18) and leucocyte function-associated antigen-1 (LFA-1). In this respect, platelet-monocyte conjugate formation is shown to induce tissue factor expression on the monocyte – a strong promoter of coagulation, thereby representing a crosslink between inflammation and haemostasis (Zhao et al., 2003b).

The crosstalk between platelets and leucocytes may have an influence on inhibition of the two cell types. For example, in the case where a patient presents with a high leucocyte count, platelet function is shown to be inhibited by the action of the surface enzyme, ecto-ADP-ase (CD39), unlike in healthy volunteers with a normal leucocyte count (Heptinstall et al., 2005). Platelets also release transforming growth factor-β (TGF-β) (Hua et al., 1998) and soluble P-selectin (Gamble et al., 1990), which have been shown to inhibit the adhesion of leucocytes to the vascular endothelium and cause generation of superoxide anions.

The presence of platelet leucocyte aggregates has been demonstrated in many cardiovascular diseases, such as stable and unstable angina, myocardial infarction and in patients undergoing percutaneous coronary intervention (PCI) (Cerletti et al., 2012).

1.1.4 Natural inhibitors of platelet activation

Once platelet aggregate formation stops the bleeding through the different mechanisms that involve adhesion, activation and secretion, it is normal for the body to then prevent continuous activation of the platelets. In this respect, many factors play an important role: nitric oxide (NO) and prostacyclin (PGI_2) are released from the vascular endothelium, both of which suppress platelet activation via specific pathways and also have vasodilatory effects (Cines et al., 1998). NO acts by elevating platelet cyclic guanosine monophosphate (GMP) levels by activating guanylate cyclase (GC) and inhibiting agonist-induced increases in cytoplasmic Ca^{2+} levels (Mendelsohn et al., 1990, Loscalzo, 2001). Endothelial cyclooxygenase (COX)-1 and prostaglandin G/H synthase-2 (PGHS-2; also known as COX-2) mediate the production of PGI_2, which activates platelet surface IP receptors through a Gs-mediated increase in platelet cyclic AMP (cAMP) and thereby inhibits platelet activation (Boie et al., 1994, Grosser et al., 2006).

Adenosine is another inhibitory agent of platelet function, which interacts with A_{2A} receptors and increases cAMP levels (Hove-Madsen et al., 2006). It is proposed that plasma adenosine levels rise during hypoxia and ischaemia; also, adenosine is shown to exert an antithrombotic effect in animal models of

thrombosis (Linden et al., 2008). In this regard, adenosine may become accessible when using dipyridamole as an antiplatelet drug, as it inhibits the adenosine uptake by erythrocytes, thereby increasing the exposure of platelets to adenosine and inhibiting platelet cyclic GMP-dependent phosphodiesterase (PDE) (Schaper, 2005).

Platelet function can be regulated by receptor desensitization – an important factor in maintaining the balance in platelet responsiveness to ADP. It has been indicated that after repeated exposure to low concentrations of the agonist, platelets become insensitive to additional stimulation with ADP, in compliance with the term 'desensitization' (O'Brien, 1965). The interaction of ADP with $P2Y_1$ and $P2Y_{12}$ receptors relies on different protein kinases, such as G protein-coupled receptor kinase (GRK), leading to their phosphorylation and desensitization (Hardy et al., 2005). Activated $P2Y_1$ and $P2Y_{12}$ receptors are then internalized using a clathrin-dependent pathway (Baurand et al., 2005). Conversely, internalized receptors are resensitised by dephosphorylation using protein phosphatase 2A (PP2A) and recovered back to the plasma membrane (Mundell et al., 2008). It is evident that the recovery of $P2Y_{12}$ receptors to the plasma membrane is a more rapid process than the recovery of $P2Y_1$ receptors (Baurand et al., 2005). Different regulatory mechanisms govern the desensitization and resensitisation of $P2Y_1$ and $P2Y_{12}$ receptors, which demonstrates their importance in maintaining the response to ADP.

The ADP platelet receptor response is also partially controlled by the enzyme ecto-ADP-ase/CD39, which is located on the surface membrane of leucocytes

and endothelial cells. CD39 acts by converting ADP into AMP, thereby decreasing the concentration of ADP when it is released from the activated platelets or erythrocytes (Atkinson et al., 2006).

1.2 Platelets and antiplatelet drugs

Platelets show a dual mechanism toward the normal haemostatic process. During primary haemostasis, platelets activate and interact with each other to favour platelet aggregation to the injured blood vessels; however, this can only stop bleeding and requires the incorporation of fibrin. During secondary haemostasis, platelets participate in the coagulation process by releasing various coagulation factors, adhesive proteins and exposing phospholipids. These are important initiator events of the coagulation cascade, which leads to stabilisation of the haemostatic plug (Franchini et al., 2008). However, unnecessary or uncontrolled platelet activation, as a result of atherothrombotic plaque, can lead to thrombosis.

The use of antiplatelet drugs helps to reduce the likelihood of intravascular thrombus formation and adverse ischaemic as they achieve platelet inhibition. All of the currently clinically available antiplatelet drugs essentially interfere with some aspect of platelet function, which will be discussed in more detail in the next section (Lippi et al., 2007).

1.2.1 Acetylsalicylic acid

Aspirin, also known as acetylsalicylic acid (ASA) is an important antiplatelet therapy for patients who have, or are at high risk of developing, atherosclerotic diseases. The main action of aspirin on the platelet is inhibition of the production of TXA_2 generation. This is accomplished by irreversible inhibition of COX-1 via irreversible acetylation of the serine residue at position 259 of the protein sequence (Roth and Majerus, 1975, Thorngren and Gustafson, 1983). ASA is absorbed very quickly from the gastrointestinal (GI) tract, achieves peak plasma concentration after 30-40 minutes and takes up to 6 days to recover half of normal platelet function. The reason for this is that platelets are anucleated; thus, they are unable to synthesise additional COX-1 and COX-1 inhibition, therefore, persists for the 5-10 day life span of platelets (Bujold et al., 2011). ASA is bioavailable in several formats: soluble, compressed, delayed release and enteric-coated (Maree et al., 2007, Patrono and Rocca, 2007).

The arachidonic acid (AA) metabolism pathway is an important target for ASA therapy. AA is an important and dominant fatty acid in the platelet plasma membrane. The liberation of the membrane phospholipids by the action of phospholipase A_2 (PLA_2), and then the conversion, via the COX pathway leads to the formation of prostaglandin G_2 (PGG_2). PGG_2 is then converted by endoperoxides to prostaglandin H_2 (PGH_2) (Moncada and Vane, 1979, Lagarde, 1988). Thromboxane synthase then rapidly converts PGH_2 into TXA_2. Both PGH_2 and TXA_2 are extremely potent agonists that can diffuse across the

plasma membrane and bind to specific Gprotein-coupled platelet receptors (Offermanns et al., 1994). Regarding the COX enzyme, it exists in two forms: COX-1, which is constitutively expressed in platelets (Ahmed et al., 2000), and COX-2, which is upregulated during inflammation, in leucocytes and in endothelial cells in response to tissue injury (Smith et al., 1996).

The antithrombotic Trialists' Collaboration revealed that ASA therapy is associated with a 15% reduction in vascular mortality, a 25% reduction in stroke in high-risk atherothrombotic disease patients and a 34% reduction in myocardial infarction (Antithrombotic Trialists, 2002). This trial has recommended a dose of ASA between 75 to 150mg for chronic therapy. However, antiplatelet properties of ASA have been shown to vary among individuals and recurrent events in some patients could be due to ASA non-responsiveness or ASA resistance (Cattaneo, 2004). ASA resistance could have resulted from patient noncompliance, drug interactions – especially with other nonsteroidal anti-inflammatory drugs (NSAIDs) – genetic polymorphisms of COX-1 and components of other pathways of platelet activation not affected by ASA (Hankey and Eikelboom, 2006).

1.2.2 $P2Y_{12}$ antagonist

P2X and P2Y receptor subtypes and tissue distribution

Purinergic receptors are categorized into adenosine (P1) and nucleotide (P2) receptors. Nucleotide receptors are then classified into two groups: ligand-gated ion channels (P2X) and G protein-coupled receptors (P2Y).

P2X receptors

The P2X receptors are ligand-gated ion channels that mediate the fast entry of Ca^{2+} in response to extracellular ATP. There are seven members of P2X receptor: $P2X_1$–$P2X_7$. $P2X_1$ is most widely expressed, with pronounced expression in platelets, megakaryocytes, smooth muscle cells and vas deferens epithelium. Though $P2X_1$ is incapable of triggering platelet aggregation alone, it has been shown to be involved in collagen and shear-induced aggregation (Oury et al., 2001); hence, it is proposed to have a significant role in platelet activation during arterial thrombosis (Mahaut-Smith et al., 2004). Synergistic interaction between $P2X_1$ and $P2Y_1$ has been described, indicating that the $P2X_1$ receptors play a priming role in the subsequent activation of $P2Y_1$ (Vial et al., 2002). $P2X_2$-$P2X_7$ receptors are predominately expressed in the central and peripheral nervous system (Harden et al., 2010).

P2Y receptor

P2Y receptors can be divided into two main subgroups: the $P2Y_1$ subfamily which consists of $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{11}$ receptor subtypes. They are all coupled to Gq to activate phospholipase C, leading to formation of IP3 and consequent mobilisation of intracellular Ca^{2+}, platelet shape change, aggregation and TXA_2 generation (Wijeyeratne and Heptinstall, 2011). They are all activated by different nucleotides (ATP, ADP, uridine di-/triphosphate; UDP/UTP) while $P2Y_{11}$ is the only receptor exclusively activated by ATP (Harden et al., 2010). These receptors confer different cellular functions in different tissues, namely the brain, autonomic nervous system, respiratory

epithelia, GI tract, kidney, eye, vascular tissue, and the immune and inflammatory cells (Harden et al., 2010, Ralevic and Burnstock, 1998). $P2Y_1$, which is expressed in vascular endothelial cells (Wang et al., 2002), is responsible for vasodilation in response to the release of endothelium-derived hyperpolarizing factor (EDHF) and NO from endothelial cells (Malmsjo et al., 1999). The second group of P2Y receptors, known as the $P2Y_{12}$ subfamily, comprises the $P2Y_{12}$, $P2Y_{13}$, and $P2Y_{14}$ subtypes, which are coupled to Gi and inhibit adenylate cyclase, leading to reduction of cAMP and indirect activation of platelets. $P2Y_{12}$ and $P2Y_{13}$ are largely activated by ADP, while $P2Y_{14}$ is activated by UDP-glucose and other nucleotide sugars (Harden et al., 2010). The $P2Y_{12}$ receptor plays a pivotal role in haemostasis, through increased expression on platelets, and in inflammation, by inducing microglia migration in the brain. $P2Y_{13}$ is also expressed in the brain and in the spleen, while $P2Y_{14}$ is largely expressed in the brain, placenta, neutrophils, lymphocytes, epithelial cells from various tissues and in the GI smooth muscle (Harden et al., 2010).

The binding of ADP with the $P2Y_{12}$ receptor plays an important role in platelet activation and thrombogenesis. It also inhibits the activation of adenylate cyclase in response to prostacyclin, a natural inhibitor of platelet aggregation, as seen in Figure 1-3(Cattaneo, 2011).

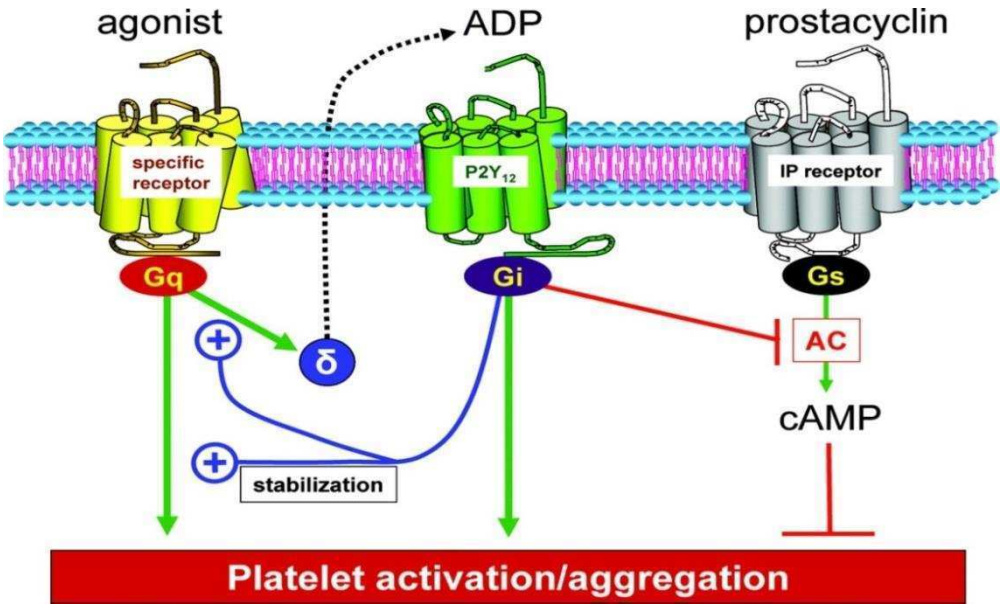

Figure 1-3: The role of ADP in platelet function.

This diagram represents the important receptors situated on the platelet and shows their role in platelet activation, which is achieved by interaction with the Gi-coupled P2Y12 receptor and the inhibition of platelet by exerting the effect on the Gs-coupled IP receptor (Cattaneo, 2011). $P2Y_{12}$ is the main receptor for ADP on platelets. It is also accountable for the amplification of platelet activation and aggregation triggered by other agonists (Storey et al., 2000b). Antagonism at $P2Y_{12}$ receptors has been the target of antiplatelet drugs in the clinic due to the tissue-selective distribution of these receptors, which are restricted to platelets and sub-regions of the brain (Hollopeter et al., 2001). There are a number of agents that have shown to interact with the $P2Y_{12}$ receptor, thereby clinically reducing platelet function. Examples of these drugs are ticlopidine, clopidogrel, prasugrel, cangrelor and ticagrelor. These drugs display more effective inhibition of platelets, however, when used in combination with aspirin.

1.2.2.1 Ticlopidine

Ticlopidine is considered to be the first $P2Y_{12}$ antagonist. It belongs to the group of drugs known as thienopyridines, which include two other drugs, clopidogrel and prasugrel. These prodrugs undergo hepatic metabolism by cytochrome P450 to be converted from the inactive to the active state. The active metabolites then bind to the cysteine residues of the $P2Y_{12}$ receptor, thereby irreversibly preventing the binding of its ligand, ADP (Herbert and Savi, 2003).

Ticlopidine has been shown to irreversibly inhibit ADP-induced platelet aggregation, with greatest effect detected after 15 days of oral administration. It has been used as an alternate to aspirin until its many side effects, such as low neutrophils and thrombotic thrombocytopenia purpura, were reported due to toxicity. Thereby, it has been completely substituted with clopidogrel in the clinic (Benoit and Dogne, 2003) .

1.2.2.2 Clopidogrel

It is prodrug that has to be converted into its active form by the action of the enzyme CYP2C19 or cytochrome P450 in the liver (Savi et al., 2000). A thienopyridine, it is most widely used as an antithrombotic drug and recommended in the clinical setting for the prevention of acute coronary syndrome and ischaemic stroke (Greenhalgh et al., 2011). The irreversible binding of clopidogrel to the $P2Y_{12}$ receptor's cysteine sulfhydryl residues

causes blockade of ADP binding, preventing receptor, and ultimately platelet activation. The drug's effect on platelet function lasts 7-10 days, which is, as mentioned before, the life span of the platelet.

The advantage of clopidogrel was studied in several trials, such as the CAPRIE and CURE trials. In the CAPRIE trial, a population of 19,185 patients with cardiovascular diseases were studied; clopidogrel was revealed to be more effective than aspirin and was established as an alternative drug (Committee, 1996). Later, in the CURE trial, clopidogrel combined with aspirin was shown be more 20% more effective than aspirin alone in reducing death from cardiovascular diseases (Yusuf et al., 2001). These days, dual oral antiplatelet therapy with aspirin and clopidogrel has been the suggested regimen for patients with acute coronary syndrome (ACS) undergoing PCI (Bertrand et al., 2000a). This was shown and clearly investigated in the more recent CHARISMA trial, which shows that dual antiplatelet therapy is superior to aspirin alone in patients with coronary artery disease (Almanaseer and Desai, 2006).

Although clopidogrel provides considerable protection against thrombotic events, it has some disadvantage toward platelet inhibition which ranges from cellular factors to clinical factors. The former include accelerated platelet turnover, an upregulated $P2Y_{12}$ pathway and increased ADP exposure in conjunction with genetic factors, such as polymorphisms of CYP3A. Clinical factors include poor absorption and patient compliance and, in particular, drug

interactions are shown to influence the ability of clopidogrel to inhibit platelet aggregation (Snoep et al., 2007). In this respect, it has been show that certain drugs, such as the proton pump inhibitor, omeprazole, can inhibit the activity of the CYP2C19 enzyme (Azam and Jozic, 2009).

A more recent drug, prasugrel, is similar to clopidogrel in that it is a prodrug; however, it has more rapid action after oral administration. Also, prasugrel is less affected by polymorphism of the CYP2C19 enzyme (Brandt et al., 2007a). The major disadvantage of prasugrel, which restrict its use, however, is the high cost.

1.2.2.3 Prasugrel

Prasugrel is a thienopyridine prodrug, like ticlopidine and clopidogrel, that needs to be converted to its active metabolite, R-138727, to inhibit platelet function. It shares the same property as clopidogrel, in which the active metabolite irreversibly binds to the sulphydryl groups of $P2Y_{12}$ receptors to prevent the platelet response to ADP. However, prasugrel differs from clopidogrel in that it has fast onset (after 30 minutes), a long duration (72 hrs) of action after oral administration and it shows greater platelet inhibition in individual patients. In this regard, most of the patients treated with prasugrel show good inhbition of platelet function, while 30% of patients treated with clopidogrel have an unsatisfactory response (Wijeyeratne and Heptinstall, 2011). The latter is down to to the more efficient in vivo generation of active metobiltes compared to clopidogrel (Sugidachi et al., 2007).

The TRITON-TIMI 38 study compared prasugrel and clopidogrel, revealing a significant improvement (18%) in composite adverse events, such as death from cardiovascular disease, associated with use of prasugrel. However, prasugrel was also associated with increased bleeding in patients, in comparison with those who took the standard dose of clopidogrel (Jakubowski et al., 2010). Despite the fact that prasugrel is shown to be a better inhibitor of platelet function than clopidogrel, the reason for limiting its clincal spread is the high cost and bleeding (Wiviott et al., 2007, Marzot and Pengo, 2009). These shortcomings have to be taken into consideration and might limit the the administration of this drug clinically.

1.2.2.4 Ticagrelor

Ticagrelor, also known as AZD6140, is a cyclopentyl-thiazole-pyrimidine. It is metabolized to its active form, ARC124910XX, which has similar pharmacokinetics to the parent compound (Husted and van Giezen, 2009). The mode of action of ticagrelor and its active metabolite is reversible, thus, it cannot inhibit the platelet function as its concentration in the blood is reduced (upon clearance of the drug from the circulation). Similar to clopidogrel, ticagrelor is also a CYP450 substrate; thus, drug-drug interactions is a likely risk (Nawarskas and Clark, 2011). Numerous clinical trials have studied the effect of ticagrelor in comparison to other antiplatelet drugs.

The DISPERSE study investigated the pharmacodynamics of ticagrelor in atherosclerotic disease patients, in comparison to two other drug regimens: aspirin (70-100mg) once daily; ticagrelor (50,100,200 or 400mg); and clopidogrel (75mg) for 28 days. These studies revealed that ticagrelor (100mg, 200mg or 400mg) inhibited platelet aggregation more effectively and rapidly, both after the first dose and after 28 days of therapy. However, ticagrelor has demonstrated a moderate to high incidence of bleeding, unlike clopidogrel (Husted et al., 2006, Storey et al., 2007). Recently, the PLATO study (platelet inhibition and patient outcomes) compared ticagrelor with clopidogrel with respect to major adverse cardiac events (MACE) in patients with non-ST and ST-elevation ACS. This study has shown that ticagrelor was associated with a decrease in MACE and mortality, compared to clopidogrel (James et al., 2009).

1.2.2.5 Cangrelor

Cangrelor, also known as AR-C69931MX, is an ATP analogue that is administrated intravenously for direct and reversible inhibition of $P2Y_{12}$ receptors (Ingall et al., 1999). Although it cannot be given orally like ticagrelor, cangrelor shows very effective inhibition of platelet function – it disappears within minutes of infusing the drug (Storey et al., 2001). This demonstrates the usefulness of cangrelor during the periprocedural period in patients undergoing PCI.

Cangrelor can be a beneficial antiplatelet agent for distinct period of time, according to the clinical situation (Ueno et al., 2010). In this respect, (Steinhubl

et al., 2008) have shown that cangrelor may counteract the effect of clopidogrel and prasugrel to act as reversible inhibitor of platelet function due to the competitive interaction between cangrelor and the active metabolite of clopidogrel at the $P2Y_{12}$ receptor level. Based on this finding, it is proposed that the clopidogrel loading dose is administered after the termination of cangrelor infusion.

Cangrelor is also used in the research setting to assess platelet inhibition via antagonism of the $P2Y_{12}$ receptor. Various small trials have investigated the effect of cangrelor on platelet aggregation in comparison with other antiplatelet agents. First, it was compared to aspirin in healthy volunteers and showed a distinguished inhibition of platelet aggregation induced by ADP, PAF, thrombin receptor activating peptide (TRAP) and collagen. This indicated the important role of $P2Y_{12}$ receptor in supporting platelet aggregation (Storey et al., 2000b). Later studies have shown the ability of cangrelor to inhibit ADP- and TRAP-induced platelet aggregation in vitro (Storey et al., 2001, Storey et al., 2002b) as well as significant inhibition of ADP-induced platelet activation via P-selectin and platelet-monocyte conjugate formation (Storey et al., 2002a). Recently, the findings of the CHAMPION-PCI clinical study have determined that cangrelor significantly decreases the rate of ischaemic events, including stent thrombosis, during PCI, with no significant increase in severe bleeding (Bhatt et al., 2013).

1.2.3 Dipyridamole

Dipyridamole was originally recognised in 1959 as an antianginal medication, as it presents vasodilation which results in increased blood flow to the coronary arteries, without affecting the heart muscle's oxygen consumption. Later, its effectivness as an antithrombotic agent was demonstrated in animals and humans (Litwack, 1962). Dipyridamole inhibits platelets by blocking platelet cyclic neucleotide phosphodiesterase (PDE)-III and PDE-V, resulting in accumulation of cAMP and cGMP, respectively, and also via the blockade of adenosine uptake, thereby preventing stimulation of adenylate cyclase and an increase in cAMP (Moncada and Korbut, 1978). In vitro and ex vivo studies confirmed that dipyridamole had an inhibitory effect on platelet aggregation in blood stimulated with PAF and AA and this inhibition was potentiated with adenosine (Heptinstall et al., 1986).

In a study with healthy volunteers, dipyridamole was shown to increase the inhibitory effect of aspirin on thrombi developed on the subendothelial matrix (Muller et al., 1990). In early clinical trials, a combination of dipyridamole with aspirin produced a small decline in the risk of stroke and increased survival, in comparison to placebo alone (group, 1987). A following, larger trial (ESPS-II; the European stroke prevention study) showed that the effect of dipyridamole combined with aspirin in patients with previous stroke or transient ischemic attack (TCA) reduced the risk of recurrent stroke and death by 13% and 15%, respectively (Diener et al., 1996).

1.2.4 GPIIb/IIIa antagonist

This group of antiplatelet agents exert their effect by inhibiting the final platelet aggregation pathways that include the interaction of fibrinogen with its main receptors on platelets, GPIIb/IIIa and integrin αIIbβ3 (Coller, 1997).

The first recognised GPIIb/IIIa inhibitor was abciximab, a monoclonal antibody fragment derived from the murine monoclonal antibody 7E3 (Coller et al., 1983). A remarkable benefit of using abciximab was the reduction of thrombotic complications in patients with refractory unstable angina undergoing percutaneous transluminal angioplasty (PTCA) (CAPTURE, 1997). Abciximab can bind up to 80% of the GPIIb/IIIa receptors within 2 hours of intravenous administration and its inhibitory action can last for up to 15 days after cessation of treatment (Mascelli et al., 1998). Tirofiban and eptifibatide are other GPIIb/IIIa antagonists which, if used with heparin or heparin with aspirin, can reduce ischaemic events in patients with unstable angina ACS or those undergoing PCI (Therapy, 2000). The major disadvantages of GPIIb/IIIa antagonists, however, are large variations in pharmacodynamics and pharmacokinetics between individuals, as well as limited safety and efficacy (Kereiakes et al., 1998).

Following platelet activation by strong agonists, such as collagen or thrombin, up to 50% of the GPIIb/IIIa receptors are exposed on the plasma membrane (Wagner et al., 1996). In resting platelets, this internal pool of receptors, located in the α-granules, is not available to interact with GPIIb/IIIa

antagonists. This influences the clinical efficacy of the drug class to completely inhibit the internal pool of GPIIb/IIIa (Gawaz et al., 1998).

Although GPIIb/IIIa inhibitors target the final aggregation pathway, they have no influence on platelet activation, as revealed by several studies (Tsao et al., 1997, Klinkhardt et al., 2000). Consequently, active platelets circulate and bind to leucocytse to form PLCs. In this regard, PLCs formation was shown to be raised where platelets were activated in the presence of GPIIb/IIIa antagonists (Zhao et al., 2003b). This observation could explain the failure of platelet inhibition by orally available agents, which restricts its use to the intravenous administration during coronary procedures. The most commonly used antiplatelet drugs and their specific targets are shown in Figure 1-4.

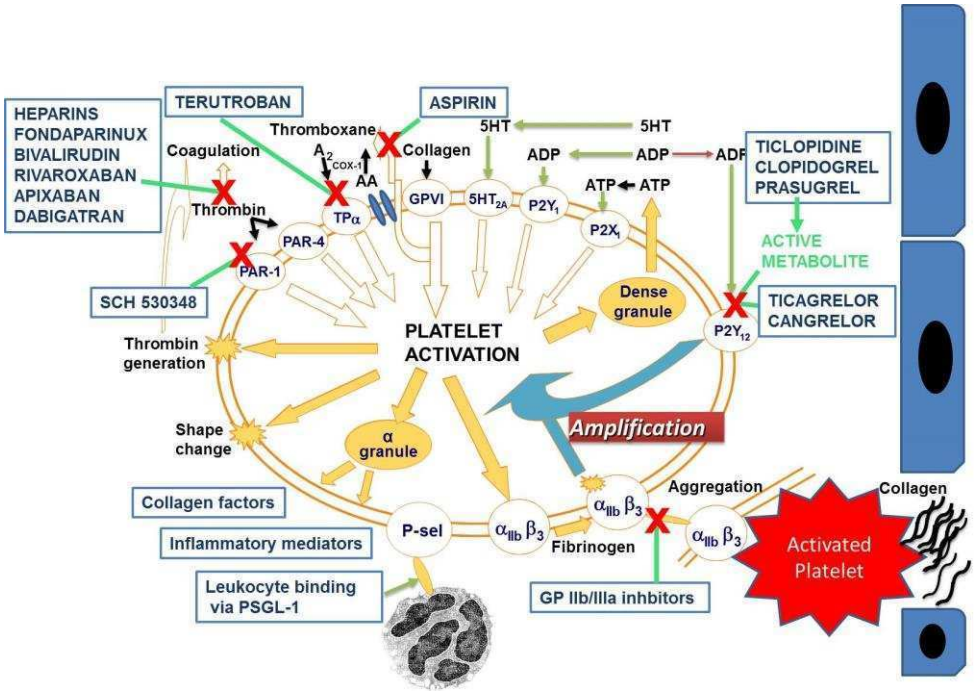

Figure 1-4: Antiplatelet agents and agonists of platelet activation.

Different platelet surface receptors initiate platelet activation, resulting in platelet aggregation, release of α- and dense granule contents, thrombin generation and its pro-coagulant activity triggering of the platelet surface. The released adenosine diphosphate (ADP) from dense granules binds to $P2Y_1$ and $P2Y_{12}$ receptors; hence, $P2Y_{12}$ acts as a powerful amplifier of platelet activation and all the functional responses associated with it. The binding of collagen to glycoprotein (GP)-VI is an important inducer of thromboxane A_2 (TXA_2) production. The contents of α-granules play a role in coagulation and mediate the inflammatory response during thrombosis. The antiplatelet agents acting on their receptors on platelets are demonstrated in the figure (Gladding et al., 2008, Storey, 2011).

1.3 Platelet Function Testing

Evaluation of platelet function is of significance in identifying patients with platelet dysfunction, such as hyperfunction, and has become important for monitoring antiplatelet therapy. The majority of platelet function testing (PFT) has been traditionally used for the diagnosis and management of patients with bleeding events. In this regard, PFT is still labour intensive, time consuming and requires specialised equipment and expert staff to run tests. The identification of haemostasis defects in the laboratory, including platelet function abnormalities, involves a multistep process. This is because platelets are involved in atherothrombosis and newer tests are increasingly used for monitoring the efficacy of antiplatelet therapies, including any variability that may occur between patients, with the purpose to predict any adverse events, such as thrombosis or bleeding in arterial thrombotic diseases. The ultimate goal of PFT is to select the optimal dose of drug for the prevention or the treatment of thrombosis, so as to also minimising haemorrhagic events (Michelson, 2004). The assessment of platelet function is a stepwise procedure that requires clinical and laboratory cooperation in order to successfully diagnose platelet function defects. Table 1-1 summarises the most recent PFT and their clinical values (Choi et al., 2014).

Table 1-1: Major platelet function tests and their clinical applications (Choi et al., 2014).

Name of test	**Principle**	**Clinical applications**
Platelet aggregometry	Platelet aggregation to a panel of agonists	Diagnosis of inherited and acquired platelet defects
PFA-100/200	High shear platelet adhesion and aggregation	Detection of inherited and acquired platelet defects, monitoring antiplatelet drugs
Flow cytometry	Measurement of platelet GP, secretion, MP and activation markers by fluorescence	Diagnosis of platelet GP defects, platelet release, PMP, platelet activation markers, monitoring antiplatelet drugs
Impact	Measurement of platelet adhesion and aggregation under high shear	Detection of inherited and acquired platelet defects, monitoring antiplatelet drugs

Thromboelastography (TEG/ROTEM)	Monitoring rate and quality of clot formation	Prediction of signal bleeding, aid to blood product usage, monitoring antiplatelet drugs
VerifyNow	Platelet aggregation	Monitoring antiplatelet drugs
Multiplate	Platelet aggregation	Monitoring antiplatelet drugs
VASP-P	Flow cytometry with phosphoprotein-phosphorylation	Monitoring $P2Y_{12}$ receptor activity
Microparticles	Flow cytometry with calibrated beads	Platelet activation markers, intracellular communication

Continued Table 1-1:

The initial step in PFT is to obtain a fresh blood sample from a patient or volunteer. As the blood tends to clot in uncoated glass, anticoagulants are used to keep the blood in a liquid state, which is important in order to study platelet function in vitro. In this respect, the preparation of platelets for aggregation studies should always be carried out in either plastic or siliconized glass tubes. Studies of secretion, adhesion and platelet aggregation necessitate an appropriately anticoagulated blood sample. A variety of anticoagulants are used for this purpose, such as citrate, heparin, hirudin and ethylenediaminetetraacetic acid (EDTA) – each with their own advantages and disadvantages.

As platelet aggregation relies on the presence of free Ca2+ in the plasma, the preferred anticoagulants for most aggregation studies are citrate and hirudin. However, EDTA is the preferable agent for measuring numbers of platelet and for morphological studies. This is because EDTA causes physical changes to the platelet, such as the dissociation of GPIIb/IIIa into its subunits, as well altering platelet function as a result of Ca^{2+} chelation. EDTA is, therefore, not suitable for aggregation testing (White et al., 1999**).** Hirudin has two advantages: first, it directly inhibits thrombin and ultimately prevents fibrinogen from being converted into fibrin. Second, it maintains divalent cations, such as Ca^{2+}, and preventing thrombin-catalysed reactions, such as activation of factors V, VIII and XIII. Citrate acts in the same way as EDTA, without damaging the platelet by means of partial chelation of Ca^{2+} **(**Sohn et al., 2001)**;** it also increases the expression of P-selectin on the platelet surface (Gurbel et al., 2007, Storey

et al., 2007). Sodium citrate also causes less spontaneous activation of platelets in vitro than EDTA (Macey et al., 2002).

The major functions of platelets are adhesion to the damaged blood vessel walls, aggregation to form a haemostatic plug, promotion of the coagulation system and induction of inflammation. Thus, PFT is important in studies of patients with platelet defects, such as Bernard-Soulier (BS) syndrome, which is characterised by deficiency of the adhesion receptor GP/Ib/IX/V, and Glanzmann thrombasthenia (GT) – a deficiency of the GPIIb/IIIa receptor. Moreover, platelets play a pivotal role in the pathophysiology of cardiovascular diseases, as inappropriate activation may cause ischaemic events. In antiplatelet therapy, it is vital to perform platelet function tests for the following purposes: 1) to distinguish between responder and non-responder patients to antiplatelet therapy, 2) to evaluate the suitability of the antiplatelet drugs for the patients and 3) as an important tool for surgeons to make the decision of stopping the drugs before surgery to prevent the occurrence of bleeding (Mueller et al., 2009).

Investigations of platelet function have included measurements of the ability of platelets to adhere, aggregate, and/or to undergo activation. The only standard clinical tests of platelet function are bleeding time and platelet aggregometry (Rodgers and Levin, 1990). Some other tests are being used in research settings, such as soluble P-selectin, β-thromboglobulin, platelet factor 4 (PF4) and plasma and urinary assays of TXA_2 metabolites (Venturinelli et al., 2006).

One of the main challenges of assessing platelet function is the complexity of recapitulating haemostasis in vitro (Harrison et al., 2007). This process is complex and very delicate as the platelets are very sensitive to manipulation and are also very susceptible to artefactual in vitro activation.

Lastly, platelets are considered multifunctional and included into many pathophysiological processes such as haemostasis and thrombosis, vessels constriction and repair, inflammation and promotion of atherosclerosis, among others. Thus, PFT could evaluate any one of these essential process, although the majority of tests focus directly on haemostasis, as seen in Figure 1-5 (Harrison, 2005).

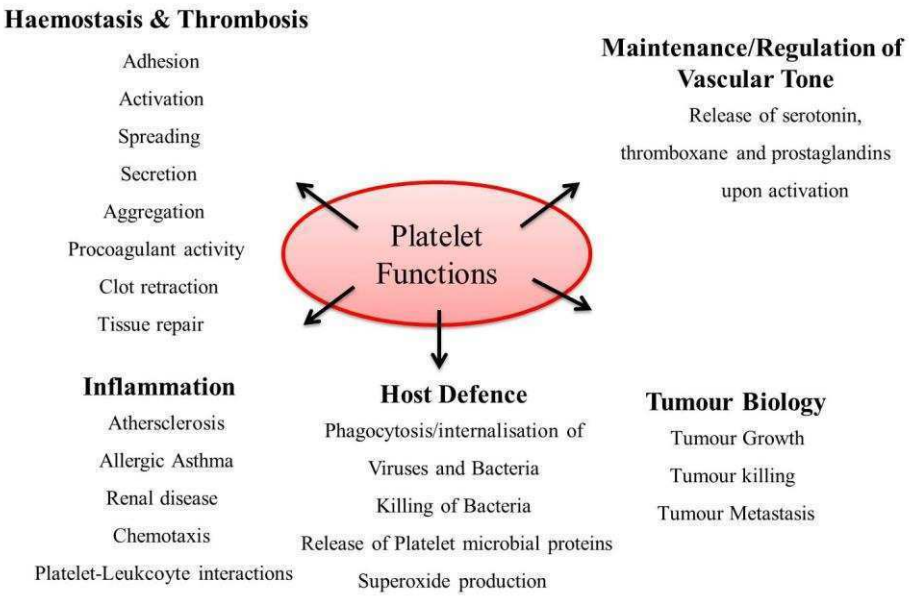

Figure 1-5: The multifunctional platelet.

Platelets are involved in many pathophysiological processes in addition to haemostasis and thrombosis, namely maintenance of vascular tone, inflammation, host defence and tumour biology, among other processes as indicated (Harrison, 2005).

1.3.1 Pre-analytical variables of platelet function testing

PFT is time-consuming and prone to various problems due to pre-analytical variables such as the following:

- An aspirin-containing compound should be avoided for at least 10 days prior to testing because it interferes with the release reaction.
- The platelet studies should not be performed after a fatty meal, especially if optical methods involving platelet-rich plasma (PRP) are used to study platelet function. This is because the presence of lipids can affect the ability of the aggregometer to measure platelet aggregates and can lead to a decrease in the percentage of aggregation.

- The blood should be processed at room temperature, or 37°C, as chilling activates platelet.
- The ratio of blood to anticoagulant should be 1:9 when citrate is the anticoagulant of choice and at least 20ml of blood is needed for a full aggregation study.
- The pH for platelet aggregation studies should be within the physiological range 7.7-8.0 (preferably 7.4) and tests have to be completed within two hours of blood collection (Woodhams et al., 2001). This is important to prevent the evaporation of carbon dioxide (CO_2) after preparation, which leads to an increase in pH (Rogers and Des Prez, 1972).
- PRP should be carefully separated after preparation to avoid contamination with red and buffy coat.

In this method, PRP is usually diluted with platelet-poor plasma (PPP) in order to adjust the platelet count to a standard count of $300X10^9$/L. However, there is a debate surrounding this issue as some researchers argue that PPP may contain some substances that affect platelet function, arguing that PRP is preferred and that the time-consuming process for the adjustment of the platelet count is unnecessary (Linnemann et al., 2008). In addition, others argue that dilution with PPP does not reflect the platelet function in vivo (Cornelia.a.k.van der stelt, 2007). However, more recent studies have shown that the platelet count is believed to be a major contributing factor to in vitro platelet aggregation (Cattaneo et al., 2007).

Another important concern to shed light on is centrifugation**.** (Merolla et al., 2011) suggest in their study that centrifugation has an impact on the platelet count; as centrifugation increases, the mean platelet volume and platelet aggregation decreases. Thus, it is important to standardise the procedure of platelet aggregation (Cattaneo et al., 2009a, Hayward, 2011).

1.3.2 Platelet adhesion

Platelet adhesion exemplifies the initial step in haemostasis and thrombosis. Platelet adhesion to a microplate coated with protein has previously been used (Bellavite et al., 1994). Following that, the technique has undertaken many developments to investigate platelet adhesion under static conditions and shows that platelet adhesion to collagen and fibrinogen is affected by the concentration of divalent cations (Whiss and Andersson, 2002). Also, it was shown that the adhesion was dependent on the surface to which platelets adhere; however, there is no difference between fibrinogen and collagen (Eriksson and Whiss, 2005). It should be noted that the measurement of platelet adhesion is complicated and the potential chance of platelet aggregation cannot be eliminated. Several techniques are available, such as IMPACT-R. This has been used and measures platelet adhesion as the percentage of the well surface covered by platelet aggregate under flow conditions. However; it is less accurate and cannot detect the efficiency of antiplatelet drugs (Bouman et al., 2011). Recently, new developments based on platelet adhesion assay by (Bouman et al., 2011) have been used. Here, a 6µm diameter spot of fibrinogen and

albumin was arrayed on a glass slide. Blood was then added and washed. To identify platelets, primary antibody (CD41) and fluorescent secondary antibody (goat anti-mouse IgG) were used. Platelet adhesion was calculated as the dot array occupancy (DAO), which represents the number of bound platelets/number of bound and unbound platelets X100. This method has the potential to personalise and improve the efficacy of antiplatelet therapy. Recently, a new assay development, based on the use of polystyrene beads coated with different protein, has shown promise as a tool to investigate platelet adhesion and inhibition in whole blood (Tynngard et al., 2015).

1.3.3 Platelet activation

1.3.3.1 Soluble activation marker

When platelets become activated, they release their granule contents. Hence, a method to assess platelet function is based on the measurement of the products released by platelets in platelet-poor plasma (PPP). The most valuable tests are the radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) for platelet specific proteins. These proteins include soluble P-selectin (sP-selectin), β-TG, PF4 and thromboxane β_2 (metabolite of TXA_2) in the urine and plasma (Cerletti et al., 2012).

1.3.3.2 Membrane-bound marker

1.3.3.2.1 Activated marker

P-selectin: selectin refers to family of molecules expressed on the following three cell types: platelets, endothelial cells and leucocytes (P-selectin, E-selectin and L-selectin, respectively) (Blann et al., 2003).

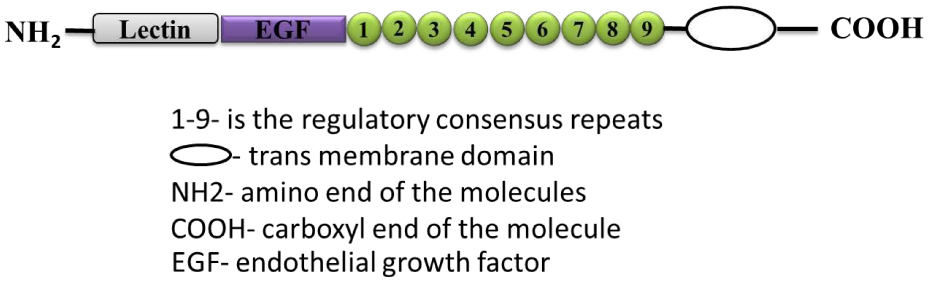

Figure 1-6: Structure of P-selectin (Blann et al., 2003)

The endothelial cell lines the inside surfaces of the blood vessels and interact with the activated platelets. The cell adhesion molecule is also found on inactive endothelial cells, although they are stored within granules, referred to as Weibel-Palade bodies, and α-granules in inactive platelets. P-selectin is also referred to as CD62P, platelet activation-dependent granule, granule membrane protein 140 (GMP-140) or the external membrane protein, PADGEM (Merten and Thiagarajan, 2000).

P-selectin enables the rolling of platelets and leucocytes along activated endothelial cells. This is accomplished by the interaction between P-

selectin with its counter-receptor on leucocytes, known as P-selectin glycoprotein ligand-1 (PSGL-1), which leads to recruitment of neutrophils and macrophages, along with other inflammatory mediators, and induces leucocytes to generate procoagulant microparticles. Also, P-selectin triggers increased expression of tissue factor on monocytes. Once the platelet is activated, P-selectin is moved from intracellular granules to the external membrane, while fibrinogen aggregates the platelets though the bridging of GPIIb/IIIa between adjacent platelets. E-selectin plays a significant role in the original recruitment or activation of leucocytes to the site of injury of inflammation. This is due to the fact that once the endothelial cells are activated, by molecules such as thrombin or histamine, during inflammation, E-selectin moves from an internal cell location to the endothelial cell surface.

Raised levels of P-selectin are detected in several vascular diseases, such as myocardial infarction, unstable angina and thrombocytic thrombocytopenia purpura (TTP). Also, it has been suggested that P-selectin may indicate the functional status of platelets and endothelial cells (Koh et al., 2004). Soluble P-selectin (sP-selectin) is a form of P-selectin relocated from secretory granules to the surfaces of platelets and endothelial cells, after these cells have been physiologically activated, which then sloughs off into the plasma (Blann et al., 2008). Increased levels of soluble P-selectin are seen in hypercholesteraemic patients (Davi et al., 1998).

The most common marker, measured by flow cytometry, to assess platelet activation is P-selectin (CD62P) since it is a constituent of α-granules and is expressed on the surface of platelets when they are degranulated and activated (Blann et al., 2003). The data is usually represented as the percentage of CD62P-positive platelets of the total platelet population, and mean fluorescence of CD62P-positive cells (Merten and Thiagarajan, 2000). This can be performed by stimulating the blood sample with an agonist and then labelling P-selectin with fluorescent antibodies. The resulting florescence is used as an indication of the degree of platelet activation. In this regard, the platelet research group in Nottingham have developed a novel strategy to study platelet activation, based on the measurement of P-selectin, which uses a commercial test kit (provided with the platelet agonists) to stimulate the platelets, followed by fixation of the blood with a novel fixative called PAMFix. The fixed samples were shown to be stable for up to nine days before testing (Fox et al., 2009). This test has two components: the $P2Y_{12}$ P-selectin test and the aspirin P-selectin test. It was used to assess cardiovascular patients with platelet reactivity, while on treatment with aspirin or clopidogrel, which enhances the risk of subsequent cardiovascular events. Platelet reactivity, which is defined as residual platelet activity after antiplatelet drug therapy, was successfully demonstrated when using the $P2Y_{12}$ P-selectin test; however, the aspirin P-selectin test revealed a low response (Thomas et al., 2014). This test has, therefore, gained interest as a tool for measuring platelet activation, rather than platelet aggregation, which has been the focus of most other commercially available platelet function tests. Clinically, GPIIb/IIIa

antagonists that block platelet aggregation were not shown to better than $P2Y_{12}$ antagonists at preventing platelet activation and aggregation. Therefore, it could be deduced that platelet activation may be a more effective target than platelet inhibition the final common pathway of aggregation. Accordingly, measurement of platelet activation via P-selectin may act as a more accurate physiological determinant of the response to antiplatelet therapy than measurement of platelet aggregation. Also, P-selectin measurements can be performed in the presence of GPIIb/IIIa antagonists, unlike tests based on platelet aggregation (Thomas et al., 2014).

1.3.4 Platelet aggregation

PFT is classified based on the underlying mechanisms used to assess platelet function, as well as the appropriateness of such methods in suitable clinical settings. These tests fall into three classes: platelet aggregometry assays, such as light transmittance aggregometry (LTA) and multiplate electrode aggregometry (MEA); flow cytometry; and point of care assays, such as VerifyNow.

1.3.4.1 Light transmittance aggregometry

The assessment of platelet function by optical aggregometry was first developed in 1962 (Born, 1962) (Bartlett, 1977, Barragry et al., 1979). This test is considered as the gold standard and is based on the stimulation of

platelets in PRP after addition of different agonists. such as ADP, epinephrine, collagen, AA and thrombin. Thrombin is often substituted by TRAP, which has the advantage of activating the platelet without converting fibrinogen to fibrin (scott D. Berkowitz, 1998). However, TRAP may not exhibit all aspects of thrombin-induced platelet activation as the bound receptor – PAR-1 – is not the only platelet receptor activated by thrombin. Other platelet receptors of thrombin include PAR-4 and GP1b (Yamamoto et al., 1991).

This method is based on the idea that when platelets aggregate in response to the addition of an agonist, the sample of PRP becomes clear as more light passes through it. This reflects the percentage of aggregation, which is calculated by dividing the distance from baseline to the maximal aggregation achieved by the distance from baseline to the theoretical 100% aggregation, then multiplying by 100 (Pakala and Waksman, 2011) .

This test can be used to determine the degree of platelet inhibition achieved by measuring the baseline of the sample prior to administration of the drug (Mahmud and Ang, 2006). ADP is considered a weak agonist of ADP receptors on the platelet cell surface. It shows biphasic aggregation curves; the initial phase represents (reversible) shape change as the platelets respond to the agonist, while the second phase represents irreversible aggregation of the platelets and degranulation. ADP is selectively secreted from dense granules, as the secondary activator, after primary aggregation induced by collagen, thrombin or high concentrations of ADP itself. Also,

ADP is passively released from injured erythrocytes and endothelial cells. As well as this, ADP can be produced from ATP by the action of CD39 on the endothelium and white blood cells (Yang et al., 2010, Stafford et al., 2003). Two types of ADP receptors, $P2Y_1$ and $P2Y_{12}$, are involved in platelet aggregation. The binding of ADP to $P2Y_1$ receptors is responsible for shape change and Ca^{2+} mobilisation, which causes the primary wave of aggregation, while the binding of ADP to $P2Y_{12}$ receptors is accountable for late or maximum aggregation via the inhibition of adenylate cyclase (Cattaneo and Lecchi, 2007). Thienopyridine drugs, such as clopidogrel, are revealed to selectively inhibit the $P2Y_{12}$, but not the $P2Y_1$, receptor. It is, therefore, more appropriate to evaluate the inhibitory effect of clopidogrel via the measurement of late aggregation, achieved by $P2Y_{12}$ receptors (Linnemann et al., 2008).

AA is considered to be a strong agonist and is converted to the active metabolite TXA_2 by COX and thromboxane synthase. Aggregation induced by AA is normally monophasic and is followed by a short lag phase.

Although LTA is considered to be the gold standard, it still has many disadvantages, such as poor reproducibility, requirement for an experienced technician, high sample volume and sample preparation (Mueller et al., 2009).

1.3.4.2 Impedance Aggregometry

This method of whole blood aggregation (WBA) was initiated in 1980 by Cardinal and Flower (Cardinal and Flower, 1980). It is based on the measurement of the electrical resistance between two electrodes immersed in whole blood. The advantage of this method is that it does not require a centrifugation step and so less damage to the platelets is expected. Moreover, the test is performed under more accurate physiological conditions and takes less time (Dyszkiewicz-Korpanty et al., 2005).

Experimental evidence has revealed that platelets aggregated onto the electrode contain leucocytes and erythrocytes, making this technique significantly more sensitive than PRP for the purpose of monitoring antiplatelet therapy (Nicholson et al., 1998). This method is also insensitive to micro-aggregation. In respect to the amount of ADP needed to induce aggregation, WB requires large amounts of ADP, compared to LTA. This is due to the effect of RBCs and WBCs, which are able to degrade ADP by the enzymes ADP-ase and 5-nucleotidase (Bartlett, 1977).

1.3.4.3 Multiplate Electrode Aggregometry

This is considered to be a new generation platelet function analyser which identifies the electrical impedance changes between two electrodes. These are immersed into stirred blood sample, which is activated either by ADP with PGE_1, AA, TRAP, and other agonists. The activated platelets adhere

to and accumulate on the electrodes. Platelet adhesion to the electrodes results in elevation of the electrical resistance, which is continuously measured for six minutes.

This multiplate technology uses two sets of electrode pairs which confirms that duplicate determination is performed on each sample. Also, it uses disposable test cells to ensure high reliability. Different anticoagulants can be used, such as hirudin, citrate and heparin. Among these, hirudin is the most favoured one as it does not affect the Ca^{2+} level in the sample, which is known to affect platelet aggregation.

The results can be obtained within a few minutes and are represented as the area under the curve (AUC) (Velik-Salchner et al., 2008). The instruments are easy to handle and samples can be tested between 30 and 180 min after venipuncture, as seen in Figure 1-7 (Toth et al., 2006b).

Turbidometric platelet aggregation has been traditionally used in clinical trials as the PFT of choice to assess the effectiveness of antiplatelet therapy and to predict MACE, although the number of MACE incidents was low (Mueller et al., 1997, Gum et al., 2003, Matetzky et al., 2004). The MADONNA study (Siller-Matula et al., 2013), which was used to identify patients with high platelet reactivity (HPR), has demonstrated the benefit of tailored antiplatelet therapy in patients undergoing PCI. The major disadvantages that prevent the use of this test for clinical assessment of

platelet function are high sample volume, the need for sample, the requirement of skilled personnel and a high cost (Modica et al., 2007). MEA has been used also to study inherited bleeding disorders and GT in two studies (Awidi et al., 2009, Albanyan et al., 2015).

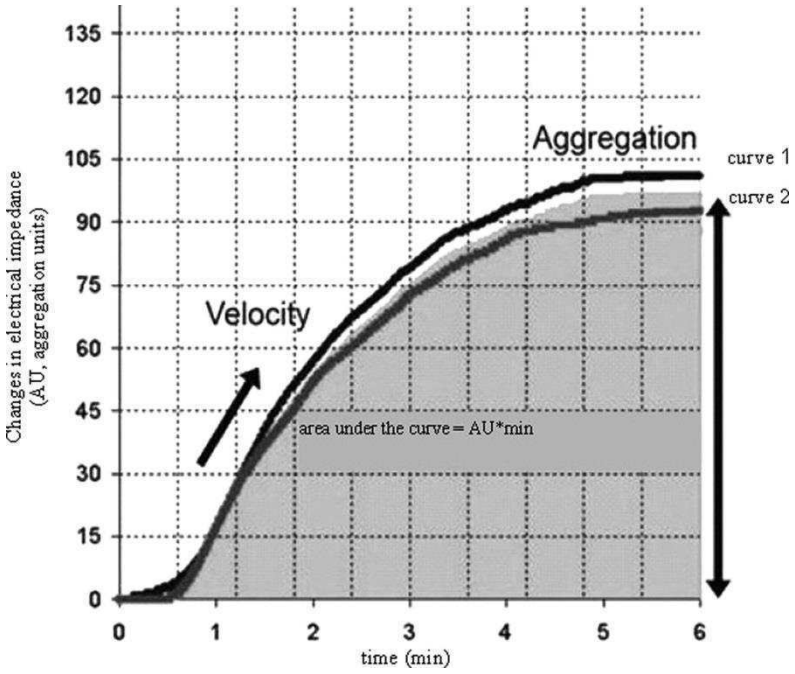

Figure 1-7: Multiplate Electrode Aggregometry data representation.

During a measurement period of 6 min, the change in electrical impedance (AU*min) is calculated from the mean values of the two curves (Toth et al., 2006a).

1.3.4.4 Plateletworks

Plateletworks® (Helena Laboratories, Texas, USA) is a point of care testing that offers an immediate platelet and WBC count, haematocrit (HCT), haemoglobin (HGB) and red cell indices. Also, it can quantify platelet aggregation in whole blood after addition of the agonist. Aggregation kits such as this are depend on the idea of comparing platelet

counts with a control, after aggregating with either ADP or collagen (Harrison et al., 2007). The principle is based on counting the decrease in single platelets using impedance counting technology.

Plateletworks® has many advantages:

1. No sample preparation – it is performed on whole blood using pre-loaded baseline and platelet agonist tubes.
2. A simple, rapid procedure with platelet count and aggregation results available under 5 min.
3. Automatic, closed tube sampling optimises safety and ease of use for any operator.

However, the fresh blood sample has to be assessed immediately, so as to minimize delay which can prevent accurate assessment of platelet aggregation.

1.3.4.5 VerifyNow®

This is a point of care test, formerly known as the Ultegra Rapid Platelet Function Analyser (RPFA). It is used for the measurement of aspirin, clopidogrel or GPIIb/IIIa antagonists-induced defects in platelet function. VerifyNow® (Accumetrics, San Diego, USA) utilises the same principle as the LTA, in which ADP is used to stimulate platelets and to monitor clopidogrel therapy, while AA is used to monitor aspirin therapy. Following activation by the agonist, fibrinogen-coated beads are able to bind to the activated platelet through GbIIb/IIIa on the platelet surface to

form the agglutinate. Three assays are currently commercially available: the GPIIb/IIIa assay (sensitive to GPIIb/IIIa antagonists); the $P2Y_{12}$ Assay (sensitive to theinopyridines); and the aspirin assay (sensitive to aspirin) (Figure 1-8). This instrument is considered as point of care technique, where the tests are fully automated, without the requirements of sample transport, time delay or a specialised laboratory.

In the aspirin assay, aspirin is capable of blocking the activity of the circulating enzyme COX-1, activated by AA. In contrast, the $P2Y_{12}$ assay uses two agonist: ADP, which interacts with $P2Y_1$ and $P2Y_{12}$ receptors; and prostaglandin E1, which suppress the activity of $P2Y_1$ receptors to reduce the intracellular free Ca^{2+} level (Michelson et al., 2006).

Aspirin and anti-$P2Y_{12}$ drugs are normally administered for the management of ACS patients, and following PCI, to lessen the prevalence of recurring cardiovascular events. In this regard, clopidogrel is viewed as one of the most commonly used $P2Y_{12}$ antagonists; however, the pharmacodynamical response to clopidogrel varies, with a significant number of patients presenting with platelet reactivity irrespective of the treatment – which is normally associated with increased risk of cardiovascular outcomes (Aradi et al., 2010). In this respect, the VerifyNow® system has been used in two large studies, GRAVITAS and TRIGGER-PCI, with the intention to show the possible benefit of personalised antiplatelet therapy in low risk patients undergoing PCI for stable coronary artery disease. The GRAVITAS study did not reveal the

benefit of tailored antiplatelet therapy, while TRIGGER-PCI demonstrated an unexpectedly small number of adverse cardiovascular events (Price et al., 2011, Trenk et al., 2012).

A decreased level of platelet inhibition, as shown by the use of the VerifyNow® GPIIb/IIIa assay, is coupled with increased incidence of MACE in patient treated with abciximab (Steinhubl et al., 2001). Using the VerifyNow® aspirin test in patients treated with aspirin before PCI, the level of platelet function or 'aspirin resistance' was associated with increased incidence of post-PCI myonecrosis (Chen et al., 2004).

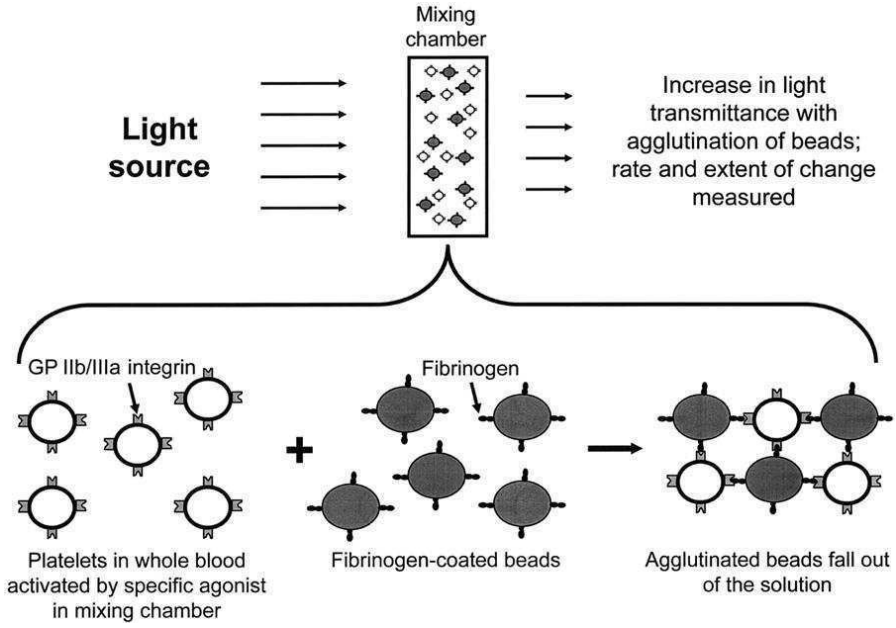

Figure 1-8: Diagrammatic representation of the platelet function test by VerifyNow®.

The mixing chambers contain a platelet agonist (arachidonic acid (AA), adenosine diphosphate (ADP) or thrombin receptor activating peptide (Iso-TRAP) and fibrinogen-coated beads. When the anticoagulated whole blood is added to the mixing chambers, the platelets are activated. Then, the activated glycoprotein (GP)-IIb/IIIa receptors on platelets bind fibrinogen on the beads and cause agglutination of the platelets and beads. Light is then passed through the chambers and measured; light intensity increases as the agglutinated platelets and beads are removed out of the solution. Inhibition of GPIIb/IIIa receptor ligation by inhibition of arachidonic acid- or ADP-induced platelet activation, or direct pharmacologic blockade of GPIIb/IIIa receptors with antagonists, reduces agglutination in proportion to the amount of platelet inhibition achieved (Michelson et al., 2006).

1.3.4.6 Platelet function analyser, PFA-100

The Platelet Function Assay (PFA)-100 (Siemens Healthcare Diagnostics, Illinois, USA) is a new point of care test that depends on the time needed for the occlusion of an aperture (known as closure time) in response to a specific stimulus, under conditions of high shear. This is designed so as to recapitulate the process that happens during vessel wall damage. Two membranes are used; collagen/epinephrine (CEPI) or collagen plus adenosine-5-diphosphate (CADP). A cartridge is introduced, PFA $P2Y_{12}$ (Siemens Healthcare Diagnostics, Illinois), specifically created for the purpose of clopidogrel monitoring (Ang and Mahmud, 2008). The advantages of this test include low sample volume, simplicity, rapidity and no requirement for sample preparation, as it depends on whole blood and the physiologically relevant high shear state. The presence of the platelet activator together with a high shear rate results in platelet attachment, activation and aggregation. CEPI was developed to detect aspirin-induced defects while CADP was developed for platelet dysfunction testing (Mueller et al., 2009).

The use of PFA-100 CEPI is useful for monitoring aspirin therapy and may exhibit plaque instability in coronary artery disease (Linden et al., 2007) . Although it is supposedly less appropriate for cases of aspirin resistance than other tests, it has been commonly used in clinical investigations of aspirin resistance (Kratzer, 2006). The test has also been show to predict MACE in patients in aspirin non-responder state with recurrent ischaemic attacks (Grundmann et al., 2003). PFA-100 has also been used to identify

inhibition with GPIIb/IIIa antagonists, as failure to detect non-closure is concurrent with increased incidence in following MACE (Madan et al., 2002). On the other hand, PFA-100 is not suggested for monitoring clopidogrel therapy (Hayward et al., 2006) The PFA-100 results are highly reliant on the platelet count and HCT, as results under 80 x 10^9/L and 30% cause substantial prolongation of the closure time (Harrison et al., 1999). PFA-100 could also be used to detect intraoperative blood loss during surgery (Toth et al., 2006b). However, PFA-100 should preferably be used in combination with other tests, such as prothrombin time (PT), partial thromboplastin time (PTT) and thrombin time (TT).

1.3.4.7 Flow Cytometry

Flow cytometry is a technique that rapidly determines the specific features of a large number of individual cells. Before flow cytometry analysis, cells in suspension are labeled with fluorescent monoclonal antibodies, then passed through a flow chamber at a rate of 1,000 to 10,000 cells/min. The cells are focused through a beam of laser and scatter the light in forward or side scatter angles, corresponding to the properties of each cell. Forward scatter light reflects the cell size, while side scattering reflects cell complexity (Roussel et al., 2010).

With regard to platelet function, flow cytometers are being used to measure activation–dependant changes on the platelet surface membrane receptors, including P-selectin (CD62b), PAC-1, GbIIb/IIIa, CD63 and GPIb/V/IX, as well as constitutively expressed molecules, such as CD42a, CD42b, CD40

and CD61 (Blann et al., 2003). In this respect, the use of flow cytometry to examine platelet activation markers before PCI can predict increased risk of acute and subacute ischaemic events after PCI (Kabbani et al., 2003). Flow cytometry is also applicable to quantification of platelet-leucocyte interactions, particularly monocytes, which are considered as markers of platelet activation in vivo platelet (Michelson et al., 2001). Increased levels of circulating platelet leucocyte aggregates (PLA), as measured by whole blood flow cytometry, are shown after PCI (Michelson et al., 2001). Platelet monocyte aggregates (PMA), measured by flow cytometry, are considered to be early markers of myocardial infarction (Furman et al., 2001). This method has the advantages of using whole blood, a small sample volume and requires minimal manipulation of the sample to prevent the loss of platelet subpopulations. However, flow cytometry has many disadvantages, such as the requirement of intricate sample, high cost of equipment and difficult maintenance (Berkowitz et al., 1998).

Flow cytometry is also of importance in studying intracellular signalling mechanisms, such as phosphorylation; for instance, the vasodilator–stimulated phosphoprotein (VASP) phosphorylation assay. VASP is an intracellular actin regulatory protein, a substrate for both cAMP- and cGMP-dependent protein kinase and is abundantly expressed in platelets. VASP is phosphorylated at two sites, serine 157 (Ser157) and serine 239 (Ser239) and reflects the level of cAMP and cGMP in the platelets (Yamamoto et al., 2008). It is considered as a marker for platelet inhibition as phosphorylation associates with inhibition of the $P2Y_{12}$ receptor and,

therefore, inhibition of platelet aggregation, while the non-phosphorylated form is associated with basal or induced activity (Horstrup et al., 1994, Schwarz et al., 1999).

Whole blood flow cytometry is of distinctive benefit to clinical trials, where samples can be taken at remote sites, with no need of specific expertise or platelet function analysis, and then dispatched to the core testing laboratory. This may result in good quality control measures, as all assays are performed in a single core laboratory. In this regard, VASP assay samples in citrated WB are shown to be stable for 72 hours, at room temperature, prior to analysis at the core laboratory. Recently, our group has invented a fixative solution termed 'VASPFix' to use for the measurement of VASP phosphorylation in platelets and to monitor the effects of $P2Y_{12}$ receptor antagonists. This fixative has shown to enable good stability of samples for up to six months, frozen (Glenn et al., 2014).

1.3.4.8 Single platelet counting

Single platelet counting (SPC) measures platelet aggregation as the decrease in single platelets, as they form aggregates in stirred whole blood sample (Fox et al., 1982). The platelet count can be measured in small sub-samples that are eliminated from the test tube and then fixed at different time points, thus, offering kinetic information about platelet aggregation.

Platelets are then labelled with specific antibodies and counted using flow cytometry. In this case, RBCs are used as the internal standard for counting

individual platelets. This method is sensitive to micro-aggregate formation and also provides evidence of disaggregation of the platelets.

1.3.5 Platelet-leucocyte conjugate formation

At the site of plaque rupture, activated platelets bind to PSGL-1, present on the leucocytes, via P-selectin. This modifies the leucocytes' activation model and their recruitment, ascertaining the role of platelets in the inflammatory process (Merhi et al., 1997, Theilmeier et al., 1999). The measurement of PLCs formation may act as a stronger indicator of platelet activation than the detection of surface P-selectin. This is because, upon platelet degranulation in vivo, they lose surface P-selectin and continue to circulate (Michelson et al., 1996a).

With respect to platelet activation, until recently, surface P-selectin (CD62P) has been considered the gold standard marker of platelet activation (Michelson et al., 2001). Different flow cytometry methods have been previously reported to measure PLCs formation. This was associate with the concern of a possibility of in vitro platelet activation that may result in artefactual platelet PLC formation, causing overestimation of the complexes formed.

There are two approaches used to measure PLCs formation. The first method involves cell separation and RBC lysis to remove erythrocytes and permit clear separation of white cell populations (Li et al., 1997, Michelson

et al., 2001). The second method does not involve RBC lysis, thus, helping minimise platelet activation. (Hagberg and Lyberg, 2000b). In this method, common anti-CD45 leucocyte markers are used to discriminate erythrocytes from leucocytes. Then, a combination of a platelet-specific, anti-CD61 and monocyte-specific anti-CD14 antibodies allow for the detection of PLCs (Barnard et al., 2003). Both methods have been used in the experimental phase of this thesis, with application of different optimisation steps.

1.3.6 Summary

There is an increased interest in the evaluation of platelet function to monitor the effect of antiplatelet therapy, as a result of the variability that occurs between patients in the response of their platelets to antiplatelet drugs. This created the goal to select the optimum dose of antiplatelet drugs that treat thrombosis and minimise bleeding side effects. Aspirin response variability, or resistance, can be measured by PFT utilising AA (VerifyNow Aspirin assay or plateletworks) or thromboxanes as the end point (serum thromboxane B_2 or urinary 11-dehydro thromboxane B_2). Theinopyridine response variability, or resistance, can be measured by PFTs that utilise ADP (VerifyNow $P2Y_{12}$ assay or plateletworks) or by signalling–dependent assays, such as the VASP phosphorylation assay (Penz et al., 2007).

Platelet aggregation, based on turbidometry, is still regarded as the gold standard platelet function test, to some degree, as most large clinical trials

of antiplatelet drugs have used this as a final stage. However, turbidometric platelet aggregation has many disadvantages, such as the requirement of large blood volumes, expert technicians and sample preparation. Whole blood flow cytometry technology, therefore, offers distinctive advantages in clinical trials as they don't require expert staff, or platelet function analysers at the local site, and samples can be sent off to a central laboratory with decent quality control.

POC assays, such as VerifyNow circumvent the limitations encountered with turbidometric platelet aggregation and reveal great potential for clinical usefulness in patients with coronary artery diseases treated with antiplatelet drugs. In those patients, the degree of platelet inhibition, as determined by platelet reactivity testing using VerifyNow® in the GRAVITAS study (Price et al., 2011), and MEA in the MADONNA study, (Siller-Matula et al., 2012) has been useful to predict MACE.

1.4 Scope of the study

PFT and the effects of drugs that inhibit platelet activity can help to facilitate the development of effective antiplatelet therapy, as well as help to identify individuals vulnerable to thrombosis. In our lab, some new approaches to platelet function testing have been developed. These techniques rely on the use of a fixative solution that stabilises platelet aggregates for up to 9 days and PLCs for up to 3 days. The studies

presented in this thesis address, in more detail the suitability of developing PFT and explore approaches that utilise small volumes of blood. The idea of using a 96-well plate approach to study platelet function appears to be promising. This, first, required that different parameters, such as platelet concentration and the effect of different fixative methods, were studied. Second, based on the established protocol to study platelet aggregation using a single platelet counting technique and flow cytometry, this method was further developed to 1) accommodate more agonists and antagonists, using the 96-well plate format and 2) to assess in vitro and ex vivo platelet inhibition after stimulation with different agonists, using small blood volumes. This modified method was found to be appropriate to study platelet aggregation and PLCs formation from the same fixed whole blood sample, thus, providing more clinical value to the effect of antiplatelet drug on the multifunctional role of platelets in haemostasis and thrombosis simultaneously.

2 Materials and Methods

2.1 Materials

2.1.1 Anticoagulants

Tri-sodium citrate dehydrate (citrate) was prepared as 3.13% (w/v) solution in distilled water and was used at a ratio of 1:9 with blood. Evacuated tubes contained 3.1% tri-sodium citrate (S-Monovette 0.106M; Sarstedt, Leicester, United Kingdom, UK). Ethyldiaminetetraacetic acid (EDTA) was prepared as a 100mM solution in water, pH 7.4, and was used at a ratio of 1:25 with blood (final concentration of 4mM).

2.1.2 Agonists

Adenosine-5'-diphosphate (ADP) and thrombin receptor activated peptide (TRAP) were obtained from Sigma Aldrich Chemical Co. (Poole, UK). They were dissolved in saline at a final concentration of 2.5mmol/l. Arachidonic acid (AA) was prepared in ethanol at a concentration of 25mM and stored at -20^{o}C. A known volume of AA was dried down under nitrogen and reconstituted in twice the volume of 0.1M Na_2CO_3 to provide a 12.5mM stock solution. This solution was stored frozen until required. U46619 was prepared in ethanol at a concentration of 10mM and stored at -20°C; this was diluted 1/400 with saline before use to provide a stock solution of 25μM. This solution was stored frozen until required. Both AA and U46619 were obtained from Sigma Aldrich Chemical Co. (Poole, UK). The agonists were dissolved in saline at a concentration of 2.5mM

(25×100µM) and final working concentrations were prepared daily by further dilution in saline. Collagen (Horm Chemie) was obtained from Biomerieux (Basingstoke, UK) at a concentration of 1mg/ml. The required concentration of collagen was prepared daily by dissolving in the provided buffer.

2.1.3 Antagonists

MK-0852, a cyclic heptapeptide based on the RGD amino acid sequence (Merck Sharp and Dohme), was prepared as a 0.25mM solution in saline. Aspirin, from Sigma Aldrich Chemical Co. (Poole, UK), was kept as a frozen stock in saline at a final concentration of 2.5mmol/l. Cangrelor (ARC-C69931) was obtained from The Medicines Company (New Jersey, USA) and stored frozen as 25µl stock. KPL-1 (anti-PSGL-1 or anti-CD162) from Abcam (Cambridge, UK) were stored a 4^{o}C short term (1-2 weeks). For long term use, aliquots were stored at -20^{o}C or -80^{o}C. Platelet antagonists were prepared in fresh saline (sodium chloride, 0.9% w/v), always incubated with blood for 30 minutes and then added to the whole blood before the agonist. Quorum sensing molecules (QSM) were obtained from Sigma Aldrich Chemical Co. (Poole, UK) and prepared in 99.9% (w/v) dimethyl sulfoxide (DMSO).

2.1.4 Antibodies

CD61-PE (MCA1227F), CD62P-PRE (MCA796PE) and CD45-PerCP (Cat.345809) were obtained from AbD Serotec (Oxford, UK). CD14-APCCy7 (Cat 557831) and Isotypic IgG-FITC (Cat. 345815) was obtained from Becton Dickinson (Oxford, UK). Mouse anti-human CD62P-FITC (Cat. MCA883F), CD42a-FITC (Cat. MCA 1227F) were from AbD Serotec (Oxford, UK).

2.1.5 General materials

96-well plate flat bottom (Co-star 3370, assay plate with low evaporation Lid, Non-Treated, sterile, polystyrene) were obtained from www.corning.com/lifesciences; 96-well plate round bottom were obtained from Thermo scientific (Cat No 611U96); and Optimul 96-well plates were prepared by Tim Warner's research group at Queen Mary University of London, as previously described (Chan et al., 2011), in which micro-titre plates were pre-coated with hydrogenated gelatine (0.75% w/v) in phosphate-buffered saline (PBS) to block the surface activation of platelets before the addition of the platelet agonists. The plates were then placed in an -80^{o}C freezer for 1 hour, before being removed into a freeze-dryer and left overnight at -40^{o}C. The plates were then removed from the freeze-dryer, vacuum-sealed, foil-packed and kept at room temperature. Plates were either used immediately or stored for 3, 6, 12 or 24 weeks. Plate stickers and adhesive film for micro-plates were obtained from VWR (Cat No 60941-062); erythrolyse RBC lysing buffer (10x) was obtained from

AbD Serotec (Oxford, UK), stored at room temperature, and diluted 1 in 10 with distilled water as required. FACS tubes, FACS flow solution (Cat. 342003) and sheath fluid for flow cytometry were obtained from Becton Dickinson (Oxford, UK). For QSM, steps were taken to ensure that the concentration of DMSO was always the same (0.1%). Saline (0.9% sodium chloride) was supplied by Baxter Healthcare Ltd (Thetford, UK).

PBS from Oxoid, ADA (adenosine deaminase) was from obtained from Sigma. Fixing solution (AggFixA) and (AggFixB) were obtained from Platelet Solutions Ltd (Nottingham, UK), while 2ml syringe, 0.2um filter and collagen coated plates were obtained from Becton Dickinson (MA01730). The CO_2 incubator, safety cabinet, electronic pipette and polystyrene tubes (64 x 11mm) were obtained from Bibby Sterilin (Cat No. 30908 and 95 x 16.8mm) and 10ml graduated polystyrene tubes were obtained from Sarstedt (Leicester , UK; Cat No (62492), FACS tubes were obtained from Becton Dickinson (Oxford, UK; Cat No 352052).

2.1.6 Devices

2.1.6.1 Multi-Sample Agitator

All the aggregation studies described that used test tubes (LP3) were performed using a dedicated multi-sample agitator (MSA), as shown in Figure 2-1. The MSA (University Of Nottingham, Nottingham, UK) is a portable, compact piece of equipment which consists of an aluminium

block that is surrounded by a heater pad and a magnetic stirring device, aimed to provide accurate temperature control and stirring. The temperature setting is determined by means of a temperature controller on the front panel of the unit. The temperature is pre-set to 37°C, but can be adjusted in the range of 0–50°C, as per the reference manual supplied with the instrument. 12 evenly spaced wells, located in the central portion of the block, in a circular arrangement, are designed to accommodate standard 64×11mm polystyrene tubes, in which small 500ml aliquots of blood are placed. A small Teflon™-coated magnetic flea is placed in the blood and agitated via a magnetic stirring device located beneath the aluminium block. The block also has 17 additional wells arranged in an outer circle for pre-incubation of blood and storage. These consist of a further 12 wells of the same size: central wells together with a further 5 wells, which can accommodate larger 95×16.8mm capped polystyrene tubes, containing up to 10ml of anti-coagulated blood. The MSA is used for two purposes: 1) to agitate the blood sample at a standard stir speed in a reproducible and consistent way and 2) to keep the blood sample at 37°C. It has been widely used by our research group in the University of Nottingham.

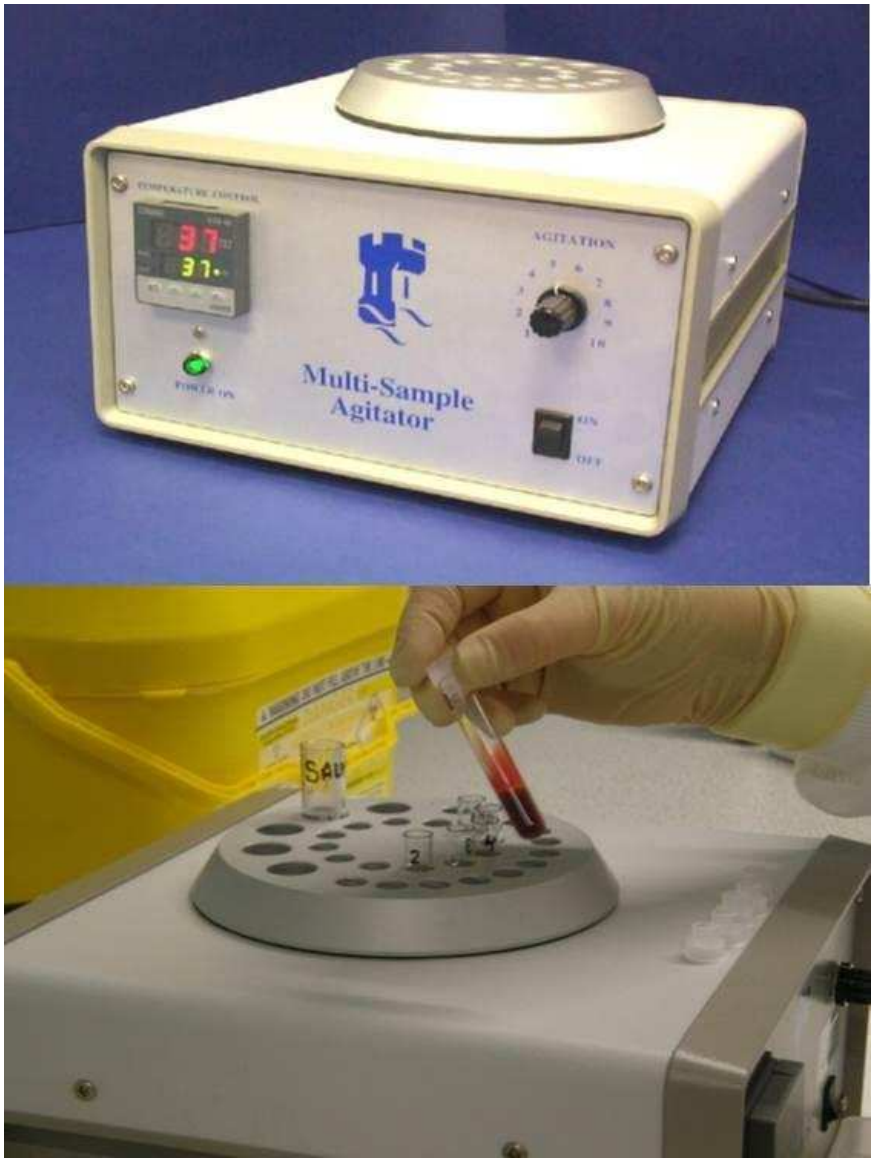

Figure 2-1: The Multi-Sample Agitator.

2.1.6.2 Bio-shaker

This small device is used to shake the 96-well plate and it has the following properties: fast and high-precision heating, from ambient to 99°C; fast shaking and mixing, up to 3,000 rpm; programmable, with two programmable soft keys; integrated vortex and short mix functions; wide

range of adapters and blocks; and a stylish and compact case made of aluminium.

For the experiments performed in this thesis, the following set up was used as follows: 5 minutes for platelet aggregation studies; 10 minutes for PLCs studies, at 1000 rpm and 37^{o}C. Later, 5 minutes were adjusted to perform platelet aggregation and PLCs.

Figure 2-2: Bio-shaker

2.1.6.3 Flow cytometry

2.1.6.3.1 FacsCantoII

The BD FACSCanto II is the new model of the FACSCanto bench top analyser. It is 64 x 62 x 91cm in size (HXDXW) and has a weight of 145Kg (320Ib). FACSCantoII uses two lasers: an argon 488-nm solid state laser with 20mW output and a 633 helium–neon laser with 17mW output.

Both lasers are air-cooled. There is an option for a third laser, which is a violet solid state laser at 405nm with 30mW output. As a bench top analyser, the optical analyser and optical alignment is fixed and requires no adjustment. The lasers are connected to the optical bench by the optical fibre. The flow cell is 180x430µm in size, a rectangular quartz. The FSC channel has a resolution of 1.0µm and SSC 0.5µm. The FSC detector is a photodiode with 488/10 bandpass (BP) filter and the SSC has photomultiplier tubes (PMTs) also with a 488/10 BP.

Figure 2-3: FacsCantoII

The FACSCantoII is equipped with 6 PMTs in a 4-2 standard configuration to detect the fluorescence signals. The optical filters and detectors are aligned octagonally and trigonally, respectively. This alignment offers better efficiency of light detection. For the FACSCantoII, the typical detector bands are:

Blue laser:

- 530nm (e.g. FITC)
- 585nm (e.g. PE)

- >670nm (e.g. PerCP)
- 780nm (e.g. PerCP-CY7)

Red laser:

- 660nm (e.g. APC)
- 780nm (e.g. APC-Cy7)

FACSCantoII has six compensated fluorescence parameters and two scatter parameters. It also has automated start, shutdown and cleaning cycles. FACSClean solution, BD FACS shutdown solution and BD FACSFlow are provided by BD to perform the cleaning cycle. Sheath fluid consumption, under normal operation, is 1-10L/hr and under 1mL/hr in standby mode. The FACSCanto uses the software BD FACSDiva (version 6.3.1) on a Microsoft Windows operation system.

2.1.6.3.2 LSR11

The BD LSR II flow cytometer is fully configurable with 1 to 4 fixed-aligned, air-cooled lasers: a UV-laser with 355nm (20mW), a violet diode-laser with 405nm (25mW), a standard solid state Coherent Sapphire blue laser with 488nm (20mW) and a red laser with 640nm (40mW). Similar to the FACSCantoII benchtop analyser, it is 56 x 157 x 86cm (HXDXW) and weighs 240Kg (525Ib). By distributing a large number of fluorochromes (up to 18 colours) over four lasers, the flow cytometer offers a wide choice of measurements, decreases spectral overlap and improves sensitivity. The

BD LSR II is the first commercially available flow cytometer to use a solid state UV laser. The collection optics on the BD LSR II flow cytometer includes patented octagon and trigon optical arrays, the same as the FACSCantoII. The optical arrays increase the sensitivity and flexibility of the BD LSR II flow cytometer, yielding more information from each sample. Light from four spatially separated laser beam spots is delivered by fibre optics to octagon and trigon detector arrays. The 488nm octagon array is capable of collecting up to seven colours plus SSC. The 633nm, 405nm and 355nm trigon arrays can each detect up to 3 fluorescent colours. The concept behind the optical arrays is that reflectance of light is more efficient than transmittance. The optical arrays transmit the highest wavelength to the first PMT and reflect the lower wavelength to the next PMT through a series of long pass dichroic mirrors. More colours can be detected with minimum light loss. The octagon and trigons have single-position insertion sockets for both inner reflectance mirrors and outer discrimination filters. Detecting more colours does not require time-consuming adjustments; the BD LSR II flow cytometer allows filter and mirror changes to be made to the optical array without diminishing maximum signal.

Figure 2-4: LSRII

2.1.6.3.3 Amnis® Flow

Amnis® imaging flow cytometers are equipped with up to 12 channels and up to 4 excitation lasers for each cell in a population; microscopic images provide qualitative and quantitative image data of every event acquired in flow. Amnis Flow Platform-FlowSight® is used in the experiments in this thesis to image PLCs. The FlowSight® Imaging Flow Cytometer has the benefit of producing up to 12 images of each and every cell. The unique image collection system simultaneously produces dark field (SSC), bright field and up to 10 fluorescence images. The FlowSight® Imaging Flow Cytometer operates at ~20X magnification, allowing visualisation of fluorescence from the membrane, cytoplasm, or nucleus. Identifying cell conjugates and single platelets was required for our experiments; single platelets were beautifully distinguished from PLCs.

Figure 2-5: Flow cytometry (Amnis®)

2.1.6.4 Ultra-Flo 100

The Ultra–Flo 100 electronic platelet counter was introduced in 1978 by the Becton & Dickinson Company to allow accurate measurement of platelet counts without separation of other blood components (Fox et al., 1982). This technique is based on a hydrodynamic focusing technique, whereby diluted WB is focused into the center of the counting aperture, where the size of cells is electronically determined.

The number of cells in two pre-set size categories are counted and referenced to platelets and RBCs. The Ultra–Flo 100 uses ratio counting, whereby for a given number of RBCs in a blood sample, a measure of the relative number of platelets is displayed. In this way, the actual RBC count (X $10^6/\mu L$) can be set up on the machine and the actual platelet count (X $10^3/\mu L$) is displayed.

The percentage of platelet aggregation is calculated as the percentage fall in the single platelets, in comparison to the initial platelet count. This technique is sensitive to micro-aggregation and aggregation responses are reproducible over a number of hours.

2.2 Methods

2.2.1 Blood collection

Venous blood was taken from healthy volunteers who denied taking any drugs, such as NSAIDs, known to affect platelet function, in the two weeks prior to donation. Blood was also taken ACS patients who were on aspirin and either clopidogrel or prasugrel. Blood was taken by forearm venipuncture using a polypropylene syringe and a 19G needle and dispensed into polystyrene tubes containing anticoagulants. In the majority of the experiments blood was anti-coagulated with sodium citrate, prepared as 3.13% (w/v) solution of tri-sodium citrate dehydrate and used at a ratio of 1:9 (v/v) with blood. For full blood counts (FBC), a further sample was taken into a commercially prepared vacutainer tube from BD that contained 72µl of 7.5 % K_2EDTA (Lavender top) (1:24, v/v) as an anticoagulant. In some tubes, antiplatelet agents were added to the anticoagulant at the required concentration. These tubes were routinely incubated at 37°C prior to experimentation, for approximately 30 min.

2.2.2 Measurement of platelet aggregation in whole blood by flow cytometry, using a single platelet counting technique

Platelet aggregation was assessed in WB from healthy volunteers, who denied taking any drugs that interfere with platelet function for two weeks, or from ACS patients, using a single platelet counting technique (SPC). This technique has been used in the division of Cardiovascular Medicine at the Queen's Medical Centre (QMC) for many years (Fox et al., 2004b). It measures the drop in the number of single platelet counts as the platelets form aggregates.

2.2.2.1 Using the MSA and test tubes

Aliquots of citrated whole blood (final volume of 480µl) were dispensed into a polystyrene (LP3) tubes that contained a stirrer bar. Then the tubes were placed into the stirring well of the MSA, operating at 37°C and 1000 rpm. Platelet aggregation was then induced by adding 20µl of the following agonists: ADP (1, 3, 10µM), collagen (1, 3, 10µg/ml) and U46619 (0.3, 1, 3µM). The final volume in the LP3 tube was kept at 500µl. after that, aliquots of blood (20µl) were removed and fixed using AggFixA at different time points (0.5, 1 and 4 minutes). 36µl of the fixed samples were analysed using the Ultra-Flo 100 – a method called single fixation Ultra-Flo 100 (SFU). The remainder of the sample was allowed to stand for 30 minutes. The fixed samples were then fixed again with AggFixB and then

analysed by FACScantoII flow cytometry using round bottom 96-well plates – a method called double fixation flow cytometry (DFF).

2.2.2.2 Using a Bio-shaker and 96-well plates

Blood obtained from subjects was anti-coagulated with sodium citrate and aliquots of 3ml were dispensed into polystyrene tubes. For the platelet inhibition study, drugs, such as aspirin (10, 30 and 100µM), the $P2Y_{12}$ inhibitor cangrelor (10, 100, 1000nM), the GPIIb/IIIa blocker MK-0852 (10µM) and KPL-1, the anti-P-selectin glycoprotein ligand-1 (anti-PSGL-1) (5µg/ml), were incubated with blood for 30 minutes prior to testing. Saline was as added to separate blood to serve as a control. The tubes were capped and inverted at least 3 times to ensure enough mixing. Platelet agonists of different concentrations – AA (0.03, 0.1, 0.3, 0.5 and 1mM), ADP (0.3, 1, 3, 10 and 30µM), collagen (0.1, 0.3, 1, 3 and 10µg/ml) and TRAP (0.1, 0.3, 1, 3 and 10µM) – were prepared and 4µl of each was added to a flat bottom 96-well plate. With respect to the optimul plate, lyophilised agonists was already prepared and loaded into the plates. Using a multi-channel pipette, whole blood (46µl) was added to each well and the plate was shaken for 5 minutes at 1000 rpm and 37ºC; a fixative solution (Platelet Solutions Ltd, Nottingham, UK) was applied to stop platelet stimulation and stabilise samples. 17µl of AggFixA was added to the blood (1/3 ratio), after shaking, and left for 15-30 min at room temperature. Using a round bottom 96-well plate, 135µl of AggFixB, and then 15µl of the AggFixA blood, was mixed with AggFixB (1/10 ratio). This allowed the

platelets to be fixed for up to 9 days. Following fixation, another round bottom 96-well was used and 10µl (1:20 dilution) of FITC-CD42a, which is a monoclonal antibody against glycoprotein IX (GPIX), used together with light scatter characteristics to identify the platelet population, was added to the plate. After that, 5µl of AggFixB blood was then mixed with the antibody (1/2 ratio) and then incubated at 4^{o}C for 25 min. Finally, 200µl of FacsFlow was added to each well and the plate was then analysed using the FacsCantoII flow cytometer (Becton Dickenson, UK; equipped with a solid state blue laser operating at 20mM power, at a wavelength of 488nm, and connected to a Hewlett Packard computer with FACSDiva acquisition software). Platelet aggregation was then measured as the percentage fall in the number of single platelet count compared with the initial platelet count in a control sample of unstimulated fixed blood anticoagulated with EDTA **(Baseline count – sample count) / Baseline count x 100.** Baseline count represents the EDTA count, while sample count represents the agonist count.

A fixed number of RBC events (50,000 events) were acquired, following which platelets were identified and quantified by a gating procedure based on their FITC fluorescence and forward/size scatter properties. The schematic diagram in Figure 2-6 shows the steps involved and Figure 2-7 shows the flow cytometry dot plot.

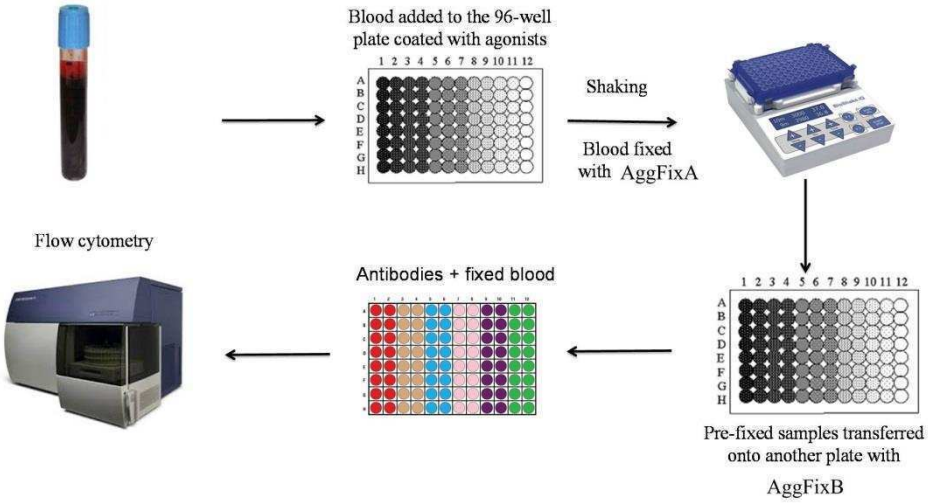

Figure 2-6: 96-well plate whole blood aggregation.

Diagrammatic presentation of platelet aggregation using single platelet counting technique and a 96-well plate by flow cytometry:

Summary of the steps involved

- Blood samples are incubated with the drugs of interest
- Platelet agonists are prepared and then loaded onto the 96-well plate
- Blood samples are then added to the plate, which is mounted on a Bio-shaker for 5 min, at 37°C and 1000rpm
- Blood samples are stabilised by AggFixA/AggFixB (Platelet solutions Ltd., Nottingham, UK)
- Fixed blood is incubated with platelet identifier FITC-CD42a for 25 min at 4^{o}C
- Finally, Facsflow is added and analysis is performed by flow cytometry

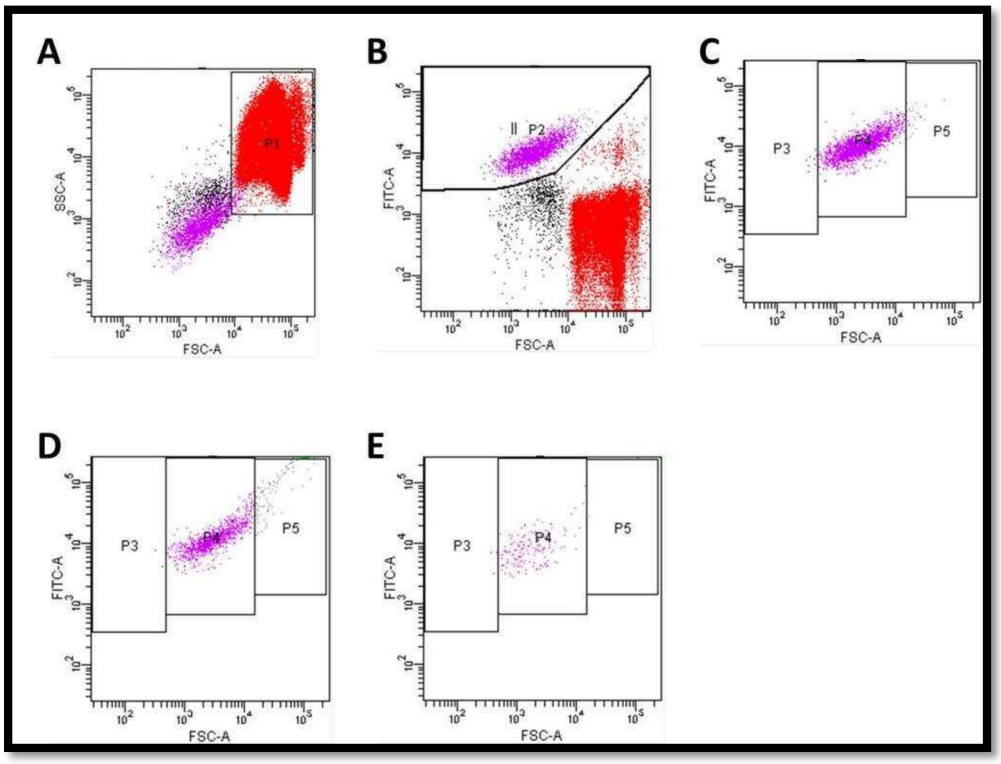

Figure 2-7: Aggregation based on immuno-counting using FACSCantoII.

Flow cytometric analysis dot plot of platelet aggregation using the single platelet counting technique.

Platelet aggregation studies are performed, using either the traditional method, which involves the use of reaction tube (LP3 tube) and MSA to stir the blood, or using the 96-well plate and the Bio-shaker to shake the blood. The final step is common to both methods and involves the readings obtained from flow cytometry.

Figure 2-7, shows the flow cytometry dot plot used to identify the platelet aggregation (quantification of single platelets) using the SPC technique. A)

50,000 red blood cell events were counted (region P1 on a forward scatter-side scatter plot), as the data acquisition stopped after that, and this is considered as a reference for the measurement of platelet aggregation. B) CD42a fluorescence was plotted against forward scatter and region P2 was selected by drawing a curved line to include all platelet-related events, excluding erythrocyte/platelet co-incidence. CD42a-FITC antibody was used to label the platelets by interacting with GPIX on platelets and by its forward scatter properties. C) CD42a-positive platelet events in P2 were then redrawn and EDTA-anticoagulated blood containing unstimulated platelets was used to set 3 rectangular gates to quantify microparticles (P3), single platelets (P4) and micro-aggregates (P5). Counting the number of single platelets, no aggregation was taking place as demonstrated by high availability of single platelets in the EDTA sample. D) Partial aggregation was possibly due to inhibition by drugs, resulting in more free single platelets, as demonstrated by incomplete inhibition. E) Complete aggregation as a result of agonist induced platelet aggregation.

Table 2-1: Single platelet count results obtained from flow cytometry.

Results for the control sample treated with EDTA (A), saline (B) and sample stimulated with ADP (30µM).

A) EDTA

Population	#Events	%Parent	%Total
All Events	52,764	####	100.0
P1	50,000	94.8	94.8
P2	2,936	5.6	5.6
P3	9	0.3	0.0
P4	2,923	99.6	5.5
P5	4	0.1	0.0

B) Saline

Population	#Events	%Parent	%Total
All Events	52,277	####	100.0
P1	50,000	95.6	95.6
P2	2,367	4.5	4.5
P3	11	0.5	0.0
P4	2,353	99.4	4.5
P5	4	0.2	0.0

C) ADP

Population	#Events	%Parent	%Total
All Events	50,197	####	100.0
P1	50,000	99.6	99.6
P2	115	0.2	0.2
P3	1	0.9	0.0
P4	109	94.8	0.2
P5	5	4.3	0.0

From the results indicated in Table 2-1, the number of single platelet counts from the sample incubated with EDTA **(A)** can be seen; in example 2923, there was no aggregation. In samples incubated with saline **(B)**, example 2353 is slightly a slightly lower value than that for EDTA, which is due to the spontaneous aggregation. In samples stimulated with ADP

(C), example 109 is a low value reflecting complete which reduces the numbers of free single platelets.

2.2.3 Measurement of platelet aggregation in whole blood using the Ultra-Flo 100 whole blood platelet counter

The blood samples fixed with AggFixA solution were gently mixed by repeated inversion and 36µl of the sample was added to a vial containing 9.1ml of saline. Cells were counted immediately after dilution. All the platelet counts were determined with the RBC setting at 600.

2.2.4 Measurement of platelet aggregation in whole blood using flow cytometry and quorum sensing molecules

These experiments were done in the same way as the previously described experiments utilising a Bio-shaker and 96-well plates (section 2.2.2.2). However, here, the anti-coagulated blood was obtained from healthy volunteers, who denied taking antiplatelet drugs, following which 3µl 10µM of quorum sensing molecules (QSM) was added to 3ml of the blood. Another tube with 0.1% DMSO served as a vehicle.

2.2.5 Measurement of platelet–leucocyte interaction in fixed and stabilised sample using lysing vs. non-lysing (fluorescent triggering)

2.2.5.1 Antibody preparation

A mixture of antibody solutions was prepared, consisting of CD14-APC-CY7 (used to distinguish monocytes and neutrophils), CD62P-PRE or CD61-PE or CD42a-FITC (used to identify platelets) and CD45-PerCP (used to identify leucocytes). The tubes were then covered with foil and stored in the fridge before use. Antibodies were prepared for no longer than 2 hours before use. Erythrolyse solution was prepared in distilled water at a 1/10 dilution and then left, covered, at room temperature.

2.2.5.2 Lysing protocol

Blood sample stimulation for PLCs was performed using a 96-well plate format and a lysing approach. This involves lysing of RBCs using the erythrolyse solution. For measurements, high concentrations of ADP (30µM), collagen (10µg/ml) and TRAP (10µM) were prepared on the day of use. As with the aggregation method, 4µl agonists was added to the 96-well plate, following which 46µl blood was dispensed into each well. The plate was then shaken for 10 min at 37^{o}C and 1000 rpm. 10 min was the optimum shaking time, as this was shown to produce enough conjugate by allowing more interaction of platelets with leucocytes (Redlich et al., 1997). After shaking the blood, 17µl AggFixA was added to each well.

Then 50µl fixed blood was transferred and mixed with 450µl AggFixB solution in FACS tube, keeping the ratio at 1:0. Then, the fixed blood was then centrifuged for 10 min at 1600rpm, using a bench centrifuge. The supernatant was removed and 20µl mixed antibody was then added to tubes, in turn, washing into the cells so as to mix thoroughly and then covered for 25-30 minutes at 4°C. After incubation, 1ml erythrolyse solution was added to allow the red cells to lyse. This would take 10-15 min, depending on the blood donor. After that, 1ml facsflow was added, vortex-mixed and then centrifuged for 10 min at 1600rpm, using a bench centrifuge. The supernatant was decanted and another 2ml facsflow was added, the sample then centrifuged once again for 10 min at. The supernatant was then decanted and resuspended in 300µl facsflow, vortex-mixed and analysed by flow cytometry.

PLCs formation was quantified using the flow cytometer, LSR II (Becon Dickinson). The LSR II is equipped with a solid state laser operating at 20mM and a wavelength of 488nM, which was used for the analysis. This was connected to a computer with FACSDiva software. Leucocytes were identified by logical gating from dot plots of forward scatter, which reflect cell size, and of side scatter, which reflect cell granularity profiles and are acquired by linear amplification. Monocytes were characterised by a forward scatter–side scatter profile and CD14 APC-CY7 positivity, while neutrophils were identified in the same way, except they were negative for CD14 expression. Fluorescence parameters were acquired using logarithmic amplification. 10,000 leucocyte events were acquired for each

sample. Platelet–monocyte (PM) and platelet neutrophil (PN) conjugates were quantified as 1) the % of monocytes or neutrophils expressing CD62-PE or CD61-PE or CD42a-FITC fluorescence greater than that of a mouse IgG control and 2) as median CD62-PE, CD61-PE or CD42a-FITC fluorescence of the monocyte or neutrophil population.

2.2.5.3 Flow cytometry dot plot:

Using the lysing technique and double label analysis, flow cytometry identifies the leucocyte population based on forward light scatter (indicative of cell size) and side scatter (indicative of cell complexity) on a log setting. In all cases, a total of 10,000 events were recorded and tested for each sample. All the fluorescence parameters were acquired using logarithmic amplification. Monocytes were differentiated from the leucocyte population by their CD14-APC-CY7-positivity. PLCs were determined by gating the neutrophils and monocyte population. IgG_1: FITC isotype matched antibody served as a negative control. The values presented are the % and median fluorescence of the platelet identifier (CD62P-PE or CD61-PE or CD42a-FITC) used in each population.

In all flow cytometry dot plots (control in Figure 2-8, EDTA in Figure 2-9, MK-0852 in Figure 2-10 and KPL-1 in Figure 2-11), A) indicates the light scatter profile to obtain the leucocyte population and B) shows a plot of CD14-APC-CY7 vs. side scattered. Monocytes were identified by their

CD14-positivity. P2 and P3 were drawn to gate neutrophils and monocytes, respectively. C) A plot is shown of CD62P-PE (an example of a platelet identifier) vs. CD14-APC-CY7 to determine the PLCs P4 and P5, for neutrophils and monocytes, respectively. D) and E) are plots of side scatter vs. CD62P-PE to distinguish PN and PM conjugates from CD62P-PE-positive platelets, respectively.

- The following four figures show the flow cytometry dot plot using the lysing technique:

1) Control

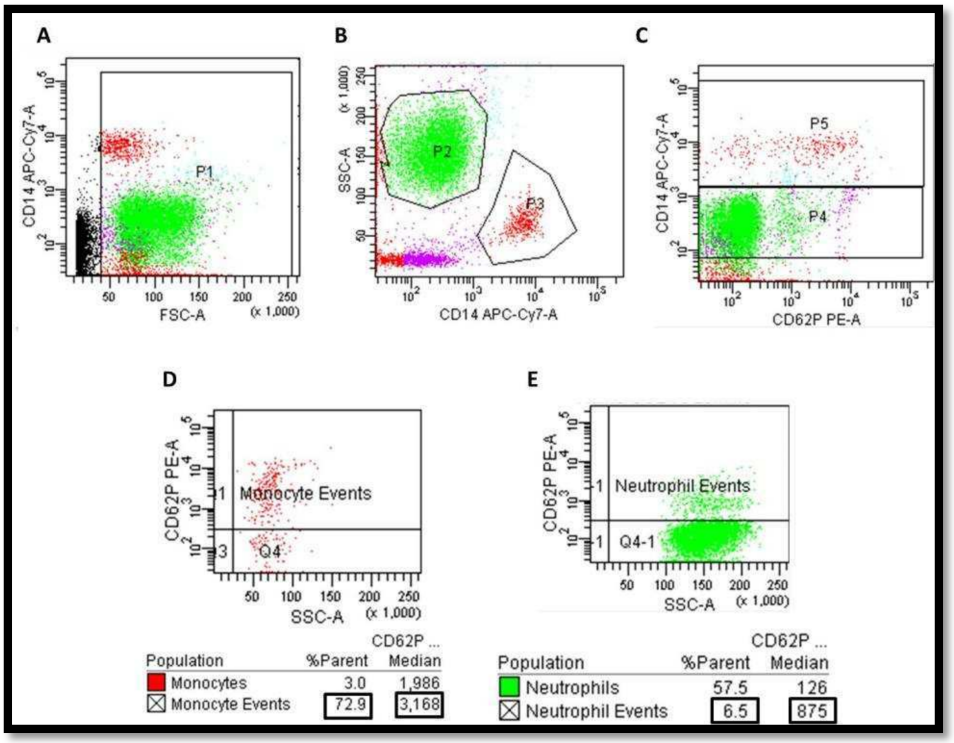

Figure 2-8: A flow cytometry dot plot to identify platelet-leucocyte conjugate formation in control samples using a lysing technique.

2) EDTA

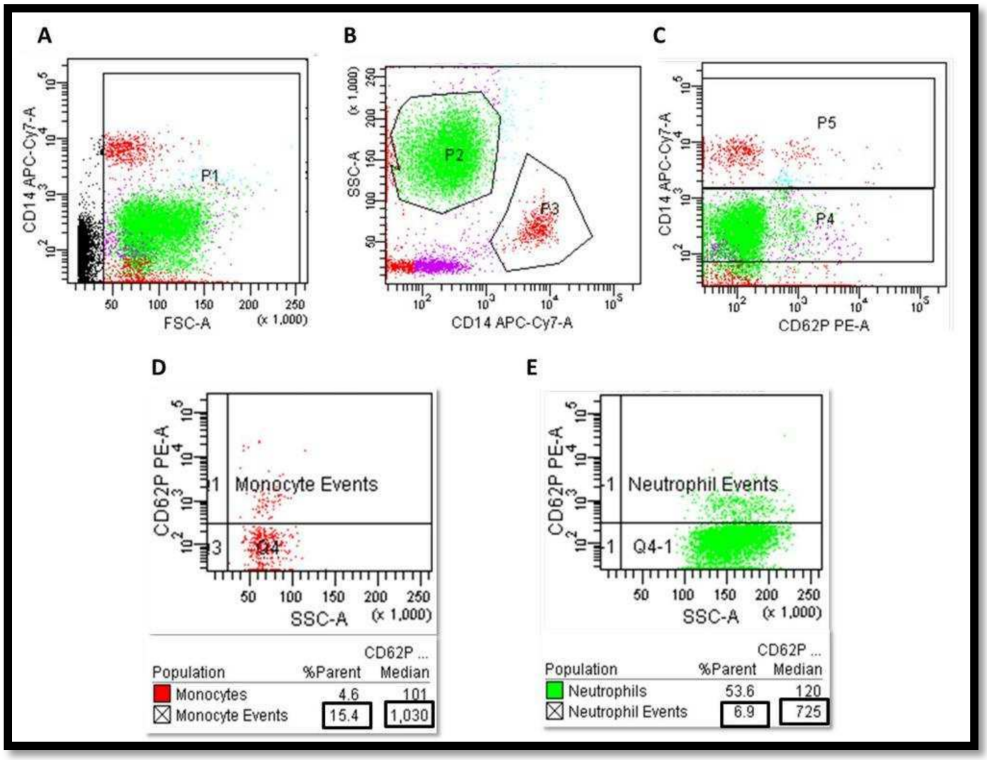

Figure 2-9: A flow cytometry dot plot to identify platelet-leucocyte conjugate formation in samples incubated with EDTA, using a lysing technique.

3) MK-0852

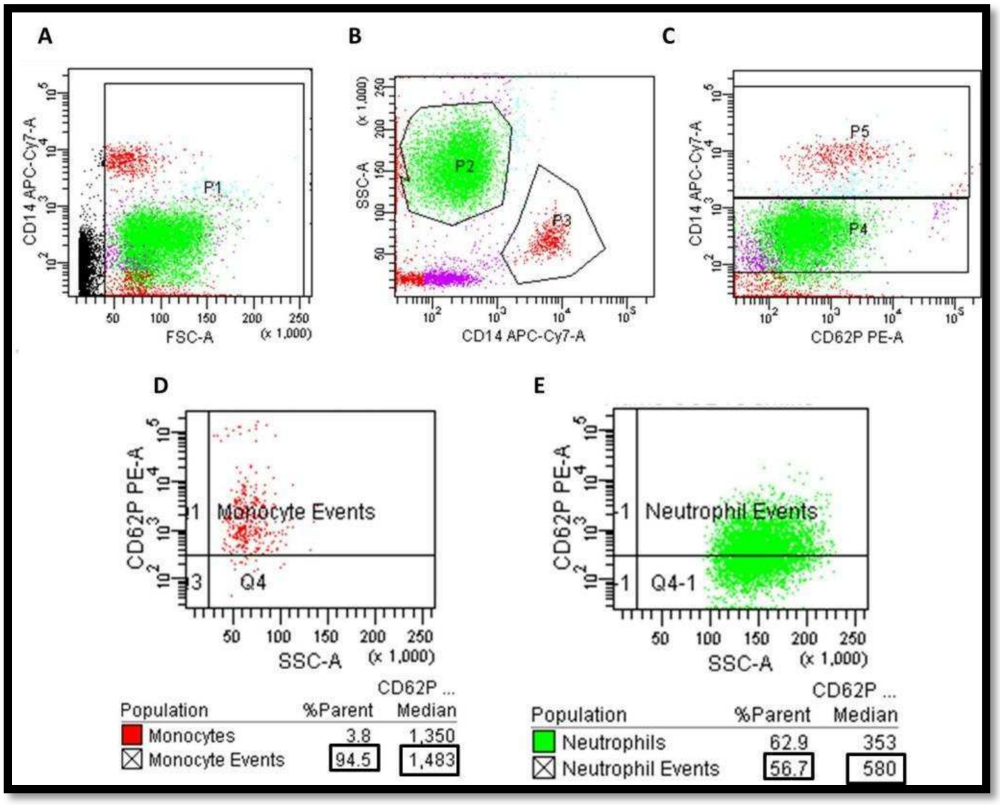

Figure 2-10: flow cytometry dot plot to identify platelet-leucocyte conjugate formation in samples incubated with MK-0852, using a lysing technique.

4) KPL-1

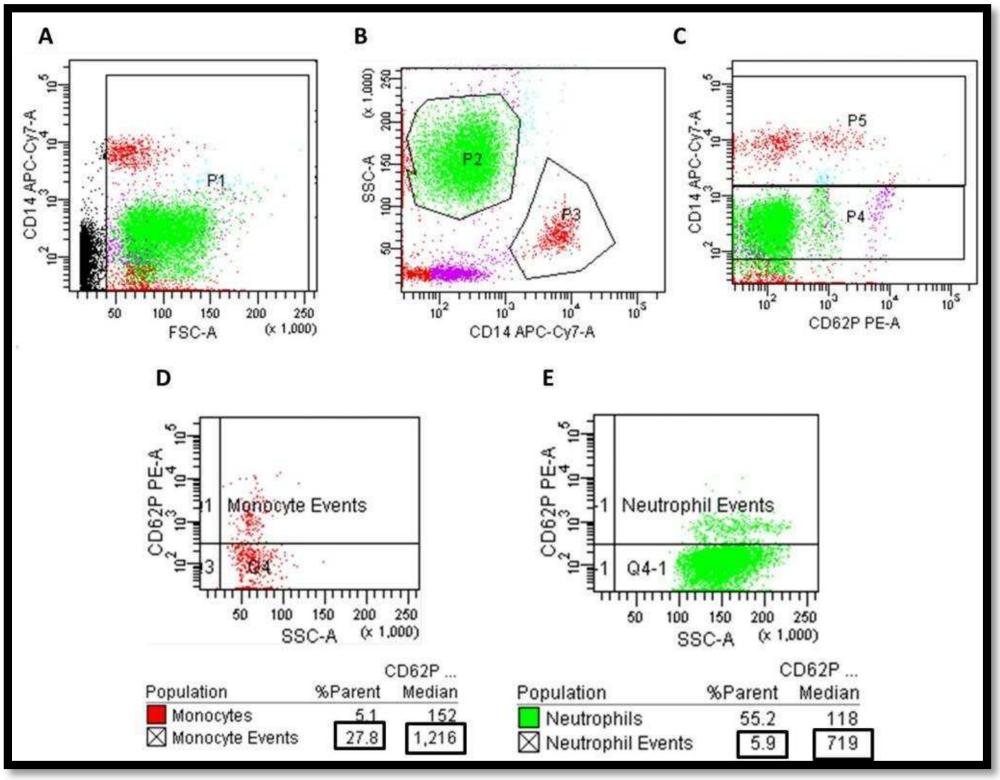

Figure 2-11: flow cytometry dot plot to identify platelet-leucocyte conjugate formation in samples incubated with KPL-1, using a lysing technique.

In the all the flow cytometry dot plot presented, for control, EDTA, MK-0852 and KPL-1, A) and B) are indicative of a light scatter profile to obtain the leucocyte population. A total of 10,000 events were collected and analysed, where region P2 represents the neutrophil population and P3 represents the monocyte population. C) A plot of CD62P-PE (as an example of a platelet identifier) against CD14-APC-CY7 was used to determine PLCs. In D) and E), the monocyte and neutrophil conjugates were presented based on the side scatter properties and positivity of the platelet marker used. Increased upshift indicates more conjugates obtained

and an increase in the CD62P % and median fluorescence is demonstrated, and is indicative of a large amount of PLCs formation.

2.2.5.4 Triggering protocol

In this method, flow cytometric analysis of PLCs is relies on unlysed whole blood samples – a technique based on fluorescent triggering. The steps involving sample stimulation with platelet agonists and fixation with AggFixA and AggFixB were exactly the same as those in the aggregation method. The AggStab blood was transferred to small Eppendorf tube (1ml) and centrifuged, using a small centrifuge, set for 3 min at 1000 rpm. The supernatant was decanted and 20µl of following antibody mix, CD45 Percp (1:10), CD14 APC-CY7 (1:20) and CD61 PE (1:400), was added and incubated in the dark for 30 min. Finally, 300µl FacsFlow solution was added and analysed by flow cytometry (LSRII). In A), the blood cell population was collected from forward and side scatter, in logarithmic mode. These included leucocytes, erythrocytes and platelet aggregates and excluded single platelets. In all cases, a total of 10,000 events were collected. In B), the leucocyte population were identified by their side scatter characteristics and CD45-positivity. In C) and D), monocytes were differentiated from neutrophils by their high CD14-positivity. PLCs were expressed as the % and median fluorescence of CD61-positivity in each subpopulation.

2.2.5.5 Flow cytometry dot plot

In all the flow cytometry dot plots (control in Figure 2-12, EDTA in Figure 2-13, MK-0852 in Figure 2-14 and KPL-1 a in Figure 2-15): A) the cell population was collected from forward and side scattering in logarithmic mode, which represents leucocytes, erythrocytes, platelet aggregates and excludes single platelets; B) P1 was generated to identify leucocytes by their side scatter characteristics and CD45-positivity; C) and D) neutrophils and monocytes were identified on the basis of CD14 expression and side scatter properties. P4 and P2 were drawn for neutrophils, whereas P3 and P5 indicated monocytes.

E) and F) PM and PN conjugates were distinguished from single platelets, positive for CD61-PE. The Figures 2-12 to 2-15 show flow cytometry dot plots using the fluorescent triggering technique:

1) Control

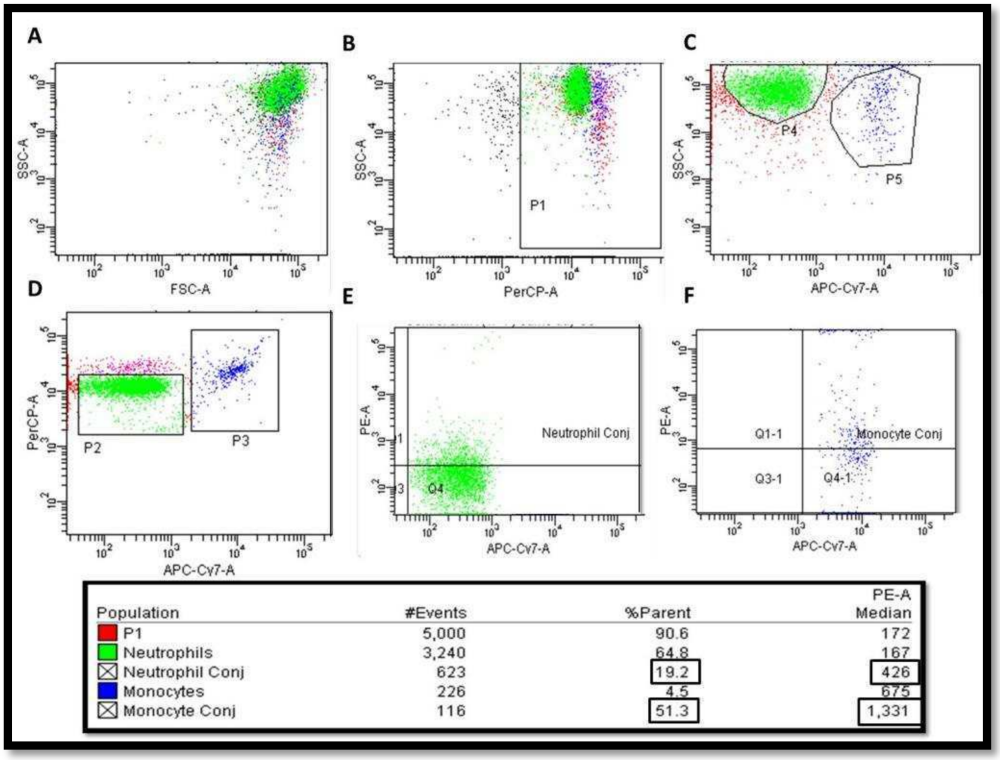

Figure 2-12: A flow cytometry dot plot to identify platelet-leucocyte conjugate formation in control samples, using a fluorescent triggering technique.

2) EDTA

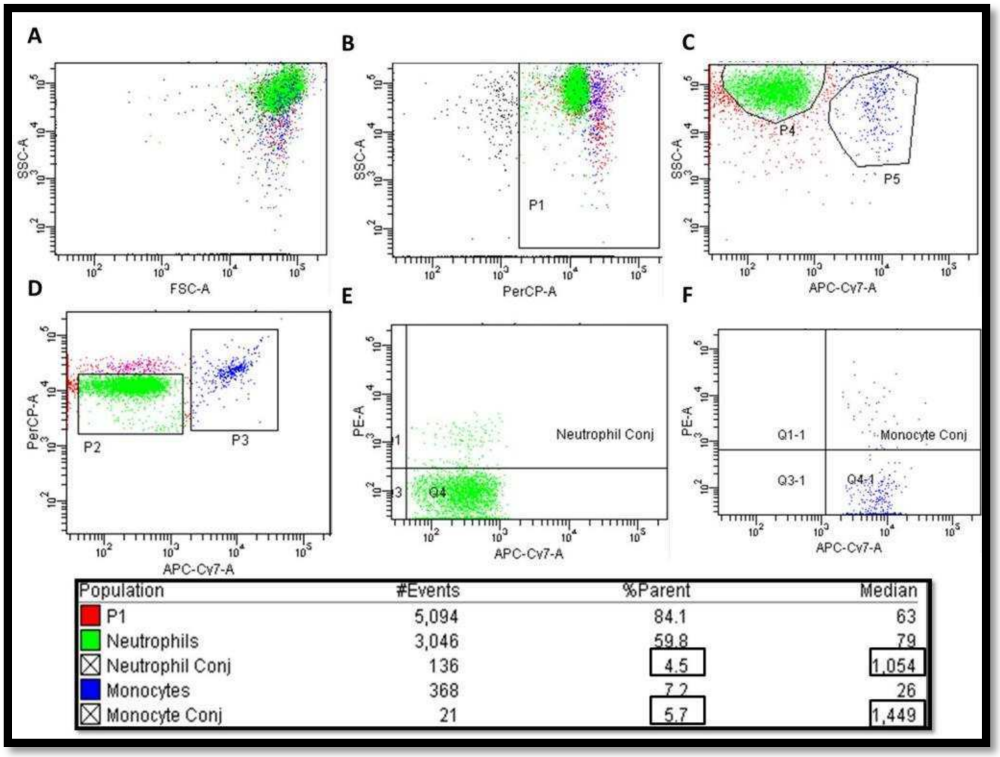

Figure 2-13: A flow cytometry dot plot to identify platelet-leucocyte conjugate formation in samples incubated with EDTA, using a fluorescent triggering technique.

3) MK-0852

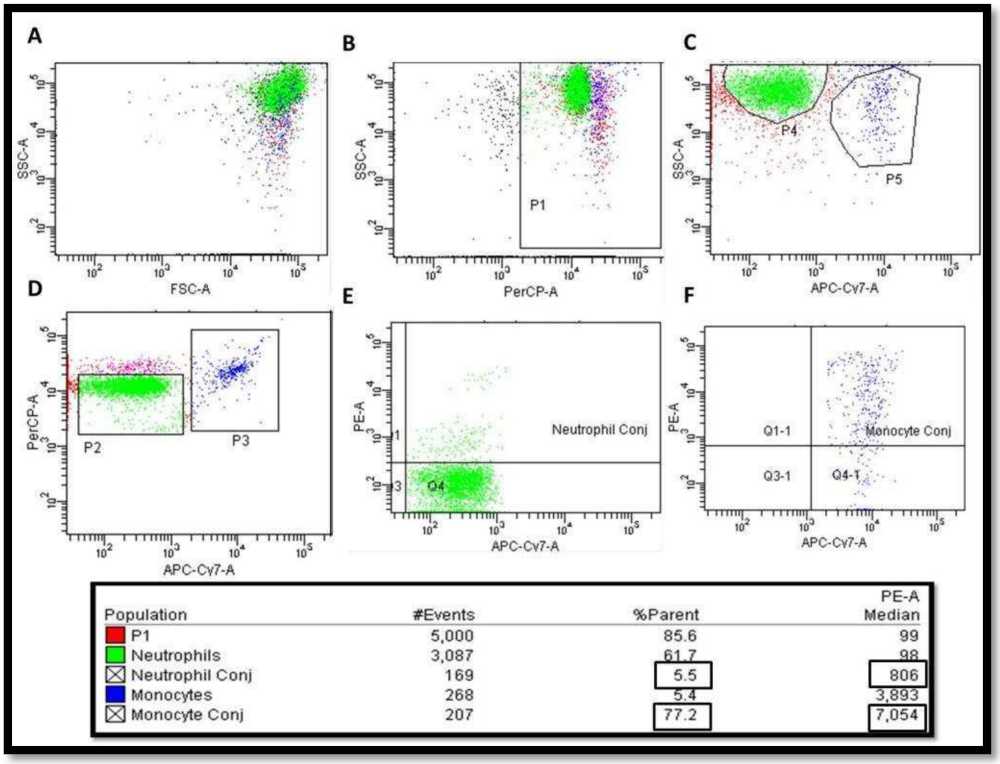

Figure 2-14: A flow cytometry dot plot to identify platelet-leucocyte conjugate formation in samples incubated with MK-0852, using a fluorescent triggering technique.

4) KPL-1

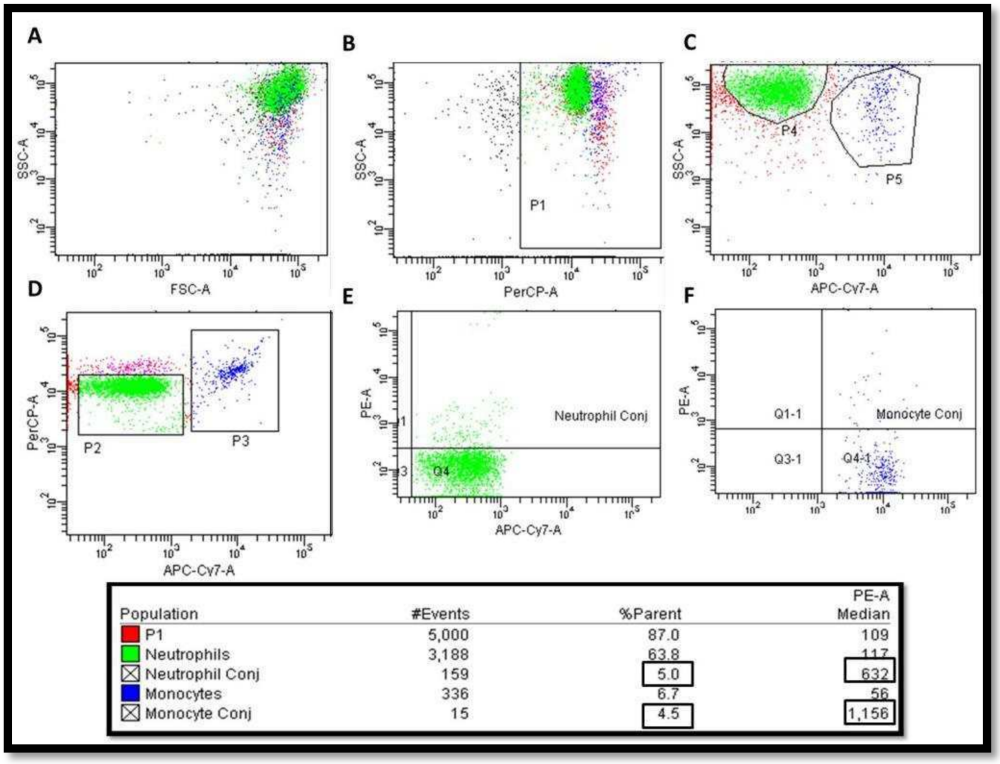

Figure 2-15: A flow cytometry dot plot to identify platelet-leucocyte conjugate formation in samples incubated with KPL-1, using a fluorescent triggering technique.

2.2.6 Simultaneous measurement of platelet aggregation and platelet leucocyte conjugate formation

The ideas that fixed blood samples can be analysed at later stage and two measurements of platelet function can be assessed seemed feasible. This led to the following experiments investigating the effect of antiplatelet drugs on platelet aggregation and PLCs formation in the same sample of fixed whole blood.

The experiments performed were based on the 96-well plate format and samples were loaded agonists and shaken for 5 min. Then, 25µl AggFixA blood was transferred to another 96-well plate with 225µl AggFixB, at a 1:10 ratio, and the volume was increased to obtain enough conjugates. Then, from this fixed blood sample, 5µl was used for the aggregation measurement (see section 2.2.2.2) and the rest of the blood was transferred to small Eppendorf tubes and tested based on a triggering approach (see section 2.2.5.4).

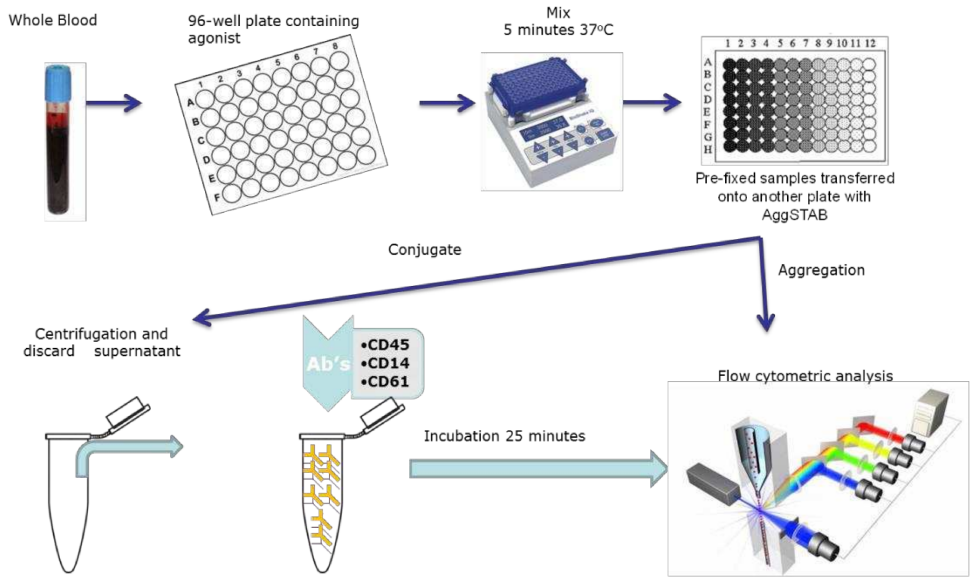

Figure 2-16: Simultaneous analysis of platelet aggregation and platelet-leucocyte conjugate formation

2.2.7 Statistics

Results were presented as either mean ± standard deviation (SD) or standard error of mean (SEM). Statistical data analysis was accomplished using Graphpad Prism software, using paired t-test for two groups and a one way or two way analysis of variance (ANOVA), combined with the Bonferroni test, for multiple comparisons. Graphpad Prism was also used to calculate the EC50, which is the required concentration of platelet agonist capable of achieving half the maximum of level of aggregation. The agonist concentration achieving EC50, where the maximal response reached the plateau, was calculated. Values with $p \leq 0.05$ were considered statistically significant. The n number represents the number of volunteers or patients recruited for specific studies.

3 Optimisation of platelet aggregation in whole blood using two methods based on stirring and shaking

3.1 Introduction

The study of platelet aggregation is one of the most widely used tests to identify and diagnose defects in platelet function that might lead to thrombosis, for example in ACS, bleeding, BS syndrome or GT. Platelet aggregation can be performed on different cell preparations, such WB, PRP, washed platelets or gel-filtered platelets. Out of these, WB has the advantage of involving less manipulation of platelets than PRP. PRP preparation involves centrifugation, which may lead to platelet activation. Using WB also tests platelet function in a milieu that is more physiological due to the presence of RBCs and WBCs (Harrison, 2005).

Blood samples acquired for platelet aggregation testing are generally treated with 3.2 or 3.8% (w/v) tri-sodium citrate dehydrate for anticoagulation (Mody et al., 1999). Heparin can also be used as an anticoagulant; however, previous studies on platelet aggregation have revealed that heparin may cause platelets to aggregate together spontaneously, making them more difficult to work with (O'Brien et al., 1969). Hirudin is another anticoagulant, which acts by directly inhibiting thrombin and keeping Ca^{2+} concentrations at physiological levels of 1.1 to 1.2 mmol/l (Storey et al., 1998). These anticoagulants influence platelet aggregation measurements differently, making the selection of anticoagulant critical and in need of more investigation. Citrate, for example, has Ca^{2+}-chelating properties and is known to decrease extracellular Ca^{2+} availability, which maintains the integrity of the

aggregation receptor, the GPIIb/IIIa complex (Fitzgerald and Phillips, 1985). As a result, GPIIb/IIIa antagonists are more potent in blood that has been anticoagulated with citrate than in blood that has been treated with other anticoagulants, such as D-Phe-ProArg-chloromethyl-ketone (PPACK), which does not affect Ca^{2+} concentrations (Rebello et al., 2000, Kereiakes et al., 2001, Voss et al., 2005). Currently, the most frequently used anticoagulant for testing platelet function is sodium citrate (Neufeld et al., 1999).

In WB, platelet aggregation can be successfully measured using single platelet counting techniques (SPC) (Butchers et al., 1980, Fox et al., 1982, Saniabadi et al., 1983). These methods involve stirring samples of WB in the presence of platelet stimulants, such as ADP, and monitoring the fall in the number of single platelets as aggregation takes place. Following this, samples may be fixed so that aggregation can be stopped at predetermined time points (Bevan and Heptinstall, 1985). The fixed blood can then be analysed using a WB platelet counter, such as an Ultra-Flo 100, or a Sysmex KX-21 analyser, or by flow cytometry (Fox et al., 2004b, Bacus et al., 1980, Iyu et al., 2011). Platelet aggregation is expressed as the decrease in SPC, compared to the initial platelet count, in a control sample of unstimulated fixed blood anticoagulated with EDTA. EDTA prevents aggregation by chelating Ca^{2+} to the degree that GPIIb/IIIa, the final aggregation receptor, becomes no longer functional. These methods of assessing platelet aggregation have been used in the Division of

Cardiovascular Medicine here at the University of Nottingham for many years (Fox et al., 1994, Heptinstall et al., 1995, Storey et al., 2002b).

The WB platelet counter, the Ultra-Flo 100, depends on a change in electrical impedance of the cells passing through a small aperture, which can detect single platelets and differentiate them from RBCs (Bacus et al., 1980). It has proved to be a useful tool in measuring platelet aggregation in WB, where the extent of aggregation is determined from the number of single platelets remaining. The Sysmex KX-21 analyser is another WB platelet counter that uses impedance technology. These early techniques do not provide comprehensive information about platelets, such as receptor function or cell signalling. Therefore, an alternative instrument, such as a flow cytometer, may be used to acquire a more in-depth understanding of platelet function and their interactions with other cells, such as leucocytes.

Flow cytometry provides a reliable, informative and rapid analysis for the assessment of platelet function. The use of flow cytometry in clinical and research settings has risen dramatically in the past decade. This is attributable to flow cytometry using WB, hence, allowing platelets to be analysed in their physiological state, as previously mentioned, due to the presence of RBCs and WBCs. It also reduces the activation of platelets in vitro, which occurs with the preparation of PRP and washed platelets (Brown and Wittwer, 2000, Saboor et al., 2013).

The use of flow cytometry in platelet studies relies upon the identification of particular proteins situated on platelet membranes or inside cells. This is accomplished by binding specific antibodies (conjugated with fluorochromes) to target proteins and visualisation of the fluorescence emitted following fluorochrome excitation with a laser. It is preferable for the antibodies used for WB flow cytometry to be monoclonal, rather than polyclonal, so as to reliably saturate the exact epitopes of interest, avoid non-specific binding and remove incubation and washing steps, if a secondary antibody is used. Flow cytometry can also solely use light scattering properties to discriminate platelets from other blood cells. Forward scatter gives information about cell size, while side scatter reflects cell complexity. As far as platelet aggregation is concerned, it can be measured by flow cytometry of WB. In this respect, an SPC technique was employed to measure platelet aggregation in WB by estimating the fall in the number of single platelets as they form aggregates. In these experiments, single platelets are identified by their positivity to the platelet identifier, FITC-CD42a, and their gating properties (Michelson et al., 2000, Michelson, 2006).

CD42a is a platelet membrane GP, also known as GPIX, which forms a non-covalent complex with GPIb and GPV, ultimately forming the complex, GPIb-IX-V, which mediates platelet adhesion (Michelson et al., 1996b). The measurement of platelet aggregation by flow cytometry, using SPC techniques, has been already established by our research group for counting the fall in single platelets in relation to 50000 RBC events (Fox et

al., 2004b). However, measurements of platelet aggregation are influenced by different factors, such as the type of anticoagulants used to prevent blood clotting, the time between venipuncture and blood testing and the volume of blood needed to carry out an analysis. The fixative solution used is another variable and whether it is used prior to or after the antibody is added is controversial. In most flow cytometric methods, the blood sample is fixed prior to incubation with antibodies. (Atar et al., 2010). However, it has been shown that certain receptors, such as the GPIIb/IIIa fibrinogen binding sites, and the binding of the antibody, PAC-1, are significantly influenced by the fixative solution.

The current perception is that studies of platelet function must be completed within about two hours, without fixation, if reliable results are to be obtained. However, fixation is beneficial in a clinical setting where there may not be immediate access to a platelet function analyser, as it allows measurements to be carried out at later stage. It also prevents subsequent artefactual platelet activation (Michelson, 1996). Fixatives, such as formaldehyde (FA) or paraformaldehyde (PFA), have been used by researchers at different concentrations to allow analysis after time periods of longer than two hours (Hu et al., 2000). Our research group has formulated a new fixative solution (AggFixA/AggFixB(S)) that has the advantage of fixing platelet aggregates and keeping them stable for up to nine days, facilitating the remote testing of platelet function (May JA, 09/05/08). The fixation method comprising the use of one fixative is referred to as single, and the one with two fixatives is referred to as double.

The single fixation approach utilises a solution known as platelet activator molecule (PAM) fixative, which is normally added to the blood at a ratio of 1:3. The single fixation method has been previously used here to successfully measure platelet aggregation using flow cytometry and Ultra-Flo 100 (Fox et al., 2004b) and, also, to assess platelet activation from the measurement of the P-selectin (CD62P) marker (Fox et al., 2009). The single fixation approach is shown to preserve platelet aggregate stability for 15-30 min, without altering the integrity or distortion of platelets. The second method involves a two-stage fixation. The first stage, AggFixA, consists of relatively high FA and no EDTA, while the second stage, AggFixB(S), consists of relatively low FA with EDTA and is normally added in the ratio of 1:10 (May JA, 09/05/08).

Temperature is an important factor that affects platelet aggregation. Many researchers have described THAT cooling at low temperatures, such as 28^{o}C, enhanced platelet aggregation in response to stimulation with ADP (Scharbert et al., 2006, Xavier et al., 2007). A possible explanation for this observation is that low temperatures increase the fragility of RBCs and, therefore, more ADP is produced. Stirring forces can have an impact on platelet aggregation, as lengthy stirring of blood without agonist can damage RBCs and result in the release of small amounts of ADP, which can cause spontaneous aggregation. This is not the case with PRP due to the lack of RBCs (Saniabadi et al., 1987, Goldsmith et al., 1995). Therefore, most platelet aggregation studies on WB are performed at 37^{o}C in a test tube, using a MSA, at 37^{o}C and 1000 rpm, which is used to agitate

the blood and the platelet stimulant. However, this has many drawbacks; in particular, that measurements are usually made only at a single time point. This method is also time-consuming and a relatively large volume of WB is required in order to get a full dose-response curve for platelet aggregation. Therefore, the use of 96-well plates has many advantages, including the requirement for only a small volume of WB, which makes it more convenient, especially when the volume of blood available is very limited. Also, several platelet activation pathways can be assessed in parallel by using a range of concentrations of different agonists; in addition, it uses a very small volume of agonist (4µl). Therefore, this approach may offer a high throughput test for assessing platelet function. By combining this with the double fixation method, samples can be stabilised so that analysis can be achieved remotely in a central laboratory. This method depends on a shaking technique using a Bio-Shaker, which is designed to mix the blood in a 96-well plate with the platelet stimulants. This differs to the previous technique that involves stirring using an MSA.

The 96-well plate aggregation methodology has already been used for fresh agonists (Armstrong et al., 2009a, Armstrong et al., 2008, Ali et al., 2009) or lyophilised agonists (Chan et al., 2011). In these studies, platelet-rich plasma was prepared and measurements were carried out using a 96-well plate reader to determine the change in light absorbance as the platelets aggregate. LTA has been previously adapted for use with 96-well plates (Fratantoni and Poindexter, 1990). In the 96-well plate method, blood was

mixed with stimulants using a Bio- Shaker, which was adapted for the 96-well plate.

Table 3-1: Comparison of existing vs. 96-well plate methods.

A summary of the main differences between the existing method and the new method in development (96-well plate). This new approach will eventually facilitate the remote testing of platelet function.

	Existing method	96-well plate method
Equipment	Test tube	96-well plate
Mixing force	Stirring	Shaking
Volume of blood/single test	480µl	46µl
Volume of agonist/single test	Large volume (20µl)	Small volume (4µl)
Number of agonists	Single agonist	Multiple agonists

The development of the 96-well plate assay, used to measure platelet aggregation, went through many improvements, including critical factors, such as the type of anticoagulant (citrate vs. hirudin), the type of 96-well bottom (flat, round and V-shaped) and the range of platelet agonists used. This work was conducted by the Platelet Research group in Birmingham. It was of particular significance to finding the most appropriate conditions and allowing further investigations after development of this assay.

The double fixation method allows the platelet aggregate to be stable and, therefore, to offer more time frames for analysing fixed blood samples. Also, there is existing evidence that flow cytometry could be applied to investigate platelet aggregation using SPC techniques. Therefore, the aims of this chapter are to compare platelet aggregation in WB using the single fixation method and the Ultra-Flo 100 to count single platelets, with a double fixation method and flow cytometry to count single platelets. Additionally, the effect of reducing blood volume on platelet aggregation, using the double fixation method and flow cytometry, was studied. Subsequent optimisation of the WB assay was performed by the Platelet Research group in Birmingham, who recommended using 46µl of blood, a 96-well flat bottom plate and a range of concentrations of AA, ADP, collagen and TRAP (wet agonists). Therefore, a further aim was to reproduce the pattern of platelet aggregation here using the recommended conditions. It was anticipated that the results obtained might provide data upon which further studies of platelet aggregation in WB in vitro and ex vivo can be based.

3.2 Results

3.2.1 Investigation of platelet aggregation in test tubes with agitation using the Multi Sample Agitator

All aggregation experiments were performed using a device called the MSA, which stirs blood samples, or PRP, at a standard speed and temperature. This device is designed to agitate the blood in a reproducible

and consistent way. For the experiments conducted here, the temperature of the MSA was set at 37^{o}C and the degree of agitation was set to 1000 rpm.

3.2.1.1 Comparison of the double-fixation and single-fixation protocols for the measurement of platelet aggregation in response to ADP

The first set of experiments was aimed at comparing the effect of these fixative solutions on the measurement of platelet aggregation, with platelet counting performed using either flow cytometry or the Ultra-Flo 100 WB platelet counter. This study demonstrates the use of two fixative methods (double vs. single) to measure ADP-, collagen- and U44619-induced platelet aggregation in WB using a range of concentrations at different times, which had been established and validated by our research group. The double fixation method uses two fixation stages and analysis was carried out on a flow cytometer using 96-well plates. However, the single fixation method uses a single fixative to measure the fall in the SPC using Ultra-Flo 100.

In this study, and subsequent studies, in the present chapter, platelet aggregation was estimated as the fall in the SPC as platelets aggregated, with reference to RBC. Baseline values were measured in unstimulated samples using blood anticoagulated with EDTA, which is a known Ca^{2+}-chelating agent, therefore providing the absolute platelet count.

Platelet aggregation was calculated using the following formula:

$$\frac{\text{(Baseline platelet count–Sample platelet count) x 100}}{\text{Baseline platelet count}}$$

Blood samples were fixed at different times (at 0.5 and 4 min) after stimulation with different concentrations of ADP (1, 3 and 10µM) and then incubated with the FITC-CD42a antibody for flow cytometry analysis. For single fixation measurements using Ultra-Flo 100, the fixed blood samples were mixed gently by repeated inversion and 36ml of the fixed blood were added to a vial containing 9.1ml saline. All platelet counts were determined with the RBC setting at 600. Aggregation results were determined using a flow cytometer for double-fixed blood and the Ultra-Flo 100 for single-fixed blood.

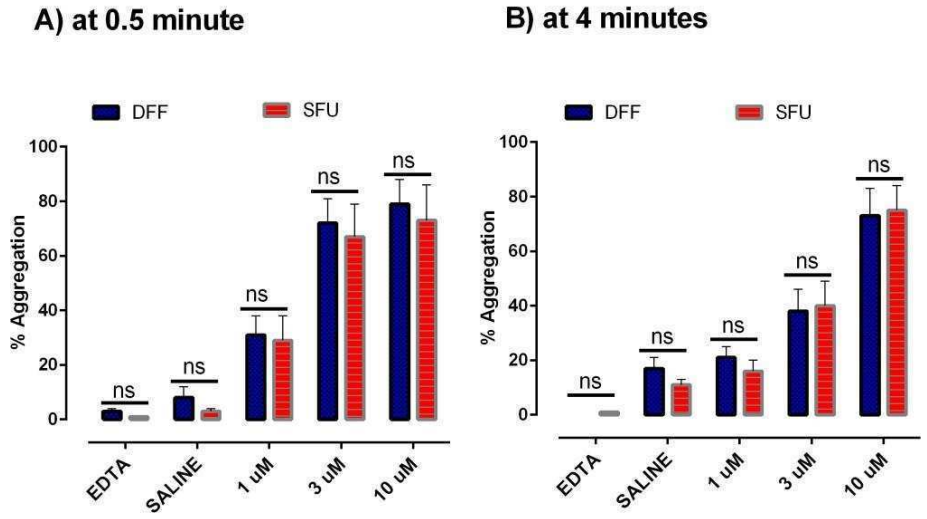

Figure 3-1: ADP-induced platelet aggregation in WB.

Samples were fixed using double fixation and flow cytometry (DFF) (blue bars) and single fixation Ultra-Flo 100 (SFU) (red bars) at 0.5 and 4 minutes. Results are expressed as mean ± SEM, n=6. Analysed with paired-t test. ns: no significance.

It is well established that the range of ADP concentrations that demonstrates an increase in aggregation in a dose-dependent manner are 1, 3 and 10μM. The time course was to be of a maximum of 4 min, as after this time the aggregation pattern does not change dramatically. The results presented in Figure 3-1 (A and B) demonstrate ADP-induced platelet aggregation at 0.5 and 4 min, using both approaches. Dose-dependent increases in platelet aggregation in response to platelet stimulation, with a range of ADP concentrations, were also evident. For blood stimulated with 10μM ADP, the percentage of platelet aggregation reached a maximum of nearly 80% at 0.5 and 4 min. However, for blood stimulated with 3μM ADP for 4 min, the aggregation appeared to be less (more reversible) than in blood stimulated for 0.5 min. Samples incubated with saline showed a small percentage of spontaneous aggregation. Paired t-test analysis performed showed no statistically significant difference between both fixation approaches at the lowest (1μM), intermediate (3μM) and highest (10μM) concentrations of ADP.

The overall pattern of the aggregation values measured using both fixation methods gave similar results. This can be seen from the correlation curve (Figure 3-2), which illustrates a high correlation between the DFF and the SFU, using Pearson's correlation curve measurement ($y = 1.0097x$, $R^2 = 0.9649$). In this respect, DFF is advantageous in fixing platelet aggregates for up to 9 days. This has been confirmed by our research group, which showed the stability of the platelet aggregate stimulated by different

agonists – ADP, collagen, U46619 and sulprastone (analogue of PGE_2). The aggregation values measured between 1-3 days were highly correlated with the values obtained between 6-9 days (Figure 3-3), (correlation values; $y = 0.987x$, $R^2 = 0.9824$) (May JA, 09/05/08).

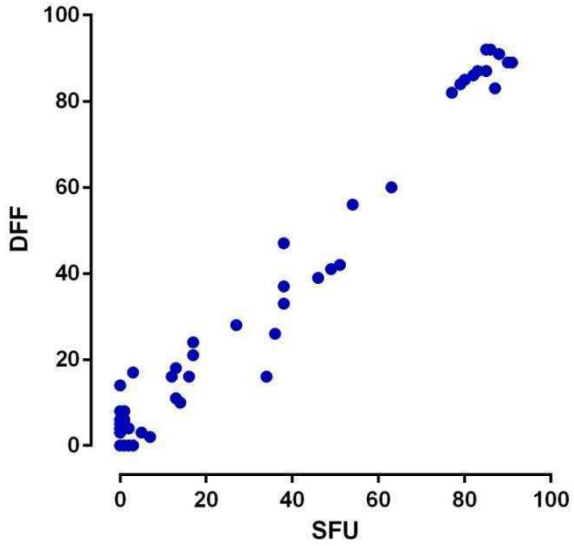

Figure 3-2: Correlation of platelet aggregation stimulated with ADP.

Blue dots represent aggregation values measured by double fixation and flow cytometry (DFF) and single fixation using the Ultra-Flo 100 (SFU) at 0.5 and 4 min using adenosine diphosphate (ADP) (n=6).

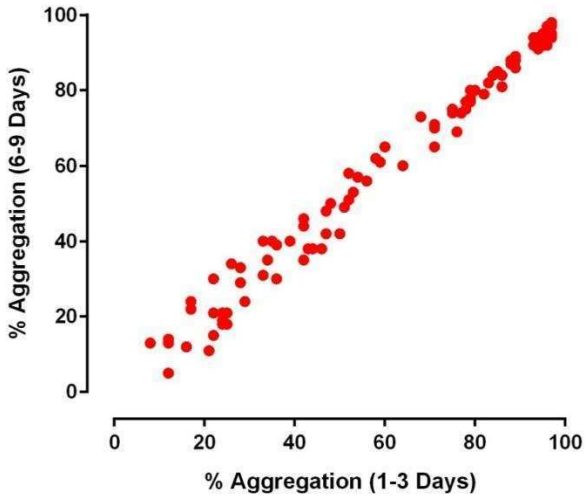

Figure 3-3: Aggregation stability obtained at 1-3 and 6-9 days.

Dots represent the aggregation values measured using adenosine diphosphate (ADP), collagen, U46619 and sulprastone.

3.2.1.2 Double fixation flow cytometry vs. single fixation Ultra-Flo 100 platelet aggregation in whole blood, using collagen as a stimulant

These experiments used the same methodology as the previous experiment, except that here collagen (Horm) was used as the stimulant at concentrations of 0.25, 1 and 4µg/ml, with platelets fixed at 1 and 4 min. This experiment was performed to determine whether the approaches used to measure platelet aggregation were comparable. It would also ensure that the results obtained using double fixative on flow cytometry correlated with the results obtained from single fixation using the Ultra-Flo 100 when another stimulant, such as collagen, was used.

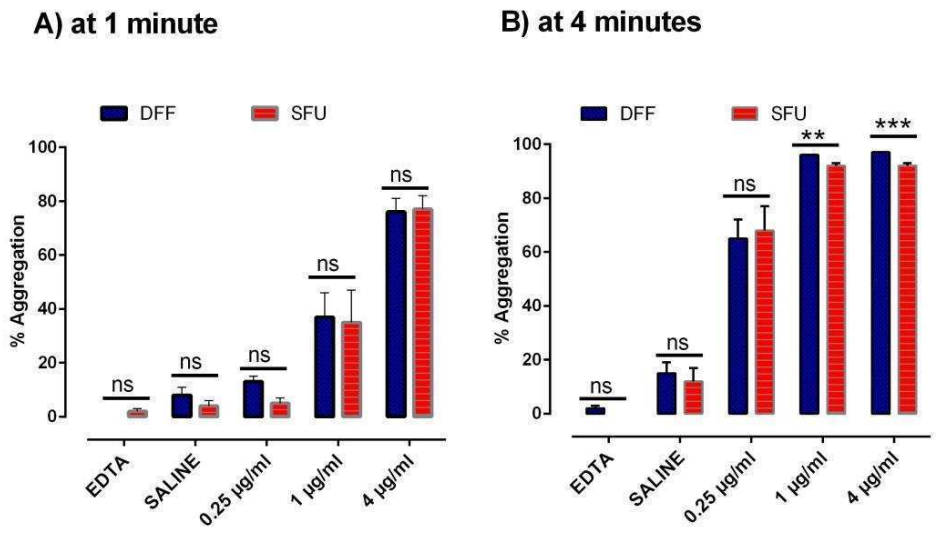

Figure 3-4: Collagen-induced platelet aggregation in whole blood.

Samples were fixed using double fixation and flow cytometry (DFF) (blue bars) and single fixation and the Ultra-Flo 100 (SFU) (red bars), measured at 1 and 4 min. Results are expressed as mean ± SEM, n=6. **P<0.01, ***P<0.001 and analysed with paired-t test

At a time course of one minute, the percentage platelet aggregation increased in, duplicate order, from 20% with blood stimulated with 0.25µg/ml collagen, to 60% with 1µg/ml, to 80% with 4µg/ml. However, at a time course of 4 min, there was a dramatic increase in the percentage of platelet aggregation, with it reaching 100% at intermediate (1µg/ml) and highest (4µg/ml) concentrations. At these two concentrations, a paired t-test showed that there was a statistically significant difference between double fixation flow cytometry and single fixation Ultra-Flo 100 (P<0.01 and P<0.001).

The overall results indicate that collagen-induced platelet aggregation in WB, measured using the two approaches, are comparable at 1 and 4 min

after platelet stimulation (Figure 3-5). The data clearly demonstrates that the two fixation approaches correlate well, using Pearson's correlation analysis ($y=1.0128x$, $R^2=0.9706$).

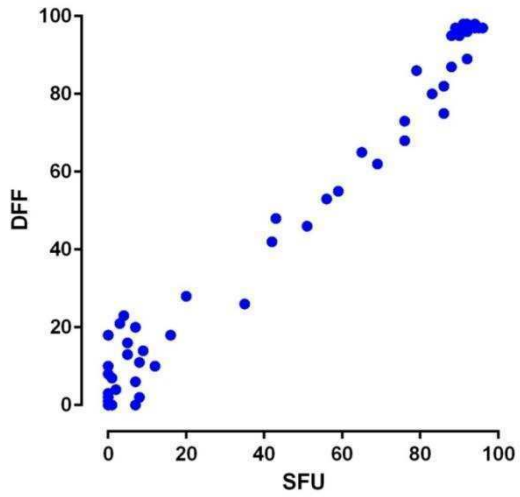

Figure 3-5: Correlation of platelet aggregation stimulated with collagen.

Dots represent values measured by double fixation and flow cytometry (DFF) and single fixation using the Ultra-Flo 100 (SFU) at 1 and 4 min, using collagen as the stimulant (n=6).

3.2.1.3 Double fixation Flow Cytometry vs. single fixation Ultra-Flo 100 whole blood aggregation, using U46619

These experiments were conducted using a different platelet stimulant known as U46619. U46619 is a stable analogue of the thromboxane receptor (TR) agonist and the endoperoxidase PGH_2 (Abramovitz et al., 2000). It shows similar properties to TXA_2, causing platelet shape change and aggregation (Coleman et al., 1981). In these experiments, blood was

stimulated with a range of U46619 concentrations (0.3, 1 and 3µM) and stimulated for 1 and 4 min.

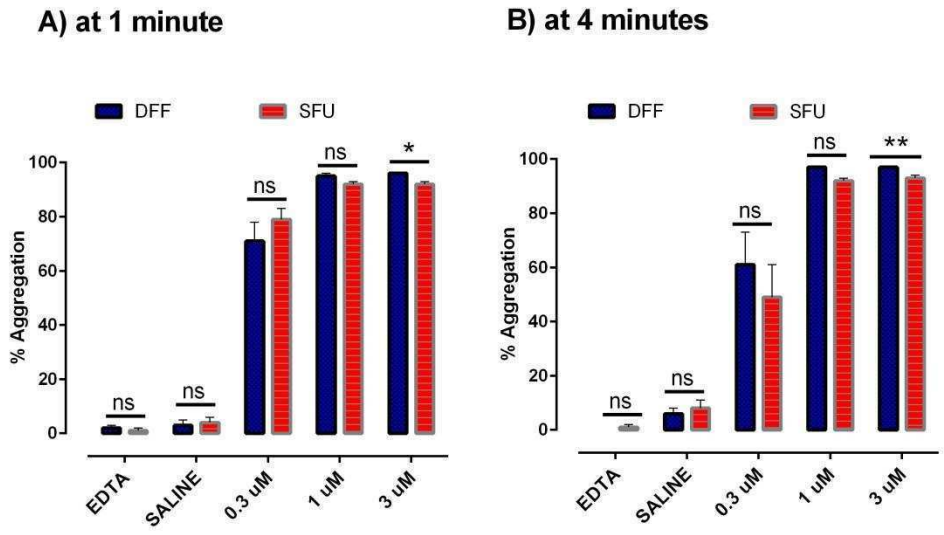

Figure 3-6: U46619-induced platelet aggregation in whole blood.

Samples were fixed using double fixation flow cytometry (DFF) (blue bars) and single fixation Ultra-Flo 100 (SFU) (red bars) at 1 and 4 min. Results are mean ±SEM, n=6. *P< 0.05, **P<0.01 analysed with the paired-t test.

This experiment utilised the same principle and methodology as the previous experiments, but with U46619 (a thromboxane analogue) as the stimulant. This experiment was carried out with the intention of determining whether the double fixation method flow cytometer and single flow method Ultra-Flo 100 were comparable.

At 1 min, platelet aggregation in the blood stimulated with 0.3µM U46619 exhibited higher values of 70% with DFF and 80% with SFU. However, at 4 min, platelet aggregation was lower, with DFF shown to preserve the aggregate more than SFU.

In blood stimulated for 1 and 4 min with 1 and 3µM U46619, platelet aggregation exhibited a similar pattern of nearly 100%. Platelet aggregation measured with DFF was demonstrated to be slightly higher than SFU. This was not statistically significant in blood stimulated with 1µM of the compound. However, in blood stimulated with 3µM, the difference between DFF and SFU was statistically significant ($p<0.05$) at 1 min and even more so at 4 min.

With both approaches, there was a high correlation between the aggregation values as analysed by Pearson's correlation curve measurement (Figure 3-7; $y=1.0125x$, $R^2=0.9799$).

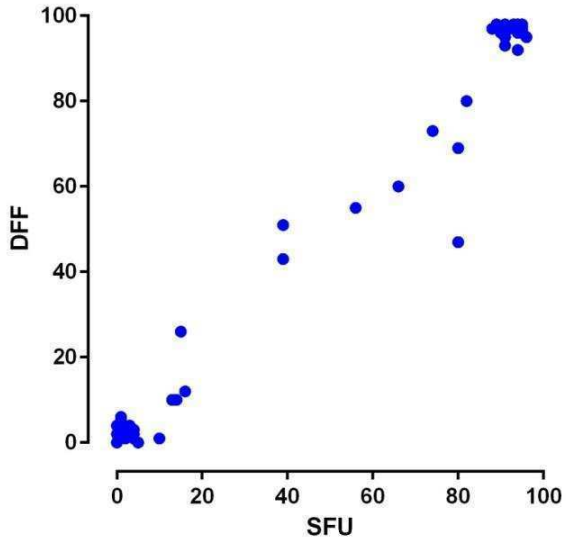

Figure 3-7: Correlation of platelet aggregation stimulated with U46619.

Dots represent values measured by double fixation and flow cytometry (DFF) and single fixation using the Ultra-Flo 100 (SFU) at 1 and 4 min, using U46619 as a stimulant (n=6).

The previous comparison of the two fixation methods, DFF and SFU, indicated that the aggregation results were highly comparable for all blood stimulants (ADP, collagen and U46619). It can, therefore, be concluded that the DFF method is comparable to the well-established electronic cell counter method and that they can be used interchangeably. Therefore, DFF can be used as the fixation method for further investigations throughout the present thesis.

It is required to minimise the blood volume used for PFT in order to carry out as many tests as possible on one sample; to address this issue, the next section focuses on studying the effects of reducing blood volume on aggregation measurements. This was carried out in an attempt to reduce the blood volume used for testing, which would make it of use for investigating as many platelet functions as possible (Chapter.6), and also to make it applicable to patients with restricted blood volumes, such as paediatric patients.

3.2.2 Measurement of platelet aggregation using different blood volumes

It has already been demonstrated that the double fixation method using flow cytometry is highly comparable to the single fixation Ultra-Flo 100 method (section 3.2.1); these experiments were carried out on WB using the double fixation method and flow cytometry only. The present study aimed at determining the effects of reducing the blood volume used for aggregation studies and to see if this had an effect on aggregation results. Different volumes of blood (125, 250 and 500µl) were stimulated with a range of ADP concentrations (1, 3 and 10µM) and fixed at 0.5 and 4 min.

3.2.2.1 Using ADP

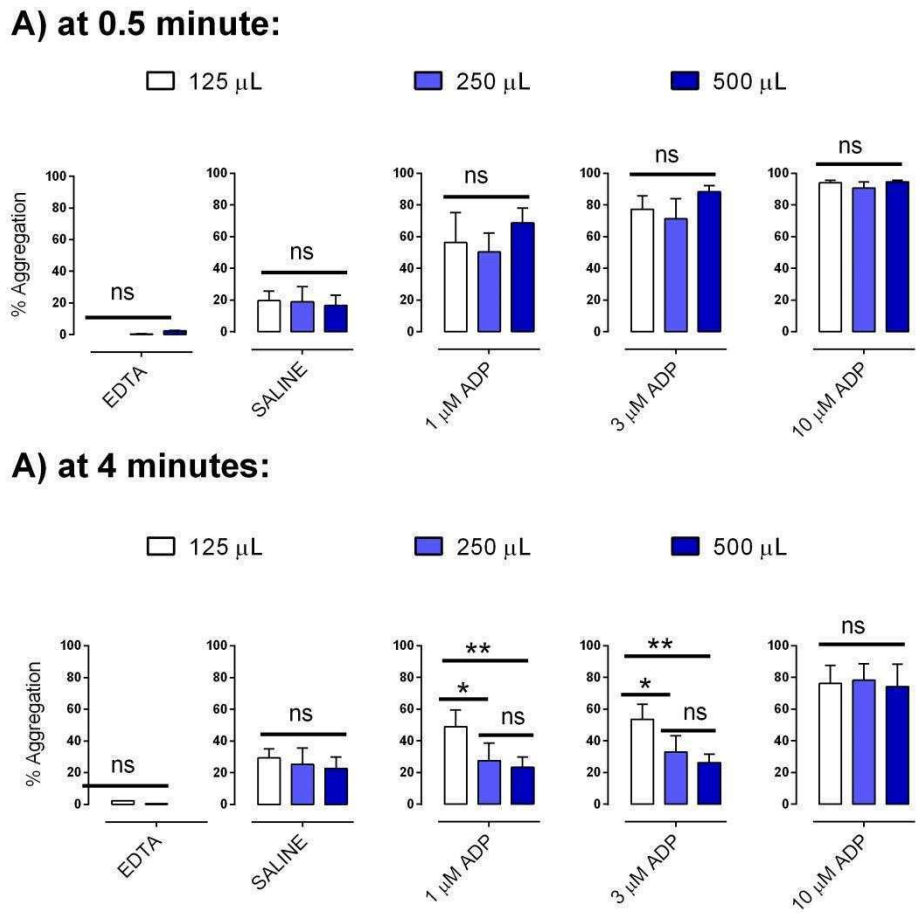

Figure 3-8: Platelet aggregation stimulated with ADP, using different blood volumes.

Adenosine diphosphate (ADP)-induced aggregation in whole blood using double fixation flow cytometry (DFF) and different blood volumes (125µl (white bars), 250µl (purple bars) and 500µl (blue bars)) at 0.5 (A) and 4 (B) min. Results are expressed as mean ± SEM (n=6). *P< 0.05; analysed by ANOVA with the Bonferroni test.

The ADP data derived at 0.5 min showed that platelet aggregation increased with different blood volumes and ADP concentrations, in a dose-dependent manner. It was also observed that at different concentrations of

ADP the percentage of platelet aggregation was very close and showed no significant difference, as indicated by ANOVA. With high ADP concentrations (10µM), aggregation was nearly 100% with the different blood volumes tested.

At 4 min, the data showed that there was more aggregation with the lowest blood volume used (125µl) and different ADP concentrations, and this appeared to make aggregation less reversible. However, with higher blood volumes (250µl and 500µl), there was less platelet aggregation, which tended to be more reversible at ADP concentrations of 1µM and 3µM – indicating that aggregation at low ADP concentrations appeared to be more reversible. ANOVA showed that the aggregation value measured for 125µl blood was statistically different from that for 250µl ($P<0.05$) and for 500µl ($P<0.01$), in samples stimulated with 1µM and 3µM ADP. There was no significant difference between aggregation values for 250µl and 500µl of blood. For blood stimulated with a high concentration of ADP (10µM), aggregation values were not significantly different from each other.

3.2.2.2 Using collagen

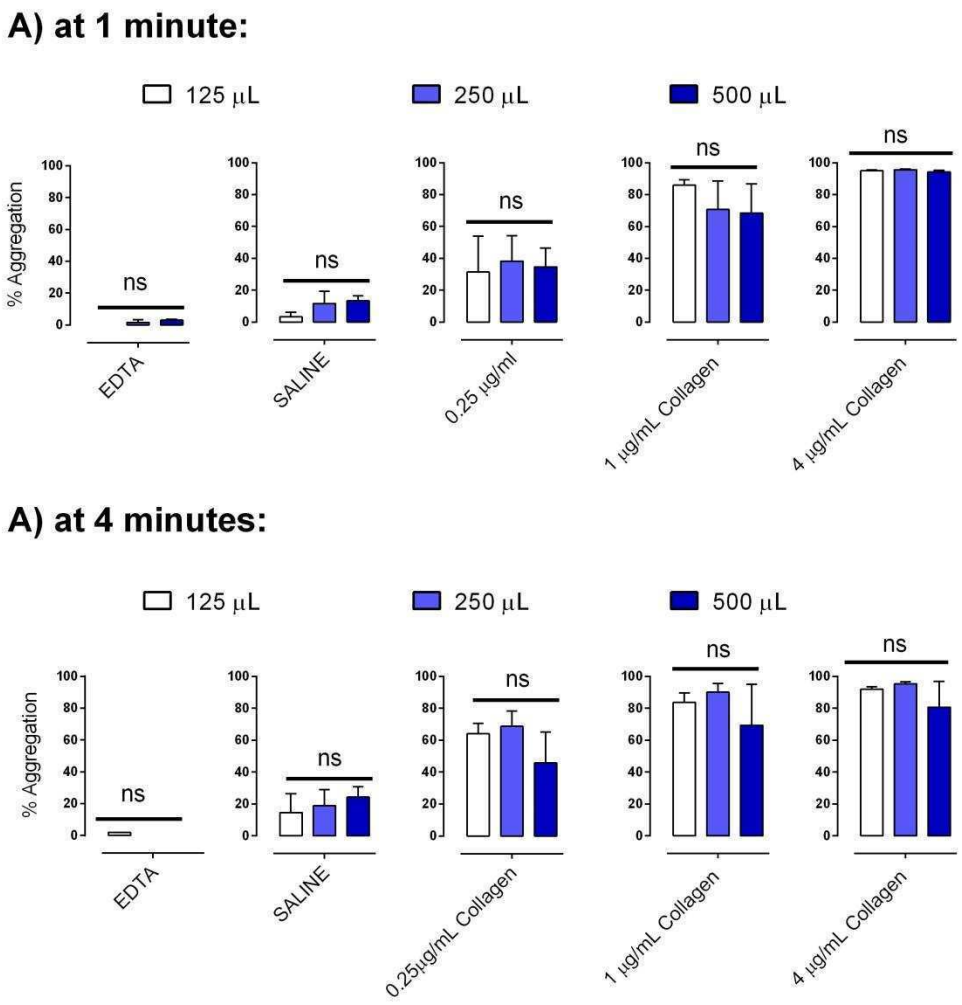

Figure 3-9: Platelet aggregation stimulated with collagen, using different blood volumes.

Collagen-induced aggregation in whole blood using double fixation flow cytometry (DFF) and different blood volumes (125µl (white bars), 250µl (purple bars) and 500µl (blue bars)) at 1 (A) and 4 (B) min. Results are expressed as mean ±SEM (n=6). *P< 0.05; analysed by ANOVA with the Bonferroni test.

With respect to blood stimulated with collagen and fixed at 1 min, it was clear that platelet aggregation was approximately the same with 500µl, 250µl and 125µl of blood, at low and high concentrations of collagen (0.25

and 4µg/ml). Intermediate collagen concentrations (1µg/ml) stimulating 125µl of blood exhibited more platelet aggregation than 250µl and 500µl of blood. Maximal aggregation to the same extent was reflected with the different volumes of blood stimulated with 4µg/ml collagen.

At 4 min, platelet aggregation appeared to increase with different blood volumes stimulated with different collagen concentrations. However, when using 250µl and 125µl of blood, there was higher platelet aggregation than when using a larger blood volume (500µl). This reflects, that with lower blood volumes, there is more platelet aggregation, which is more stable and less reversible.

3.2.2.3 Using U46619

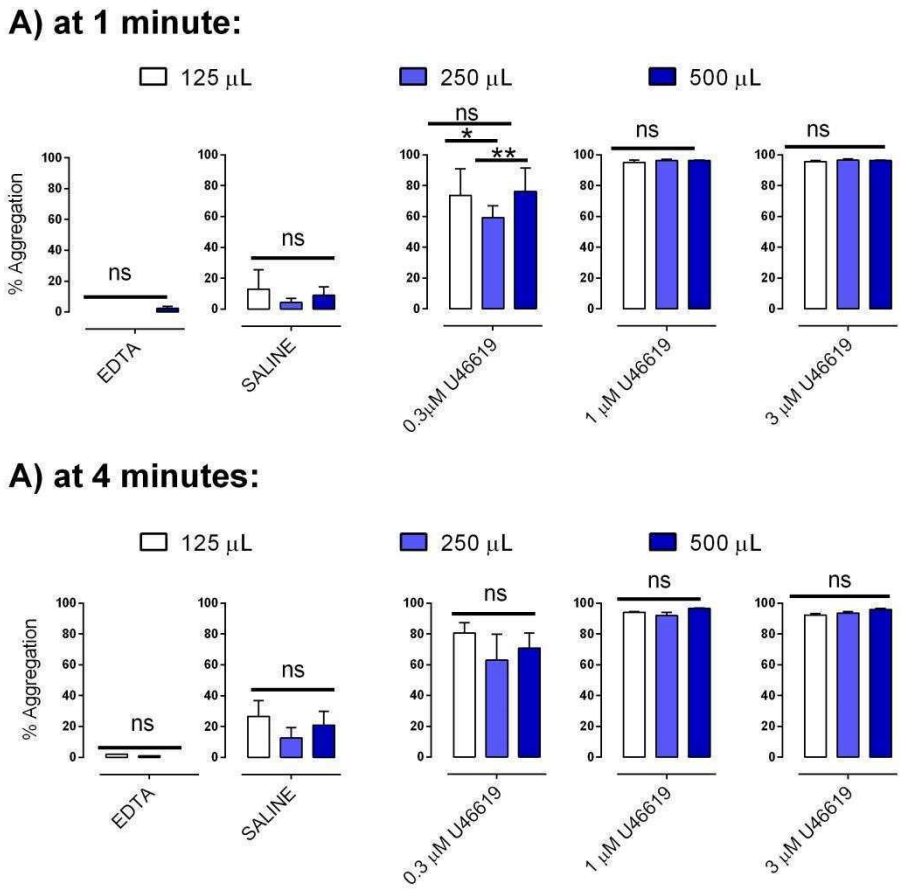

Figure 3-10: Platelet aggregation stimulated with U46619, using different blood volumes.

U46619-induced aggregation in whole blood using double fixation flow cytometry (DFF) and different blood volumes (125µl (white bars), 250µl (purple bars) and 500µl (blue bars)) at 1 (A) and 4 (B) min. Results are expressed as mean ±SEM (n=6). *P< 0.05; analysed by ANOVA with the Bonferroni test.

When U46619 was used as the platelet stimulant, platelet aggregation was the same with different volumes at concentrations of 1µM and 3µM, at 1 and 4 min. At a concentration of 0.3µM U46619, results tended to be

variable, with 500µl and 125µl exhibiting a higher percentage of platelet aggregation at 1 and 4 min, respectively.

From the experiments above, which were performed to investigate the effect of reducing blood volume on platelet aggregation, it can be concluded that blood aggregation was very similar at the highest agonist concentrations used and variable at low and intermediate concentrations. With respect to blood volume, the aggregation results did not differ hugely between the different volumes used.

The work in this chapter demonstrated that the double fixation method has the great advantage of allowing measurements to be performed within a longer time frame (9 days) and anywhere (remote setting). Investigations into the influence of the volume of blood used showed that aggregation is more stable at lower blood volumes. Therefore, the following section connects these finding with others made by the Platelet Research group in Birmingham, leading to the use of the 96-well plate for even lower blood volumes (of 46µl per well or condition) – allowing many platelet stimulants to be investigated in parallel.

3.2.3 Measurement of platelet aggregation in 96-well plates by a shaking technique, using a WB shaker

The traditional methods for measuring platelet aggregation are either labour-intensive or time-consuming; also, many platelet activation pathways cannot be determined in parallel with them. To overcome these short-comings, the aim of this work was to develop a method for assessing platelet function, in response to different platelet stimulants, using a relatively small volume of a WB sample. This was carried out using a Bio-shaker, which was set at 37^{o}C and 1000 rpm, with the analysis carried out using 96 well-plates. Fixation was performed after 5 min of shaking, using the double fixation approach, thereby allowing analysis to be carried out at a later stage. Measurements of platelet aggregation were achieved at a later stage using the SPC technique and flow cytometry, where the platelets were identified by their positivity for CD42a FITC.

3.2.3.1 96-well plates with wet agonists

96-well flat-bottomed plates were used and different concentrations of agonists were prepared freshly (wet agonists) on the day before experiments were carried out. Blood was obtained from healthy volunteers who declared that they did not take drugs that interfere with platelet function. Blood was anticoagulated with sodium citrate. The results are presented as percentage of platelet aggregation, measured as decreases in single platelets as they aggregate.

3.2.3.2 Optimisation

This work was carried out in collaboration with the Birmingham Platelet Research group in order to optimise the technique and ensure that it is suitable for measuring platelet aggregation in WB. Optimisation involved the following:

- Type of the plate to be used (round-, flat- or v-shaped-bottomed).
- Volume of blood to be used.
- Agonist and concentration range to be used.

3.2.2.1 Measurements of platelet aggregation in healthy volunteers

After we had optimised the different factors needed to ensure that the conditions were optimal, the following experiments were carried out to obtain a full dose-response curve for platelet aggregation in response to a range of concentrations of platelet stimulants (AA, ADP, collagen and TRAP). Experiments were also carried out to establish that small volumes of whole blood (~ 1.2 ml) could be used to generate this amount of data. The percentage platelet aggregation was measured as the fall in the single platelet count against that of blood anticoagulated with EDTA or saline, to determine the spontaneous aggregation without platelet stimulation. Aggregation data measures vs. EDTA were presented throughout.

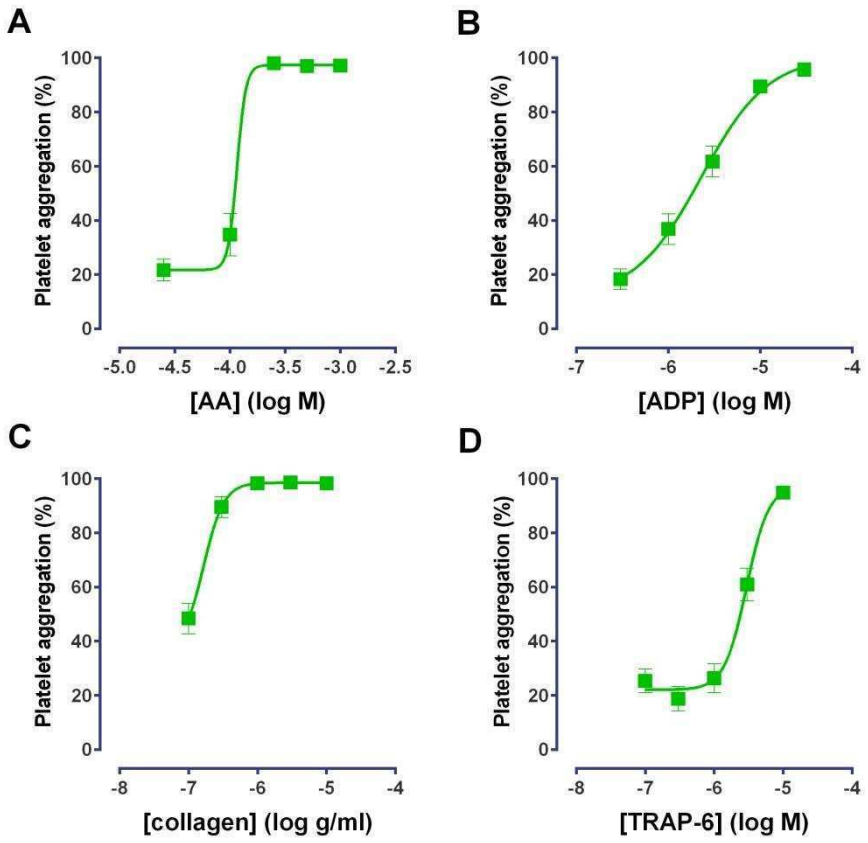

Figure 3-11: Platelet aggregation in healthy volunteers in response to wet agonists.

Effects of arachidonic acid, AA (0.03-1mM), adenosine diphosphate, ADP (0.3-30µM), collagen (0.1-10µg/ml) and thrombin receptor activated peptides, TRAP (0.1-10µM) on platelet aggregation. Results are expressed as mean ± SEM, (n= 20), vs. EDTA.

When samples of WB were shaken with either AA, ADP, collagen or TRAP in 96-well plates, the extent of platelet aggregation that occurred depended on the concentration of the particular agonist used. In all cases, aggregation was complete (nearly 100%) at the highest concentration of agonist used (vs. EDTA and saline). EC50 was calculated for each donor using Graphpad Prism software to indicate the concentration of agonists

achieving 50% of maximum platelet aggregation. Mean EC50±sem was estimated as follows; AA= 0.13 ± 0.048 mM (n=10), ADP= 2.45 ± 0.073 µM (n=10), collagen= 0.14 ± 0.06 µg/ml (n=10) and TRAP= 2.62 ± 0.06 µM (n=10).

Overall the aggregation pattern of the values measured with respect to EDTA was slightly higher than that with respect to saline. The aggregation values, which were measured in duplicate, were robust and reproducible (CV<10%).

3.3 Discussion

The study of the role of platelets in haemostasis and thrombosis, as in ACS, and when monitoring the effects of administered drugs on platelets, requires reliable PFT. One measure for assessing platelet function is by measuring platelet aggregation. This can be done by LTA on PRP, or in WB, by using a variety of methods, such as with point-of-care testing devices, Multiplate, VerifyNow® or Platelet Function Analyser-100 (PFA-100). It can also be measured by flow cytometry using a SPC technique.

The overall objective of the present chapter was to further optimise measurements of platelet aggregation in WB using a single platelet counting technique (used here in the University of Nottingham for many years (Storey and Heptinstall, 1999), in terms of effectiveness of fixation,

reducing blood volume for analysis and using a 96-well plate format. WB is more physiological than PRP, as erythrocytes and leucocytes are present, and centrifugation involved in the preparation of PRP can result in platelet activation (Fox et al., 2004b).

In circumstances where there is no immediate access to a flow cytometer, fixation is preferable for the prevention of platelet activation in vitro (Tschoepe et al., 1991, Shattil et al., 1987). However, fixation is an important variable that may also involve alteration of platelet receptors. The stage at which fixation is performed is controversial, i.e. whether it should be before or after antibody labelling (Shattil et al., 1987). Using both methods, samples have been shown to be stable for at least 24 hrs (Hagberg and Lyberg, 2000a). FA or PFA of different concentrations can be used to fix blood for platelet aggregation measurements, with PFA preferred, as FA can directly induce the expression of platelet activation markers CD62P or CD63 (Cahill et al., 1993). For most flow cytometry protocols, blood samples are fixed before labelling with antibody; however, certain receptors such as CD41, which is specific for platelets (Michelson et al., 2000), or GPIIb/IIIa, which is specific for the final aggregation step (George et al., 1986), are affected by the fixation process.

A solution based on PFA, at a final concentration of 0.5-1% (w/v), is the standard reagent used for fixing and stabilising blood samples for flow

cytometry analysis (Michelson et al., 2000). FA at a final concentration of 0.16% (w/v) was previously established here to be optimal for platelet function analysis using flow cytometry (Fox et al., 2009). The first sets of data presented in this chapter were focused on the use of two novel approaches to fixation for measuring platelet aggregation in WB: double DFF and SFU. Blood and platelet stimulants at different concentrations were stirred together in a test tube using MSA and fixed at different times, between 0.5 minutes and 4 minutes; after this time, the percentage of aggregation does not change. Experiments were performed with the intention of checking whether both fixation methods are comparable to each other. If they were then double fixation could be used in further investigations. Single fixation involved one stage of fixation with PAM fix; this stabilises the platelets aggregates, thereby allowing measurements of free single platelets to be made. DFF offered the advantage of fixing platelets and keeping aggregates stable for up to 9 days, thereby allowing remote platelet function testing.

Previously, our research group showed that there was a high correlation between single platelet counting (using flow cytometry vs. Ultra-Flo 100) after single fixation of ADP-stimulated samples in citrated anticoagulated blood (Fox et al., 2004b). In light of this, a comparison between double fixative solutions and flow cytometry, and the well-established electronic counting technique (Ultra-Flo 100) with single fixation method was performed, using different agonists. The results obtained (Figure 3-2, Figure 3-5 & Figure 3-7) indicated that the aggregation values measured by

double fixation and flow cytometry were highly correlated with those obtained using the established method of using an Ultra-Flo 100 whole blood platelet counter.

ADP-induced platelet aggregations using both fixation methods were highly correlated. However, at 4 min of stirring, the percentage of aggregation tended to be lower due to reversible aggregation. When collagen was used, the same correlation pattern was shown; however, the DFF method tended to produce slightly higher aggregation values at the highest two concentrations (1 and 4µg/ml) of agonist, which was statistically significant. This could be explained by the fact that a double fixative is able to preserve the aggregate better than a single fixative solution. The same thing applied to blood stimulated with U46619. The overall results indicate that the DFF method correlates well with the SFU method. Therefore, a decision was made to use DFF in further experiments investigating the effects of reducing the volume of blood used on platelet aggregation.

The effects of using different blood volumes on measurements of platelet aggregation have not been previously investigated. The aim here was to minimise the blood volume used for aggregation studies. The traditional method of measuring platelet aggregation uses a large volume of blood and it can be even greater when investigating the effects of antiplatelet drugs. Therefore, experimentation on reducing volumes was of importance here.

Different volumes of WB were stimulated by ADP, collagen and U46619, fixed with double fixative solution and analysed with flow cytometry. The results showed that for blood stimulated with ADP, the aggregation values were the same, except for lower concentrations (1 and 3µM). There was also a trend of greater aggregation in low blood volume samples. This suggests that in small blood volumes aggregation seems to be less reversible. Collagen produced a similar pattern of aggregation values for the different volumes used. In the case of U46619, the percentage of platelet aggregation remained the same, except for the lowest concentration used. The results indicate that the lower the blood volume, the more stable the platelet aggregate. Therefore, the next experiments were conducted using low blood volumes that were fixed using the DFF method in a 96-well format.

The 96-well plate format has been developed as a high throughput technique for measuring platelet aggregation, where blood volume can be even smaller. This was carried out using a WB shaker to shake plates with blood and agonist. Prior to use, different factors were investigated, such as the choice of anticoagulant, the type of bottom of the 96-well plates, and the range of concentrations of platelet agonists used. A decision was made to use citrate as the anticoagulant. Plates with flat bottoms were chosen, as this design allows blood to be warmed evenly at 37^{o}C and inhibits sedimentation of blood at the bottom of plates (this could happen with V-or U-shaped 96-well plates. Afterwards, combining double fixation with 96-

well plates enabled the assessment of platelet aggregation and its inhibition by a wide range of agonists and antagonists.

The aggregation measurements conducted on healthy volunteers showed that all of the agonists investigated produced significantly higher platelet aggregation responses in a dose-dependent manner (Figure 3-11). This shows that the assay is able to demonstrate different platelet activation pathways by different agonists, which platelet antagonists can act upon. This corroborates previous studies, where investigators were able to scan platelet responses to different platelet agonists. However, those investigations were done in PRP, using measurements of light absorbance to measure platelet aggregation (Armstrong et al., 2009b, Peace et al., 2008). The assay developed here requires only a small volume of blood (46µl) per well and approximately 1.2 ml of blood for the whole test. Clearly, this would be of great benefit in circumstances when the volumes of blood allowed to be taken are very small, such as from neonates and infants. Being able to fix the blood and stabilise it for a longer may allow measurements in areas remote from the patients, where there is no immediate access to flow cytometry.

In this chapter, I have described a simple method for measuring platelet aggregation in response to different agonists, using double fixation flow cytometry. The novel method is comparable to using the traditional SFU method, using a SPC technique. Data obtained from optimisation

experiments demonstrate the appropriate protocol for assessing platelet aggregation using 96-well plates and flow cytometry. In the next chapter, results of whole blood aggregation assays based on SPC techniques, which can be used with a very small volume of blood, will be presented. In combination with the 96-well plate format and shaking method, this represents a promising tool for the study of platelet function and inhibition by antiplatelet drugs in vitro and ex vivo.

4 Inhibition of platelet aggregation *in vitro* and *ex vivo* using a 96-well plate format and Bio-shaker

4.1 Introduction

We checked the parameters that influence the measurement of platelet aggregation, such as the appropriateness of the double fixation approach and the 96-well plate format in normal volunteers. More importantly, we minimised the volume of blood and agonists used for the measurement of platelet aggregation. We then decided to develop this assay further in order to investigate platelet inhibition by commonly-used antiplatelet drugs, in vitro and ex vivo.

In particular, 96-well plate optimisation processes showed that this assay could be used further to investigate platelet inhibition by antiplatelet drugs in vitro and ex vivo. In clinical settings, platelet aggregation can be inhibited by drugs that target either their activation or the final aggregation pathway. The most commonly used inhibitors of platelet aggregation target the COX1 enzyme, the ADP receptor, $P2Y_{12}$, and GPIIb/IIIa. Aspirin acts by inhibiting COX1, thereby, blocking the production of TXA_2 in platelets (Vane and Botting, 2003). ADP receptor antagonism can be achieved by many drugs, such as cangrelor, clopidogrel and prasugrel. Cangrelor is a direct and reversible antagonist of the $P2Y_{12}$ receptor, which does not require conversion to an active metabolite for its antiplatelet action (Bouman et al., 2009). Clopidogrel is another $P2Y_{12}$ receptor antagonist, which needs to be hepatically converted to its active metabolite, allowing it to bind irreversibly to $P2Y_{12}$ receptors (Storey, 2006). Like clopidogrel,

prasugrel has to be converted to an active metabolite in order to inhibit platelet function. Prasugrel, however, differs from clopidogrel in that it has a more rapid onset of action after oral administration (Michelson, 2009). Drugs such as abciximab, tirofiban, eptifibatide and MK-0852 are antagonists of GPIIb/IIIa receptors and interfere with the attachment of the GPIIb/IIIa receptor to fibrinogen, thereby blocking the final common pathway of platelet aggregation (Kereiakes et al., 1997).

Blood platelets play a significant role during the vessel-obstructing cascade of ACS, as a result of atherosclerotic plaque rupture, or during PCI. To inhibit this cascade, dual antiplatelet therapy (DAPT), comprising both aspirin and $P2Y_{12}$ antagonists, is routinely administered to ACS patients (Schulz and Massberg, 2012). This has been previously demonstrated in the CURE study, which highlighted the advantage of using clopidogrel to inhibit platelet function when it was administered concomitantly with aspirin (Fox et al., 2004a). To study the role of platelets in the pathogenesis of this disease, and monitor antiplatelet drug effects, requires reliable PFT; different techniques are available to measure platelet aggregation.

The current technique for investigating platelet inhibition, LTA, is considered by many researchers to be the gold standard method for assessing platelet reactivity to different agonists, such as AA and ADP, after the administration of aspirin and clopidogrel (Hankey and Eikelboom,

2006, Michelson, 2004). However, it is time-consuming, laborious, requires expert staff and a specialized laboratory - these drawbacks limit its use in a clinical setting. Also, the preparation of PRP and the practice of adjusting the platelet count with PPP could introduce another factor, such as ADP, which is introduced due to high speed centrifugation and may inhibit platelet aggregation, due to receptor desensitization (Cattaneo et al., 2007). In light of this, LTA lacks the required sensitivity for determining the effect of $P2Y_{12}$ antagonists. It is very important to use WB, as this offers the advantage of testing blood in a more physiological environment, due to the presence of RBCs and WBCs. Also, an easy and rapid test to assess platelet function in vitro and ex vivo might prove to be useful in clinical practice. At present, a range of near-patient testing, POC testing devices, are used to assess platelet function in whole blood, such as: the VerifyNow® system (Accumetrics, San Diego, CA), the Platelet Function Analyser-100 (PFA-100, Dade Behring, Marburg, Germany) (Michelson, 2005, Harrison et al., 2007) and the Multiplate analyser (Dynabyte, Munich, Germany) (Paniccia et al., 2009). Although these methods have been in use in the clinical setting, their value remains to be proved (Mani et al., 2004). These tests of platelet function cannot provide extensive information about platelet-drug interactions as the results obtained are single numerical values.

Therefore, it is of importance to find a robust, easily performed and reproducible test for measuring platelet aggregation and assessing platelet

inhibition by antiplatelet drugs (Lordkipanidze et al., 2007, Cattaneo, 2007). The idea of adapting 96-well plates for measuring platelet aggregation was important. The SPC technique was employed for measurement of platelet aggregation, and the loss of platelets after stimulation of anti-coagulated blood, using flow cytometry. A 96-well plate was adapted as a tool for measuring platelet aggregation using a Bio-Shaker. As previously explained, a 96-well plate requires only a small volume of blood to investigate various platelet activation pathways in response to a range of platelet agonists.

In this chapter, the use of 96-well plates with wet platelet agonists will be used to investigate platelet inhibition by antiplatelet drugs, in WB, in vitro and ex vivo. Also, the use of 96-well plates with the lyophilised platelet agonist, Optimul, will be investigated in parallel with wet agonists.

4.2 Methods

4.2.1 In vitro studies

Blood was obtained from healthy volunteers who reported that they had not taken any antiplatelet drugs for the past two weeks. Blood was then incubated with the tested drug at 37^{o}C for 30 min. During the incubation period, a range of different platelet agonists was prepared and 4µl added to flat-bottomed 96-well plates. Using a multi-channel pipette, 46µl blood

was transferred to the plate and the plate was shaken for 5 min using a Bio-Shaker (37^{o}C and 1000 rpm). To fix the blood, 17µl AggFixA was added and it was left for 10-15 min. Using another round-bottomed 96-well plate, 135µl AggFixB solution was added. Then, 15µl AggFixed blood was added to the round-bottomed 96-well plate containing AggFixB blood. The final step involved incubation with a FITC-CD42a-labeled antibody, where 10µl antibody was incubated with 5µl AggFixB blood.

The Optimul plate is a flat-bottom 96-well microtiter plate, pre-coated with hydrogenated gelatine in PBS, to block the surface activation of platelets before the addition of platelet agonists. The agonists used were AA, ADP, epinephrine, collagen, TRAP-6, U46619, and ristocetin (in lyophilised formats), where the same procedure applied.

4.2.2 Ex vivo studies

ACS patients who were on dual antiplatelet therapy (aspirin with either clopidogrel or prasugrel) were recruited to the ACS clinic in the Division of Cardiovascular Medicine in Nottingham.

4.3 Results

The preliminary data presented in the previous chapter, which was obtained using 96-well plates, with added wet agonists, and the double fixation flow cytometry method, indicated that the 96-well plate assay development

offered an advantage as an alternative tool for detecting platelet inhibition with antiplatelet drugs. The chapter demonstrated a platelet aggregation curve with a range of platelet agonists, which are used regularly in research and clinical practice for assessing platelet function. Aggregation increased in a dose-dependent manner. Therefore, the following experiments will look at the effects of the antiplatelet drugs, such as aspirin, the $P2Y_{12}$ antagonist cangrelor and the GPIIb/IIIa antagonist MK-0852 in vitro. Also, the ex vivo inhibition of platelets in patients, particularly those with ACS, on dual therapy will be assessed.

The results were presented as a percentage of platelet aggregation, calculated from the following formula:

$$\frac{\text{(Baseline platelet count–Sample platelet count) x 100}}{\text{Baseline platelet count}}$$

Baseline count was obtained from a sample treated with either EDTA or saline. Therefore, the percentage of aggregation results calculated from EDTA and saline analyses were termed ‘aggregation vs. EDTA’ and ‘aggregation vs. saline’, respectively. The reason for this is to see if there is any difference between the aggregation measurements with EDTA, which chelates Ca^{2+}, thereby prevents aggregation, and those with saline, which could cause the platelets to spontaneously aggregate and affect the results. As the results obtained showed a similar pattern of platelet responses, aggregation measurements vs. EDTA were only presented.

4.3.1 Effects of aspirin on platelet aggregation

One method of inhibiting platelet activation is with the use of aspirin. Aspirin decreases formation of TXA_2 by blocking the COX enzyme. The aim of the following experiments was to determine the effect of a range of concentrations of aspirin on platelet aggregation, in response to a range of different agonist concentrations. Blood was incubated with a range of aspirin concentrations (10, 30 and 100μM) for 30 min and then stimulated with a range of concentrations of the agonists, AA, ADP, collagen and TRAP. After that, plates were shaken for 5 min at 37°C and 1000 rpm.

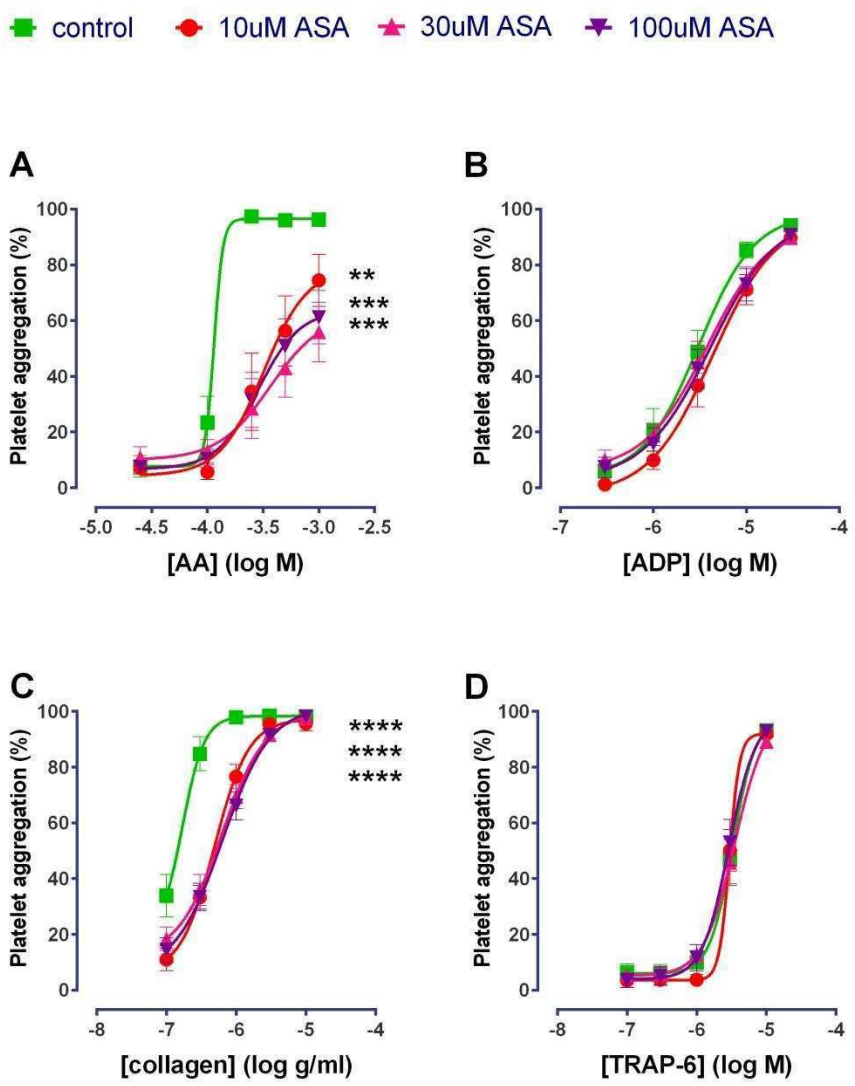

Figure 4-1: Effects of aspirin on platelet aggregation.

Platelet aggregation induced by a range of concentrations of arachidonic acid (AA) (0.03- 1mM), adenosine diphosphate (ADP) (0.3-30µM), collagen (0.1-10µg/ml) and thrombin receptor activating peptide (TRAP) (0.1-10µM) and the effects of 10, 30 and 100µM aspirin. Results are expressed as mean ± SEM (n=10) vs. EDTA. *P<0.05, repeated measures two-way ANOVA followed by Bonferroni's post-test. **P<0.01 (10 µM ASA vs. saline) using AA. *** P<0.001 (30 and 100µM vs. saline) using AA. **** P<0.0001 (10, 30 and 100µM vs. saline) using collagen.

The results obtained show that aspirin inhibited AA-induced platelet aggregation to the same extent, except at the highest concentration of AA.

Repeated two-way ANOVA indicated that inhibition using 10µM ASA vs. saline was statistically significant ($P<0.01$), as well as inhibition using 30 and 100µM ASA vs. saline ($P<0.001$). Less inhibition was observed with high concentrations (0.5 and 1mM) of AA. ASA-inhibited collagen-induced platelet aggregation was statistically significant ($P<0.0001$) for all concentrations of ASA (10, 30 and 100µM) vs. saline. Inhibition by any concentration of ASA was not, however, statistically significant in blood stimulated by ADP and TRAP. The pattern of aggregation and inhibition was similar vs. EDTA or saline, but slightly less with saline.

Aspirin reduced the extent of the aggregation induced by lower concentrations of AA and collagen. Higher concentrations of AA or collagen overcame the inhibition of aggregation seen with aspirin. The results obtained were similar irrespective of the concentration of aspirin used. In contrast, there was little or no effect of aspirin on aggregation induced by ADP or TRAP. To summarise, the results of the present study show that aspirin significantly inhibited platelet aggregation induced by AA, but the degree of inhibition decreased at higher concentrations of AA (0.5 and 1mM). Aspirin partly inhibited platelet aggregation induced by collagen and had little or no effect on ADP- and TRAP-induced aggregation. Subsequent experiments were focused on looking at the effect of the $P2Y_{12}$ antagonist, cangrelor, on platelet aggregation.

4.3.2 Effects of cangrelor on platelet aggregation

These experiments were aimed at checking the suitability of the developed 96-well plate assay for detection of platelet inhibition induced by a range of concentrations of cangrelor. In these experiments, blood was incubated with a range of cangrelor concentrations (10, 100 and 1000nM) and then stimulated with a range of concentrations of the agonists, AA, ADP, collagen and TRAP.

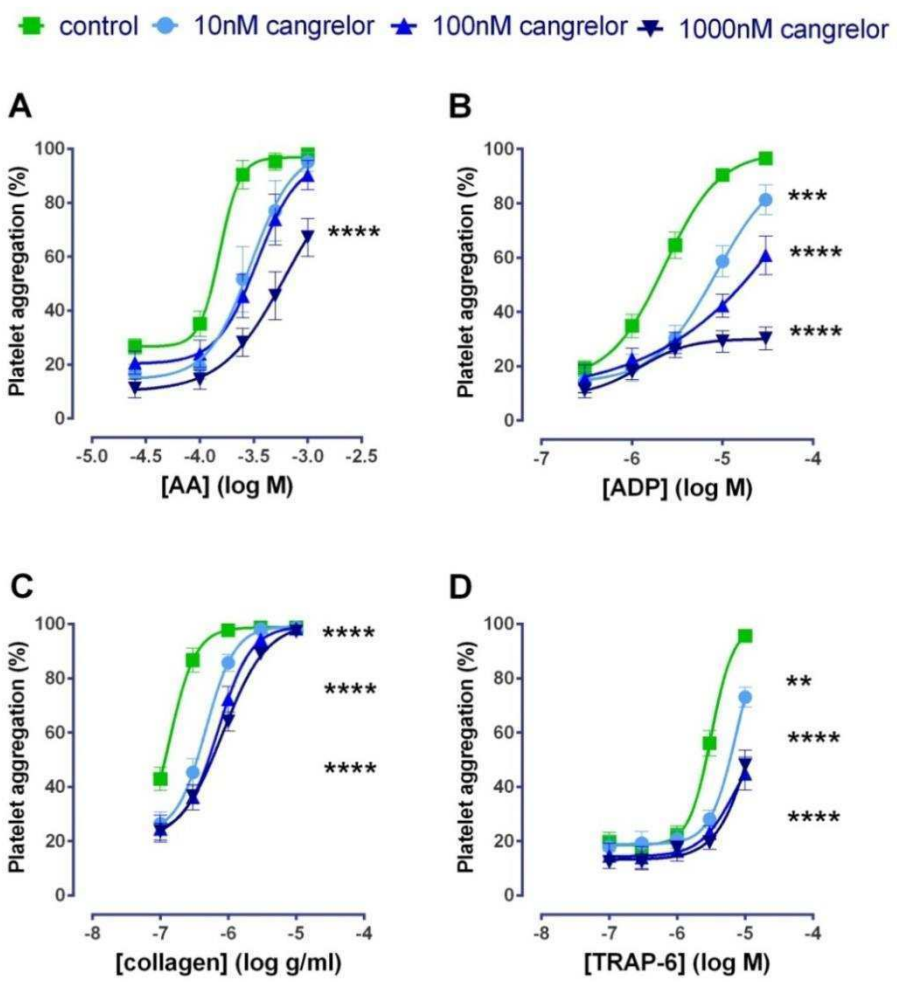

Figure 4-2: Effects of cangrelor on platelet aggregation.

Platelet aggregation induced with a range of concentrations of arachidonic acid (AA) (0.03-1mM), adenosine diphosphate (ADP) (0.3-30µM), collagen (0.1-10µg/ml) and thrombin receptor activating peptide (TRAP) (0.1-10µM) and the effects of 10, 100 and 1000nM cangrelor. Results are expressed as mean ± SEM (n=10) vs. EDTA. *P<0.05, repeated measures two-way ANOVA followed by Bonferroni's post-test. **** P<0.0001 (1000nM vs. saline) using AA. *** P<0.001 (10nM vs. saline) using ADP. **** P<0.0001 (100 and 1000nM vs. saline) using ADP. **** P<0.0001 (10, 100 and 1000nM vs. saline) using collagen. ** P<0.01 (10nM vs. saline) using TRAP. **** P<0.0001 (100 and 1000nM vs. saline) using TRAP.

The results show that cangrelor inhibited ADP-induced platelet aggregation in a dose-dependent manner and inhibition was statistically significant as follows: $P<0.001$ for 10nM cangrelor vs. saline; and $P<0.0001$ for 100 and 1000nM cangrelor vs. saline. Inhibition of TRAP-induced platelet aggregation showed a similar pattern to that of ADP for blood incubated with 100 and 1000nM cangrelor. However, in blood incubated with 10nM, TRAP was inhibited to a lesser extent ($P<0.01$). When using collagen, similar, strong inhibition of platelet aggregation was observed with different blood concentrations (10, 100 and 1000nM vs. saline) ($P<0.0001$). Inhibition of AA-induced platelet aggregation was not evident with 10 or 100nM cangrelor. However, blood incubated with 1000nM demonstrated greater inhibition, which was statistically significant ($P<0.0001$).

In conclusion, cangrelor reduced the extent of aggregation induced by all concentrations of AA, with 1000nM cangrelor producing the strongest inhibition (Figure 4-2). Cangrelor also reduced the extent of the aggregation induced at lower collagen concentrations. Cangrelor inhibited aggregation induced by ADP in a dose-dependent manner and also inhibited aggregation induced by TRAP.

4.3.3 Effects of a combination of aspirin and cangrelor on platelet aggregation

As previously indicated in section 4.3.1, platelet inhibition induced by aspirin in blood stimulated with high AA concentrations (>0.5mM) was to be less than expected. One possible explanation for this might be the presence of endogenous ADP in WB, which may have leaked from RBCs from highly concentrated AA. Therefore, these experiments were carried out to investigate the hypothesis that ADP is present at higher concentrations of AA (0.5 and 1mM), as demonstrated by incomplete inhibition. Also, the additional inhibition observed when both drugs were used together with all agonists could be tested.

Blood was incubated with a range of cangrelor concentrations (10, 100 and 1000nM) plus 100µM aspirin, and then stimulated with a range of concentrations of the agonists: AA (A), ADP (B), collagen (C) and TRAP (D).

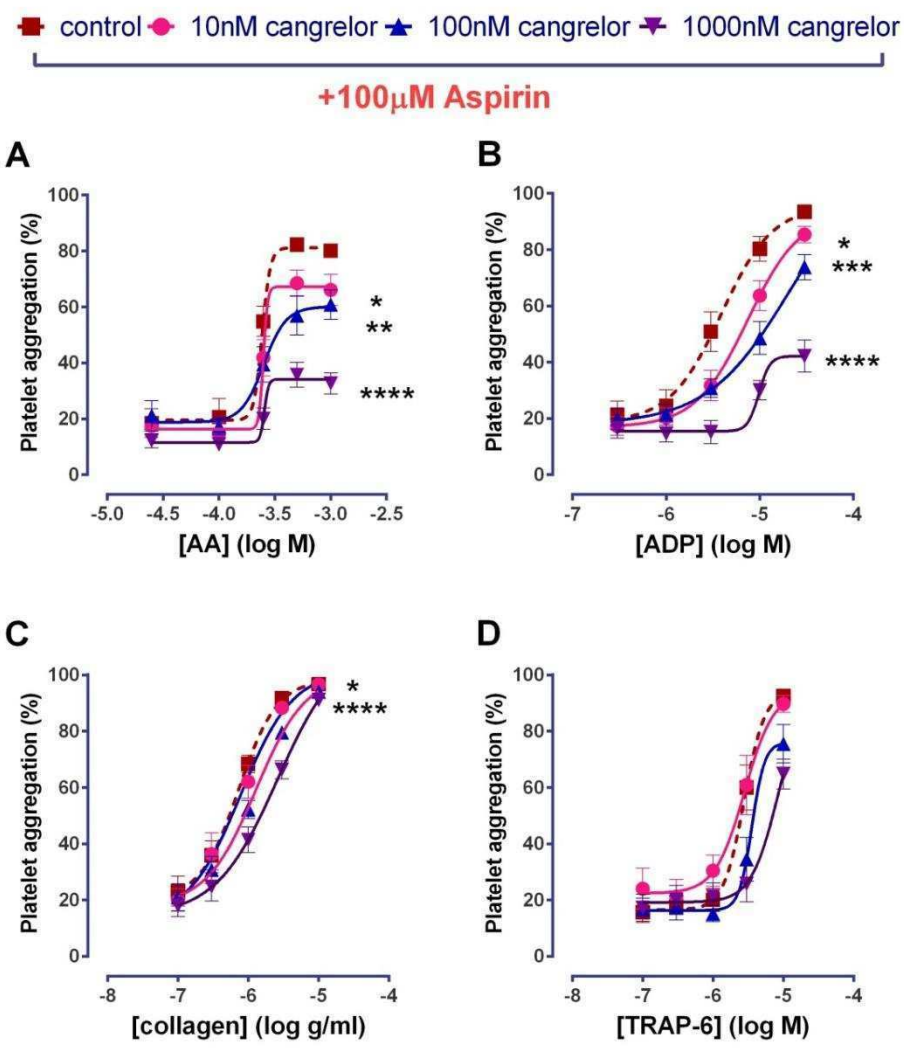

Figure 4-3: Effects of aspirin and cangrelor on platelet aggregation.

Platelet aggregation induced by a range of concentrations of arachidonic acid (AA) (0.03-1mM), adenosine diphosphate (ADP) (0.3-30μM), collagen (0.1-10μg/ml) and thrombin receptor activating peptide (TRAP) (0.1-10μM) and the effects of 100uM aspirin alone or in combination with 10, 100 and 1000nM cangrelor. Results are expressed as mean ± SEM (n=10) vs. EDTA. *P<0.05, repeated measures two-way ANOVA followed by Bonferroni's post-test. * P<0.05 (10nM vs. saline) using AA. **P<0.01 (100nM vs. saline) using AA. ****P<0.0001 (1000nM vs. saline) using AA. * P<0.05 (10nM vs. saline) using ADP. ***P<0.001 (100nM vs. saline) using ADP. **** P<0.0001 (1000nM vs. saline) using ADP. * P<0.05 (100nM vs. saline) using collagen. **** P<0.0001 (1000nM vs. saline) using collagen.

The results obtained from blood incubated with 10 and 100nM cangrelor were statistically significant different to those from blood incubated with saline and stimulated with AA ($P<0.05$ and $P<0.01$, respectively). Strong inhibition of blood incubated with 1000nM was obtained, in comparison to saline. This confirms that ADP is involved at high concentrations of AA, as its effect was reduced with cangrelor in a concentration-dependent manner. The results revealed slight inhibition of blood incubated with 10nM cangrelor vs. saline and stimulated with ADP ($P<0.05$). More inhibition was obtained with blood incubated with 100nM vs. saline and even greater inhibition of the blood incubated was achieved with the highest concentration of cangrelor used (1000nM) vs. saline. Collagen-induced platelet aggregation was not inhibited by the lowest concentration of cangrelor (10nM). Slight inhibition ($P<0.05$) of collagen-induced platelet aggregation was observed when blood was incubated with 100nM cangrelor, with greater inhibition achieved by the maximum concentration of cangrelor ($P<0.0001$). Finally, there was no inhibition observed in the blood stimulated with TRAP.

In summary, this study indicates that a combination of aspirin and cangrelor produced additive inhibition of aggregation, compared to aspirin alone. This can be seen clearly with all four agonists used. Cangrelor, when added to the maximal concentration of aspirin (100μM), inhibited platelet aggregation induced by AA further, confirming that ADP plays a significant role in the mechanism of platelet aggregation measured in WB.

This may explain the incomplete inhibition of AA-induced aggregation in WB by aspirin alone.

4.3.4 Effects of MK-0852 (anti-GPIIb/IIIa) on platelet aggregation

The following experiments were carried out to investigate the suitability of the 96-well plate assay for detecting inhibition of the final platelet aggregation pathway, which involves the GPIIb/IIIa receptor. The antagonist MK-0852 was used. To conduct this experiment, blood was incubated with 10μM MK-0852 (anti-GPIIb/IIIa) and then stimulated with a range of concentrations of the agonists: AA (A), ADP (B), collagen (C) and TRAP (D).

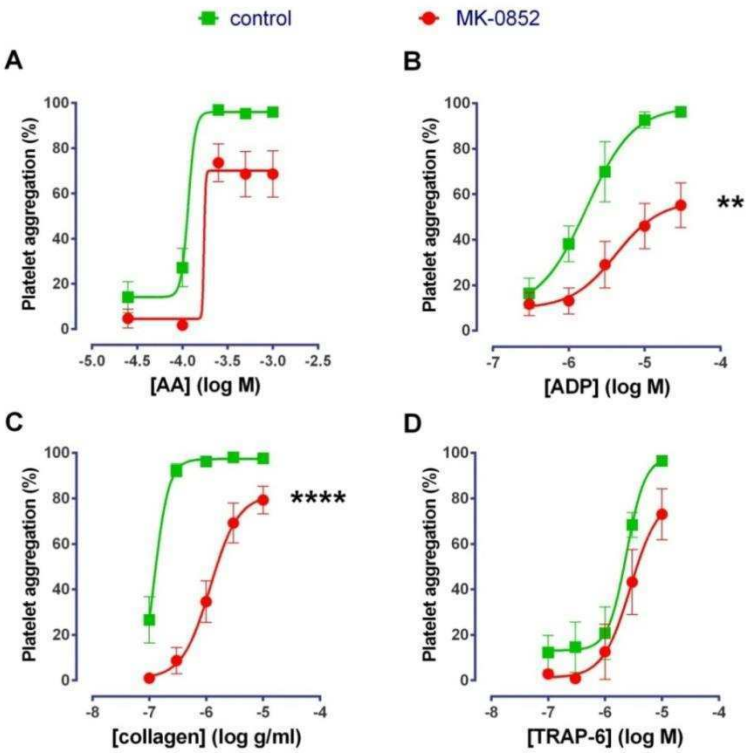

Figure 4-4: Effects of MK-0852 on platelet aggregation.

Platelet aggregation induced by a range of concentrations of arachidonic acid (AA), adenosine diphosphate (ADP), collagen and thrombin receptor activating peptide (TRAP) and the effects of 10µM MK-0852 (anti-IIb/IIIa). Results are expressed as mean ± SEM (n=7) vs. EDTA. Repeated two-way ANOVA followed by Bonferroni's post-test. **P<0.01 (10µM MK-0852 vs. saline) using ADP. ****P<0.0001 (10µM MK-0852 vs. saline) using collagen.

The results obtained show that incubation of blood with 10µM MK-0852 caused slight inhibition when blood was stimulated with ADP, as indicated in (B); inhibition was statistically significant in blood stimulated with collagen (P<0.0001). Repeated two-way ANOVA demonstrated that inhibition of blood stimulated with AA (A) and TRAP (D) was not statistically significant. Overall, the results indicate that platelet

aggregation in WB, subjected to the SPC technique and 96-well plates, is not completely inhibited by MK-0852. This result requires further investigation. I anticipated that delays in fixing blood could have resulted in some inhibition, as reflected by the increase in the number of the single platelets. The following experiments were designed to investigate this observation.

4.3.5 Effects of MK-0852 and MK-0852 fixed after a 10 min delay in stimulation

Previous results showed that inhibition of platelets by antagonism with GPIIb/IIIa resulted in partial inhibition. A possible explanation for this observation may be delays in fixing blood treated with MK-0852, as it took place after fixing saline-treated blood. Therefore, the following experiments were performed to investigate the effects of delaying the fixation of blood incubated with MK-0852 after stimulation with a range of concentrations of the agonists: AA (A), ADP (B), collagen (C) and TRAP (D). One set of stimulated blood was fixed soon after shaking and another was fixed after a 10 min delay in stimulation.

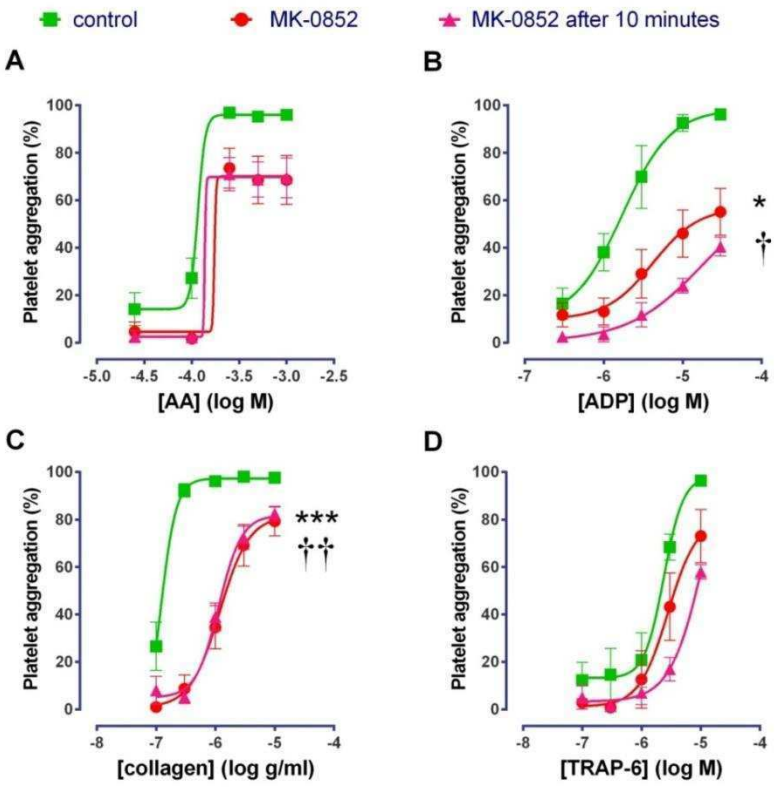

Figure 4-5: Effects of MK-0852 on platelet aggregation fixed at different times.

Platelet aggregation induced by a range of concentrations of arachidonic acid (AA), adenosine diphosphate (ADP), collagen and thrombin receptor activating peptide (TRAP) and the effects of 10µM MK-0852 (anti-IIb/IIIa). Results are expressed as mean ± SEM (n=7) vs. EDTA. *P<0.05 (MK-0852 vs. saline) and † P<0.05 (MK-0852 after 10 minutes vs. saline). Analysed with repeated measures two-way ANOVA followed by Bonferroni's post-test.

The results demonstrate that the pattern of inhibition was similar for blood stimulated with ADP (B) (P<0.05), whether blood was fixed straight after stimulation or after 10 min. The inhibition was shown to be statistically significant (P<0.001 and P<0.01) with MK-0852, and after 10 min, when using collagen vs. saline. Inhibition was not statistically significant with

TRAP (D) and AA (A). Also, differences in inhibition were not statistically significant between WB incubated with 10µM MK-0852 and with fixation 10 min after blood stimulation. These experiments show that a slight delay in fixing the blood treated with MK-0852 after shaking (by 5 min) did not lead to different results from those observed with fixation straight after shaking, confirming that fixation delay is not the problem in these experiments. Alternatively, this could imply that partial inhibition was due to the fact that the fall in single platelets is a result of the binding of platelets to other cells and not to the time delay after stimulation. This observation will be investigated more thoroughly in the next chapter.

4.3.6 Effect of Quorum Sensing Molecules on platelet aggregation

After demonstrating the benefit of the 96-well plate assay in investigating the effect of antiplatelet drugs on platelet function, the following studies were aimed to look at the possible effect of quorum sensing molecules (QSMs), produced by Pseudomonas aeruginosa, on platelet function by measuring platelet aggregation. Molecules, such as 3O-C12-HSL, act through multiple signalling pathways, e.g., Ca^{2+} mobilization, activation of Rho GTPases and mitogen activated protein kinases (MAPK) – all of which control diverse functions in host cells, such as cytoskeleton remodelling, chemotaxis and migration (Williams and Camara, 2009). Chemotaxis and migration toward quorum sensing are paralleled by cytoskeletal F-actin accumulation in the leading edge of neutrophils;

specifically, by increased F-actin to G-actin ratio and via activation of Rho GTPases Rac1 and Cdc42 (Karlsson et al., 2012). Blood was obtained from healthy volunteers, who denied taking antiplatelet drugs, and 3µl of the following QSM (3O-C12-HSL, PQS and Methyl PQS) was added to 3ml of blood. Blood was then stimulated by the following agonists; AA, ADP, collagen and TRAP.

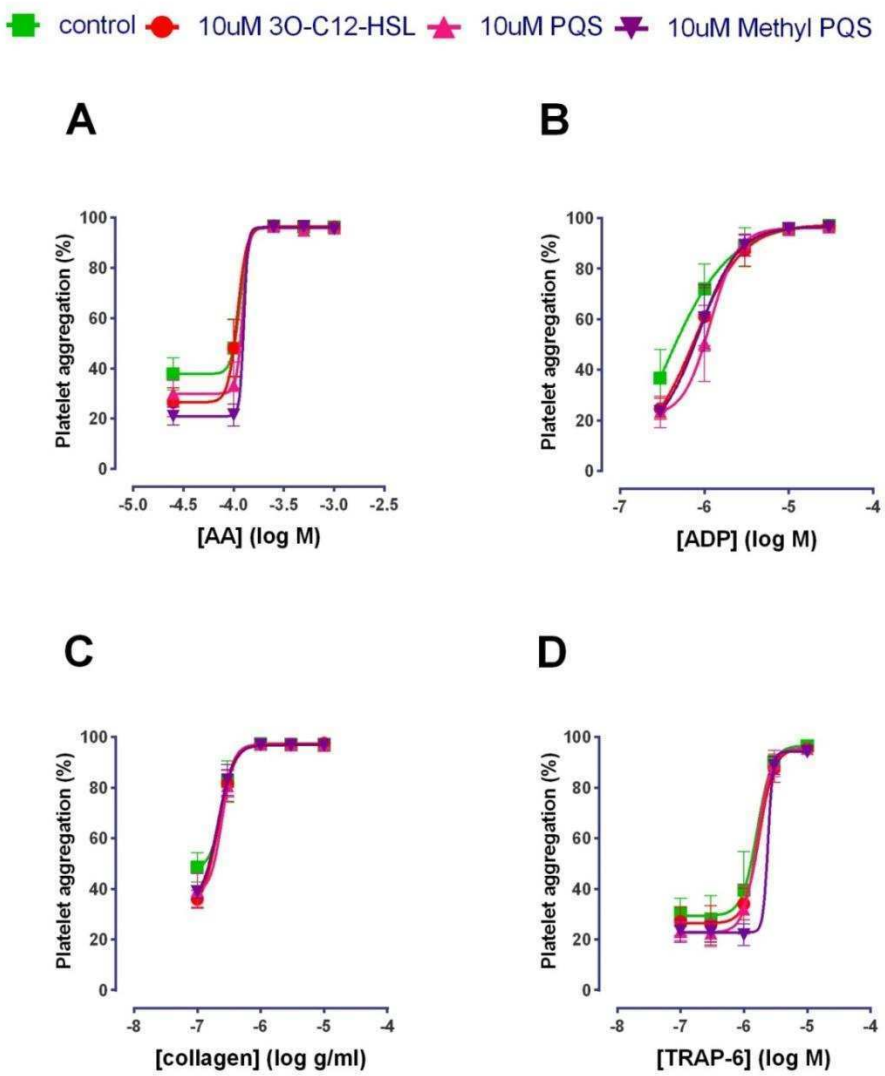

Figure 4-6: Effects of QSM on platelet aggregation.

Platelet aggregation induced by a range of concentrations of arachidonic acid (AA) (0.03-1mM), adenosine diphosphate (ADP) (0.3-30µM), collagen (0.1-10µg/ml) and thrombin receptor activating peptide (TRAP) (0.1 - 10µM) and the effects of 10µM of 3O-C12-HSL, 10µM of PQS and 10µM of Methyl PQS. Results are expressed as mean ± SEM (n=6) vs. EDTA.

As can be seen in Figure 4-6, blood incubated with different QSM had no significant effect on platelet aggregation induced by the different agonists.

4.3.7 Platelet aggregation in patients receiving dual antiplatelet therapy

After demonstrating that the 96-well assay could be used to study the effects of aspirin and cangrelor in vitro, the following set of experiments were conducted to see if this assay could detect platelet inhibition in ACS patients who were on aspirin (75mg) plus either clopidogrel (75mg) or prasugrel (10mg). Blood obtained from ACS patients was transferred to the lab as soon as possible after venipuncture and then stimulated with a range of concentrations of the agonists: AA (A), ADP (B), collagen (C) and TRAP (D).

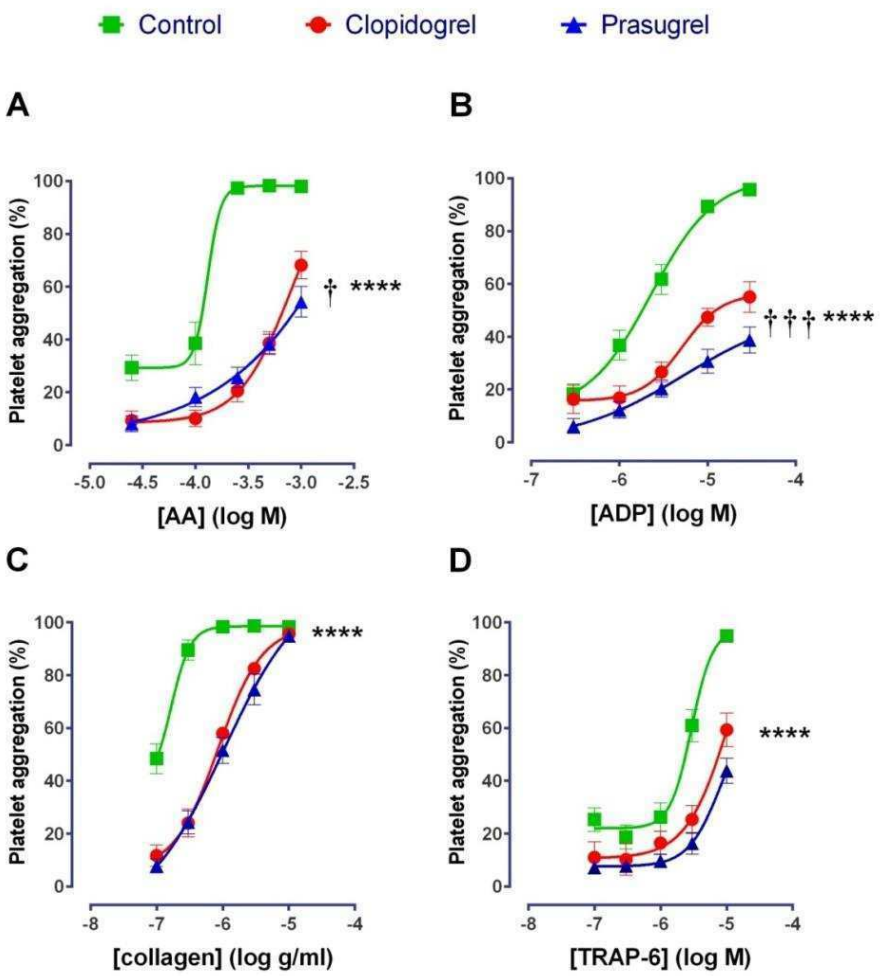

Figure 4-7: Ex vivo inhibition of platelet aggregation using wet agonists.

Platelet aggregation induced with a range of concentrations of arachidonic acid (AA), adenosine diphosphate (ADP), collagen and thrombin receptor activating peptide (TRAP) in patients receiving dual antiplatelet therapy with aspirin plus either clopidogrel (n=14) or prasugrel (n=14). Results are expressed as means ± SEM (n=28) vs. EDTA. *P<0.05 (clopidogrel or prasugrel vs. saline) and † P<0.05 (clopidogrel vs. prasugrel). Analysed with mixed two-way ANOVA.

The results illustrated significant platelet inhibition in blood stimulated with AA, ADP, collagen and TRAP, which was obtained from patients on prasugrel (10mg) or clopidogrel (75mg) vs. saline. Both drugs block the

interaction of ADP with its receptor, $P2Y_{12}$, on platelets and therefore reduce ADP-induced aggregation to a different extent. There was a slight difference in the inhibition observed between blood from patients on clopidogrel and prasugrel ($P<0.05$) in blood stimulated by AA. A greater difference in inhibition was observed in blood stimulated with ADP ($P<0.001$). This indicated that blood from patients on prasugrel exhibited more inhibition than clopidogrel, as seen in blood stimulated by AA and ADP. Inhibition of blood from patients on prasugrel or clopidogrel was not statistically significant with either collagen or TRAP stimulation.

Overall, the results obtained showed that blood from patients on clopidogrel inhibited platelet aggregation to the same extent as prasugrel. However, blood from patients on prasugrel showed a more pronounced inhibition than clopidogrel in blood stimulated with ADP and slight inhibition with AA. In summary, the results obtained show that the novel assay developed could be used to investigate platelet inhibition in patients receiving dual antiplatelet therapy.

We have demonstrated that the 96-well plate assay using wet agonists could be used as a useful tool for studying platelet aggregation in WB and detecting platelet inhibition in vitro and ex vivo. However, there may be some variation between laboratories with respect to preparation of the agonists. The following experiments were performed using Optimul plates

(96-well plates with a lyophilised agonist). This study involved in vitro and ex vivo inhibition of platelets with antiplatelet drugs.

4.3.8 Lyophilised agonists with 96-well plates (Optimul)

This method involved the use of Optimul plates, where all platelet agonists had been prepared previously and were in a lyophilised form. Optimul plates offer more choice of platelet agonists, similarly to wet plates plus epinephrine and ristocetin, which induce agglutination of platelets in the presence of von Willebrand factor and U46619. This also had the advantage of removing the unnecessary step of preparing the agonist, thereby providing a faster and more effective test of platelet function. The protocol for this is exactly the same as for the one used for wet plates, except for agonist preparation.

4.3.8.1 Measurement of platelet aggregation in healthy volunteers

Platelet function was assessed in blood from 20 healthy volunteers who did not take drugs that interfere with platelet function. Blood was shaken with a range of different concentrations of various agonists (AA, ADP, collagen, TRAP, epinephrine, U46619 and ristocetin), which were all in a lyophilised form. The aims of these experiments were to determine the effectiveness of using lyophilised agonist for investigating platelet aggregation and inhibition by antiplatelet drugs in vitro and ex vivo, using flow cytometry.

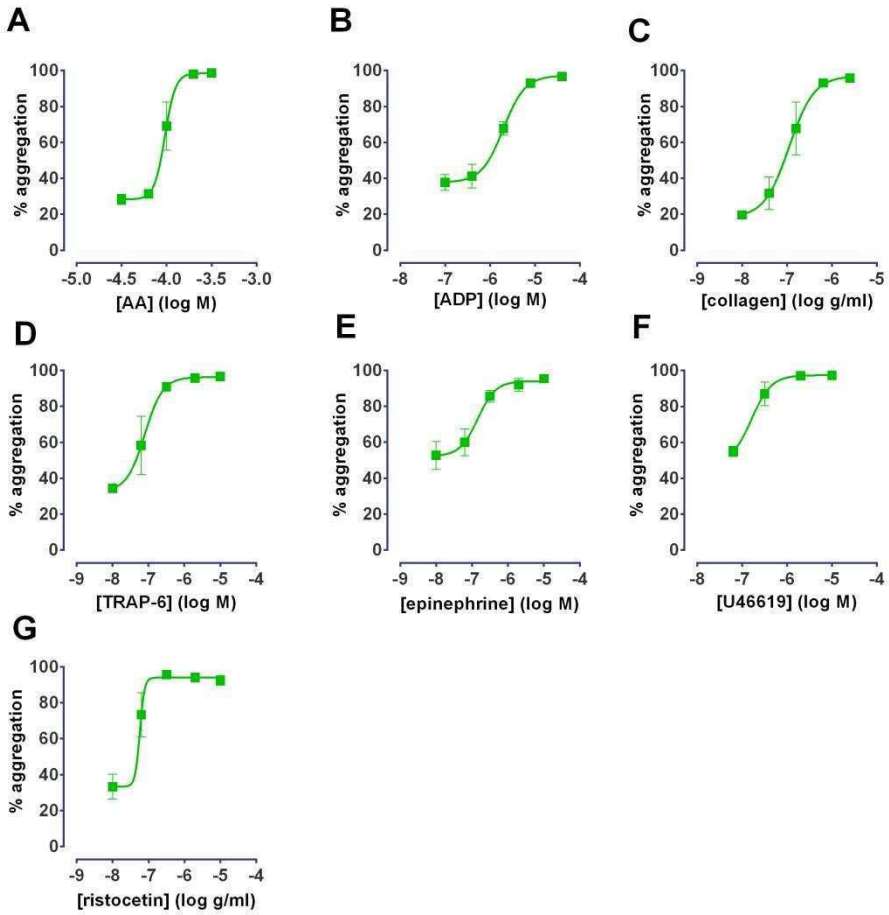

Figure 4-8: Platelet aggregation in healthy volunteers in response to lyophilised agonists.

Effects of arachidonic acid (AA), adenosine diphosphate (ADP), collagen, thrombin receptor activating peptide (TRAP), epinephrine, U46619 and ristocetin on platelet aggregation. Results are expressed as mean ± SEM (n=20) vs. EDTA.

The dose curve analysis showed that the extent of platelet aggregation that occurred depended upon the concentration of the particular agonist used. EC50 was calculated for each donor using GraphPad Prism software, indicating the concentration of agonists achieving 50% of maximum platelet aggregation. Mean EC_{50} ±sem was estimated as follow: AA =0.11

± 0.03 mM (n=20), ADP =1.56 ± 0.08 µM (n=20), collagen =0.33 ± 0.14 µg/ml (n=20), TRAP =0.17± 0.1 µM (n=20), epinephrine =0.01± 0.18 µM (n=20), U46619=0.16± 0.21 µM, (n=20) and ristocetin =53 ± 0.11 ng/ml (n=20).

The same applied here, when a small volume of blood was used for testing. This was similar to 96-well plate aggregation using fresh agonist.

4.3.8.2 In vitro platelet inhibition with antiplatelet drugs

This set of experiments was carried out by the Birmingham Platelet Research group and the aim was to examine the use of lyophilised agents to study platelet inhibition by antiplatelet drugs. The results obtained show that aspirin alone resulted in inhibition of AA- and collagen-induced aggregation. Cangrelor induced a shift in the dose-response to most agonists, in addition to profound inhibition of the ADP response.

4.3.8.3 Effect of antiplatelet drugs in ACS patients

Platelet inhibition by antiplatelet drugs ex vivo was assessed using 96-well plates and lyophilised agonist. This was designed to investigate the usefulness of this assay for detecting platelet inhibition in ACS patients. Blood was obtained from 20 ACS patients, who were receiving aspirin and either clopidogrel (11) or prasugrel (9), and was transferred to the lab as soon as possible after venipuncture. Blood was then stimulated with a

range of concentrations of the following agonists: AA (A), ADP (B), collagen (C), TRAP (D), epinephrine (E) , U46619 (F) and ristocetin (G).

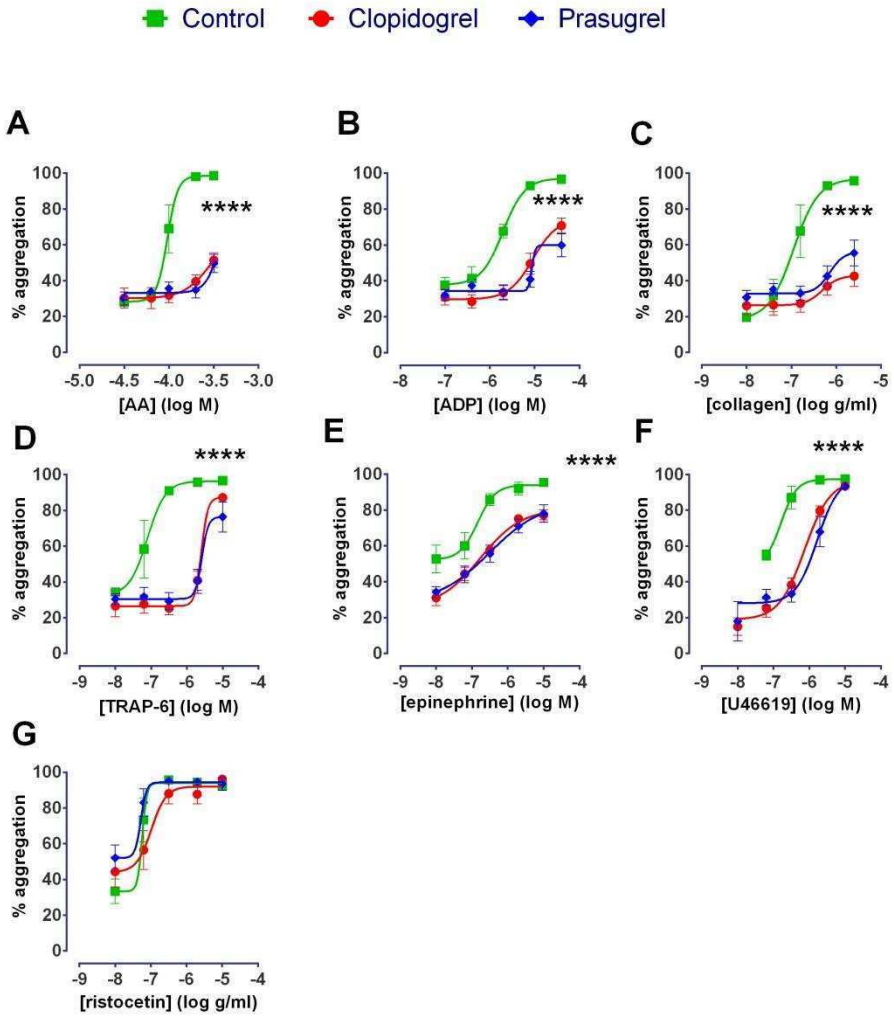

Figure 4-9: Ex vivo inhibition of platelet aggregation using lyophilised agonists.

Platelet aggregation induced by a range of concentrations of arachidonic acid (AA), adenosine diphosphate (ADP), collagen, thrombin receptor activating peptide (TRAP), epinephrine, U46619 and ristocetin in patients receiving dual antiplatelet therapy with aspirin and either clopidogrel (n=11) or prasugrel (n=9). Results are expressed as mean ± SEM (n=20) vs. saline. Results are expressed as mean ± SEM, n=20 vs EDTA. *P<0.05 (clopidogrel or prasugrel vs. normal controls. Analysed with mixed two-way ANOVA.

The results showed that there was large inhibition of platelet aggregation in blood from patients on prasugrel or clopidogrel vs. control, which was stimulated with the agonists AA, TRAP, ADP, U46619, collagen, epinephrine and ristocetin ($P<0.0001$). There was no difference in platelet inhibition in blood from patients on clopidogrel and prasugrel. In summary, this study clearly shows inhibition of platelet aggregation by both $P2Y_{12}$ receptor antagonists, to a similar extent, with different platelet stimulants However, it did not demonstrate the strong inhibition obtained in blood from patients on prasugrel, when using fresh agonists.

4.4 Discussion

We had conducted further optimisation of platelet aggregation in WB, carried out previously, with respect to the fixation approach, the anticoagulants, and type of 96-well plate used. Therefore, this chapter focuses solely on 96-well plates and whether they could be used in further developments involving a 96-well plate format, which would offer the advantage of using less blood volume. After this, combining double fixation with 96-well plates allowed an assessment of platelet aggregation and inhibition by a wide range of agonists and antagonists. To our knowledge, this is the first study that characterises platelet aggregation and inhibition in vitro and ex vivo using a 96-well format and flow cytometry.

In terms of 96-well plate assay development, the aggregation measurements conducted on blood from healthy volunteers in the previous chapter showed that all of the agonists investigated produced a dose-dependent increase in platelet aggregation responses, where it reaches the maximum at the highest concentrations of the agonist used. This reflects that the assay is able to demonstrate different platelet activation pathways, which platelet antagonists can act upon. This is in agreement with other studies, where investigators were able to scan the responses of platelets to different platelet agonists. However, these investigations were carried out on PRP using measurements of light absorbance to measure platelet aggregation (Armstrong et al., 2009b, Peace et al., 2008). This novel assay requires only a small blood volume (46µl) per well and approximately 1.2ml of blood for the whole test, as well as a small volume of agonists.

Clearly, this would be of great benefit in circumstances where the volume of blood that can be taken is very small, as with neonates and infants. Also, it would be very advantageous to perform more than one platelet function test on a single sample, such as platelet aggregation and PLCs formation. The results obtained indicate that this assay could be used further to detect platelet inhibition using antiplatelet drugs in vitro and ex vivo.

The first results focused on the use of aspirin, which blocks the COX enzyme downstream of the AA pathway and, therefore, decreases the

production of TXA_2, thereby reducing aggregation (Burke et al., 2003). The results obtained demonstrate a dose-dependent inhibition of AA-induced platelet aggregation at concentrations below 0.5mM. Higher concentrations of AA appeared to overcome the inhibition, irrespective of the aspirin concentration used. A possible explanation could be that high concentrations of AA may lead to increases in the activity of PLA_2, which can cause RBC lysis, resulting in the release of ADP (Stoykova et al., 2013). RBCs are regarded as a source of ADP, which causes platelets to aggregate, and, therefore, may account for the minute inhibition observed at high AA concentrations (Hollopeter et al., 2001). In these experiments, involving AA-induced platelet aggregation, RBC haemolysis was visualised macroscopically on the 96-well plate's well, indicating the occurrence of haemolysis with 0.5 and 1mM AA. Another study (Greenberg et al., 2000) showed variability in platelet aggregation in response to stimulation by 1mM AA and that the inhibition of platelet aggregation by low doses of aspirin can be overcome with high AA concentrations, making the selection of the concentration of this agonist critical in clinical studies.

Assessing platelet function in WB needs to be carried out with caution; many factors could be implicated in the findings, such as the use of WB and the mixing forces that rely on the shaking technique. The current guidelines for AA concentrations state between 0.5-1mM in PRP, and

could be even as high as 1.6mM AA, which was shown to be the optimum , as indicated by one study (Burke et al., 2003).

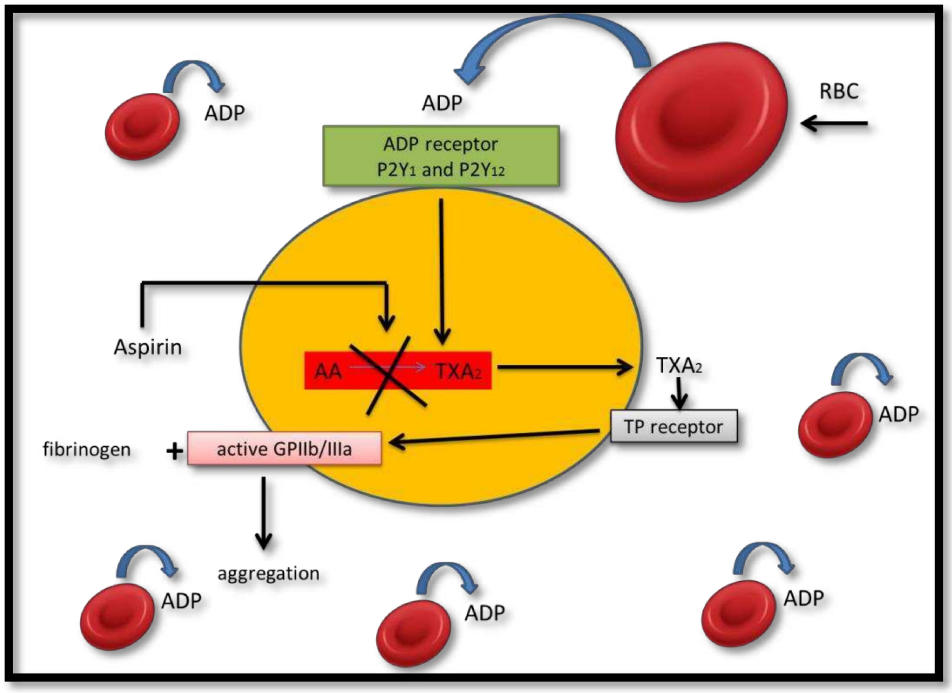

Figure 4-10: Effects of haemolysis on platelet aggregation induced by AA. Schematic representation of the effect of red blood cell (RBC) lysis at arachidonic acid (AA) concentrations >0.5mM on platelet aggregation and inhibition with aspirin. Haemolysed RBCs release adenosine diphosphate (ADP) into the milieu, which interacts with ADP receptors to produce thromboxane A_2 (TXA_2), thereby causing the platelet to aggregate and counteracting the effect of aspirin to inhibit the formation of TXA_2.

Aspirin partially inhibited collagen-induced platelet aggregation, specifically at low concentrations. Aspirin had no effect when ADP or TRAP was used as platelet agonists. Concerning the type of anticoagulants, and inhibition with aspirin, a study by (Storey et al., 2000b)) indicated that

the inhibition of platelet aggregation by aspirin was more prominent in blood that had been anti-coagulated with citrate.

Using the novel assay, cangrelor produced a dose-dependent inhibition of ADP-induced platelet aggregation. This is in line with previous findings (Storey et al., 2000a), which have shown that the inhibitory effect of the selected antagonist was concentration-dependent. In this regard, another $P2Y_{12}$ antagonist, prasugrel, behaves similarly as shown by (Frelinger et al., 2007). Cangrelor also inhibited collagen-induced platelet aggregation at low concentrations, suggesting that secondarily-released ADP is important in collagen-induced platelet aggregation (Herbert et al., 1999). At high collagen concentrations, aggregation becomes ADP-independent, as demonstrated by the inhibition being overcome. Inhibition was dose-dependent when TRAP was used as an agonist and cangrelor had no effect on AA-induced aggregation.

As previously demonstrated, in studying the effect of aspirin on AA-induced platelet aggregation, there is a possibility that ADP could be implicated in overcoming the inhibition, as shown in Figure 4-3. To investigate this finding, a combination of different concentrations of cangrelor with the maximal aspirin concentration (100µM) resulted in an additive inhibition of both ADP- and AA-induced aggregation. This demonstrates the involvement of an endogenous ADP component in WB and makes it critical to evaluate inhibition with high concentrations of AA

agonist. The present study also shows augmentation brought about when aspirin and cangrelor were used concomitantly.

Platelet agonists initiate platelet aggregation through binding to GPCRs, which leads to an inside-out signaling mechanism, thus, resulting in conformational changes in GPIIb/IIIa receptors. In this way, the receptors are made available for fibrinogen binding. Fibrinogen forms bridges between platelets leading to aggregation.

Therefore, in order to block the final aggregation pathway, a blocking agent, MK-0852, which inhibits the GPIIb/IIIa receptor, was used. High concentrations of platelet inhibitors at concentrations of 10µM were used to obtain a given inhibition of platelet aggregation (Nurden, 1997). This was needed because potent platelet agonists, such as collagen and thrombin, are known to increase the surface expression of GPIIb/IIIa receptors by up to 50%, requiring high concentrations of inhibitors (Wagner et al., 1996). The results obtained from this experiment, as shown in Figure 4-4, show incomplete inhibition using the novel assay utilising SPC. SPC was more sensitive, detecting inhibition at low concentrations of collagen (<1µg/ml). However, at high collagen concentrations, inhibition tended to be less. On the contrary, when using the Multiplate, which relies on MEA technology, GPIIb/IIIa receptor antagonists had significant inhibitory effects at higher collagen concentrations of 2.5µg/ml. This may be due to the fact that SPC techniques measure the enrolment of platelets into aggregates (micro-aggregates), which may be as small as two platelets,

while MEA is reliant on the firm attachment of platelets to a metal sensor (macro-aggregate) (Toth et al., 2006a). Our findings could be explained as follows: the blockade of the GPIIb/IIIa receptor can result in the production of extra free single platelets that can go through other routes by binding to other cells, such as leucocytes, causing a false reduction in the number of single platelets, which is detected as more aggregation or as incomplete inhibition. In other words, platelet interaction with, for example, leucocytes leads to a fall in the single platelet count, which gives rise to a low platelet count – seen as high platelet aggregation when assessed with the SPC technique. This agrees with previous work conducted by our research group (unpublished data), where a similar pattern of incomplete inhibition using the SPC technique was shown. However, when MEA was compared to the standard SPC technique for measuring platelet aggregation in WB, it reflected good inhibition, indicating that MEA is more sensitive for detecting macro-aggregates than micro-aggregates. However, mixing forces relied on stirring rather than shaking. (Mascelli et al., 1997) demonstrated greater sensitivity to platelet inhibition by GPIIb/IIIa antagonism, similarly to our findings.

Using SPC in PRP, the antagonism of GPIIb/IIIa receptors results in more effective inhibition of platelet aggregation compared to whole blood (Storey et al., 1998). This is due to the fact that PRP has no leucocytes available for the activated single platelets to interact with. An increase in active platelets, as demonstrated by CD62P markers, has been reported in vitro and ex vivo following the inhibition of GPIIb/IIIa receptors

(Klinkhardt et al., 2000, Matzdorff et al., 2000, Schneider et al., 2000). This incomplete inhibition will be investigated further in the following chapter.

A combination of drugs, DAPT, that interfere with more than one activation pathway, such as aspirin and $P2Y_{12}$ inhibitors, is effective in a clinical settings for inhibiting platelet aggregation in ACS patients (Hamm et al., 2012). Following our demonstration of in vitro inhibition of platelet aggregation using this novel assay, the next set of experiments were aimed at investigating the suitability of this assay for detecting inhibition of platelet aggregation in ACS patients. This assay showed inhibition of platelet aggregation in these patients, when compared to drug-free control, with all agonists used. However, prasugrel produced more inhibition than clopidogrel in ADP- and AA-induced platelet aggregation. Several studies have shown that prasugrel provides greater, and more consistent, platelet inhibition than clopidogrel due to earlier and more extensive formation of its active metabolite, thus, indicating that prasugrel is approximately 10-fold more potent than clopidogrel at inhibiting platelet aggregation (Brandt et al., 2007b, Weerakkody et al., 2007).

The new assay based on a 96-well format seems feasible to investigate the effect of new compound on platelet function. Molecules known as QSM, produced by Pseudomonas aeruginosa, were investigated to see if they had any effect on platelet aggregation. Based on the fact that QSM in their effect on neutrophil chemotaxis and increase calcium mobilisation has a

relevant signalling pathway to that occurring in platelets especially that involve tyrosine activation (Holm and Vikstrom, 2014). The results obtained showed no significant effect of these molecules on platelet aggregation.

Although the 96-well approach developed was shown to be superior to the existing method, it still requires platelet stimulants to be freshly prepared. This means that the technique would be limited to specialised laboratories and may show some variation. To address this issue, platelet aggregation and inhibition were investigated in vitro and ex vivo, using lyophilised agonists, by using Optimul plates. This was aimed to investigate the potential to reduce variation, especially in agonist preparation.

Optimul plates were able to detect concentration-dependent platelet aggregation with all agonists studied. The AA dose-response curve for lyophilized vs. wet agonists was approximately similar, as calculated form the mean EC50. Collagen-induced aggregation demonstrated a slightly higher concentration of the lyophilized agonist needed to achieve 50% of maximum aggregation than wet agonist. ADP and TRAP-induced platelet aggregation showed a lower concentration of the lyophilized agonist, achieving 50% of maximum platelet aggregation than wet agonists. Paired-t test analysis of the mean EC50 of the blood stimulated with AA, ADP, and collagen showed no statistical difference between wet vs. lyophilized

agonists. However, blood stimulated with TRAP, showed a statistically significant difference (P=0.0024) difference between them.

The main finding from the ex vivo study was that the pattern of responses seen in blood obtained from patients receiving $P2Y_{12}$ antagonists was similar to that seen in blood obtained from normal volunteers, which had been incubated with these agents in vitro. Also, the effect of antiplatelet agents was seen beyond their pharmacological targets, which indicated shifts in the dose-response curve with the majority of platelet agonists.

The in vitro study with aspirin detected inhibition of platelet aggregation in response to AA and collagen, while cangrelor induced profound inhibition of ADP-induced aggregation. Optimul plates also successfully detected the inhibition of platelet aggregation in ACS patients. This series of experiments implies that the Optimul approach could be useful for assessing antithrombotic drugs for inhibiting platelet aggregation in vitro and ex vivo.

In summary, the development of a SPC technique using a 96-well plate format, which requires a relatively small volume of whole blood, has been assessed. The assay technique uses a more physiological milieu for assessing platelet function through the measurement of platelet aggregation. The assay developed is capable of detecting platelet aggregation and inhibition using a range of agonists and antagonists. Platelet antagonism using MK-0852, which blocks the GPIIb/IIIa receptor, indicated incomplete inhibition, possibly due to the binding of platelets to leucocytes. Further measurements will be conducted using the initial step of the 96-well plate format.

5 Measurement of platelet-leucocyte interactions using a standard protocol with red blood cell lysis

5.1 Introduction

Out of the results presented in the previous chapter, the ones specifically examining the effects of GPIIb/IIIa antagonism will be examined further here. The results showed incomplete inhibition using the SPC technique, which could be due to the fact that, although GPIIb/IIIa receptors were inhibited, platelet activation was not, thus, more active single platelets could then adhere to leucocytes, as reflected by the increase in platelet-leucocyte interactions. This has been studied extensively and demonstrated previously by our Nottingham research group. Our group and others have previously demonstrated that PLCs formation is enhanced when platelets in WB are activated in the presence of MK-0852 and other GPIIb/IIIa antagonists (Sanderson et al., 1998, Scholz et al., 2002, Zhao et al., 2003c). GPIIb/IIIa antagonism partially inhibits platelet aggregation, but does not have an effect on P-selectin.

Platelet activation in injured arteries can result in the formation of a thrombus. Upon vascular injury, sub-endothelium layer interruption leads to exposure of collagen, which in turn results in platelet activation. This can lead to the release of the contents of platelet granules, such as ADP and TXA_2. These chemicals can activate other platelets and induce platelet aggregation. As a result of this, formation of platelet/fibrin deposits obstructs blood vessels and results in various clinical manifestations such as heart attacks, unstable angina and strokes (Heptinstall et al., 2006, Ruggeri, 2002). Also, activated platelets may bind to leucocytes, mainly

monocytes, in vivo to form PLCs. This is achieved by the binding of platelet P-selectin (CD62P) to its receptor on the leucocyte P-selectin glycoprotein ligand-1 (PSGL-1), or via fibrinogen or thrombospondin (Entman and Ballantyne, 1996, Sarma et al., 2002). PSGL-1 is constitutively expressed on the surface of circulating leucocytes (Yip et al., 2013). In this respect, an earlier study in developed mice, deficient in PSGL-1, has been used to approve the concept that P-selectin-PSGL-1 binding is needed for the early attachment of leucocytes to the injured vessel wall (Yang et al., 1999).

Beyond the initial attachment, P-selectin-PSGL-1 is also involved in signal transduction. With respect to monocytes, this binding, for example, can induce tissue factor expression, which initiates the coagulation system in vivo and results in the release of inflammatory mediators, such as tumour necrosis factor-α (TNF-α) and the chemokine monocyte chemotactic protein 1 (MCP-1) (Lindmark et al., 2000, Weyrich et al., 1995, Koike et al., 2000). Stabilisation of PLCs is achieved by numerous mechanisms, comprising the binding of glycoprotein Ibα and leucocytes Mac-1 (CD11b/CD18) (Simon et al., 2000), as shown in Figure 5-1. Cross-talk and binding of platelets to leucocytes have been investigated in various cardiovascular diseases. Also, they have been suggested as potential predictive markers for clinical outcomes (Klinkhardt and Harder, 2005). Circulating PLCs are considered to be a sensitive marker of the interaction that exists between inflammation and thrombosis. This interaction provokes WBC activation with upregulation of CD11b/CD18, cytokine

production, and expression of tissue factors and pro-coagulant activity in monocytes.

It has been recognised that when platelets are in the active state, they also express the CD40 ligand on their surfaces (Henn et al., 1998). The attachment of CD40L to CD40 on monocytes leads to their activation and production of cytokines, including IL-6, which is related to unstable angina and also increases the production of C-reactive protein (CRP) (Bataille and Klein, 1992, Alderson et al., 1993, Biasucci et al., 1996). Taking PSGL-1 into account as an important receptor for the initial attachment of platelets and leucocytes, in a study by (Bournazos et al., 2008), it was detected that, in the absence of apparent activation of PSGL-l, P-selectin binding of platelets to monocytes represents a normal physiological process, which has a minor influence on the possibility of monocytes to cause vascular injury.

Measurements of platelet-leucocyte conjugates are believed to be a more sensitive marker of platelet activation than the detection of surface P-selectin on single platelets. This is due to the fact that degranulated platelets promptly lose surface P-selectin in vivo but continue to circulate (Michelson et al., 1996a). It has been shown that PM conjugates remain detectable in the peripheral blood for a considerably longer time (Michelson et al., 2001). Binding of unstimulated platelets has no effect on receptor expression and chemokine production. However, binding of active

platelets does promote the inflammatory response. It has been determined that increased levels of P-selectin on the surface of active platelets, or the binding of various platelets to a monocyte, is required to initiate monocyte activation via PSGL-1. In this respect, it has been shown that inhibiting platelet adhesion through P-selectin decreases inflammation. The extent of platelet monocyte activation is highly dependent on platelet activation (Furman et al., 1998) and to a lesser extent on monocyte activation (Li et al., 2000). Based on this assumption, GPIIb/IIIa antagonism increases agonist-induced PLCs by preventing platelet aggregation, thus, leading to more activated platelets being available to adhere to (Zhao et al., 2003c). In addition to this, there is also the probability that MK-0852 may participate in platelet activation through outside–in signalling at the level of GPIIb/IIIa receptors (Scarborough et al., 1999). Formed PLCs were more frequently monocytes than neutrophils. In this regard, it has been indicated that monocytes were more active than neutrophils (80% of monocytes compared to half that proportion of neutrophils) in forming conjugates (Xiao and Theroux, 2004).

As indicated in the previous chapters, measurement of platelet aggregation is influenced by pre-analytical variables. With respect to the measurement of PLCs, technical and methodological considerations must be taken into account. Different methods have been used for the measurement of PLCs formation utilising flow cytometry. The high sensitivity of the assay makes it highly vulnerable to artefactual activation in vitro. There are many factors, which have been studied and revealed to have a possible effect on

measurements, such as type of anticoagulant and the blood sample collection and processing technique (Li et al., 1997, Michelson et al., 2000, Barnard et al., 2003). EDTA anticoagulation significantly decreases platelet-leucocyte interactions in vitro by cation chelation. On the other hand, unfractionated heparin activates platelets and increases PLC formation by a P-selectin-dependent mechanism (Sarma et al., 2002). PPACK, a direct thrombin inhibitor, does not cause cation chelation and has been established to be reliable for the measurement of PLCs formation (Harding et al., 2007). Anticoagulants based on CTAD (0.109M buffered sodium citrate, or sodium citrate 0.106M, 15mM theophylline, 3.7mM adenosine and 0.198mM dipyridamole) prevent platelet activation following venipuncture (Pearson et al., 2009).

It is important to process samples for PLCs measurements as soon as possible, as (Harding et al., 2007) demonstrated that PLCs increase in a time-dependent fashion, irrespective of the type of the anticoagulant used. The anticoagulant used for PLCs measurements in this thesis is based on citrate. It has been shown that the rate of increase in PLCs appears slow, and this is very advantageous in a situation where there is a possible delay in sample fixation and staining with antibodies. They have also indicated that if the samples are kept at 4^{o}C, after fixation and immune-staining, they remain stable for 24 hrs. In this respect, a fixation process based on AggFixA and AggFixB solutions, by Platelet Solution, has been used to fix blood for PLCs measurements, as it has been shown to allow analysis for up to 3 days. The method of blood collection may also have an effect on

PLCs measurement. Samples obtained by intravenous cannula increase PLCs with time; however, they do not change with time when samples are collected by venipuncture.

To study PLCs formation, many techniques have been described (Li et al., 1997, Hagberg and Lyberg, 2000b, Barnard et al., 2003) and, in this chapter, the standard protocol involving RBC lysis will be considered. There are different views regarding RBC lysis performed during sample processing. The aforementioned study (Harding et al., 2007) indicated that RBC lysis allows effective and accurate discrimination of leucocyte subpopulations and, therefore, flow cytometry can be performed easily. In this respect, performing RBC lysis along with immediate fixation does not result in an increase in PLCs and is less time consuming to perform. (Li et al., 1997) have, however, suggested that the practise of RBC lysis may lead to artificial increases in platelet-leucocyte aggregation, although in the presence of erythrocytes, analysis of PLC formation by flow cytometry becomes difficult and cumbersome.

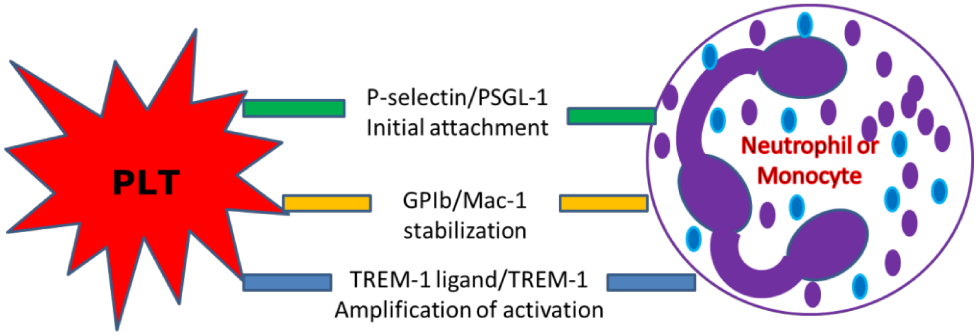

Figure 5-1: Receptor-counter receptor pairs involved in platelet-neutrophil and monocyte interactions. TREM, triggering receptor expressed on myeloid cells. GPIb, glycoprotein Ib. PLT, platelet. PSGL, P-selectin glycoprotein ligand 1. Mac-1, macrophage-1 antigen.

Adapted from (Michelson and Newburger, 2007) .

The aim of the study described in this chapter was to evaluate 96-well plates as tools for investigation of PLCs formation, which utilising a shaking technique rather than the usual stirring method. This will add another application to 96-well plate use and fixative solutions. Also, the use of different antibody labelling to characterise PLCs, and study the in vitro effect of MK-0852 and the ex-vivo effect of antiplatelet drugs, were tested. PLCs formation was performed using the standard lysing protocol with RBC lysis (Zhao et al., 2003c, Sarma et al., 2002, Barnard et al., 2003, Harding et al., 2007).

5.2 Methods

Blood was obtained from healthy volunteers, who reported that they had not taken antiplatelet drugs in the past two weeks. The blood was placed in polystyrene tube containing tri-sodium citrate (3.2%), as an anticoagulant, in the presence of saline (control) and antiplatelet drugs, such as MK-0852 (10µM). An agent, KPL-1 (5µg/ml), was also used to block leukocyte PSGL-1 (Snapp et al., 1998). Blood was incubated with the drug of interest at 37^{o}C for 25-30 min and then transferred into 96-well plates with platelet agonists at maximal concentrations: ADP (30µM), collagen (10µg/ml) and TRAP (10µM). Blood was shaken for 10 min (set up for 37^{o}C and 1000 rpm), as this time is indicated to be optimal for conjugate formation and, thereafter, there is a tendency for the number of conjugates to decrease (Redlich et al., 1997). Then blood was fixed using AggFixA solution and left for 15 min. Fixed blood was then transferred into FACS tubes and stabilised with AggFixB. Then, the lysing approach was performed on samples, as explained in the methods section 2.2.5.2. Results are presented as mean ± SD. *P<0.05 was considered to be statistically significant. Data was analysed with paired t-tests or one way–ANOVA repeated measures, as indicated in the legend of each figure.

5.2.1 In vitro

Blood was taken from healthy volunteers, who refrained from taking any drugs known to interfere with platelets, two weeks prior to analysis.

5.2.2 Ex vivo

Blood was obtained from ACS patients in the ACS clinic of the Division of Cardiovascular Medicine in Nottingham. These patients were on aspirin and either clopidogrel or prasugrel. Samples were immediately transferred to the lab for PLCs measurements.

Flow cytometry

PLCs formation was measured using a flow cytometer (LSR-II) equipped with a 5W laser, operating at 15mW power and a wavelength of 488nM. To determine PLCs, a total of 10000 leucocyte events were collected using a combination of forward scatter (reflection of cell size) and side scatter (reflection of cell granularity). Subsets of leucocytes were differentiated from one another on the basis of their differential light scattering properties and CD14-positivity.

5.3 Results

PLCs were presented as 1) the percentage of neutrophils (%PN) or monocytes (%PM), which were positive for platelet markers (CD62P, CD42a or CD61), thus, reflecting the number of leucocytes with platelets bound to them or 2) the median (CD62P-PE, CD42a-FITC or CD61-PE) fluorescence of the whole monocyte (M-MF) and neutrophil (N-MF) population.

5.3.1 Uses of CD62P-PE as a platelet identifier to study the effect of MK-0852 on platelet–leucocyte conjugate formation

These experiments were designed to study the effects of MK-0852 (10µM) as a fibrinogen receptor antagonist on platelet leucocyte (neutrophil and

monocyte) conjugate formation (PLCs; PN; PM). CD62P was used in these experiments as an identifier of the platelets forming the conjugate. P-selectin (CD62P), is a component of the α-granule membrane of resting platelets, expressed only on platelet surfaces during or after platelet degranulation and secretion (Woollard, 2005). In this respect, P-selectin is considered to be the gold standard marker of platelet activation (Michelson, 1996, Ault et al., 1999).

5.3.1.1 Neutrophils median fluorescence and percentage platelet-positive neutrophils

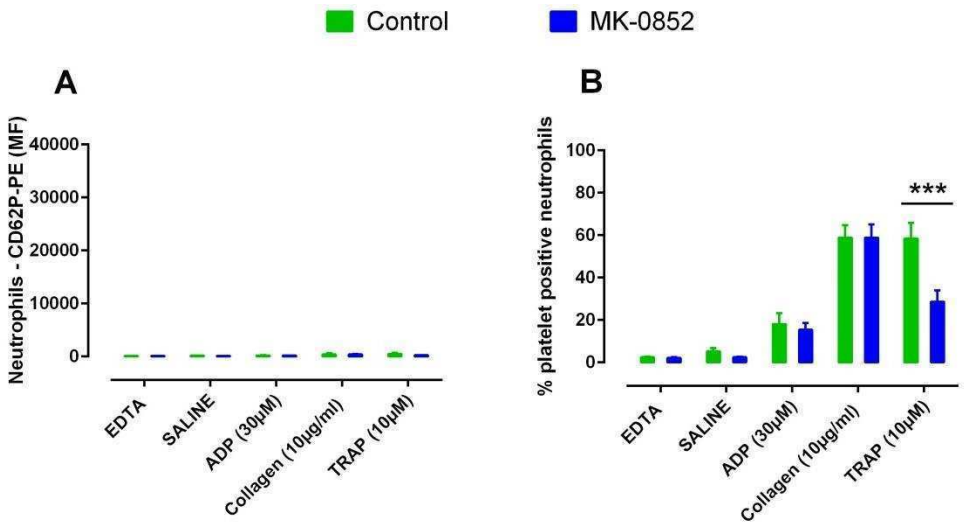

Figure 5-2: Detection of platelet-leucocyte conjugates (neutrophils) formation using CD62P-PE.

Effects of MK-0852 (10µM) on platelet neutrophil conjugate (PLCs) formation based on neutrophil median fluorescence (N-MF) (A) and platelet-positive neutrophils (%PN) (B) using CD62P-PE. Results are expressed as mean ± SD (n=6). *** P< 0.001 (MK-0852 vs. control) of (%PN) using TRAP.

Figure 5-2 demonstrates that the median fluorescence of CD62P-PE was not enhanced by antagonism with MK-0852 and reflected by low MF values with different blood stimulation. %PN was high, but demonstrated no potentiation of conjugate formation in blood incubated with MK-0852. However, blood stimulated with TRAP and incubated with MK-0852 revealed a significant inhibition of %PN formed (P<0.001).

5.3.1.2 Monocyte median fluorescence (M-MF) and percentage platelet positive monocytes (%P/M):

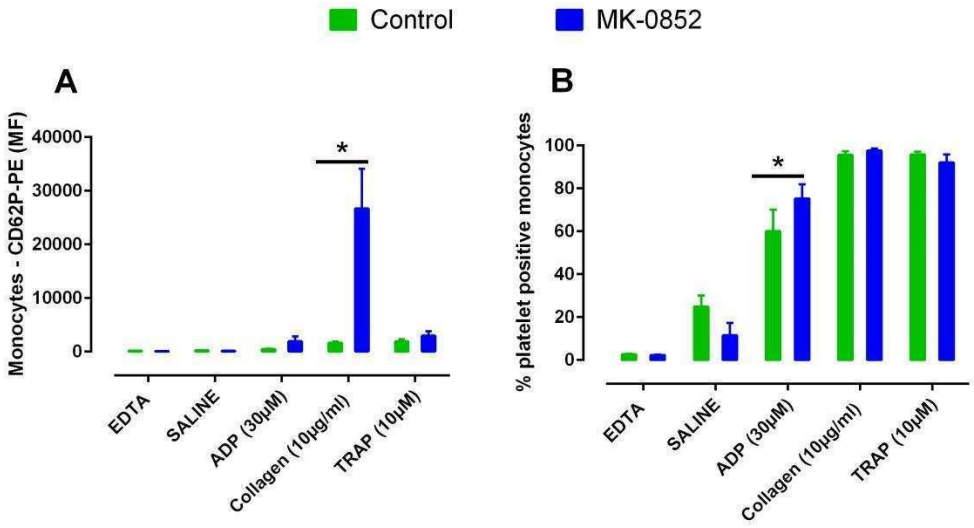

Figure 5-3: Detection of platelet-leukocyte conjugate formation (monocytes) formation using CD62P-PE.

Effects of MK-0852 (10µM) on platelet-monocytes (PM) conjugate formation based on monocyte median fluorescence (M-MF) (A) and %PM (B) using CD62P-PE. Results are expressed as mean ± SD (n=6). * P< 0.05 MK-0852 vs. control analysed with paired t-test. * P< 0.05 (control vs. MK-0852) of monocyte MF using collagen and %P/M using ADP.

Platelets stimulated with different agonist in control samples demonstrated a low M-MF value. However, the sample incubated with MK-0852 and stimulated with collagen, has shown a distinct increase in the amount of PM conjugates formed and presented as MF. %PM was slightly enhanced following shaking of blood with collagen stimulation. This enhancement was higher in blood incubated with MK-0852 and stimulated only with ADP. Again, blood stimulated with TRAP and incubated with MK-0852 was inhibited, although to a lesser extent than neutrophils.

Overall, it can be seen from these experiments that PM conjugate formation is more extensive than PN conjugate formation, with the latter more readily inhibited by MK-0852 antagonism, especially in blood stimulated with TRAP.

5.3.2 Uses of CD42a-FITC as a platelet identifier to study the effect of MK-0852 and KPL-1 on platelet–leucocyte conjugate formation

These experiments were performed to determine the suitability of using the CD42a platelet marker and the effects of MK-0852, as a fibrinogen receptor antagonist, on PLCs formation (PN and PM). Also, in order to evaluate the contribution of PSGL-1 to PLCs formation, and the effect of KPL-1, a mouse anti-PSGL-1 monoclonal antibody was used, which blocks the PSGL-1 receptor, thereby inhibiting the interaction of P-selection (expressed upon the activation of platelets) with its counter receptor on

leucocytes. MK-0852 and KPL-1 were incubated with blood for 30 min to allow the interaction of these drugs with both receptors; this has been established to be the optimum time to block receptors (Snapp et al., 1998).

5.3.2.1 Neutrophil median fluorescence and percentage platelet-positive neutrophils

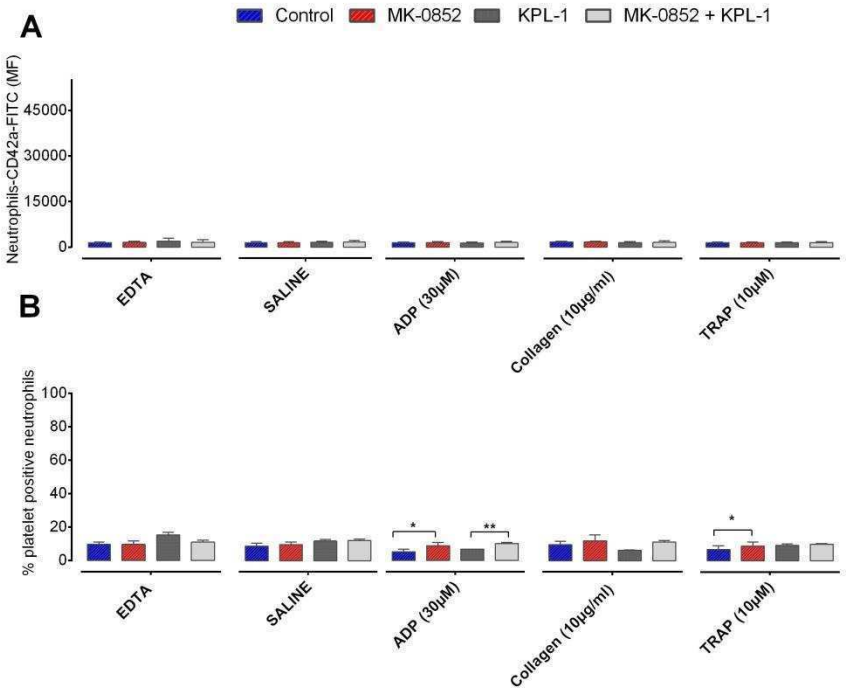

Figure 5-4: Detection of platelet-leucocyte conjugate (neutrophils) formation using CD42a-FITC.

Effects of MK-0852 (10µM) and KPL-1 (5µg/ml) on platelet neutrophil conjugate (PLCs) formation based on neutrophil median fluorescence (N-MF) (A) and percentage platelet-positive neutrophils (%PN) (B) using CD42a-FITC. Results are expressed as mean ± SD (n=6). * P< 0.05, analysed by one-way ANOVA repeated measures.

All of the results obtained showed low N-MF intensity and %PN in blood stimulated with ADP, collagen and TRAP and incubated with saline and MK-0852 ± KPL-1. There was no potentiation of conjugate formation upon treatment with MK-0852. There was no difference between samples treated with saline and MK-0852 and with the addition of KPL-1.

The number of neutrophils with bound platelets were few, with slight increases observed in blood stimulated with ADP and TRAP, which was shown to be statistically significant ($P<0.05$). There was also a subtle increase in blood stimulated with collagen; however, ANOVA showed that this was not statistically significant. A reduction in the percentage of PN was observed in blood incubated with KPL-1 and stimulated with ADP and collagen, but not with TRAP. Samples incubated with MK-0852 plus KPL-1, and stimulated with ADP, showed an increase in conjugates compared to samples incubated only with KPL-1.

5.3.2.2 Monocyte median fluorescence and percentage platelet positive-monocytes

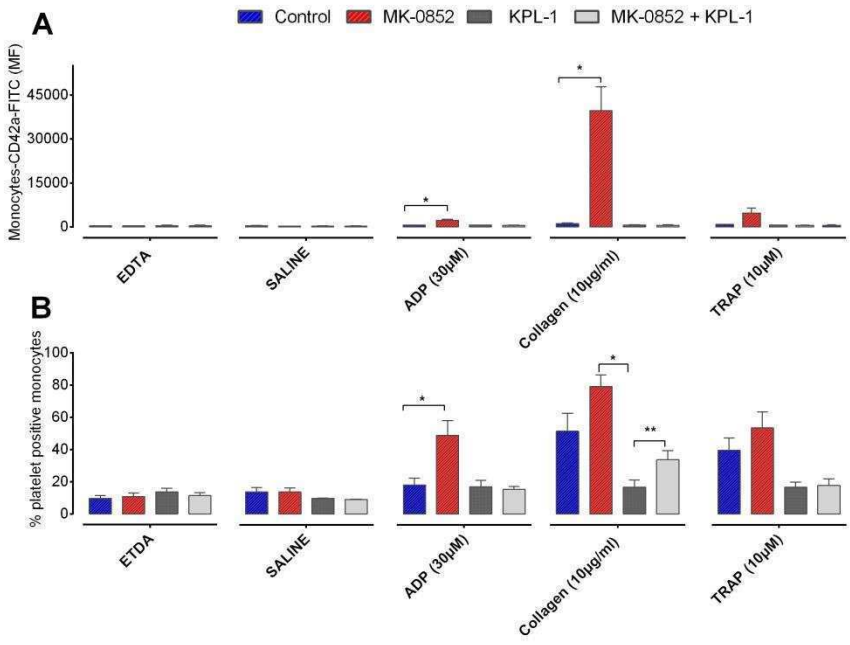

Figure 5-5: Detection of platelet-leucocyte conjugate (monocytes) formation using CD42a-FITC.

Effects of MK-0852 (10µM) and KPL-1 (5µg/ml) on platelet monocyte conjugate (PLCs) formation based on monocyte median fluorescence (M-MF) (A) and percentage platelet-positive monocytes (%PM) (B) using CD42a-FITC. Results are expressed as mean ± SD (n=6). * P< 0.05 analysed by one-way ANOVA repeated measures.

As expected, with respect to monocytes, EDTA showed a low M-MF value due to the fact that it chelates the Ca^{2+} required to form platelet-leucocyte aggregates. The same pattern was demonstrated with saline.

Blood incubated with MK-0852 and stimulated with ADP and TRAP showed a slight increase in the median fluorescence intensity. A dramatic increase in the median florescence was exhibited with collagen. Blood incubated with KPL-1 decreased the median fluorescence to control levels. Addition of MK-0852 + KPL-1 showed similar values to those seen with KPL-1 alone.

Different blood stimulation produced an increase in %PM in blood incubated with MK-0852. Addition of KPL-1 abrogated the %PM, irrespective of the agonist used. This scenario was the same with blood incubated with MK-0852 plus KPL-1, and stimulated with ADP and TRAP, but not collagen. Collagen produced an increase in the conjugates formed, which was shown to be significantly different ($P<0.01$) from KPL-1 alone.

To summarise, M-MF using CD42a antibody to identify platelets show similar results to the previous antibody used (CD62P), with respect to MF. %PM increased when MK-0852 was present in samples stimulated with ADP, TRAP and collagen – prominent in producing conjugate formation. Addition of KPL-1 led to a reduction in the %PM in blood stimulated by different agonists.

5.3.3 Uses of CD61-PE as a platelet identifier to study the effect of MK-0852 and KPL-1 on platelet–leucocyte conjugate formation

After demonstrating the use of different markers, such as CD62P and CD42a, as platelet identifiers and studying their usefulness for investigating PLCs formation, the experiments described here were aimed at using CD61 as the platelet identifier, in order to select the most appropriate marker for further studies. CD61 comprises the IIIa component of the GPIIb/IIIa receptor (Konopka et al., 2009). The CD61 antigen is constitutively expressed on platelet membranes that associate with CD41 (IIb) to form the heterodimeric complex, GPIIb/IIIa (CD41/CD61), which is expressed predominantly in platelets and megakaryocytes. The samples were incubated and stimulated in a similar manner as in previous experiments, with the intention of observing increases in conjugate formation with MK-0852 and blockade of PSGL-1 by KPL-1, in order to confirm the binding of platelets to leucocytes via PSGL-1.

5.3.3.1 Neutrophil median fluorescence and percentage platelet-positive neutrophils

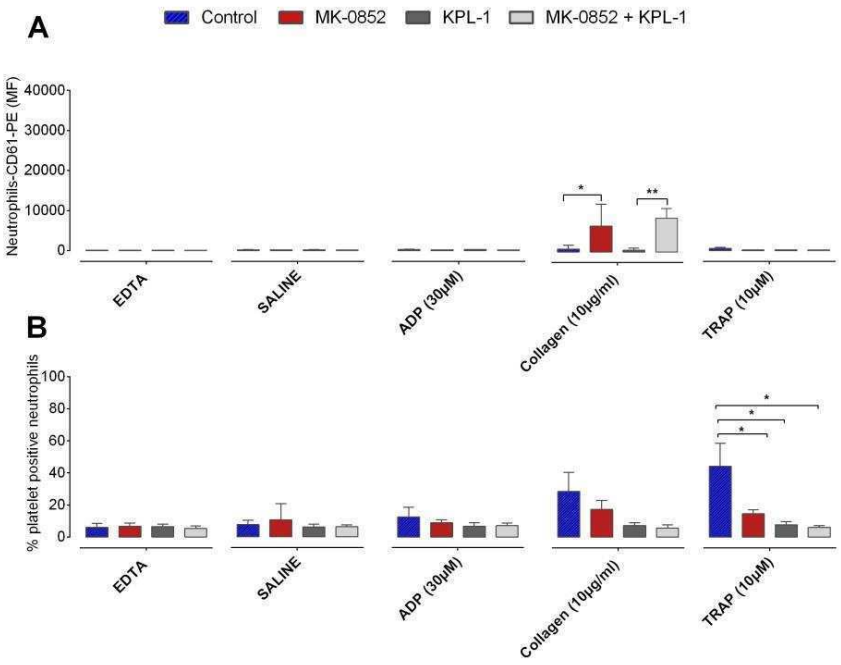

Figure 5-6: Detection of platelet-leucocyte conjugate (neutrophil) formation using CD61-PE.

Effects of MK-0852 (10µM) and KPL-1 (5µg/ml) on platelet neutrophil conjugate (PLCs) formation based on neutrophil median fluorescence (N-MF) (A) and percentage platelet-positive neutrophils (%PN) (B) using CD61-PE. Results are expressed as mean ± SD (n=6). * P< 0.05 analysed by one-way ANOVA repeated measures. * P<0.05 of blood incubated with MK-0852, KPL-1 and MK-0852+KPL-1 vs. controls in blood stimulated with TRAP.

In these experiments, the incubation of blood with MK-0852 did not result in an increase in the median intensity of fluorescence and demonstrated very low values, except with collagen. KPL-1 inhibited the conjugate formed by the different stimulants used. Interestingly, when collagen was

used, MK-0852 plus KPL-1 exhibited a similar pattern to that seen with MK-0852. %PN did not increase in conjugates formed in blood treated with MK-0852; however, the results indicated a decrease in %PN with blood stimulated with ADP and collagen (ns) and TRAP ($P<0.05$). This was even less with KPL-1 and MK-0852 in blood stimulated with collagen and TRAP.

5.3.3.2 Monocyte median fluorescence and percentage platelet-positive monocytes

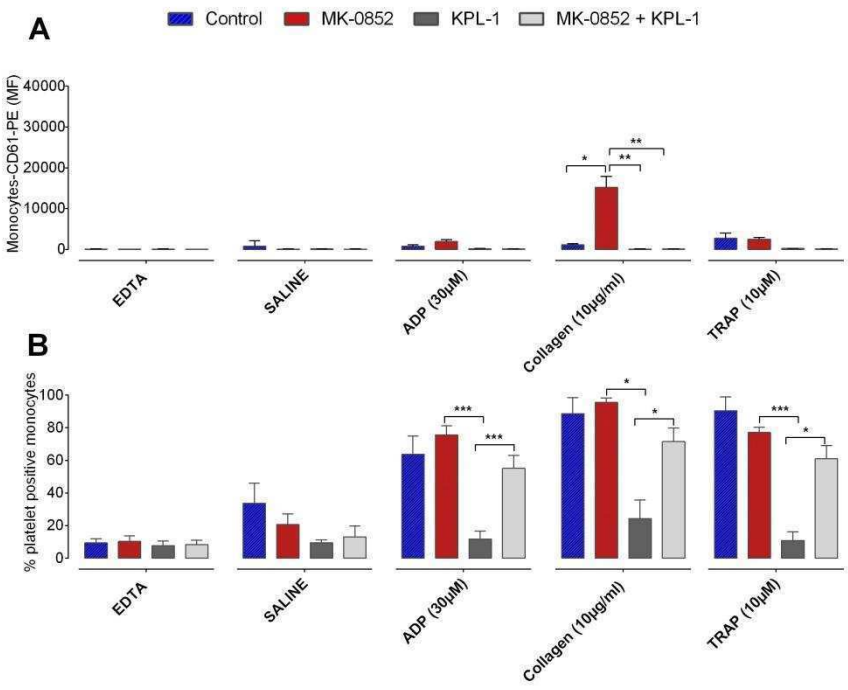

Figure 5-7: Detection of PLCs (monocyte) formation using CD61-PE.

Effects of MK-0852 (10µM) and KPL-1 (5µg/ml) on platelet monocyte conjugate formation based on M-MF (A) and %P/M (B) using CD61-PE. Results are expressed as means ± SD, n=6. * $P< 0.05$ analysed by one-way ANOVA repeated measures.

Collagen produced a dramatic increase in M-MF, in blood incubated with MK-0852. This did not occur with blood stimulated with ADP and TRAP as the agonist. In blood treated with KPL-1, M-MF dropped significantly with different blood stimulations. MK-0852+KPL-1 exhibit a similar pattern to results with KPL-1 alone.

Blood with saline treatment (control) showed a higher percentage of conjugates (nearly 70 % with ADP and 90 % with collagen and TRAP). The antagonism of GPIIb/IIIa with MK-852 resulted in a slight increase in the amount of conjugate formed from control, which was not statistically significant. With TRAP, there was slightly less conjugate with MK-0852 from control.

Addition of KPL-1 resulted in a significant reduction in %PM conjugate formation in samples stimulated with ADP, TRAP ($P<0.001$) and collagen ($P<0.05$) – this clearly indicates that platelets stick to leucocytes. Samples incubated with MK-0852 plus KPL-1 in the differential blood stimulations resulted in reverse increases in conjugates formed, thereby supporting the idea that the GPIIb/IIIa receptor is not contributing in a significant way to the formation of circulating PLCs**.**

Overall, PM conjugate formation was much higher than PN. All results with differential blood treatments incubated with EDTA showed a reduction in %PM, which is attributed to the fact that EDTA chelates Ca^{2+},

which is necessary for the formation of platelet conjugates. However, saline induced less conjugation with neutrophils than with monocytes.

For the following experiments, CD61-PE was used as the platelet identifier, investigating the in vitro (not shown) and ex vivo effects of antiplatelet drugs on PLCs formation.

5.3.4 Ex vivo effect of antiplatelet drugs on platelet-leucocyte conjugate formation in ACS patients

The study described here aimed at checking the usefulness of the new assay based on the 96-well format, using the standard protocol involving RBC lysis to look at the ex-vivo inhibition of PLC formation in ACS patients who were on aspirin and either clopidogrel (n=11) or prasugrel (n=9).

5.3.4.1 Neutrophil median fluorescence and percentage platelet-positive neutrophils

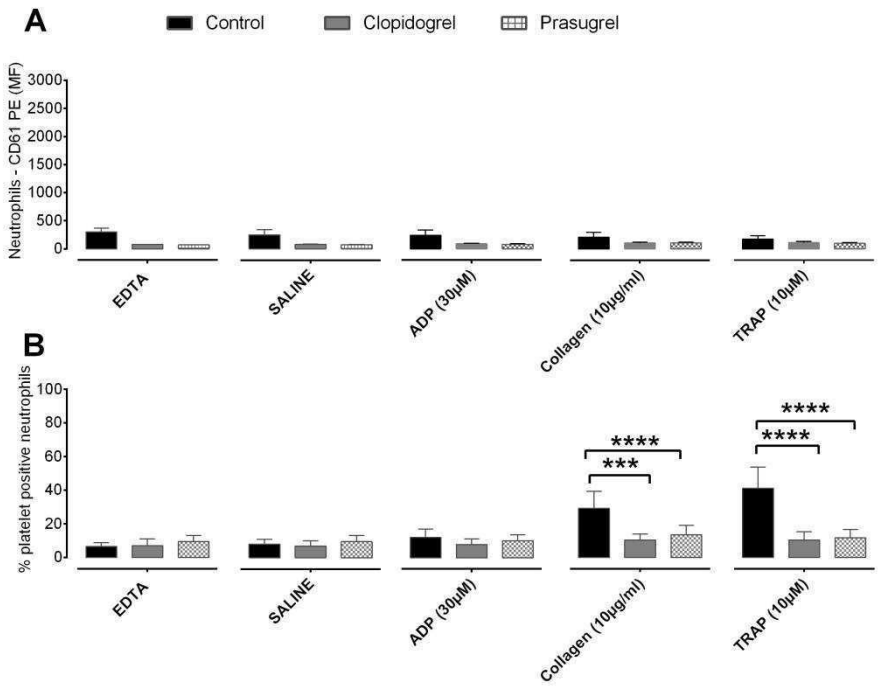

Figure 5-8: Inhibition of platelet-leucocyte conjugate (neutrophils) formation in ACS patients.

Ex-vivo inhibition of platelet-neutrophil (PN) conjugate formation based on neutrophil median fluorescence (N-MF) (A) and %PN (B). Results are expressed as mean ± SD (n=11) clopidogrel and (n=9) prasugrel. * P< 0.05 analysed by simple one-way ANOVA.

N-MF values tend to be small with low reduction of the conjugate formation in both patients group (clopidogrel and prasugrel). %PN indicated that there was no effect on platelet conjugates formed in clopidogrel and prasugrel patients, in blood stimulated with the maximal concentration of ADP. Using collagen as the stimulant, patients who were

on clopidogrel and prasugrel exhibited a significant reduction in PN conjugates. In blood stimulated with TRAP, a similar pattern of inhibition was observed in ACS patients who were on clopidogrel and prasugrel.

5.3.4.2 Monocyte median fluorescence and percentage platelet-positive monocytes

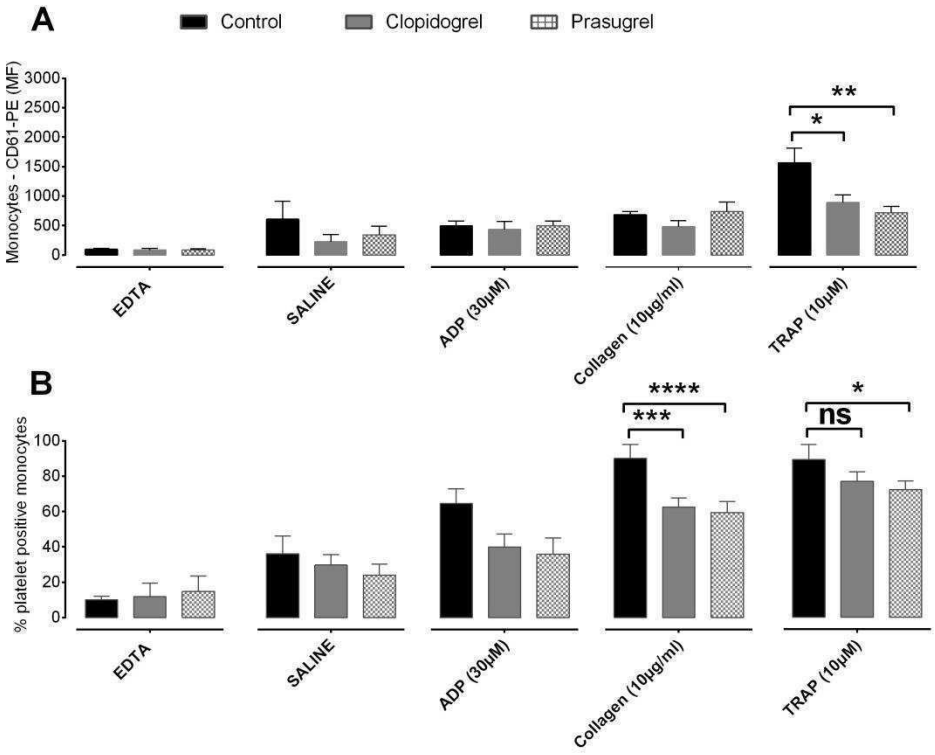

Figure 5-9: Inhibition of platelet-leucocyte conjugate (monocytes) formation in ACS patients.

Ex vivo inhibition of platelet-monocyte (PM) conjugate formation based on monocyte median fluorescence (M-MF) (A) and %PM (B). Results are expressed as mean ± SD, (n=11) clopidogrel and (n=9) prasugrel. * P< 0.05 analysed by simple one-way ANOVA.

With respect to M-MF, a similar level of conjugates were obtained in the control samples and stimulated with ADP and collagen. Platelets

stimulated with TRAP have shown the greatest amount of conjugate. Patients on clopidogrel, and stimulated with TRAP, demonstrated less conjugate, compared to control, which was even less in blood from patients on prasugrel.

%PM in blood stimulated with ADP shows a reduction in conjugates to approximately 40% in both patient categories. Blood stimulation with collagen resulted in a significant reduction in conjugates formed in patients who were on clopidogrel and prasugrel. Both reductions were shown to be statistically significant ($P<0.001$ and $P<0.0001$). When blood was stimulated with TRAP, conjugates were also greater and similar to those observed with collagen; however, less reduction was observed in clopidogrel patients and prasugrel patients, which was not significantly different in both groups.

From the results of the experiments described here, it can be seen that the level of monocyte conjugates is higher than that of neutrophil conjugates. In ACS patients, the extent of the reduction in the conjugate formed with neutrophils or monocytes was shown to be similar in both groups. However, statistical analysis reveals more inhibition in blood from prasugrel- than in clopidogrel-treated patients.

5.3.5 Platelet-leucocyte conjugate formation using the Amnis Imaging Flow Cytometer

From the experiments performed previously, PLC formation, specifically with monocytes, is high – particularly in blood stimulated with collagen, with slight increases upon treatment with MK-0852. State of the art technology, such as the Amnis Imaging Flow Cytometer, is available. The aim of the experiments performed in this section, therefore, was to investigate platelet binding to human monocytes using the Amnis Flow Cytometer so as to visualise platelet binding. In these experiments, samples were incubated with platelet idenfying markers in order to identify postive platelets (CD61-PE-positive platelets) and markers to identify monocytes (CD14-positive monocytes).

5.3.5.1 Separation of single cells from the debris and multicellular aggregates

Single cells were separated from debris and multicellular aggregates in the bright field channel, using the IDEAS features aspect ratio and area. The aspect ratio is the minor axis divided by the major axis and, thus, a measure of an image's roundness (a perfect circle will have an aspect ratio of 1; doublets typically have aspect ratios of around 0.5). The area is measured by counting the number of pixels in the image, which are converted to square microns. Typically, multicellular aggregates have areas three or more times the mean area of single cells. Cell debris will have a small area and a range of aspect ratios, depending upon the shape of the debris. Figure

5-10 demonstrates the separation and discrimination of individual cells from debris and multiple aggregates.

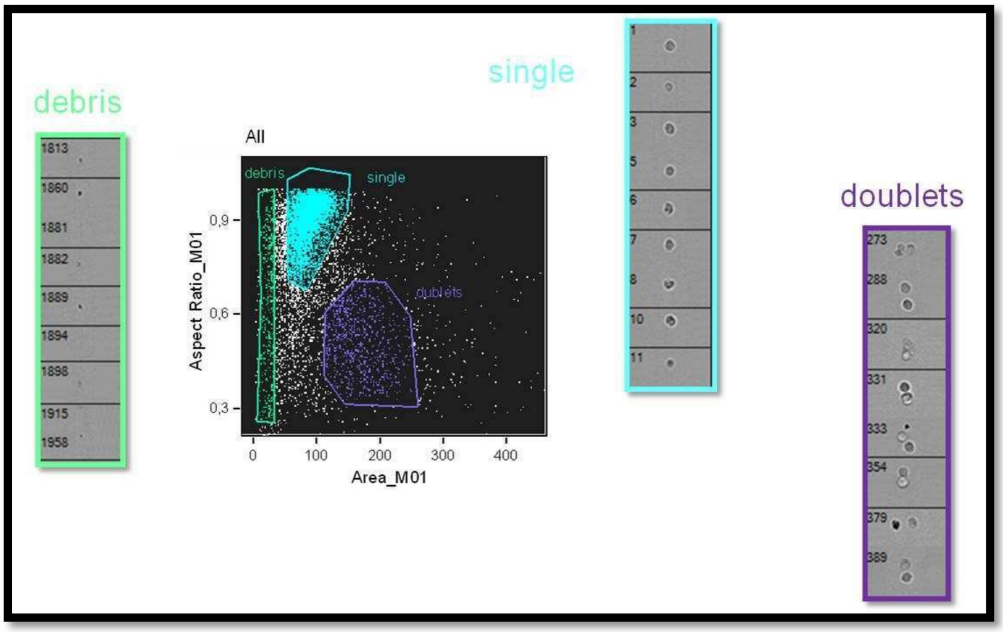

Figure 5-10: Separation of single cells from debris and multicellular aggregates.

5.3.5.2 Monocyte and platelet gating strategy

The cells in focus were gated initially, then single cells were gated based on size/aspect ratio (for single cell selection). Then, CD14-positive cells were plotted against CD61 to observe any platelet binding.

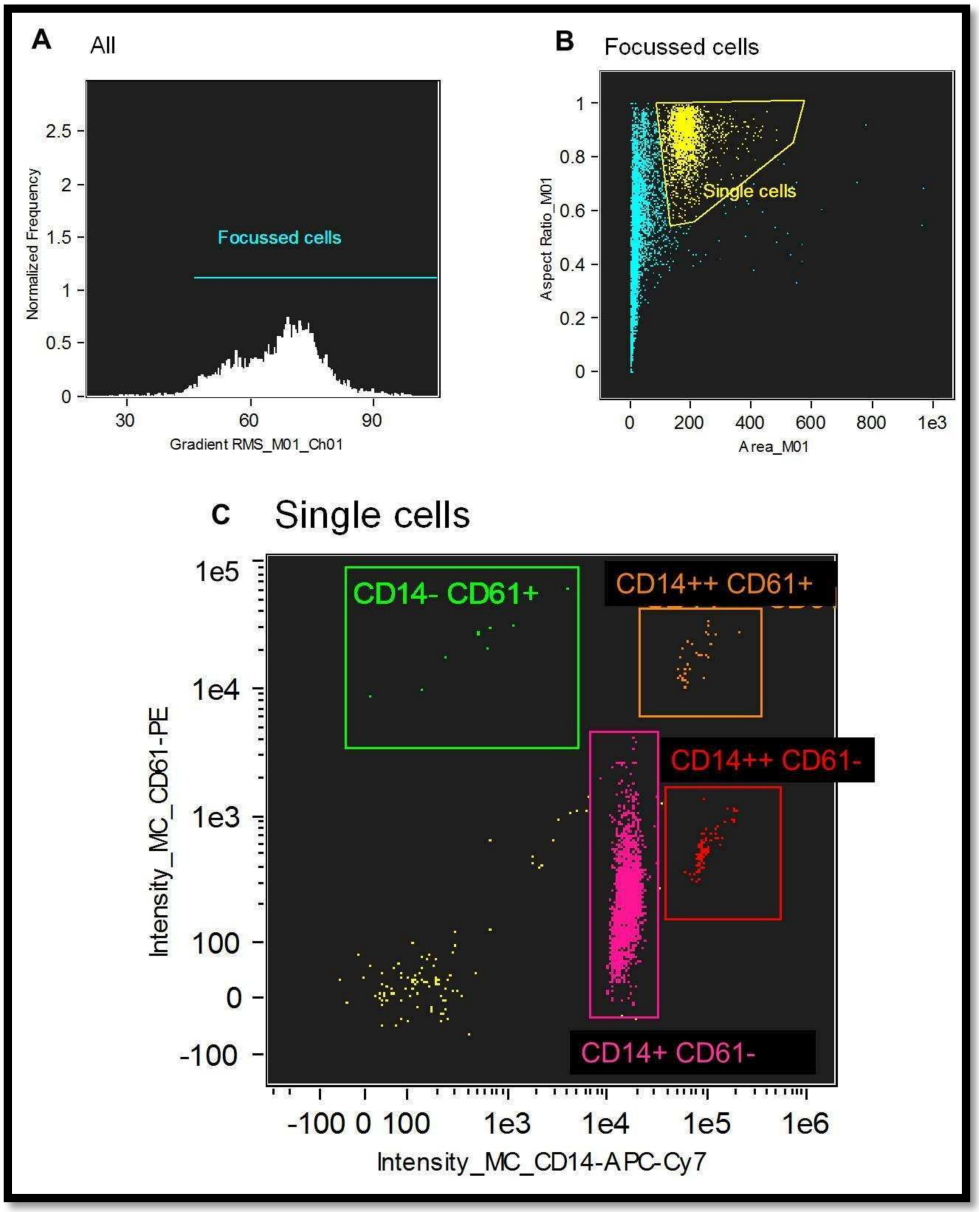

Figure 5-11: Monocyte and platelet gating strategy

5.3.5.3 Images of cell populations from a selected donor

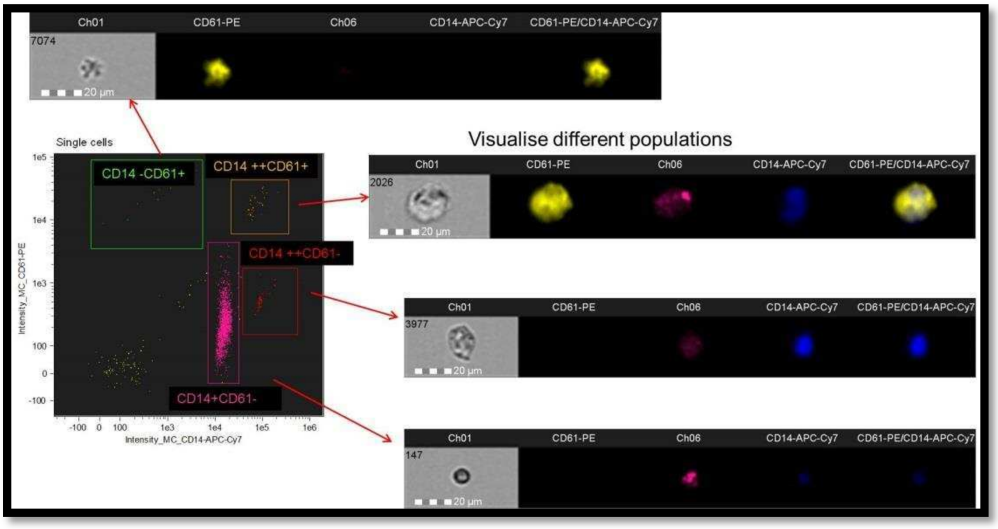

Figure 5-12: Image of different cell populations.

Platelets identified by anti-CD61 (yellow), monocytes by anti-CD14 (blue), and erythrocytes by anti-Ter119 (red) in Ch06**.** As can be seen in the channel containing anti-CD61 and anti-CD14, there were platelet-monocyte conjugates, demonstrated by the colocalisation of the yellow (indicative of platelets) and blue (indicative of monocytes) channels.

5.4 Discussion

In the introduction to this chapter, it was indicated that when blocking platelet –platelet interactions by using MK-0852 (GPIIb/IIIa antagonist), and then measuring platelet inhibition using the SPC technique, the results showed partial inhibition of platelet aggregation. It was anticipated that blocking platelet-platelet interactions would reflect antagonism of the final pathway that involves GPIIb/IIIa receptors; however, platelets may still be active and therefore, interact with leucocytes to form PLCs. PLCs formation is a consequence of the interactions between activated platelets and leucocytes. Previously, our research group demonstrated that the fibrinogen receptor antagonist, MK-0852, does not completely inhibit platelet aggregation using SPC in WB aggregation. The SPC approach measures the drop in the number of single platelets as they form aggregates; however, platelets could also form PLCs after activation in whole blood (Scholz et al., 2002).

Platelet aggregation in the formation of a thrombus, as a result of plaque rupture (as demonstrated in Figure 5-13), supports the accumulation of leucocytes – neutrophils and monocytes. This implies that activated platelets express the complete adhesive and signaling machinery necessary for the multistep adhesion cascade essential for leucocyte recruitment under shear conditions (Palabrica et al., 1992, Kirchhofer et al., 1997). Different mechanisms have been proposed to interfere with platelet binding to leucocytes.

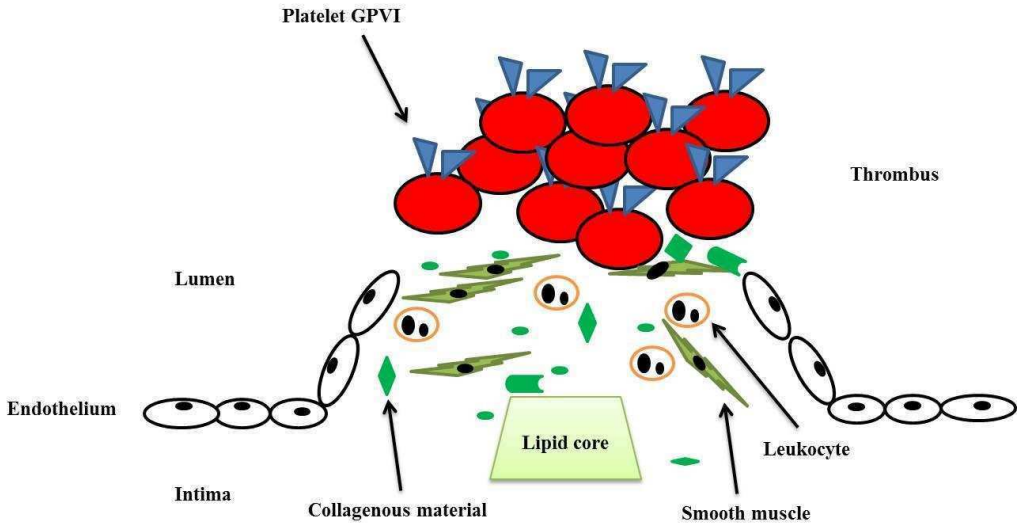

Figure 5-13: Plaque rupture.

At the site of vascular damage, platelets attract leucocytes to promote inflammation. After plaque rupture, exposed collagen induces platelet aggregation via the release of platelet stimulants, such as adenosine diphosphate. Activated platelets express glycoprotein (GP)-VI, which stimulates thrombus formation. Platelets also bind to heterotypic cells, such as leucocytes, via a P-selectin-P-selectin glycoprotein ligand-1 mechanism.

Normally, agonist-induced platelet activation is associated with the translocation of CD62P from α-granule membranes to the cell surface, and this is important for effective PLC formation through PSGL-1 (Jungi et al., 1986, Rinder et al., 1991b, de Bruijne-Admiraal et al., 1992, Spangenberg et al., 1993). However, leucocyte activation, as reflected by measuring CD11b on both monocytes and neutrophils, does not lead to the formation of platelet-leucocyte aggregate formation (Chen et al., 1994).

When using MK-0852 with platelet stimulation, PLCs formation tends to be higher. In this respect, the first set of data reported here focused on the

measurement of PLCs using different platelet markers. This demonstrated that inhibition of platelets by IIb/IIIa antagonism resulted in an increase in PLCs, with predominately monocytes rather than neutrophils. This could be due to possible differences in the expression of PSGL-1, the ligand for P-selectin, on the two cell types and/or the involvement of other receptor ligand interactions, which may stabilise the adhesion of platelets to monocytes. In the experiments performed, collagen induced PLCs formation, resulting in increased conjugates than with ADP and TRAP – presented as M/N-MF or %PM/PN. This is consistent with previous findings by our group, in which we observed a dramatic increase in PLCs when blood was incubated with MK-0852 and stimulated with collagen (Zhao et al., 2003a). However, in a study by (Kling et al., 2013) activation with TRAP resulted in high P-selectin expression and provided higher PLCs formation than ADP, collagen and AA. It is important to note that mixing forces relied upon shaking, rather than normal stirring, and this was not an issue. Also, the time was set to 10 min, as the conjugate formation was shown to be maximal at this time point (Redlich et al., 1997). From the experiments conducted, CD61PE was established to be the preferred marker as it demonstrates good stability (data not shown) and, hence, was used for further experiments.

Figure 5-14 demonstrates that antagonism by the GPIIb/IIIa receptor results in more free platelets being available to adhere to leucocytes, while Figure 5-15 shows the blockade of platelet-leucocyte interactions by KPL-1. As the mechanism that governs PLCs formation in adults, via P-selectin-PSGL-1, is

well characterized, it is notable that conjugate formation in healthy children is enhanced without increasing the expression of P-selectin, thus suggesting a different mechanism, independent of P-selectin (Yip et al., 2013). In the same study, it was also confirmed that the antagonism of P-selectin (CD62P) on platelets did not affect PLCs formation.

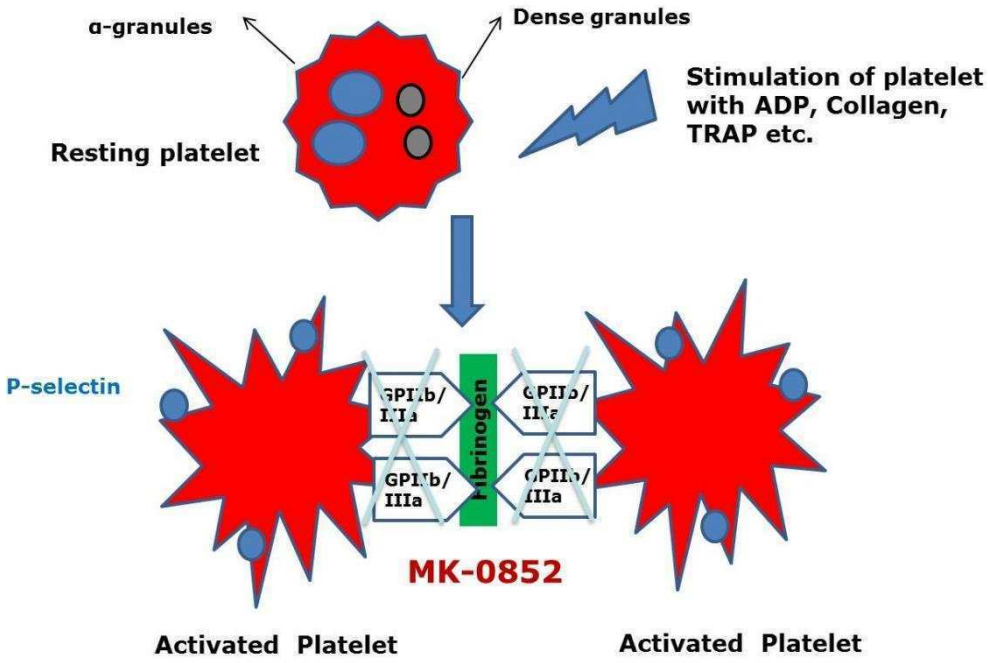

Figure 5-14: Blockade of the glycoprotein (GP)-IIb/IIIa receptor using MK-0852.

Upon platelet activation, the platelet α-granules release their contents and P-selectin is expressed on the activated platelet surface.

To confirm PLCs formation, an agent called KPL-1 was used to block the PSGL-1 receptor at the leucocyte end (Snapp et al., 1998). In all the in vitro studies on the effect of PSGL-1 blockade, KPL-1 has been shown to be sufficient for inhibiting and reversing the formation of PLCs (neutrophils &

monocytes). This finding is consistent with the study by (Fernandes et al., 2003) and the results suggest that PLC formation is mostly dependent on PSGL-1. Also, PLCs (neutrophil or monocyte) formation was suppressed irrespective of the use of ADP, collagen or TRAP to stimulate platelets. In line with this finding, PSGL-1 inhibition alone decreased PLC formation to pre-stimulation levels, also resulting in the disengagement of cells after complexes had formed. It could be assumed that if GPIIb/IIIa-mediated firm adhesion played a significant role in the formation of PLCs, blockade of PSGL-1 alone would not be able to disaggregate PLC complexes. This is clearly illustrated in Figure 5-15. A direct WB smear stained with Wright's stain, as an additional confirmation of the presence of PLCs, showed the binding of platelets to leucocytes (monocytes), as demonstrated previously (Zhao et al., 2003c), where KPL-1 successfully prevented the formation of PM complexes, irrespective of the platelet agonist used.

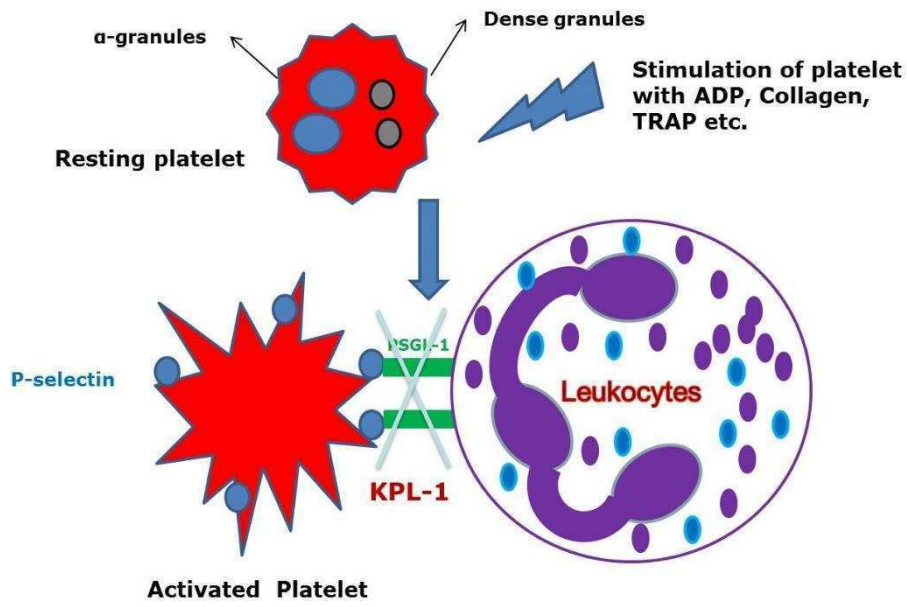

Figure 5-15: Platelet- leucocyte conjugate formation.

Platelets become attached to leucocytes through PSGL-I. P-selectin/PSGL-I mediates this interaction; however, there are several mechanisms by which platelet-leucocyte complexes (PLCs) are formed. KPL-1 blocks P-selectin glycoprotein ligand 1 (PSGL-1) receptors on leucocytes, confirming the dependence of PLCs formation on the P-selectin-PSGL-1 mechanism.

Interestingly, when MK-0852 was incubated with KPL-1, PLCs formation increased slightly again, which may suggest the binding of platelets to leucocytes via a different mechanism that does not involve PSGL-1. Also, EDTA consistently and successfully inhibited PLCs formation, consistent with earlier findings, suggesting the dependence of conjugate formation on divalent cations (Spangenberg et al., 1993). Previous studies have shown that Ca^{2+} is required for leucocytes to bind platelet P-selectin (Jungi et al., 1986). The binding of Ca^{2+} to P-selectin mediates a conformational change in the lectin domain that is essential for leucocyte adhesion (Geng et al., 1991).

There are many ways to block PLCs formation and in the study conducted here KPL-1 was used. However, other blockers are available, such as those that prevent the mechanism involving P-selectin (CD62P) on platelets. In this respect, a novel human anti-P-selectin antibody, inclacumab, has been developed for therapeutic treatments and for the prevention of cardiovascular disease (Kling et al., 2013, Tardif et al., 2013). The results obtained from this study showed that inclacumab successfully inhibited the PLC formation induced by platelet stimulation due to different agonists (ADP, collagen, AA and TRAP) and this suggests that inclacumab prevents formation of new PLCs irrespective of the pathway contributing to PLCs formation.

The initial section of the results presented in this chapter was aimed at optimising the method for measuring PLC formation, in order to select the most suitable antibodies for identifying platelets**.** With CD42a-FITC and CD62P-PE, the number of CD42a-positive neutrophils increased with time. CD61-PE appeared to be the most suitable marker, as it demonstrated good stability (data not shown) and clear inhibition with KPL-1. Having established a method based on the lysing of RBCs, and utilising the 96-well plate format with a fixation method to quantify the formation of platelet – monocyte and platelet–neutrophil conjugate formation, this method indicated the increase in PLCs formation in blood samples incubated with MK-0852. This validates our assay, especially in the first set, which involved blood stimulation using a 96-well plate. The next sections were aimed at studying the in vitro and ex vivo effects of antiplatelet drugs on PLCs formation.

The in vitro study involving the inhibition of PLCs formation using aspirin and cangrelor alone did not show PLCs formation inhibition. Then a combination of both drugs was used, based on the fact that a combination of different antiplatelet drugs, with different mechanisms of action ,may afford a better protection than an individual drug used on its own; this is frequently used in the prevention of sub-acute thrombosis – a seemingly safe and effective therapy (Kolansky et al., 2000, Muller et al., 2000). However, a combination of both drugs tended to show slight and variable inhibition. This may suggest that this 96-well plate assay, which involves blood shaking, is not favorable for investigating PLCs formation in vitro (data not shown). Previous work by our group, where stirring with variable force was used, indicated that a combination of two agents is not as effective as a triple antiplatelet in preventing PLCs formation (Zhao et al., 2001).

The connection of platelet-leucocyte interactions ACS has been well established in various clinical settings (Furman et al., 2001, Sarma et al., 2002). This is recognized to be a potential target for antiplatelet therapy in ACS patients. The data on the ex vivo effects of antiplatelet drugs on PLCs formation in ACS patients indicated that significant inhibition was obtained when clopidogrel was used with different agonists. Clopidogrel reduced spontaneous aggregation of PLCs. This is in line with previous studies, showing the clinical significance of clopidogrel, as an anti-inflammatory agent, in reducing the number of PLCs formed (Storey et al., 2002a, Klinkhardt et al., 2003). However, in this study, collagen and TRAP-induced PLCs formation was sensitive to the effect of clopidogrel. This supports previous

documentation of the significance of ADP and $P2Y_{12}$ receptors in augmenting and modulating the effects of various platelet agonists, including the effect of thrombin on PAR-1(Trumel et al., 1999). In all the ex vivo experiments conducted, blood from ACS patients taking prasugrel showed further inhibition of PLCs formation. This indicates that, in our experiments, prasugrel was better at inhibiting PLCs formation possibly due to the generation of more active metabolites in vivo (Totani et al., 2012).

Perhaps the main message arising from the work conducted in this chapter is that the flow cytometric procedure, based on RBC lysis, using 96-well plates and fixation, can be used to obtain information on PLCs formation. Also, the effect of different agonists and antagonists on PLCs formation was investigated. However, the data from experiments examining the in vitro effect of antiplatelet drugs on PLCs formation is not promising, and could imply that the methodology is not suitable for this purpose.

The effect of the different agonists and inhibitors suggests the involvement of certain adhesive proteins in the cell-cell interactions that occur. My project is based on the development and optimisation of PFT. Previously, PLCs formation was assessed by stirring; however, in this study, blood was mixed by shaking 96-well plates and fixed using AggFixA and AggFixB, allowing fixed blood to be processed for up to three days. Blood was shaken for 10 min and PLCs contained predominately monocytes, rather than neutrophils. Using

different platelet stimulants (ADP, collagen and TRAP), collagen induced the greatest PLC formation, specifically of monocytes, rather than neutrophils. KPL-1 appears to inhibit PLCs formation, irrespective of the conjugate formed. In vitro data may not be suitable for developing this assay. Ex vivo studies clearly demonstrate that inhibition of PLCs formation in ACS patients taking prasugrel was slightly greater than in blood from those taking clopidogrel. These results may reflect another application of the 96-well plate.

In conclusion, flow cytometry measurements of PLCs, using standard protocols involving RBC lysis and a 96-well assay format, might prove advantageous over existing PFT, especially in clinical situations where drugs that affect degranulation might be associated with an anti-inflammatory effect, by reducing PLCs formation. In the following chapter, experiments aimed at optimising the technique further will be described; these will use a triggering approach and the 96-well plate format. A comparison of the effect of MK-0852 on inducing PLCs formation in blood, when stimulated by ADP, collagen or TRAP and shaken for 5 or 10 min, will be performed. Finally, more investigations of the simultaneous assessment of platelet aggregation and PLCs formation from the same blood sample will be considered.

6 Measurement of platelet aggregation and leucocyte interaction using a triggering approach

6.1 Introduction

WB flow cytometric studies of platelet activation (Michelson, 1996) and platelet aggregation (Fox et al., 2004b) have several advantages. These include less manipulation and RBC loss, as a result of sample handling and a separation process that involves centrifugation. Flow cytometry can also be used to investigate PLCs formation. As explained in the previous chapter, PLCs formation can be studied by flow cytometry using different methods, such as the standard lysing protocol, which involves the lysing of RBCs, or a non-lysing protocol (triggering technique). Non-lysing, as the name implies, involves no RBC lysis and uses the pan-leucocyte MAb CD45 marker to identify the overall leucocyte scatter population, without distinguishing leucocyte sub-populations, such as neutrophils and monocytes. This method has many advantages over the lysis method, in which RBC lysis can result in the release of large amounts of ADP. This may activate platelets and lead, artefactually, to enhanced PLCs formation. Also, RBC lysis may enhance leucocyte integrin expression, thereby increasing PLCs formation (Forsyth and Levinsky, 1990, Rinder et al., 1991a).

Analysis of samples for PLCs within three hours appears to provide a reliable measurement of PLCs formation. However, PLCs formation tends to increase after three hours if a sample is not fixed. Therefore, a fixative solution, based on PFA, was used in the 96-well plate assay developed to fix the blood and to indicate the stability of leucocyte conjugate formation, for up to three days (May JA, 09/05/08).

ADP, thrombin and collagen activate platelets via different mechanisms, involving different platelet receptors (Offermanns, 2006), and their ability to induce PLCs formation varies, with collagen being most potent, as indicated previously by our group (Zhao et al., 2003a) and by the results obtained in this thesis.

There is clinical proof that combining antiplatelet agents which have differing means of action yields more favourable antithrombotic effect than using agents alone (Diener et al., 1996, Yusuf et al., 2001, Steinhubl et al., 2002). This is achieved by blocking agents that inhibit different platelet activation pathways and the aggregation that results. These agents reduce the availability of GPIIb/IIIa receptors on the platelet surface, ultimately resulting in the binding of fibrinogen and causing platelets to aggregate. It can also be achieved by directly inhibiting GPIIb/IIIa receptors using agents such as eptifibatide, abciximab and tirofiban. These drugs do not interfere with platelet activation, as a result of platelet stimulation, but block the binding of fibrinogen to the activated GPIIb/IIIa receptor (Topol et al., 1999). By blocking both activation and the final aggregation pathway, better inhibition of platelet aggregation can be obtained.

In order to make the assay for measuring PLCs easy to perform, and avoid complex sample manipulation within the standard protocol, a fluorescent triggering technique was used to capture the leucocyte population – rather than lysing RBCs, as in the original protocol. If the results obtained were

comparable to those using the standard method, the adapted protocol would be used in all further experiments.

In our assay to study platelet function, 96-well plates were shaken for 5 min. This was optimised to study platelet aggregation. To investigate PLCs formation, 96-well plates were shaken for 10 min, based on a previous study (Redlich et al., 1997) and our own laboratory – both of which demonstrated that 10 min is optimum for PLCs formation measurement. Therefore, for studies into the difference between 5 vs. 10 min shaking for PLCs measurement, it is highly desirable to combine both measurements from the same fixed whole blood.

The studies described in this chapter were also aimed at investigating PLCs formation using our 96-well plate assay and fluorescent triggering protocol, which has been widely used previously (Hagberg and Lyberg, 2000b). Also these studies were concerned with the optimum blood shaking time to investigate PLCs formation, lowering it to a convenient time of 5 min. Finally, studies were conducted, and described here, to study the effect of MK-0852±KPL-1, aspirin or cangrelor on platelet aggregation and PLCs formation simultaneously from the same fixed WB sample.

6.2 Results

6.2.1 Effects of MK-0852 (10µM) and KPL-1 (5µg/ml) on platelet-leucocyte conjugate formation using CD61-PE as a platelet identifier

These experiments aimed to investigate the effects of MK-0852, which has been shown to increase PLCs formation, and KPL-1, which blocks PLCs formation. They also aimed to translate the findings of a method, other than the standard method, which involves RBC lysing. In these experiments, blood was incubated with saline and MK-0852 ± KPL-1, then stimulated with a range of collagen concentrations and shaken for 10 min. Blood was fixed using AggFixA and AggFixB. The triggering (non-lysing) approach was used to study PLCs formation. Results, based on M-MF and %PM, will be shown.

6.2.1.1 Monocyte median fluorescence and percentage platelet-positive monocytes

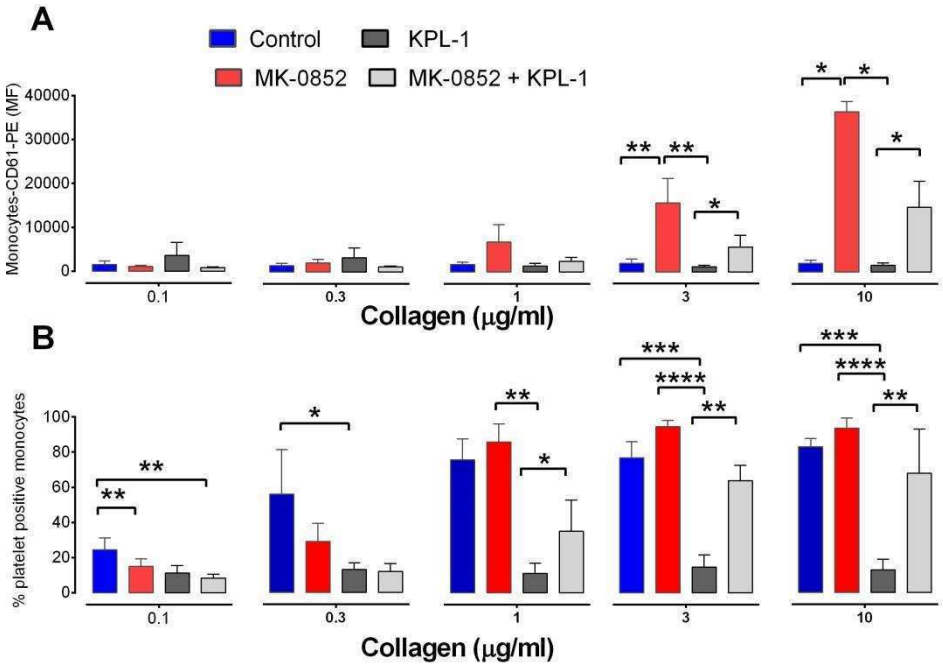

Figure 6-1: Detection of platelet-leucocyte conjugate (monocytes) formation using CD61-PE and triggering.

Effects of MK-0852 (10μM) and KPL-1 (5μg/ml) on platelet-monocyte (PM) conjugate formation based on monocyte median fluorescence (M-MF) (A) and %PM (B) using CD61-PE. Results are expressed as mean ± SD (n=6). * P< 0.05 analysed by one-way ANOVA repeated measures.

With respect to M-MF, MK-0852 enhances the increase of PLCs in a dose-dependent manner from control (saline). Considering %PM, the amount of conjugates tends to be high in the control sample, with different platelet stimulations. Addition of MK-0852 decreases the conjugate formed in blood stimulated with 0.1 and 0.3µg/ml collagen. In blood stimulated with 1, 3, and 10µg/ml, there was a dose-dependent increase in conjugates formed, with no potentiation of PLCs upon treatment with MK-0852 from control. Blocking PLCs formation with KPL-1 resulted in a significant decrease in PLCs formation, which was noticeable with the following concentrations of collagen (1, 3 and 10µg/ml), presented as M-MF and %PM. Concurrent antagonism using MK-0852 + KPL-1, which involves blocking platelet-platelet and platelet-leucocyte aggregates, showed a reverse increase in PLCs formation. This finding is similar to that obtained when investigating PLCs formation using the lysing protocol.

In conclusion, this technique, utilising fluorescent triggering and the adaptation of 96-well plates, has been shown to successfully measure PLCs formation, as the results obtained indicate an increase in the conjugate formed – especially with monocytes. KPL-1 inhibited conjugate formation, with a slight increase when added together with MK-0852.

6.2.2 Comparison of triggering and lysing methods for the detection platelet-leucocyte conjugate formation

The value obtained from the pooled data of PLCs formation induced by the maximal collagen concentration (10µg/ml) of agonists (saline, MK-0852, KPL-1, MK-0852+KPL-1), and analysed using lysing and triggering approaches, were compared. These values were expressed and plotted as N-MF, %PN, M-MF and %PM. This was done to see if both approaches (lysing vs. triggering) were comparable. The results will assure if the new triggering approach is useful for further development.

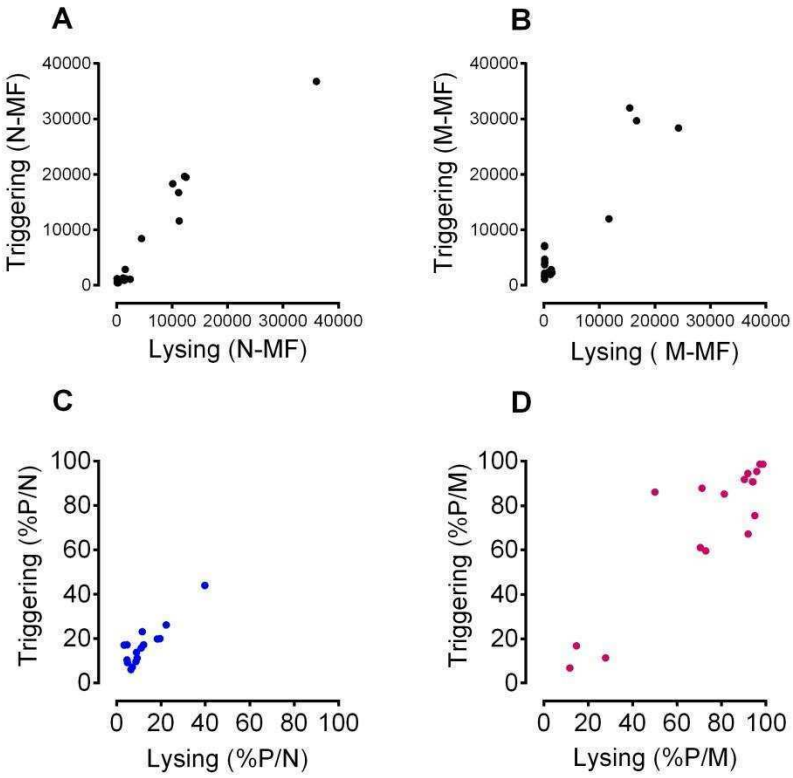

Figure 6-2: The correlation of platelet-leucocyte conjugate (neutrophils and monocytes) formation.

Platelet leucocyte conjugates (PLCs) values presented as neutrophil median fluorescence (N-MF) (A), monocyte median fluorescence (M-MF) (B), % platelet-neutrophil (%PN) (C) platelet-monocyte (%PM) conjugates and (D) analysed using the triggering vs. lysing technique.

Pearson's correlation curve analysis shows a high correlation of the data, representing conjugate formation as MF or % platelet-positive, which was analysed using triggering vs. lysing. This value was estimated as N-MF (0.9284), %P/N (0.7897), M-MF (0.8664) and %P/M (0.8007). More detailed comparisons was estimated using a paired t-test for the different conditions (Figure 6-3).

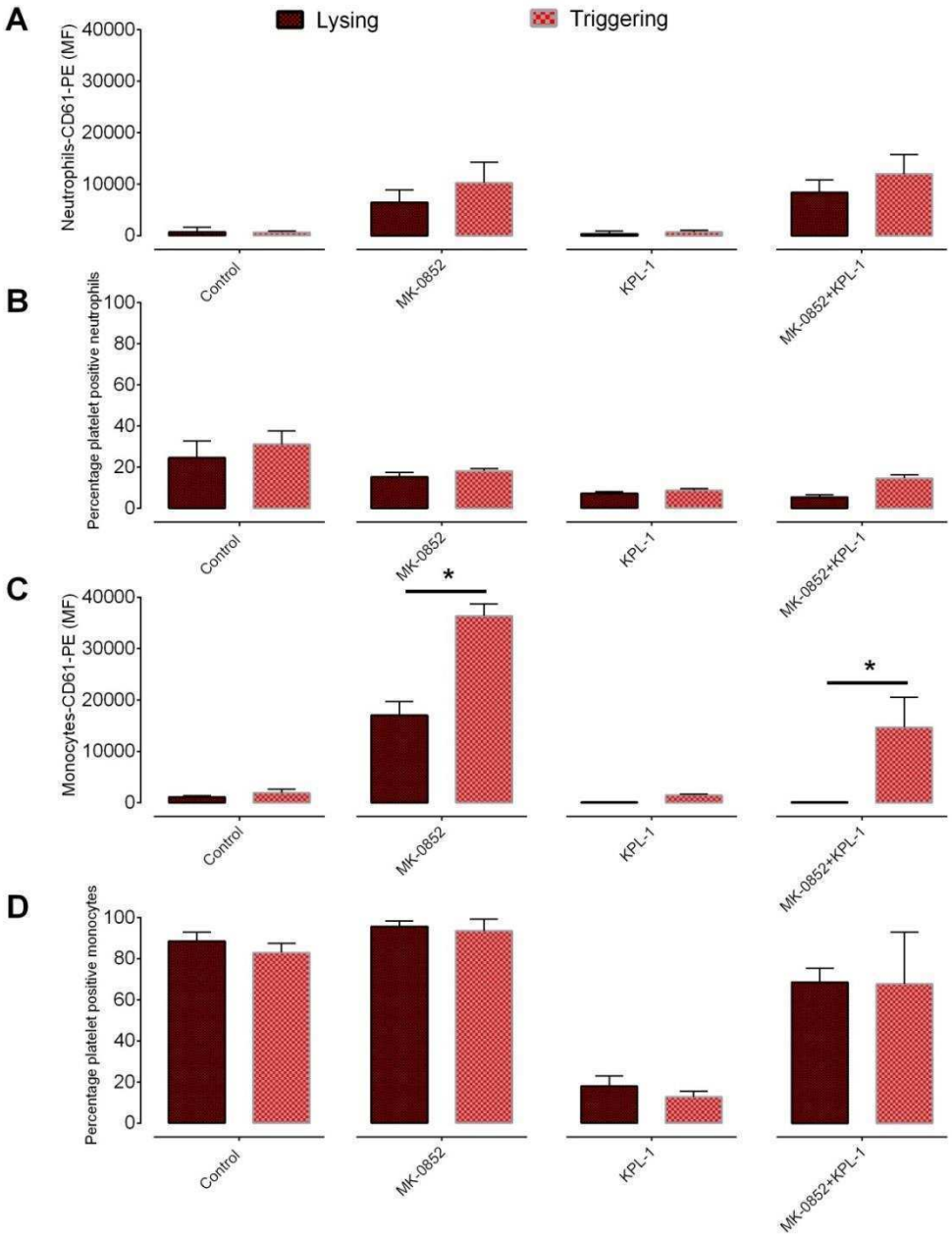

Figure 6-3: The comparisons of platelet-leucocyte conjugate (neutrophil and monocyte) formation.

Platelet-leucocyte conjugate (PLCs) values presented as neutrophil mean fluorescence (N-MF) (A), % platelet-neutrophils (%PN) (B), monocyte median fluorescence (M-MF) (C) and % platelet-monocytes (%PM) (D) and analysed using the triggering vs. lysing technique and the effect of (saline, MK-0852, KPL-1 and MK-0852+KPL-1) in the blood stimulated with collagen (10µg/ml). Results are expressed as mean ± SD (n=6). * $P<0.05$ of (MK-0852 and MK-0852+KPL-1 of triggering vs. lysing) and presented as M-MF, analysed with paired t-test.

The results obtained from the comparison indicated that triggering is highly comparable to the standard lysing approach and can be used for further investigation. However, in blood incubated with MK-0852 and MK-0852+KPL-1, PLCs formation, represented as M-MF, was higher than estimated using the lysing technique.

Then, a comparison of 5 min (optimised for aggregation measurements) and 10 min (appropriate for conjugate measurements) blood shaking was made. This would facilitate combining both measurements from the same fixed whole blood.

6.2.3 The effect of different shaking times of whole blood on platelet-leucocyte conjugate formation

These experiments were aimed at comparing the effect of blood shaking at different time points, 5 min vs. 10 min, on PLCs formation. In these experiments, blood samples were stimulated with ADP (30µM), collagen (10µg/ml) and TRAP (10µM) and then fixed using AggFixA and AggFixB. Two separate sets of 96-well plates were used and the same experimental protocol for measuring PLCs, using the triggering, was used, as explained previously – except for shaking the blood at different times. If the results after 5 min and 10 min shaking were not hugely different, platelet aggregation and conjugate formation experiments could be performed concomitantly on the same fixed WB sample for 5 min.

6.2.3.1 Neutrophil median fluorescence and percentage platelet-positive neutrophils

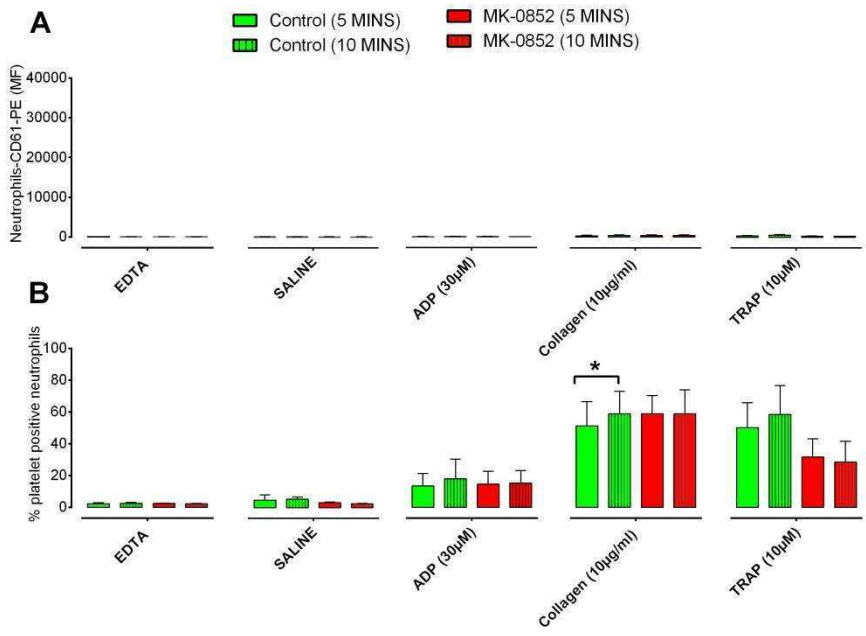

Figure 6-4: Detection of platelet-leucocyte conjugate (neutrophil) formation in sample shaken for 5&10 minutes.

Effects of MK-0852 (10µM) on platelet-neutrophil (PN) conjugate formation based on neutrophil mean fluorescence (N-MF) (A) and %PN (B) in blood samples shaken for 5 vs. 10 min and incubated with EDTA, saline, ADP (30µM), collagen (10µg/ml) and TRAP (10µM). Results are expressed as mean ± SD (n=6).* P< 0.05 analysed with paired t-test.

The amounts of platelets bound to neutrophils (N-MF) were low in blood incubated with saline and MK-0852, following stimulation with different agonists. Blood samples that were shaken for 5 min exhibited a similar pattern to those shaken for 10 min. The amount of PN conjugates (%PN) was higher, specifically under stimulation with collagen, with no further increase upon

treatment with MK-0852. However, in blood stimulated with TRAP, MK-0852 decreased t conjugate formation to the same level in 5 and 10 min shaking protocols.

6.2.3.2 Monocyte median fluorescence and percentage platelet-positive monocytes

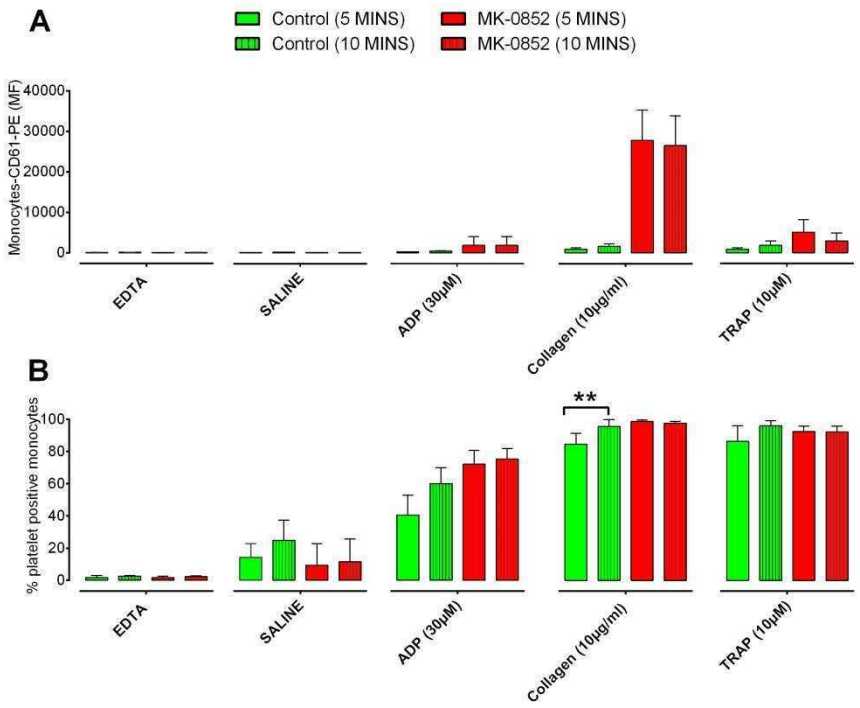

Figure 6-5: Detection of platelet-leucocyte conjugate (monocyte) formation in sample shaken for 5 and 10 min.

Effects of MK-0852 (10µM) on platelet-monocyte (PM) conjugate formation based on monocyte median fluorescence (M-MF) (A) and %PM (B) in blood samples shaken for 5 vs. 10 min and incubated with EDTA, saline, ADP (30µM), collagen (10µg/ml) and TRAP (10µM). Results are expressed as mean ± SD (n=6). * P< 0.05 analysed with paired t-test.

With respect to M-MF, PLCs formation was extremely high in blood incubated with MK-0852 and stimulated with collagen at 5 and 10 min of shaking, in which the values were very comparable. The data obtained for %PM exhibits higher conjugates, which further increased when blood was incubated with MK-0852 and stimulated with only ADP. This was estimated by ANOVA to be statistically significant ($P<0.05$), to the same extent in blood shaken for 5 vs 10 min. Blood samples incubated with saline and shaken for 10 min exhibited more conjugates compared to blood shaken for 5 min.

From the results obtained, it may be concluded that 5 min vs. 10 min blood shaking does not make a huge difference; therefore, simultaneous assessment of platelet aggregation and PLCs can be carried out reliably on blood shaken for 5 min.

6.2.4 Simultaneous measurement of platelet aggregation and platelet-leucocyte conjugate formation

I have demonstrated the usefulness of the platelet aggregation assay, based on the platelet counting technique and the 96-well format, to generate platelet activation pathways upon stimulation with the most commonly used agonists (AA, ADP, collagen and TRAP) and platelet inhibition with aspirin and/or the $P2Y_{12}$ antagonist, cangrelor (Dovlatova et al., 2013). Furthermore, fixing blood using AggFixA and AggFixB enables the measurement of platelet function to be carried out at a later stage, facilitating remote testing for up to 9 days after

(for platelet aggregation) and up to 3 days after (for PLC formation) (Fox et al., 2013). Additionally, I have confirmed the agreement of the triggering approach with the standard lysis protocol, while also checking the suitability of 5 min blood shaking, in performing PLCs formation studies with combined PA and PLCs measurements. I then decided to use fixed blood to perform both platelet aggregation and PLCs measurements of the same blood. Platelet aggregation requires only 5µl of fixed blood, while the remainder of the blood can be utilised for PLCs measurements.

6.2.4.1 Stability of PA and PLCs formation in vehicle-treated blood samples analysed on the same and next day

In these studies, the stability of both measurements of platelet aggregation and PLCs was tested on the same day, and the next day, of analysis – as a way of validating the 96-well format and blood fixation protocol, when both measurement were carried out simultaneously. Blood samples were incubated with saline and then stimulated with a range of collagen concentrations (0.1, 0.3,1,3 and 10µg/ml). Then, the experiment was done as described in section 2.2.6.

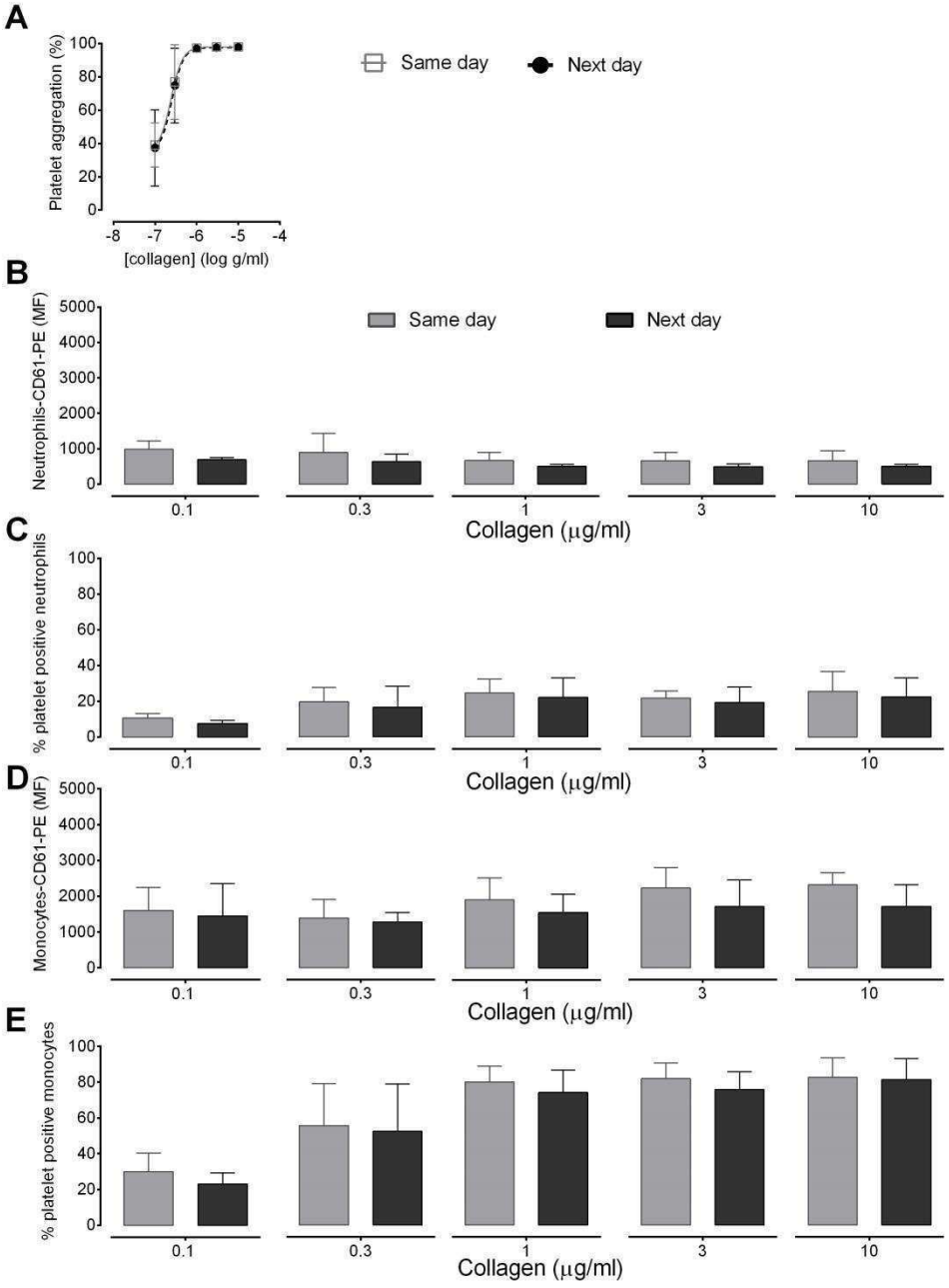

Figure 6-6: Analysis of platelet aggregation and platelet-conjugate formation performed on the same vs. the next day.

Percentage of platelet aggregation (A) induced by a range of collagen concentrations (0.1, 0.3, 1, 3, 10µg/ml) and comparison of samples performed on the same day vs. the next day. Results are expressed as mean ± SD, (n=6) vs. EDTA and analysed using non-linear regression curve. Platelet-leucocyte conjugate (PLCs) formation was based on neutrophil median fluorescence (N-MF) (B), % platelet-neutrophils (%PN) (C), monocyte median fluorescence (M-MF) (D), % platelet-monocytes (%PM) (E) induced by a range of collagen concentrations (0.1, 0.3, 1, 3, 10µg/ml) and comparison of samples analysis performed on the same day vs. the next day. Results are expressed as mean ± SD, (n=6) and analysed with paired t-test.

Aggregation measurements, when performed on the same vs. the next day, demonstrated the same pattern of increased platelet aggregation with a range of collagen concentrations in a dose-dependent manner, thereby assuring stability. In vehicle-treated blood, in response to the range of collagen concentrations, PLCs formation was judged by % CD61-positive monocytes and neutrophils, or by median CD61 fluorescence. The degree of PM conjugate formation was much greater than the degree of PN conjugate formation, especially with the highest three concentrations. Also, the PLCs measured on the same day was not statistically different than those measured on the next day, as estimated by the paired t-test.

Based on this finding, which confirms that samples are stable for analysis performed simultaneously and at different times (same vs. next day), the next set of experiments will be looking at the effect of MK-0852 on platelet aggregation and PLCs formation from the same fixed blood.

6.2.4.2 The effect of MK-0852 on platelet aggregation and platelet-leucocyte conjugate (monocytes) formation

It has been demonstrated in this thesis that MK-0852 failed to achieve complete inhibition, using the SPC technique to measure platelet aggregation. This was confirmed later, by the involvement of leucocytes, to be implicated in the loss of single platelets, especially at high collagen concentrations. Also, having checked the stability of the platelet-counting platelet activation assay in WB and fluorescent triggering, to measure PLCs simultaneously, the following experiments investigated the effect of MK-0852 on platelet aggregation and PLCs formation, translating this finding when both measurement were done together. PLCs formation data based, on M-MF and %PM will, be shown.

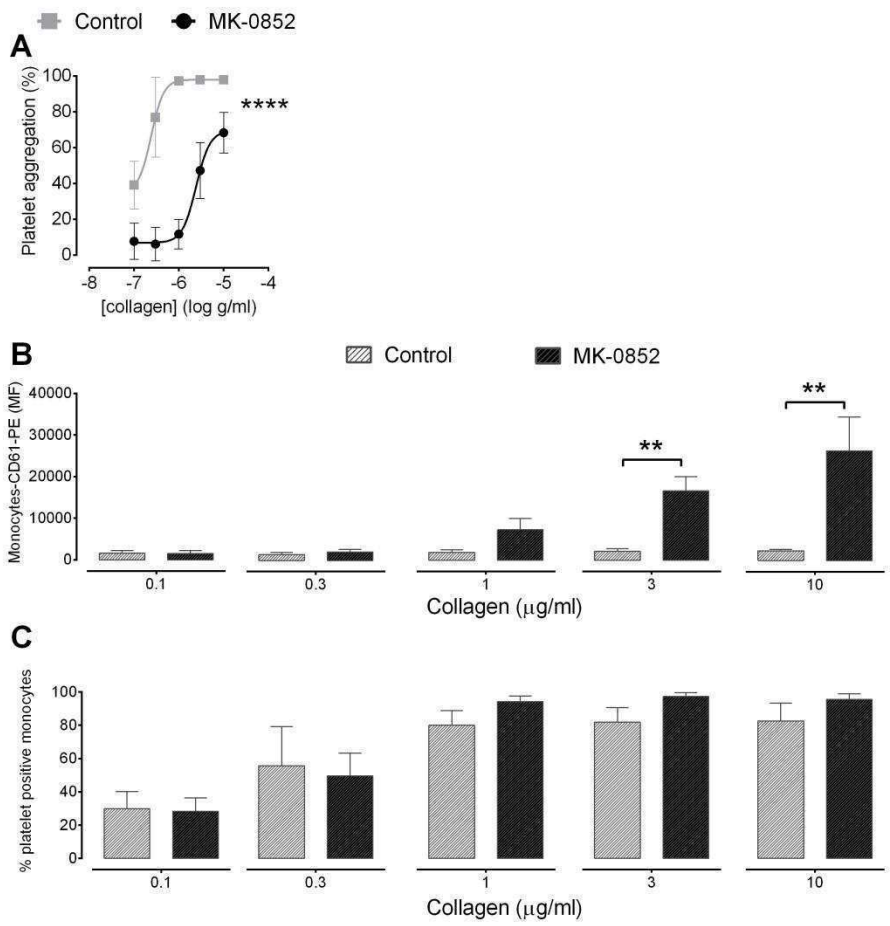

Figure 6-7: Effects of MK-0852 on platelet aggregation and platelet-leucocyte conjugate (monocyte) formation.

Platelet aggregation (A) and platelet-leucocyte conjugate formation based on monocyte median fluorescence (M-MF) (B) and % platelet-monocytes (PM) (C) induced by a range of collagen concentrations, and the effect of MK-0852 (10μM). Results are expressed as mean ± SD, (n=6) vs. EDTA. * $P<0.05$, repeated measures two-way ANOVA followed by Bonferroni's post-test.

Successfully, the results obtained here mirror the previous findings, which showed inhibition of platelet aggregation in blood stimulated with low concentrations of collagen. At high collagen concentrations, there seemed to be less aggregation, consistent with high PLCs (monocytes) formation in the sample incubated with MK-0852 from saline. This was reflected by the M-MF,

which increased in a dose-dependent manner. The data presented here is part of the data presented in Figure 6-8.

6.2.4.3 The effect of MK-0852 and KPL-1 on platelet aggregation and platelet-leucocyte conjugate formation

The previous subchapter described the importance of this assay in performing platelet aggregation and PLCs studies on the same fixed WB, indicating that platelets become bound to leucocytes following activation with collagen (with more platelets binding to monocytes than neutrophils). I then investigated the sensitivity of the assay, in order to confirm the involvement of PLCs formation as a decrease in the number of single platelets and, thus, a reduced inhibitory effect of MK-0852.

Blood samples were incubated with saline and MK-0852 ± KPL-1 and stimulated with a range of collagen concentrations. Blood was shaken for 5 min and fixed using AggFixA and AggFixB, which allowed stability of blood platelet aggregates, thereby allowing the analysis to be carried out at a later stage.

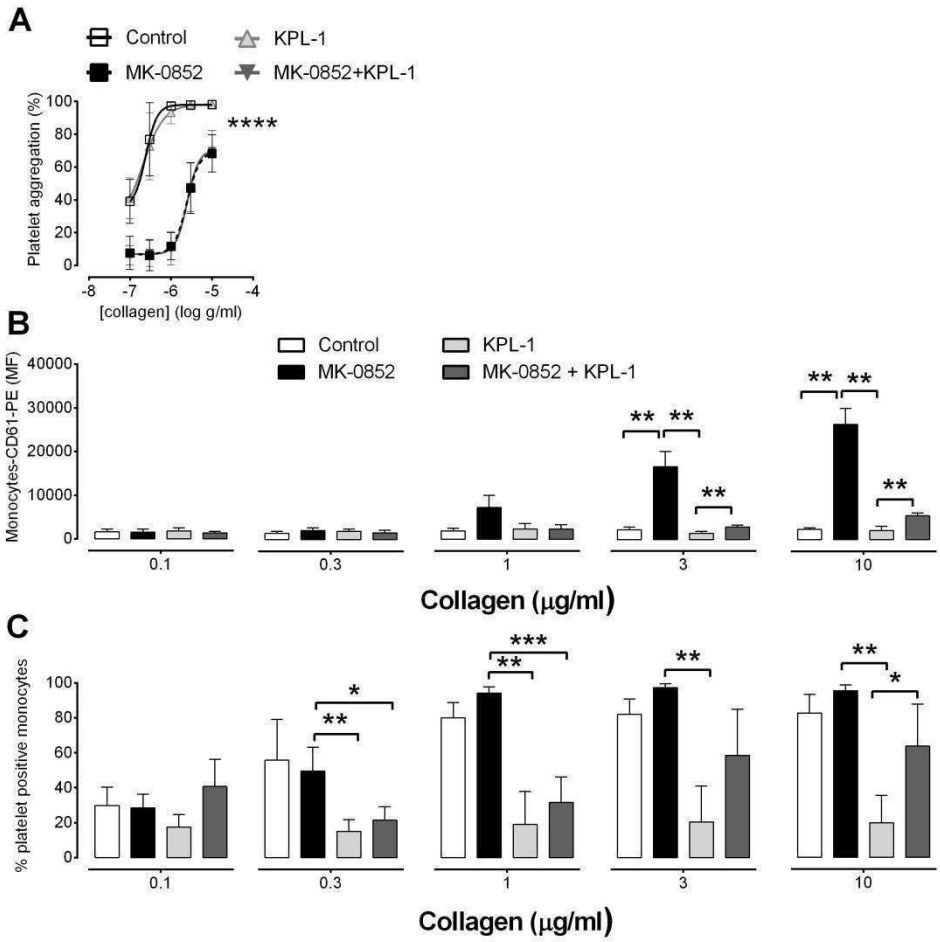

Figure 6-8: Effects of MK-0852 and KPL-1 on platelet aggregation and platelet-leucocyte conjugate (monocyte) formation.

Platelet aggregation (A) and platelet-monocyte (PM) conjugate formation based on monocyte median fluorescence (M-MF) (B) and % platelet-monocytes (PM) (C) induced by a range of collagen concentrations and the effect of MK-0852 (10μM) and KPL-1 (5μg/ml). Results are expressed as mean ± SD, (n=6) vs. EDTA. * P<0.05, repeated measures two-way ANOVA followed by Bonferroni's post-test.

With high collagen concentrations (3 and 10μg/ml), there was incomplete inhibition of aggregation by MK-0852. This was concomitant with high conjugate formation. To confirm the latter, 5μg/ml of specific agent KPL-1, which blocks the PSGL-1 receptor on leukocytes, inhibited PLCs and did not

affect the aggregation measurement. However, when combined with MK-0852, it did not show further inhibition – instead inhibition was slightly reduced.

In terms of measuring M-MF, there was no increase in PLCs formation with respect to 1) the blood incubated with saline and MK-0852 and stimulated with the lowest concentrations (0.1 and 0.3µg/ml) and 2) slight increases in the blood incubated with MK-0852 and stimulated with intermediate concentrations (1µg/ml). However, a similar pattern was observed in blood stimulated with 3 and 10µg/ml, in which PLCs formed were readily inhibited by KPL-1 and increased again slightly following addition of MK-0852.

With respect to the %PM, there was generally a greater amount of conjugate formed and a slight increase in the conjugate formed upon treatment with MK-0852 for all agonists, except for 0.1 and 0.3µg/ml collagen. Incubating blood with KPL-1 decreased the amount of conjugate formed significantly for all agonists, except for 0.1µg/ml. Blood incubation with MK-0852+KPL-1 again increased conjugate formation.

These results proved to be successful for the measurement of platelet aggregation and PLCs formation, to study the effect of MK-0852 ± KPL-1 simultaneously.

6.2.4.4 In vitro effect of antiplatelet drugs on platelet aggregation and platelet-leucocyte conjugate formation

After I have validated the advantages of the new t 96-well plate assay format for the measurement of platelet aggregation and PLCs formation, to study the effect of MK-0852 and KPL-1 in the same fixed WB, the following experiments were aimed to investigate the in vitro effect of the maximal concentration of aspirin (100μM) and cangrelor (1000nM) on 1) platelet aggregation using AA and ADP over a range of concentrations and 2) PLCs formation, using maximal and submaximal concentrations of AA and ADP as platelet stimulants.

6.2.4.4.1 Detection of the antiplatelet effect of aspirin and the $P2Y_{12}$ antagonist cangrelor on AA-and ADP-induced platelet aggregation and platelet-leucocyte conjugate formation:

Effects of aspirin

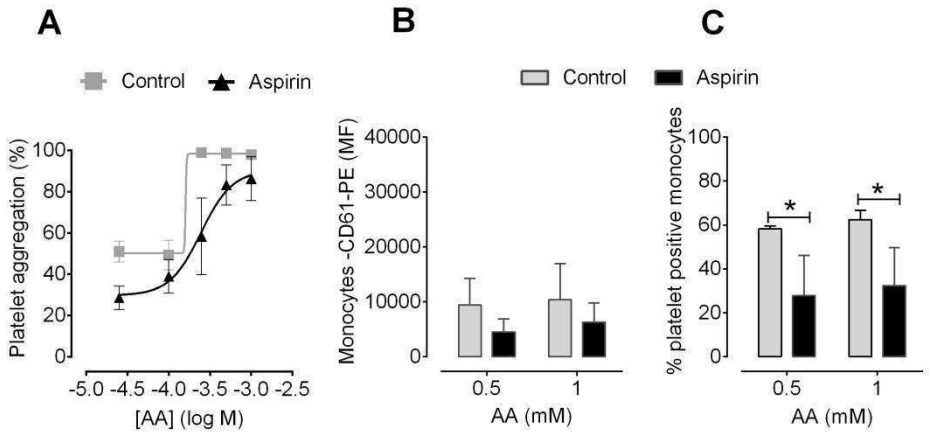

Figure 6-9: Effects of aspirin on platelet aggregation and platelet-leucocyte conjugate (monocyte) formation.

Platelet aggregation (A) induced by a range of AA concentrations (0.03 - 1mM) analysed using non-linear regression curve. Platelet-monocyte (PM) conjugate formation based monocyte median fluorescence (M-MF) (B) and %PM (C) induced by 0.5 and 1mM concentrations of AA and the effects of aspirin (100µM). Results are expressed as mean ± SD, (n=3) vs. EDTA.* $P<0.05$, repeated measures two-way ANOVA followed by Bonferroni's post-test.

In the 96-well plate assay, it was perceived that inhibition of platelet aggregation is dependent on the concentration used, with low response to high concentration of AA 0.5 and 1mM, as demonstrated before. In contrast, aspirin

demonstrated good inhibition of PLCs formation in blood stimulated with maximal and submaximal AA.

Effects of cangrelor

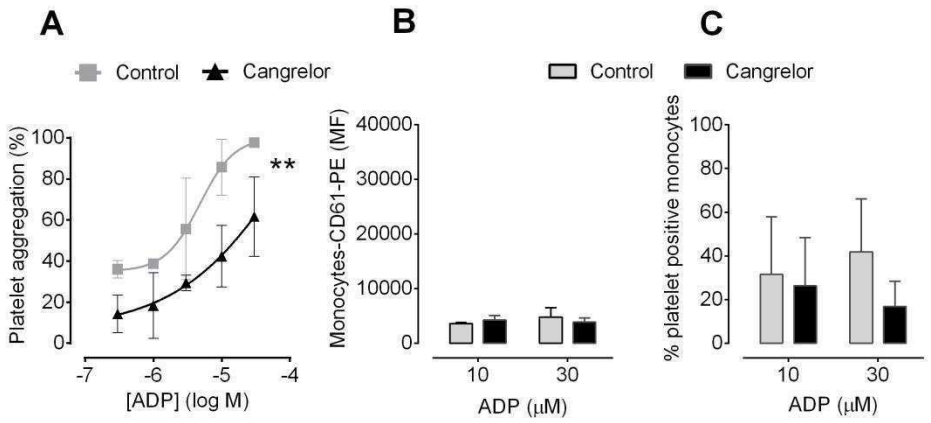

Figure 6-10: Effects of cangrelor on platelet aggregation and platelet-leucocyte conjugate (monocytes) formation.

Platelet aggregation (A) induced by a range of ADP concentrations (0.3-30μM) and analysed using non-linear regression curve. Platelet-monocyte (PM) formation based on monocyte median fluorescence (M-MF) (B) and %P/M (C) induced by 10 and 30μM concentrations of ADP and the effects of cangrelor (1000nM). Results are expressed as mean ± SD, (n=3) vs. EDTA. * $P<0.05$, repeated measures two-way ANOVA followed by Bonferroni's post-test.

Cangrelor inhibited platelet aggregation induced by ADP. However, minimal inhibition of PLC formation was observed.

6.3 Discussion

The triggering method seems to be the most desirable for studying platelet PLCs formation using WB and less sample manipulation. The study conducted by (Li et al., 1997) indicated that RBC lysis, with continuous washing and centrifugation, results in a 3-5 fold increase in PLCs formation. On these grounds, it was suggested that the triggering method may reflect in vivo PLCs formation as closely as possible. It has been known that ADP, collagen and TRAP activate platelets by different receptor-mediated pathways (Takano et al., 2004). The ability to produce PLCs varied with platelet agonist potency. When collagen was used as a stimulant in the presence of MK-0852, it showed the production of a large number of conjugates. In this respect, previous studies by our group indicated the same findings, as well as with other stimulants, such as PAF (Zhao et al., 2003a).

In this chapter, a whole new blood flow cytometric method has been described, involving less manipulation of cells and adaptation of 96-well plates. This method used a fixative solution that allows the measurement of both aggregation and PLCs formation to be carried out simultaneously at a later stage. The method is simple, rapid and may be appropriate for studies of platelet aggregation and PLCs formation in vivo. Also, the effects of blocking platelet aggregation and PLCs formation, concomitantly, was examined in order to elucidate the extent of artefactual in vitro aggregate formation, as well

as to investigate the ligands and receptors which may be involved in aggregate formation induced by agonist stimulation.

The initial data obtained demonstrated that the triggering approach is suitable for studying PLCs formation, as it clearly shows good agreement with the standard lysing protocol – justifying its use for further development. 5 min of blood shaking was proven to be comparable to 10 min for detection of PLCs formation. This facilitates the use of the same blood for measuring PA and PLC formation.

Previous work has used 96-well plates to study either platelet aggregation or PLCs, with the use of a cell-fixing solution patented by our Platelet research group in Nottingham, which has been successfully used and favors the stability of the sample. Therefore, a longer time is available for measurements. The next work aimed to carry out both measurements, on the same fixed sample of blood, as a measure of validating the assay and to confirm the stability of the data, when performed at different time interval. The results, shown in Figure 6-6, demonstrated a high comparison and assure the stability of the measurement when performed at different time intervals. Then, sensitivity of the assay was examined by studying the effect of M-0852, which has been shown to cause incomplete inhibition of platelet aggregation, along with increased PLCs formation. This measurement was achieved from the same fixed blood. With incomplete inhibition of aggregation, we tended to observe a significant increase in PLCs formation. This confirmed that the apparent lack

of the inhibitory effect of MK-852 is due to a reduction in the number of single platelets, as they bind to leucocytes. Then, PLCs formation was inhibited with the specific agent, KPL-1. Additionally, we wanted to check if any PLC formation was taking place in the normal aggregation measurements, thereby potentially compromising measurements. The aggregation measurements generated indicated that the decrease in single platelet counting was, indeed, only caused by platelet–platelet aggregate formation.

GPIIb/IIIa antagonism with drug, such as tirofiban and abciximab, is administered concurrently with $P2Y_{12}$ antagonists, clopidogrel and aspirin, in some patients PCI (Shattil et al., 1997). Therefore, it was of importance to investigate the in vitro inhibition of platelet aggregation and PLCs simultaneously to study the effect of aspirin and/or cangrelor ± MK-0852, in order to assess the interaction between these antiplatelet agents. It has already been demonstrated that GPIIb/IIIa antagonists are less potent inhibitors of platelet micro-aggregation than macro-aggregation, and substantial micro-aggregation occurs at therapeutic levels of these antagonists, yielding up to 100% inhibition of macro-aggregation (Storey et al., 2001). The data obtained clearly indicated that additional inhibition occurred when either aspirin or cangrelor were combined with GPIIb/IIIa antagonism (results not shown). This was observed equally with respect to both AA- and ADP-induced platelet aggregation. Also, triple antiplatelet therapy, comprising aspirin and cangrelor ± MK-0852, has shown similar effects to either aspirin or cangrelor ± MK-0852 in inhibiting AA- and ADP-induced platelet aggregation. This finding concurs with other studies that have demonstrated the benefits of dual

antiplatelet therapy, such as GPIIb/IIIa and $P2Y_{12}$ antagonism (Judge et al., 2005). However, in this study, maximal concentrations of collagen and TRAP were used as it was found to be independent of TXA_2 formation, whereas ADP was secreted at the highest concentration of the agonist studied. The method used here to assess the effect of antiplatelet agents relied upon the measurement of a single loss, which is a sensitive technique allowing the detection of platelet micro-aggregates that may consist of only doublets of platelets. The lack of a complete effect of GPIIb/IIIa antagonists, when studied alone, is due to the existence of platelet-leucocytes aggregates (not detected by this technique), which can result in incomplete inhibition, as we have demonstrated previously. However, GPIIb/IIIa antagonists add to the inhibition observed with aspirin or cangrelor, when studied in combination, as this clearly removes distinct pathways involved in the amplification of platelet activation.

The broader application of GPIIb/IIIa antagonists in combination with $P2Y_{12}$ antagonists may have clinical benefits. For example, the results obtained from a previous trial (Valgimigli et al., 2012) showed that when the $P2Y_{12}$ antagonist, prasugrel, was administered with or without the GPIIb/IIIa antagonist, tirofiban, in ST-segment elevation acute myocardial infarction patients taking aspirin, platelet inhibition was suboptimal (as measured by ADP-induced aggregation). This effect lasted for at least two hours with prasugrel alone, whereas the addition of tirofiban led to a significantly higher degree of inhibition. These studies, combined with ours, indicate that GPIIb/IIIa antagonists are beneficial in inhibiting platelet function when used in combination with aspirin or $P2Y_{12}$ antagonists. The effect of these drugs alone,

or in combination, demonstrate that the use of combinations of antiplatelet agents, which block three discrete pathways involved in the amplification of platelet activation, leads to greater inhibition of platelet functions, such as aggregation and PLCs, thereby reducing inflammatory responses.

To conclude, the results presented in this chapter demonstrate the advantage of the triggering method for assessing PLCs formation, as it causes less harm to cells. The adaptation of the 96-well plate as a tool for investigating the effect of antiplatelet drugs on platelet aggregation and PLCs, concomitantly, from the same fixed WB has also been demonstrated.

7 General Discussion

7.1 General discussion

Platelets play a fundamental role in haemostasis, by sustaining the integrity of the blood vessel wall via physiological haemostatic plug formation, to stop bleeding when vascular injury occurs. However, hyperplatelet activity can develop in pathological thrombosis, and hence, the subtle regulation of platelet function in vivo is crucial to maintain the balance between platelet behaviour causing thrombosis and haemostasis. Platelet agonists interact with their receptors on the platelet plasma membrane to alter platelet function and trigger outside-inside signalling, which eventually leads to a conformational change of the platelet GPIIb/IIIa receptor and platelet aggregation, through bridging with fibrinogen. Platelets can also interact with leucocytes and this is facilitated via the interaction of P-selectin (CD62P) on the surface of an active platelet with its counter receptor PSGL-1, present on leucocytes (Dole et al., 2007, Harding et al., 2007). This interaction represents the link between inflammation and thrombosis, with the former counted as the main contributor to the formation of pathophysiological atherosclerotic plaques on blood vessel walls (Singh et al., 2002). Sequentially, atherosclerosis in the walls of coronary vessels, with resulting plaque rupture and thrombus formation, exemplify the main underlying events of ACS. Platelet involvement in the pathogenesis of ACS is well documented and has been established by the clinical benefit of antiplatelet drugs in ACS (Kumar and Cannon, 2009).

After platelets enter the active condition, they release the content of their α- and dense granules, such as ADP, ATP, PF4, coagulation factor V, and Von

Willebrand Factor (VWF). Furthermore, the liberation of phospholipid from the plasma membrane through the action of PLA_2 produces AA, which is then converted by the COX1 enzyme into TXA_2. These molecules bind with their receptors present on platelets and represent an appropriate drug target for interference in their platelet activation role. Such molecules are referred to as 'antiplatelet drugs' and have been used interchangeably to prevent cardiovascular diseases.

Aspirin (ASA) is a well-recognised non-steroidal anti-inflammatory drug (NSAID) used to cure inflammation, fever, pain and also to prevent platelet activation and aggregation. The latter effect inhibits thrombus formation, thereby providing cardio protection – the basis for the use of ASA in the prevention of myocardial infarctions. Low dose ASA (75 to 100 mg) yields an antithrombotic effect by irreversibly acetylating COX-1 and, thus, inhibiting platelet generation of TXA_2. Higher doses of ASA also inhibit COX-2, which blocks prostaglandin production, leading to analgesic and antipyretic effects (Antithrombotic Trialists et al., 2009, Patrono and Baigent, 2009). Platelet inhibition reduces the likelihood of coronary artery thrombosis, the pathogenic mechanism of acute ischaemic events.

ADP plays a critical role in thrombosis via the interaction of two purinergic receptors, $P2Y_1$ and $P2Y_{12}$ (Murugappa and Kunapuli, 2006), and the antagonism of these receptors results in the significant inhibition of ADP-mediated platelet function (Wijeyeratne and Heptinstall, 2011). Antagonism at

the $P2Y_{12}$ receptor has been presented to be more efficient in the clinical setting, due to the selective distribution of this receptor in comparison to that of $P2Y_1$. The $P2Y_{12}$ receptor plays an important role in providing continual ADP-induced aggregation and increasing the platelet response to other agonists (Silvain et al., 2014).

Numerous drugs have been presented to block the $P2Y_{12}$ receptor, which represents one of the main targets of antiplatelet therapy. Clopidogrel and ticlopidine are prodrugs that are commonly used to treat patients with cardiovascular diseases. However, in clinical practice, ticlopidine has been substituted with clopidogrel, as a result of its toxic effects and its role in causing neutropenia and TTP (Cattaneo, 2006). Cangrelor is a direct, fast-acting $P2Y_{12}$ receptor inhibitor, but is obtainable only in an intravenous format. It has mainly been used in the research setting, although Storey et al. (2002b) indicated that the addition of cangrelor to blood samples from patients on clopidogrel has resulted in nearly complete inhibition of platelet aggregation.

A new $P2Y_{12}$ antagonist, prasugrel, is characterised by its reliable and faster onset of action of platelet inhibition. These features are the cause for less MACE and higher bleeding episodes among ACS patients arranged for PCI, compared to clopidogrel, as exhibited in the TRITON TIMI 38 randomised clinical trial. Therefore, prasugrel appears to be a valid replacement for clopidogrel, although its use has been shown to be unsuccessful in about 30% of treated patients. Therefore, it would be important to measure the effect of the

various $P2Y_{12}$ antagonists, in order to be able to show which drugs are achieving better inhibition without causing bleeding (Bellemain-Appaix et al., 2010).

Collagen is another platelet activator and represents an influential inducer substrate of platelet adhesion, activation and aggregation. There are more than 20 forms of collagen in the human genome; fibrillar type I and III are the major components of the extracellular matrix (ECM) of blood vessels and have been the focus of attention. The network forming type IV collagen is the major form within the subendothelial basement membrane. A number of receptors exist on the platelet membrane which are known to interact with collagen – most remarkably, the immunoglobulin superfamily member GPVI, which is the main platelet collagen receptor to mediate activation and aggregation, in addition to α2β1 (Nieswandt and Watson, 2003, Farndale et al., 2004).

TRAP-6 is a synthetic peptide ligand of the thrombin-receptor PAR-1 family, and a member of the GPCRs found on platelets. Investigations have demonstrated that TRAP-6-induced platelet aggregation can be inhibited by antagonism of the $P2Y_{12}$ receptor in vitro and ex vivo, but not when ASA is present. This finding clearly shows that activated PAR-1 directly activates Gαq protein and enhances the release reaction. The released ADP enhances platelet aggregation primarily due to $P2Y_{12}$, even though inhibition at $P2Y_1$ by MRS2179 has little effect on platelet aggregation, since $P2Y_1$ receptor-linked Gαq is already in the active state. The influence of the antagonism has been

found to be reliant on the concentration of TRAP-6, through an inversely proportional relationship; hence, suggesting that the release ADP is of significant importance at a lower concentration of TRAP-6, which is in agreement with previous finding by Nylander et al. (2003).

Elevation or reduction of platelet function testing can reflect any abnormalities that may occur either through inheritance or are acquired. Decreased platelet function can resulted from an inherited defect of platelet function, such as in BS or GT syndrome, or as a result of the use of antiplatelet agents that are directed against specific receptors present on platelets, to decrease the risk of a heart attack or stroke (Harrison et al., 2011). In this regard, the majority of PFT is limited to the research setting, rather than the clinical setting, due to the deficiency of reliability and standardisation of the techniques involved. Therefore it was the aim of the work in this thesis to conduct a test which could provide an easy to use assay to assess platelet function – preferably near a patient, without the need for sophisticated instruments or experienced staff.

For decades, LTA was regarded by many researchers as the gold standard for measuring platelet function, as it is an informative technique with many benefits, such as indicating the dynamic information about platelet aggregation. However, it also has many shortcomings, such as the requirement for a large volume of WB and the multiple steps required to prepare PRP. Besides, it is labour-intensive, time consuming and there is variation in the performance of LTA between different laboratories (Jennings et al., 2008, Cattaneo et al.,

2009b). These obstacles have restricted the value of using this assay in the clinical setting.

In order to circumvent these issues, new technologies have been developed, such as Multiple Electrode Aggregometry (MEA, Multiplate®, Roche Diagnostics International Ltd, Rotkreuz, Switzerland) and the VerifyNow® system (Accumetrics, San Diego, CA). These technologies are much simpler and are less time- and labour-consuming, compared to LTA; however, they utilise extremely costly consumables to conduct the tests, which makes them an unfavourable alternative. Another avenue that indicates the importance of PFT is when assessing the effect of a new drug on platelets, which requires a simple, non-expensive test kit – such as the one created by our research group, which is ideal and allows a sample to be processed within a convenient timeframe.

The overall aim of the research presented in this thesis was to develop a high throughput technique to measure platelet function testing, such as platelet adhesion, aggregation and PLCs. This mainly focused on the utility of the 96-well plate format as a tool, or vesicle, to incubate the blood sample and the platelet stimulants in small reaction wells. In addition, the utility of this format to measure platelet aggregation and PLCs formation from the same fixed WB was explored, together with the use of flow cytometry-based techniques, which have the advantage of using a small volume of WB and can cover multiple parameters of platelet function. Fixative solution was used throughout the

experiments performed in this thesis to indicate the suitability of it to stabilise the sample for longer than 2 hrs.

Our group at the University of Nottingham have invented a test kit to measure the activation state of platelets via the measurement of the activation marker P-Selectin (CD62P). This technique utilises a solution known as PAMFix (patent reference (PCT/GB2008/050169), which has been shown to stabilise the activation marker on platelets in WB for up to 9 days; recent studies have even achieved stability for as long as 28 days (Dovlatova et al., 2015). As previously demonstrated by our group (Fox et al., 2009), the measurement of platelet activation via the expression of the P-selectin marker on the platelet surface, in response to stimulation with a specific agonist, has been shown to offer a sensitive and robust means of estimating the level of platelet reactivity. Using this approach, the measurement of HPR was also shown to envisage the risk of recurrent thrombotic events in patients who developed ACS during treatment with the antiplatelet drug, clopidogrel (Thomas et al., 2014).

There is a wide range of techniques accessible to measure platelet function; however, these are constrained in that measurements have to be conducted on a fresh blood sample within 2-4 hours following venipuncture (Cattaneo et al., 2013). Consequently, this limits the testing of platelet function to a laboratory which is fully equipped with the appropriate instruments and has highly skilled personnel to perform the tests.

The first experiments (not presented) mainly focused on the study of platelet adhesion, which represents the initial event in haemostasis and is the most important physiological function of platelets. In addition, platelet adhesion may have an effect on plaque progression and stability in the development of atherosclerosis (Ruggeri, 2002). Therefore, early work focused on the development of an assay to investigate the measurement of platelet adhesion in fixed WB or PRP, using 96-well collagen-coated plates under static conditions; the influence of various platelet activators and inhibitors was also investigated. Collagen was chosen as the protein to which the platelets were to adhere as it represents up to 60% of all the proteins present in atherosclerotic plaques (Bou-Gharios et al., 2004). These experiments were unsuccessful when using WB, as the blood sediment in the base of the wells obscured the reading measurements. When PRP was used, different platelet counts were obtained, demonstrating an increase in platelet adhesion with increased platelet counts. This was used to establish the range at which platelet adhesion can be detected and the upper and lower limits.

In order to investigate the effect of MK-0852, a blocker of platelet aggregation, treatment of PRP with MK-0852 surprisingly resulted in a reduction in platelet adherence, which clearly demonstrated the existence of platelet adhesion and aggregation within the same well. However, the trend of an increase in adhered platelets was present. Several experiments were conducted to look at the effect of hydrogen sulphide (H_2S) on platelet adhesion, on the basis of its effect in

inhibiting platelet aggregation (Zagli et al., 2007), but the results showed no effect of H_2S on platelet adhesion.

To circumvent the limitation of this development, and the necessity to use a more physiological form of sample (i.e. WB), the concept of using polystyrene beads coated with collagen and fibrinogen or fibronectin appeared promising. This novel method uses flow cytometry and different protein-coated beads, such as collagen, fibrinogen and bovine serum albumin (BSA). The same approach has already been used to study P-selectin expression and up-regulation of fibrinogen receptors on adherent platelets (Kao et al., 2003). The data obtained by Tynngard et al. (2015) revealed that collagen beads showed moderate platelet adhesion, which was unaffected by platelet activation or inhibition. Fibrinogen beads also showed moderate platelet adhesion; however, in contrast to collagen, the adhesion increased significantly when platelets were activated by TRAP-6 or ADP and decreased when $P2Y_{12}$ or GPIIb/IIIa was inhibited. This finding demonstrates that fibrinogen is sensitive to platelet activation or inhibition in this assay. It would be of interest to investigate phosphotidyl serine (PS) exposure on platelets in response to platelet collagen binding, as demonstrated previously (Cauwenberghs et al., 2007). This would represent another application of the developed assay, in addition to adhesion and activation through the measurement of PS, which could be a further application in transfusion medicine to 1) study the exposure of PS on platelets and 2) to determine platelet viability during storage. Such studies have been undertaken by Albanyan et al. (2009), using probes, such as annexin V and

lactadhrin. Unfortunately platelet adhesion using beads was very challenging due to the difficulty in replicating the assay in Nottingham.

One of the major limitations of the available technique to assess platelet function is that testing has to be accomplished using a fresh blood sample within 2-4 hrs of blood collection (Cattaneo et al., 2013). Therefore the work presented in Chapter 3 aimed to assess the suitability of fixation techniques using two different approaches, DFF and SFU, as results indicate that there is good agreement between them. The utilisation of state of the art flow cytometry techniques to measure platelet aggregation, using the SPC technique on fixed blood samples, offers a huge advantage, as only a small volume of WB is required and there is good sensitivity. The fixative solution tested demonstrated good stability of the sample for up to 9 days. This method has been widely used by our research group, and in work generated from this thesis, and is based on a fall in the SPC, as platelets aggregated in stimulated and stirred whole blood (Fox et al., 2004b). The platelet count can be measured in a small blood volume, removed from a test tube, and then fixed at different time points, thereby providing in-depth information about the dynamics of platelet aggregation. This method is extremely sensitive to micro-aggregate formation and can provide information on platelet disaggregation. Another technique which relies on counting the decrease in single platelets, is the commercially available Plateletworks® (Helena Laboratories, Beaumont, TX), which is used to measure platelet aggregation in WB; although, this technique relies on impedance technology, which is less accurate than flow cytometry,

based counting and analysis which has to be performed immediately on a fresh, unfixed blood sample. Therefore, the flow cytometry method is superior, as the sample can be fixed and then shipped to a central laboratory, where sophisticated instruments and experienced staff are available. The measurement of platelet aggregation in WB using flow cytometry is highly advantageous and the stirring technique using a test tube can exhibit the kinetics of aggregation; however, the restriction of the minimal sample concentration to be used and, therefore, required to investigate a range of drug actions on platelets would require a very large volume of blood – thus, less information would be acquired.

The existing method for measuring platelet aggregation has many drawbacks, such as only being able to examine a few samples at any one time, leading to the requirement for a large volume of blood, together with local rules, such that generally, only a small number of different agonists can be tested at a limited range of concentrations. A broader range of agonists may be tested using a 96-well plate method, but this is also limited by the need to use freshly prepared agonists.

The adoption of the 96-well plate format offers a simple measure for the simultaneous assessment of several platelet activation pathways, using only a very small volume of WB, as previously demonstrated by Lordkipanidze et al. (2013). Using this novel assay, WB can be activated by a range of the most commonly used agonists, such as AA, ADP, collagen and TRAP, requiring just

1.2 ml of WB; hence making it attractive in cases where blood volumes are very limited. It would also be ideal for examining the effect of the most commonly used antiplatelet drugs or in new drug development. In our assay, flow cytometry was used and the mixing forces were based on shaking; however, platelet aggregation can only be measured at only one time point - 5 min.

Based on the previous results, the data presented in Chapter 4 demonstrated that the new assay, based on a 96-well format, can detect platelet inhibition by ASA and cangrelor in vitro, in blood stimulated with the most commonly used agonists. It also indicated the additive effect when a combination of ASA and cangrelor are used together. There was an additive effect when ASA was used in conjunction with cangrelor, leading to greater inhibition, specifically with AA- and ADP-induced platelet aggregation. Previous studies within our research group have demonstrated even further inhibition when triple antiplatelet therapy is utilised (Zhao et al., 2001). This reveals that combinations of three anti-platelet drugs, which act via different mechanisms, are more effective than single drugs or pairs of drugs in altering the activity of platelets (causing homo- or hetero-platelet aggregation). Ex vivo data has also demonstrated the suitability of this assay in detecting platelet inhibition in ACS patients and has clearly shown greater inhibition in patients taking prasugrel, compared to patients taking clopidogrel, upon stimulation with AA and ADP.

As the new assay relies on the use of fresh agonist, this may be responsible for variation in the results obtained. To overcome this effect, a 96-well plate with a lyophilised agonist was used, together with flow cytometry, and a dose-response curve was observed, which offers a greater agonist choice. In vitro results showed platelet inhibition with ASA and cangrelor, as well as in the ex vivo study, which demonstrated platelet inhibition in ACS patients. These findings clearly indicate the suitability of the Optimul plate as an alternative to the 96-well plate with freshly prepared agonist. A 96-well plate using lyophilised agonist is vulnerable to interpatch variation. The Optimul 96-well platelet aggregation assay has a high level of sensitivity, specificity for detecting platelet defects that result in bleeding. The requirement for a small volume of blood, and the uncomplicated nature and rapidity of the assay, make Optimul a favourable screening test in bleeding patients. To date, the adaptation of a 96-well plate using fresh or lyophilised agonist, and flow cytometry, has not been previously reported.

The final studies performed in Chapter 4 were aimed at blocking the final aggregation pathway using the antagonist of GPIIb/IIIa, an agent that interferes with the binding of fibrinogen to GPIIb/IIIa receptors. These drugs have been widely used as antithrombotic agents for the management of patients with ACS (Kereiakes et al., 1996, Kereiakes et al., 1997). For example, MK-0852 has demonstrated incomplete inhibition of platelet aggregation, when assessed using SPC, which could be due to a different environment to where platelet aggregation occurs. Previous work by our group using PRP has revealed complete platelet inhibition; it has also been demonstrated that incomplete inhibition in WB would not inhibit platelet activation and that there was an

increase in P-selectin expression (Klinkhardt et al., 2000, Matzdorff et al., 2000, Schneider et al., 2000), therefore resulting in more single active platelets. Platelets positive for P-selectin can adhere to the leucocyte PSGL-1 receptor, under WB conditions. Therefore, the formation of PLCs could lead to a fall in the SPC and lead to a low platelet count being described as a high level of aggregation, when measured by SPC techniques. In other words, platelet aggregation measured by SPC comprises o both platelet-platelet aggregates and platelet-leucocyte aggregates. In PRP there are no leucocytes available for activated single platelets to interact with and previous studies by our group have demonstrated better effectiveness of GPIIb/IIIa antagonism to inhibit platelet aggregation. In addition to the different environment in which testing is taking place, the type of test employed may reflect different findings, as previous work performed by our group using MEA – another technique to measure platelet aggregation – demonstrated complete inhibition by the antagonism of GPIIb/IIIa (unpublished data). This finding supports the notion that the SPC technique, using flow cytometry, could be used as a measure of micro-aggregation, whilst impedance through the use of MEA is a measure of macro-aggregation.

It is very important to hypothesize on the pathological process of GT, which presents with a GPIIb/IIIa deficiency and results in bleeding, with in vitro antagonism of the GPIIb/IIIa receptor with MK-0852. Platelet aggregation in response to different stimulants is absent in patients with GT because of the defect in the binding of platelets to fibrinogen (Nair et al., 2002, Michelson et

al., 2006). LTA is regarded as the gold standard technique for detecting patients with GT; however, it has many downsides which limit its use. Therefore, a reliable, fast and appropriate standardised method to test for platelet function for the diagnosis of GT would be of great importance. A new method that has lately become available is MEA (Dynabyte, Munich, Germany), which measures platelet aggregation in WB and may circumvent some of the disadvantages presented by LTA (Toth et al., 2006a). MEA is currently the focus of interest in antiplatelet therapy studies and two studies, by Awidi et al. (2009) and Albanyan et al. (2015), have shown its value in diagnosing patients with GT. It would have been interesting to investigate platelet aggregation in GT patients using 96-well plates and SPC in flow cytometry; however the limited availability of patients made this impossible. In addition, Burgess-Wilson et al. (1987) showed a normal aggregation pattern when platelet agonists were added to WB from patients with GT.

Although platelet aggregation is prevented, platelet activation was not; thus, P-selectin-positive single platelets are available to bind to leucocytes, specifically monocytes. If this in vitro observation were to occur in vivo, then this may account for the thrombocytopenia that sometimes occurs following the administration of GPIIb/IIIa antagonists to patients with vascular disease.

The last set of data demonstrates the value of the fixation approach to simultaneously assess both platelet aggregation and PLCs formation from the same fixed WB sample. The latter was performed with limited manipulation of the cells, as it involved the fluorescent triggering approach. GPIIb/IIIa

antagonism with MK-0852 markedly promoted platelet adhesion to monocytes, while adhesion of platelets to neutrophils, in response to all the agonists investigated, was not significantly increased. This may show that the monocytes had a competitive advantage over neutrophils for adhesion to the activated platelets in the experiments performed. This finding shows the failure of GPIIb/IIIa to prevent platelet activation. This is regarded as a major drawback of GPIIb/IIIa antagonism, as the intensity of thrombi is not inhibited and, accordingly, there is an increased formation of PLCs.

The success of GPIIb/IIIa antagonists in the clinical setting of acute ischaemic syndrome and PCI occurs when a combination of antiplatelet, anticoagulants or thrombolytic agents are used together (Berkowitz, 2000, Bertrand et al., 2000b). In these conditions, the use of these agents seems to be an effective regimen for counteracting the milieu of platelet activation, thrombin generation and fibrin formation in patients treated with GPIIb/IIIa antagonists. The in vitro data obtained confirmed that the combination of these three agents had an inhibitory effect on platelet aggregation and showed a reduction in PLCs.

To conclude**,** the existing platelet function tests have many disadvantages, such as in the VerifyNow® assay, which uses a single agonist concentration and, therefore, produces only a single numerical result. This cannot be probed and offers no opportunity to examine the dynamic range of platelet responses in any one sample. Therefore, this thesis has aimed to simplify the tests being

performed and to increase the amount of information obtained from a small volume of WB, which is more physiologically relevant.

Many discrepancies in platelet responses using a variety of agonists have been associated with the venipuncture technique: anticoagulants, temperature, pH, platelet count, posture, sex, race, genetics, smoking, and alcohol consumption (Taylor et al., 1992, Tiffany, 1983, O'Donnell et al., 2001). Sodium citrate is the most commonly used anti-coagulant for platelet activation studies (Schemitz et al., 1998) and it was used throughout the experiments performed in this thesis. Therefore the focus in Chapter 3 was to further extend the optimisation of platelet aggregation in WB and to compare the fixation methods used. The experiments conducted demonstrated the advantage of the fixation when using flow cytometry and was used to further the development process. In Chapter 4, a 96-well plate method was shown to be rapid, easy to perform and enable simultaneous measurement of a panel of platelet activation markers both in vitro and ex vivo. This was achieved through flow cytometric analysis of platelet activation and aggregation, with WB methods demonstrating several advantages (Michelson, 1996), including minimisation of artefacts and cell damage introduced by sample handling and separation procedures. A great body of experimental evidence reveals that the pathogenesis of thrombosis is multifactorial and includes interactions between blood cells and the vessel wall, as well as between different cells within the blood (Li et al., 1997). An important observation from the studies conducted is that MK-0852 markedly enhanced the number of platelets adhered to

leucocytes and this has led to the assessment of platelet aggregation and PLCs formation, simultaneously. The ex vivo study showed that clopidogrel reduces platelet aggregation and PLC formation in blood samples from ACS patients (Xiao and Theroux, 2004). Fixation solution (platelet solution) was used throughout the experiments, which has already been optimised to maintain platelet aggregate stability for up to 9 days and PLCs for up to 3 days. However, fixation has been implicated in increasing PLCs formation as a result of a reverse in fibrinogen binding (Janes et al., 1993) and an increase in platelet P-selectin expression (Cahill et al., 1993).

Future work arising from this thesis

- Comparison of the 96-well plate WB aggregation method to the gold standard LTA could be beneficial
- Comparison of the 96-well plate WB aggregation method to the MEA method to study the effect of MK-852
- Studying effect of QSM produced by Pseudomonas aeruginosa on PM or PN conjugate formation
- Platelet adhesion, using collagen coated beads, could be tested again as there is potential for their use in the 96-well plate format
- Checking the usefulness of the 96-well WB aggregation method to study platelet aggregation in GT patients

8 References

ABBATE, R., CIONI, G., RICCI, I., MIRANDA, M. & GORI, A. M. 2012. Thrombosis and acute coronary syndrome. Thromb Res, 129**,** 235-40.

ABRAMOVITZ, M., ADAM, M., BOIE, Y., CARRIERE, M. C., DENIS, D., GODBOUT, C., LAMONTAGNE, S., ROCHETTE, C., SAWYER, N., TREMBLAY, N. M., BELLEY, M., GALLANT, M., DUFRESNE, C., GAREAU, Y., RUEL, R., JUTEAU, H., LABELLE, M., OUIMET, N. & METTERS, K. M. 2000. The utilization of recombinant prostanoid receptors to determine the affinities and selectivities of prostaglandins and related analogs. Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids, 1483**,** 285-293.

AHMED, A. B., LATIMER, R. D. & VUYLSTEKE, A. 2000. Cardiovascular pharmacology: new drugs and new indications. Curr Opin Anaesthesiol, 13**,** 5-13.

ALBANYAN, A., AL-MUSA, A., ALNOUNOU, R., AL ZAHRANI, H., NASR, R., ALJEFRI, A., SALEH, M., MALIK, A., MASMALI, H. & OWAIDAH, T. 2015. Diagnosis of Glanzmann thrombasthenia by whole blood impedance analyzer (MEA) vs. light transmission aggregometry. Int J Lab Hematol, 37**,** 503-8.

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.

ALDERSON, M. R., ARMITAGE, R. J., TOUGH, T. W., STROCKBINE, L., FANSLOW, W. C. & SPRIGGS, M. K. 1993. CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. J Exp Med, 178**,** 669-74.

ALI, F. Y., ARMSTRONG, P. C. J., DHANJI, A. R. A., TUCKER, A. T., PAUL-CLARK, M. J., MITCHELL, J. A. & WARNER, T. D. 2009. Antiplatelet Actions of Statins and Fibrates Are Mediated by PPARs. Arteriosclerosis Thrombosis and Vascular Biology, 29**,** 706-711.

ALMANASEER, Y. & DESAI, D. M. 2006. Clopidogrel for the prevention of atherothrombotic events. N Engl J Med, 355**,** 420; author reply 420-1.

ANG, L. & MAHMUD, E. 2008. Monitoring oral antiplatelet therapy: is it justified? Ther Adv Cardiovasc Dis, 2**,** 485-96.

ANTITHROMBOTIC TRIALISTS, C. 2002. Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients. BMJ, 324**,** 71-86.

ANTITHROMBOTIC TRIALISTS, C., BAIGENT, C., BLACKWELL, L., COLLINS, R., EMBERSON, J., GODWIN, J., PETO, R., BURING, J., HENNEKENS, C., KEARNEY, P., MEADE, T., PATRONO, C., RONCAGLIONI, M. C. & ZANCHETTI, A. 2009. Aspirin in the primary and secondary prevention of vascular disease: collaborative meta-analysis of individual participant data from randomised trials. Lancet, 373**,** 1849-60.

ARADI, D., KOMOCSI, A., VOROBCSUK, A., RIDEG, O., TOKES-FUZESI, M., MAGYARLAKI, T., HORVATH, I. G. & SEREBRUANY, V.

L. 2010. Prognostic significance of high on-clopidogrel platelet reactivity after percutaneous coronary intervention: systematic review and meta-analysis. Am Heart J, 160**,** 543-51.

ARMSTRONG, P. C., TRUSS, N. J., ALI, F. Y., DHANJI, A. A., VOJNOVIC, I., ZAIN, Z. N., BISHOP-BAILEY, D., PAUL-CLARK, M. J., TUCKER, A. T., MITCHELL, J. A. & WARNER, T. D. 2008. Aspirin and the in vitro linear relationship between thromboxane A2-mediated platelet aggregation and platelet production of thromboxane A2. J Thromb Haemost, 6**,** 1933-43.

ARMSTRONG, P. C. J., DHANJI, A. R., TRUSS, N. J., ZAIN, Z. N. M., TUCKER, A. T., MITCHELL, J. A. & WARNER, T. D. 2009a. Utility of 96-well plate aggregometry and measurement of thrombi adhesion to determine aspirin and clopidogrel effectiveness. Thromb Haemost, 102**,** 772-778.

ARMSTRONG, P. C. J., DHANJI, A. R., TRUSS, N. J., ZAIN, Z. N. M., TUCKER, A. T., MITCHELL, J. A. & WARNER, T. D. 2009b. Utility of 96-well plate aggregometry and measurement of thrombi adhesion to determine aspirin and clopidogrel effectiveness. Thrombosis and Haemostasis, 102**,** 772-778.

ATAR, O. D., EISERT, C., POKOV, I. & SEREBRUANY, V. L. 2010. Stability validation of paraformaldehyde-fixed samples for the assessment of the platelet PECAM-1, P-selectin, and PAR-1 thrombin receptor by flow cytometry. J Thromb Thrombolysis, 30**,** 79-83.

ATKINSON, B., DWYER, K., ENJYOJI, K. & ROBSON, S. C. 2006. Ecto-nucleotidases of the CD39/NTPDase family modulate platelet activation and thrombus formation: Potential as therapeutic targets. Blood Cells Mol Dis, 36**,** 217-22.

AULT, K. A., CANNON, C. P., MITCHELL, J., MCCAHAN, J., TRACY, R. P., NOVOTNY, W. F., REIMANN, J. D. & BRAUNWALD, E. 1999. Platelet activation in patients after an acute coronary syndrome: Results from the TIMI-12 trial. Journal of the American College of Cardiology, 33**,** 634-639.

AWIDI, A., MAQABLAH, A., DWEIK, M., BSOUL, N. & ABU-KHADER, A. 2009. Comparison of platelet aggregation using light transmission and multiple electrode aggregometry in Glanzmann thrombasthenia. Platelets, 20**,** 297-301.

AZAM, S. M. & JOZIC, J. 2009. Variable platelet responsiveness to aspirin and clopidogrel: role of platelet function and genetic polymorphism testing. Transl Res, 154**,** 309-13.

BACUS, J. W., WATT, S. & TROBAUGH, F. E., JR. 1980. Clinical evaluation of a new electrical impedance instrument for counting platelets in whole blood. Am J Clin Pathol, 73**,** 655-63.

BARNARD, M. R., KRUEGER, L. A., FRELINGER, A. L., 3RD, FURMAN, M. I. & MICHELSON, A. D. 2003. Whole blood analysis of leukocyte-platelet aggregates. Curr Protoc Cytom, Chapter 6**,** Unit 6 15.

BARRAGRY, J. M., FRANCE, M. W., BOUCHER, B. J. & COHEN, R. D. 1979. Metabolism of intravenously administered cholecalciferol in man. Clin Endocrinol (Oxf), 11**,** 491-5.

BARTLETT, G. R. 1977. Metabolism by man of intravenously administered adenine. Transfusion, 17**,** 367-73.

BATAILLE, R. & KLEIN, B. 1992. C-Reactive Protein-Levels as a Direct Indicator of Interleukin-6 Levels in Humans Invivo. Arthritis and Rheumatism, 35**,** 982-983.

BAURAND, A., ECKLY, A., HECHLER, B., KAUFFENSTEIN, G., GALZI, J. L., CAZENAVE, J. P., LEON, C. & GACHET, C. 2005. Differential regulation and relocalization of the platelet P2Y receptors after activation: a way to avoid loss of hemostatic properties? Mol Pharmacol, 67**,** 721-33.

BELLAVITE, P., ANDRIOLI, G., GUZZO, P., ARIGLIANO, P., CHIRUMBOLO, S., MANZATO, F. & SANTONASTASO, C. 1994. A colorimetric method for the measurement of platelet adhesion in microtiter plates. Anal Biochem, 216**,** 444-50.

BELLEMAIN-APPAIX, A., BRIEGER, D., BEYGUI, F., SILVAIN, J., PENA, A., CAYLA, G., BARTHELEMY, O., COLLET, J. P. & MONTALESCOT, G. 2010. New P2Y12 inhibitors versus clopidogrel in percutaneous coronary intervention: a meta-analysis. J Am Coll Cardiol, 56**,** 1542-51.

BENOIT, P. & DOGNE, J. M. 2003. Platelet ADP receptors and their antagonists. Mini Rev Med Chem, 3**,** 145-8.

BERKOWITZ, S. D. 2000. Current knowledge of the platelet glycoprotein IIb/IIIa receptor antagonists for the treatment of coronary artery disease. Haemostasis, 30 Suppl 3**,** 27-43.

BERKOWITZ, S. D., FRELINGER, A. L., 3RD & HILLMAN, R. S. 1998. Progress in point-of-care laboratory testing for assessing platelet function. Am Heart J, 136**,** S51-65.

BERTRAND, M. E., RUPPRECHT, H. J., URBAN, P., GERSHLICK, A. H. & INVESTIGATORS, C. 2000a. Double-blind study of the safety of clopidogrel with and without a loading dose in combination with aspirin compared with ticlopidine in combination with aspirin after coronary stenting : the clopidogrel aspirin stent international cooperative study (CLASSICS). Circulation, 102**,** 624-9.

BERTRAND, M. E., SIMOONS, M. L., FOX, K. A., WALLENTIN, L. C., HAMM, C. W., MCFADDEN, E., DE FEYTER, P. J., SPECCHIA, G. & RUZYLLO, W. 2000b. Management of acute coronary syndromes: acute coronary syndromes without persistent ST segment elevation; recommendations of the Task Force of the European Society of Cardiology. Eur Heart J, 21**,** 1406-32.

BEVAN, J. & HEPTINSTALL, S. 1985. Serotonin-induced platelet aggregation in whole blood and the effects of ketanserin and mepyramine. Thromb Res, 38**,** 189-94.

BHATT, D. L., STONE, G. W., MAHAFFEY, K. W., GIBSON, C. M., STEG, P. G., HAMM, C. W., PRICE, M. J., LEONARDI, S., GALLUP, D.,

BRAMUCCI, E., RADKE, P. W., WIDIMSKY, P., TOUSEK, F., TAUTH, J., SPRIGGS, D., MCLAURIN, B. T., ANGIOLILLO, D. J., GENEREUX, P., LIU, T., PRATS, J., TODD, M., SKERJANEC, S., WHITE, H. D., HARRINGTON, R. A. & INVESTIGATORS, C. P. 2013. Effect of platelet inhibition with cangrelor during PCI on ischemic events. N Engl J Med, 368**,** 1303-13.

BIASUCCI, L. M., VITELLI, A., LIUZZO, G., ALTAMURA, S., CALIGIURI, G., MONACO, C., REBUZZI, A. G., CILIBERTO, G. & MASERI, A. 1996. Elevated levels of interleukin-6 in unstable angina. Circulation, 94**,** 874-7.

BLANN, A. D., MOHAN, J. S., BAREFORD, D. & LIP, G. Y. 2008. Soluble P-selectin and vascular endothelial growth factor in steady state sickle cell disease: relationship to genotype. J Thromb Thrombolysis, 25**,** 185-9.

BLANN, A. D., NADAR, S. K. & LIP, G. Y. 2003. The adhesion molecule P-selectin and cardiovascular disease. Eur Heart J, 24**,** 2166-79.

BOIE, Y., RUSHMORE, T. H., DARMON-GOODWIN, A., GRYGORCZYK, R., SLIPETZ, D. M., METTERS, K. M. & ABRAMOVITZ, M. 1994. Cloning and expression of a cDNA for the human prostanoid IP receptor. J Biol Chem, 269**,** 12173-8.

BORN, G. V. 1962. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature, 194**,** 927-9.

BOU-GHARIOS, G., PONTICOS, M., RAJKUMAR, V. & ABRAHAM, D. 2004. Extra-cellular matrix in vascular networks. Cell Prolif, 37**,** 207-20.

BOUMAN, H. J., VAN WERKUM, J. W., BREET, N. J., TEN CATE, H., HACKENG, C. M. & TEN BERG, J. M. 2011. A case-control study on platelet reactivity in patients with coronary stent thrombosis. J Thromb Haemost, 9**,** 909-16.

BOUMAN, H. J., VAN WERKUM, J. W., HACKENG, C. M., CLAPPERS, N. & TEN BERG, J. M. 2009. Cangrelor increases the magnitude of platelet inhibition and reduces interindividual variability in clopidogrel-pretreated subjects. Netherlands Heart Journal, 17**,** 195-198.

BOURNAZOS, S., RENNIE, J., HART, S. P., FOX, K. A. & DRANSFIELD, I. 2008. Monocyte functional responsiveness after PSGL-1-mediated platelet adhesion is dependent on platelet activation status. Arterioscler Thromb Vasc Biol, 28**,** 1491-8.

BRANDT, J. T., CLOSE, S. L., ITURRIA, S. J., PAYNE, C. D., FARID, N. A., ERNEST, C. S., 2ND, LACHNO, D. R., SALAZAR, D. & WINTERS, K. J. 2007a. Common polymorphisms of CYP2C19 and CYP2C9 affect the pharmacokinetic and pharmacodynamic response to clopidogrel but not prasugrel. J Thromb Haemost, 5**,** 2429-36.

BRANDT, J. T., PAYNE, C. D., WIVIOTT, S. D., WEERAKKODY, G., FARID, N. A., SMALL, D. S., JAKUBOWSKI, J. A., NAGANUMA, H. & WINTERS, K. J. 2007b. A comparison of prasugrel and clopidogrel loading doses on platelet function: magnitude of platelet inhibition is related to active metabolite formation. Am Heart J, 153**,** 66 e9-16.

BROOS, K., DE MEYER, S. F., FEYS, H. B., VANHOORELBEKE, K. & DECKMYN, H. 2011. Blood platelet biochemistry [Online]. Available: http://www.ncbi.nlm.nih.gov/pubmed/22119499.

BROWN, M. & WITTWER, C. 2000. Flow cytometry: principles and clinical applications in hematology. Clin Chem, 46**,** 1221-9.

BUJOLD, E., TAPP, S., AUDIBERT, F., FERREIRA, E., FOREST, J. C., REY, E., FRASER, W. D., CHAILLET, N. & GIGUERE, Y. 2011. Prevention of adverse pregnancy outcomes with low-dose ASA in early pregnancy: new perspectives for future randomized trials. J Obstet Gynaecol Can, 33**,** 480-3.

BURGESS-WILSON, M. E., COCKBILL, S. R., JOHNSTON, G. I. & HEPTINSTALL, S. 1987. Platelet aggregation in whole blood from patients with Glanzmann's thrombasthenia. Blood, 69**,** 38-42.

BURKE, J., KRAFT, W. K., GREENBERG, H. E., GLEAVE, M., PITARI, G. M., VANBUREN, S., WAGNER, J. A. & WALDMAN, S. A. 2003. Relationship of arachidonic acid concentration to cyclooxygenase-dependent human platelet aggregation. J Clin Pharmacol, 43**,** 983-9.

BUTCHERS, J., HUMPHREY, P. P. A., HYDE, J. J., LUMLEY, P. & SPURLING, N. W. 1980. The Evaluation of a New Electronic Counting Technique for Measurement of Platelet-Aggregation in Human Whole-Blood Invitro. British Journal of Pharmacology, 70**,** P160-P161.

CAHILL, M. R., MACEY, M. G. & NEWLAND, A. C. 1993. Fixation with formaldehyde induces expression of activation dependent platelet membrane glycoproteins, P selectin (CD62) and GP53 (CD63). Br J Haematol, 84**,** 527-9.

CAPTURE 1997. Randomised placebo-controlled trial of abciximab before and during coronary intervention in refractory unstable angina: the CAPTURE Study. Lancet, 349**,** 1429-35.

CARDINAL, D. C. & FLOWER, R. J. 1980. The electronic aggregometer: a novel device for assessing platelet behavior in blood. J Pharmacol Methods, 3**,** 135-58.

CATTANEO, M. 2004. Aspirin and clopidogrel: efficacy, safety, and the issue of drug resistance. Arterioscler Thromb Vasc Biol, 24**,** 1980-7.

CATTANEO, M. 2006. ADP receptors: inhibitory strategies for antiplatelet therapy. Timely Top Med Cardiovasc Dis, 10**,** E22.

CATTANEO, M. 2007. Laboratory detection of 'aspirin resistance': what test should we use (if any)? Eur Heart J, 28**,** 1673-5.

CATTANEO, M. 2011. The platelet P2Y receptor for adenosine diphosphate: congenital and drug-induced defects. Blood, 117**,** 2102-12.

CATTANEO, M., CERLETTI, C., HARRISON, P., HAYWARD, C. P., KENNY, D., NUGENT, D., NURDEN, P., RAO, A. K., SCHMAIER, A. H., WATSON, S. P., LUSSANA, F., PUGLIANO, M. T. & MICHELSON, A. D. 2013. Recommendations for the Standardization of Light Transmission Aggregometry: A Consensus of the Working Party from the Platelet Physiology Subcommittee of SSC/ISTH. J Thromb Haemost.

CATTANEO, M., HAYWARD, C. P., MOFFAT, K. A., PUGLIANO, M. T., LIU, Y. & MICHELSON, A. D. 2009a. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report

from the platelet physiology subcommittee of the SSC of the ISTH. J Thromb Haemost, 7**,** 1029.

CATTANEO, M., HAYWARD, C. P. M., MOFFAT, K. A., PUGLIANO, M. T., LIU, Y. & MICHELSON, A. D. 2009b. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. Journal of Thrombosis and Haemostasis, 7**,** 1029-1029.

CATTANEO, M. & LECCHI, A. 2007. Inhibition of the platelet P2Y12 receptor for adenosine diphosphate potentiates the antiplatelet effect of prostacyclin. J Thromb Haemost, 5**,** 577-82.

CATTANEO, M., LECCHI, A., ZIGHETTI, M. L. & LUSSANA, F. 2007. Platelet aggregation studies: autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. Haematologica, 92**,** 694-7.

CAUWENBERGHS, S., FEIJGE, M. A., THEUNISSEN, E., HEEMSKERK, J. W., VAN PAMPUS, E. C. & CURVERS, J. 2007. Novel methodology for assessment of prophylactic platelet transfusion therapy by measuring increased thrombus formation and thrombin generation. Br J Haematol, 136**,** 480-90.

CERLETTI, C., TAMBURRELLI, C., IZZI, B., GIANFAGNA, F. & DE GAETANO, G. 2012. Platelet-leukocyte interactions in thrombosis. Thromb Res, 129**,** 263-6.

CHAN, M. V., ARMSTRONG, P. C., PAPALIA, F., KIRKBY, N. S. & WARNER, T. D. 2011. Optical multichannel (optimul) platelet aggregometry

in 96-well plates as an additional method of platelet reactivity testing. Platelets, 22**,** 485-94.

CHEN, H. M., ZHANG, P., VOSO, M. T., HOHAUS, S., GONZALEZ, D. A., ZHANG, D. E. & TENEN, D. G. 1994. Neutrophils and Monocytes Express High-Levels of Pu.1 (Spi-1) but Not Spi-B. Blood, 84**,** A371-A371.

CHEN, W. H., LEE, P. Y., NG, W., TSE, H. F. & LAU, C. P. 2004. Aspirin resistance is associated with a high incidence of myonecrosis after non-urgent percutaneous coronary intervention despite clopidogrel pretreatment. J Am Coll Cardiol, 43**,** 1122-6.

CHOI, J. L., LI, S. & HAN, J. Y. 2014. Platelet function tests: a review of progresses in clinical application. Biomed Res Int, 2014**,** 456569.

CINES, D. B., POLLAK, E. S., BUCK, C. A., LOSCALZO, J., ZIMMERMAN, G. A., MCEVER, R. P., POBER, J. S., WICK, T. M., KONKLE, B. A., SCHWARTZ, B. S., BARNATHAN, E. S., MCCRAE, K. R., HUG, B. A., SCHMIDT, A. M. & STERN, D. M. 1998. Endothelial cells in physiology and in the pathophysiology of vascular disorders. Blood, 91**,** 3527-61.

COLEMAN, R. A., HUMPHREY, P. P. A., KENNEDY, I., LEVY, G. P. & LUMLEY, P. 1981. Comparison of the Actions of U-46619, a Prostaglandin H2-Analogue, with Those of Prostaglandin-H2 and Thromboxane-A2 on Some Isolated Smooth-Muscle Preparations. British Journal of Pharmacology, 73**,** 773-778.

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. A murine monoclonal antibody that completely blocks the binding of fibrinogen to platelets produces a thrombasthenic-like state in normal platelets and binds to glycoproteins IIb and/or IIIa. J Clin Invest, 72**,** 325-38.

COMMITTEE, C. S. 1996. A randomised, blinded, trial of clopidogrel versus aspirin in patients at risk of ischaemic events (CAPRIE). CAPRIE Steering Committee. Lancet, 348**,** 1329-39.

CORNELIA.A.K.VAN DER STELT, J. W. V. W., TOINE H. SEESING, JURRIEN M. TEN BERG, & CHRISTIAN M HACKENG 2007. To adjust or not to adjust the platelet count in light transmission aggregometery in patients recieving dual aspirin/clopidogrel treatment

Platelets**,** 550-553.

DAVI, G., ROMANO, M., MEZZETTI, A., PROCOPIO, A., IACOBELLI, S., ANTIDORMI, T., BUCCIARELLI, T., ALESSANDRINI, P., CUCCURULLO, F. & BITTOLO BON, G. 1998. Increased levels of soluble P-selectin in hypercholesterolemic patients. Circulation, 97**,** 953-7.

DE BRUIJNE-ADMIRAAL, L. G., MODDERMAN, P. W., VON DEM BORNE, A. E. & SONNENBERG, A. 1992. P-selectin mediates Ca(2+)-dependent adhesion of activated platelets to many different types of leukocytes: detection by flow cytometry. Blood, 80**,** 134-42.

DIENER, H. C., CUNHA, L., FORBES, C., SIVENIUS, J., SMETS, P. & LOWENTHAL, A. 1996. European Stroke Prevention Study. 2. Dipyridamole and acetylsalicylic acid in the secondary prevention of stroke. J Neurol Sci, 143**,** 1-13.

DOLE, V. S., BERGMEIER, W., PATTEN, I. S., HIRAHASHI, J., MAYADAS, T. N. & WAGNER, D. D. 2007. PSGL-1 regulates platelet P-selectin-mediated endothelial activation and shedding of P-selectin from activated platelets. Thromb Haemost, 98**,** 806-12.

DORSAM, R. T. & KUNAPULI, S. P. 2004. Central role of the P2Y12 receptor in platelet activation. J Clin Invest, 113**,** 340-5.

DOVLATOVA, N., ALGAHTANI, M., LORDKIPANIDZE, M., MAY, J., WATSON, S. P. & FOX, S. C. 2013. A 96 well plate-based whole blood assay assessing multiple platelet activation pathways appears promising in the evaluation of antiplatelet therapy. Journal of Thrombosis and Haemostasis, 11**,** 762-763.

DOVLATOVA, N., MAY, J. A. & FOX, S. C. 2015. Remote platelet function testing - Significant progress towards widespread testing in clinical practice. Platelets, 26**,** 399-401.

DYSZKIEWICZ-KORPANTY, A. M., FRENKEL, E. P. & SARODE, R. 2005. Approach to the assessment of platelet function: comparison between optical-based platelet-rich plasma and impedance-based whole blood platelet aggregation methods. Clin Appl Thromb Hemost, 11**,** 25-35.

ENTMAN, M. L. & BALLANTYNE, C. M. 1996. Association of neutrophils with platelet aggregates in unstable angina. Should we alter therapy? Circulation, 94**,** 1206-8.

ERIKSSON, A. C. & WHISS, P. A. 2005. Measurement of adhesion of human platelets in plasma to protein surfaces in microplates. J Pharmacol Toxicol Methods, 52**,** 356-65.

FARNDALE, R. W., SIXMA, J. J., BARNES, M. J. & DE GROOT, P. G. 2004. The role of collagen in thrombosis and hemostasis. J Thromb Haemost, 2**,** 561-73.

FERNANDES, L. S., CONDE, I. D., WAYNE SMITH, C., KANSAS, G. S., SNAPP, K. R., BENNET, N., BALLANTYNE, C., MCINTIRE, L. V., O'BRIAN SMITH, E., KLEM, J. A., MATHEW, S., FRANGOGIANNIS, N., TURNER, N. A., MARESH, K. J. & KLEIMAN, N. S. 2003. Platelet-monocyte complex formation: effect of blocking PSGL-1 alone, and in combination with alphaIIbbeta3 and alphaMbeta2, in coronary stenting. Thromb Res, 111**,** 171-7.

FITZGERALD, L. A. & PHILLIPS, D. R. 1985. Calcium regulation of the platelet membrane glycoprotein IIb-IIIa complex. J Biol Chem, 260**,** 11366-74.

FORSYTH, K. D. & LEVINSKY, R. J. 1990. Preparative procedures of cooling and re-warming increase leukocyte integrin expression and function on neutrophils. J Immunol Methods, 128**,** 159-63.

FOX, K. A., MEHTA, S. R., PETERS, R., ZHAO, F., LAKKIS, N., GERSH, B. J., YUSUF, S. & CLOPIDOGREL IN UNSTABLE ANGINA TO

PREVENT RECURRENT ISCHEMIC EVENTS, T. 2004a. Benefits and risks of the combination of clopidogrel and aspirin in patients undergoing surgical revascularization for non-ST-elevation acute coronary syndrome: the Clopidogrel in Unstable angina to prevent Recurrent ischemic Events (CURE) Trial. Circulation, 110**,** 1202-8.

FOX, S. C., BURGESS-WILSON, M., HEPTINSTALL, S. & MITCHELL, J. R. 1982. Platelet aggregation in whole blood determined using the Ultra-Flo 100 Platelet Counter. Thromb Haemost, 48**,** 327-9.

FOX, S. C., KILBY, M. D., SANDERSON, H. M. & HEPTINSTALL, S. 1994. Investigations on spontaneous aggregation and platelet responses to streptokinase and to adrenaline during pregnancy. Platelets, 5**,** 139-44.

FOX, S. C., MAY, J. A., JOHNSON, A., HERMANN, D., STRIETER, D., HARTMAN, D. & HEPTINSTALL, S. 2013. Effects on platelet function of an EP3 receptor antagonist used alone and in combination with a P2Y12 antagonist both in-vitro and ex-vivo in human volunteers. Platelets, 24**,** 392-400.

FOX, S. C., MAY, J. A., SHAH, A., NEUBERT, U. & HEPTINSTALL, S. 2009. Measurement of platelet P-selectin for remote testing of platelet function during treatment with clopidogrel and/or aspirin. Platelets, 20**,** 250-9.

FOX, S. C., SASAE, R., JANSON, S., MAY, J. A. & HEPTINSTALL, S. 2004b. Quantitation of platelet aggregation and microaggregate formation in whole blood by flow cytometry. Platelets, 15**,** 85-93.

FRANCHINI, M., LIPPI, G., VENERI, D., TARGHER, G., ZAFFANELLO, M. & GUIDI, G. C. 2008. Inherited platelet disorders. Clin Chim Acta, 387**,** 1-8.

FRATANTONI, J. C. & POINDEXTER, B. J. 1990. Measuring Platelet-Aggregation with Microplate Reader - a New Technical Approach to Platelet-Aggregation Studies. American Journal of Clinical Pathology, 94**,** 613-617.

FRELINGER, A. L., 3RD, JAKUBOWSKI, J. A., LI, Y., BARNARD, M. R., FOX, M. L., LINDEN, M. D., SUGIDACHI, A., WINTERS, K. J., FURMAN, M. I. & MICHELSON, A. D. 2007. The active metabolite of prasugrel inhibits ADP-stimulated thrombo-inflammatory markers of platelet activation: Influence of other blood cells, calcium, and aspirin. Thromb Haemost, 98**,** 192-200.

FURMAN, M. I., BARNARD, M. R., KRUEGER, L. A., FOX, M. L., SHILALE, E. A., LESSARD, D. M., MARCHESE, P., FRELINGER, A. L., 3RD, GOLDBERG, R. J. & MICHELSON, A. D. 2001. Circulating monocyte-platelet aggregates are an early marker of acute myocardial infarction. J Am Coll Cardiol, 38**,** 1002-6.

FURMAN, M. I., BENOIT, S. E., BARNARD, M. R., VALERI, C. R., BORBONE, M. L., BECKER, R. C., HECHTMAN, H. B. & MICHELSON, A. D. 1998. Increased platelet reactivity and circulating monocyte-platelet aggregates in patients with stable coronary artery disease. J Am Coll Cardiol, 31**,** 352-8.

GAMBLE, J. R., SKINNER, M. P., BERNDT, M. C. & VADAS, M. A. 1990. Prevention of activated neutrophil adhesion to endothelium by soluble adhesion protein GMP140. Science, 249**,** 414-7.

GAWAZ, M., NEUMANN, F. J., DICKFELD, T., KOCH, W., LAUGWITZ, K. L., ADELSBERGER, H., LANGENBRINK, K., PAGE, S., NEUMEIER, D., SCHOMIG, A. & BRAND, K. 1998. Activated platelets induce monocyte chemotactic protein-1 secretion and surface expression of intercellular adhesion molecule-1 on endothelial cells. Circulation, 98**,** 1164-71.

GENG, J. G., MOORE, K. L., JOHNSON, A. E. & MCEVER, R. P. 1991. Neutrophil recognition requires a Ca(2+)-induced conformational change in the lectin domain of GMP-140. J Biol Chem, 266**,** 22313-8.

GEORGE, J. N. 2000a. Platelets hematology, 335**,** 1531-39.

GEORGE, J. N. 2000b. Platelets. Lancet, 355**,** 1531-1539.

GEORGE, J. N., PICKETT, E. B., SAUCERMAN, S., MCEVER, R. P., KUNICKI, T. J., KIEFFER, N. & NEWMAN, P. J. 1986. Platelet surface glycoproteins. Studies on resting and activated platelets and platelet membrane microparticles in normal subjects, and observations in patients during adult respiratory distress syndrome and cardiac surgery. J Clin Invest, 78**,** 340-8.

GLADDING, P., WEBSTER, M., ORMISTON, J., OLSEN, S. & WHITE, H. 2008. Antiplatelet drug nonresponsiveness. Am Heart J, 155**,** 591-9.

GLENN, J. R., DOVLATOVA, N., WHITE, A. E., DHILLON, K., HEPTINSTALL, S. & FOX, S. C. 2014. 'VASPFix' for measurement of VASP

phosphorylation in platelets and for monitoring effects of P2Y(12) antagonists. Thrombosis and Haemostasis, 111**,** 539-548.

GOLDSMITH, H. L., BELL, D. N., BRAOVAC, S., STEINBERG, A. & MCINTOSH, F. 1995. Physical and chemical effects of red cells in the shear-induced aggregation of human platelets. Biophys J, 69**,** 1584-95.

GREENBERG, H. E., GOTTESDIENER, K., HUNTINGTON, M., WONG, P., LARSON, P., WILDONGER, L., GILLEN, L., DORVAL, E. & WALDMAN, S. A. 2000. A new cyclooxygenase-2 inhibitor, rofecoxib (VIOXX), did not alter the antiplatelet effects of low-dose aspirin in healthy volunteers. J Clin Pharmacol, 40**,** 1509-15.

GREENHALGH, J., BAGUST, A., BOLAND, A., MARTIN SABORIDO, C., OYEE, J., BLUNDELL, M., DUNDAR, Y., DICKSON, R., PROUDLOVE, C. & FISHER, M. 2011. Clopidogrel and modified-release dipyridamole for the prevention of occlusive vascular events (review of Technology Appraisal No. 90): a systematic review and economic analysis. Health Technol Assess, 15**,** 1-178.

GROSSER, T., FRIES, S. & FITZGERALD, G. A. 2006. Biological basis for the cardiovascular consequences of COX-2 inhibition: therapeutic challenges and opportunities. J Clin Invest, 116**,** 4-15.

GROUP, E. 1987. The European Stroke Prevention Study (ESPS). Principal end-points. The ESPS Group. Lancet, 2**,** 1351-4.

GRUNDMANN, K., JASCHONEK, K., KLEINE, B., DICHGANS, J. & TOPKA, H. 2003. Aspirin non-responder status in patients with recurrent cerebral ischemic attacks. J Neurol, 250**,** 63-6.

GUM, P. A., KOTTKE-MARCHANT, K., WELSH, P. A., WHITE, J. & TOPOL, E. J. 2003. A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. J Am Coll Cardiol, 41**,** 961-5.

GURBEL, P. A., BLIDEN, K. P., DICHIARA, J., NEWCOMER, J., WENG, W., NEERCHAL, N. K., GESHEFF, T., CHAGANTI, S. K., ETHERINGTON, A. & TANTRY, U. S. 2007. Evaluation of dose-related effects of aspirin on platelet function: results from the Aspirin-Induced Platelet Effect (ASPECT) study. Circulation, 115**,** 3156-64.

HAGBERG, I. A. & LYBERG, T. 2000a. Blood platelet activation evaluated by flow cytometry: optimised methods for clinical studies. Platelets, 11**,** 137-50.

HAGBERG, I. A. & LYBERG, T. 2000b. Evaluation of circulating platelet-leukocyte conjugates: a sensitive flow cytometric assay well suited for clinical studies. Platelets, 11**,** 151-60.

HAMM, C. W., BASSAND, J. P., AGEWALL, S., BAX, J., BOERSMA, E., BUENO, H., CASO, P., DUDEK, D., GIELEN, S., HUBER, K., OHMAN, M., PETRIE, M. C., SONNTAG, F., UVA, M. S., STOREY, R. F., WIJNS, W., ZAHGER, D. & EUROPEAN SOCIETY OF, C. 2012. [ESC guidelines for the management of acute coronary syndromes in patients presenting without

persistent ST-segment elevation. The Task Force for the management of acute coronary syndromes (ACS) in patients presenting without persistent ST-segment elevation of the European Society of Cardiology (ESC)]. G Ital Cardiol (Rome), 13**,** 171-228.

HANKEY, G. J. & EIKELBOOM, J. W. 2006. Aspirin resistance. Lancet, 367**,** 606-17.

HARDEN, T. K., SESMA, J. I., FRICKS, I. P. & LAZAROWSKI, E. R. 2010. Signalling and pharmacological properties of the P2Y receptor. Acta Physiol (Oxf), 199**,** 149-60.

HARDING, S. A., DIN, J. N., SARMA, J., JESSOP, A., WEATHERALL, M., FOX, K. A. & NEWBY, D. E. 2007. Flow cytometric analysis of circulating platelet-monocyte aggregates in whole blood: methodological considerations. Thromb Haemost, 98**,** 451-6.

HARDY, A. R., CONLEY, P. B., LUO, J., BENOVIC, J. L., POOLE, A. W. & MUNDELL, S. J. 2005. P2Y1 and P2Y12 receptors for ADP desensitize by distinct kinase-dependent mechanisms. Blood, 105**,** 3552-60.

HARRISON, P. 2005. Platelet function analysis. Blood Rev, 19**,** 111-23.

HARRISON, P., FRELINGER, A. L., 3RD, FURMAN, M. I. & MICHELSON, A. D. 2007. Measuring antiplatelet drug effects in the laboratory. Thromb Res, 120**,** 323-36.

HARRISON, P., MACKIE, I., MUMFORD, A., BRIGGS, C., LIESNER, R., WINTER, M., MACHIN, S. & BRITISH COMMITTEE FOR STANDARDS

IN, H. 2011. Guidelines for the laboratory investigation of heritable disorders of platelet function. Br J Haematol, 155**,** 30-44.

HARRISON, P., ROBINSON, M. S., MACKIE, I. J., JOSEPH, J., MCDONALD, S. J., LIESNER, R., SAVIDGE, G. F., PASI, J. & MACHIN, S. J. 1999. Performance of the platelet function analyser PFA-100 in testing abnormalities of primary haemostasis. Blood Coagul Fibrinolysis, 10**,** 25-31.

HAYWARD, C. P. 2011. Diagnostic evaluation of platelet function disorders. Blood Rev, 25**,** 169-73.

HAYWARD, C. P., HARRISON, P., CATTANEO, M., ORTEL, T. L., RAO, A. K., PLATELET PHYSIOLOGY SUBCOMMITTEE OF THE, S., STANDARDIZATION COMMITTEE OF THE INTERNATIONAL SOCIETY ON, T. & HAEMOSTASIS 2006. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. J Thromb Haemost, 4**,** 312-9.

HENN, V., SLUPSKY, J. R., GRAFE, M., ANAGNOSTOPOULOS, I., FORSTER, R., MULLER-BERGHAUS, G. & KROCZEK, R. A. 1998. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. Nature, 391**,** 591-4.

HEPTINSTALL, S., FOX, S., CRAWFORD, J. & HAWKINS, M. 1986. Inhibition of platelet aggregation in whole blood by dipyridamole and aspirin. Thromb Res, 42**,** 215-23.

HEPTINSTALL, S., JOHNSON, A., GLENN, J. R. & WHITE, A. E. 2005. Adenine nucleotide metabolism in human blood--important roles for leukocytes and erythrocytes. J Thromb Haemost, 3**,** 2331-9.

HEPTINSTALL, S., MAY, J., FOX, S., KWIK-URIBE, C. & ZHAO, L. 2006. Cocoa flavanols and platelet and leukocyte function: recent in vitro and ex vivo studies in healthy adults. J Cardiovasc Pharmacol, 47 Suppl 2**,** S197-205; discussion S206-9.

HEPTINSTALL, S., MAY, J. A., GLENN, J. R., SANDERSON, H. M., DICKINSON, J. P. & WILCOX, R. G. 1995. Effects of ticlopidine administered to healthy volunteers on platelet function in whole blood. Thromb Haemost, 74**,** 1310-5.

HERBERT, J. M. & SAVI, P. 2003. P2Y12, a new platelet ADP receptor, target of clopidogrel. Semin Vasc Med, 3**,** 113-22.

HERBERT, J. M., SAVI, P. & MAFFRAND, J. P. 1999. Biochemical and pharmacological properties of clopidogrel: a new ADP receptor antagonist. European Heart Journal, 20**,** A31-A40.

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**,** 202-7.

HOLM, A. & VIKSTROM, E. 2014. Quorum sensing communication between bacteria and human cells: signals, targets, and functions. Front Plant Sci, 5**,** 309.

HORSTRUP, K., JABLONKA, B., HONIG-LIEDL, P., JUST, M., KOCHSIEK, K. & WALTER, U. 1994. Phosphorylation of focal adhesion vasodilator-stimulated phosphoprotein at Ser157 in intact human platelets correlates with fibrinogen receptor inhibition. Eur J Biochem, 225**,** 21-7.

HOVE-MADSEN, L., PRAT-VIDAL, C., LLACH, A., CIRUELA, F., CASADO, V., LLUIS, C., BAYES-GENIS, A., CINCA, J. & FRANCO, R. 2006. Adenosine A2A receptors are expressed in human atrial myocytes and modulate spontaneous sarcoplasmic reticulum calcium release. Cardiovasc Res, 72**,** 292-302.

HU, H., DALESKOG, M. & LI, N. L. 2000. Influences of fixatives on flow cytometric measurements of platelet P-selectin expression and fibrinogen binding. Thrombosis Research, 100**,** 161-166.

HUA, C. T., GAMBLE, J. R., VADAS, M. A. & JACKSON, D. E. 1998. Recruitment and activation of SHP-1 protein-tyrosine phosphatase by human platelet endothelial cell adhesion molecule-1 (PECAM-1). Identification of immunoreceptor tyrosine-based inhibitory motif-like binding motifs and substrates. J Biol Chem, 273**,** 28332-40.

HUSTED, S., EMANUELSSON, H., HEPTINSTALL, S., SANDSET, P. M., WICKENS, M. & PETERS, G. 2006. Pharmacodynamics, pharmacokinetics, and safety of the oral reversible P2Y12 antagonist AZD6140 with aspirin in patients with atherosclerosis: a double-blind comparison to clopidogrel with aspirin. Eur Heart J, 27**,** 1038-47.

HUSTED, S. & VAN GIEZEN, J. J. 2009. Ticagrelor: the first reversibly binding oral P2Y12 receptor antagonist. Cardiovasc Ther, 27**,** 259-74.

INGALL, A. H., DIXON, J., BAILEY, A., COOMBS, M. E., COX, D., MCINALLY, J. I., HUNT, S. F., KINDON, N. D., TEOBALD, B. J., WILLIS, P. A., HUMPHRIES, R. G., LEFF, P., CLEGG, J. A., SMITH, J. A. & TOMLINSON, W. 1999. Antagonists of the platelet P2T receptor: a novel approach to antithrombotic therapy. J Med Chem, 42**,** 213-20.

IYU, D., GLENN, J. R., WHITE, A. E., FOX, S. C. & HEPTINSTALL, S. 2011. Adenosine derived from ADP can contribute to inhibition of platelet aggregation in the presence of a P2Y12 antagonist. Arterioscler Thromb Vasc Biol, 31**,** 416-22.

JAKUBOWSKI, J. A., LI, Y. G., SMALL, D. S., PAYNE, C. D., TOMLIN, M. E., LUO, J. & WINTERS, K. J. 2010. A comparison of the VerifyNow P2Y12 point-of-care device and light transmission aggregometry to monitor platelet function with prasugrel and clopidogrel: an integrated analysis. J Cardiovasc Pharmacol, 56**,** 29-37.

JAMES, S., AKERBLOM, A., CANNON, C. P., EMANUELSSON, H., HUSTED, S., KATUS, H., SKENE, A., STEG, P. G., STOREY, R. F., HARRINGTON, R., BECKER, R. & WALLENTIN, L. 2009. Comparison of ticagrelor, the first reversible oral P2Y(12) receptor antagonist, with clopidogrel in patients with acute coronary syndromes: Rationale, design, and baseline characteristics of the PLATelet inhibition and patient Outcomes (PLATO) trial. Am Heart J, 157**,** 599-605.

JANES, S. L., WILSON, D. J., CHRONOS, N. & GOODALL, A. H. 1993. Evaluation of whole blood flow cytometric detection of platelet bound fibrinogen on normal subjects and patients with activated platelets. Thromb Haemost, 70**,** 659-66.

JENNE, C. N., URRUTIA, R. & KUBES, P. 2013. Platelets: bridging hemostasis, inflammation, and immunity. Int J Lab Hematol, 35**,** 254-61.

JENNINGS, I., WOODS, T. A., KITCHEN, S. & WALKER, I. D. 2008. Platelet function testing: practice among UK National External Quality Assessment Scheme for Blood Coagulation participants, 2006. J Clin Pathol, 61**,** 950-4.

JENNINGS, L. K. 2009. Mechanisms of platelet activation: need for new strategies to protect against platelet-mediated atherothrombosis. Thromb Haemost, 102**,** 248-57.

JUDGE, H. M., BUCKLAND, R. J., HOLGATE, C. E. & STOREY, R. F. 2005. Glycoprotein IIb/IIIa and P2Y12 receptor antagonists yield additive inhibition of platelet aggregation, granule secretion, soluble CD40L release and procoagulant responses. Platelets, 16**,** 398-407.

JUNGI, T. W., SPYCHER, M. O., NYDEGGER, U. E. & BARANDUN, S. 1986. Platelet-leukocyte interaction: selective binding of thrombin-stimulated platelets to human monocytes, polymorphonuclear leukocytes, and related cell lines. Blood, 67**,** 629-36.

KABBANI, S. S., WATKINS, M. W., ASHIKAGA, T., TERRIEN, E. F., SOBEL, B. E. & SCHNEIDER, D. J. 2003. Usefulness of platelet reactivity

before percutaneous coronary intervention in determining cardiac risk one year later. Am J Cardiol, 91**,** 876-8.

KAO, S., TURNER, N. A., MOAKE, J. L. & MCINTIRE, L. V. 2003. A novel flow cytometric analysis for platelet activation on immobilized von Willebrand factor or fibrillar collagen. J Thromb Haemost, 1**,** 347-54.

KAPLAN, D. R., CHAO, F. C., STILES, C. D., ANTONIADES, H. N. & SCHER, C. D. 1979. Platelet alpha granules contain a growth factor for fibroblasts. Blood, 53**,** 1043-52.

KARLSSON, T., MUSSE, F., MAGNUSSON, K. E. & VIKSTROM, E. 2012. N-Acylhomoserine lactones are potent neutrophil chemoattractants that act via calcium mobilization and actin remodeling. J Leukoc Biol, 91**,** 15-26.

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**,** 1117-21.

KEREIAKES, D. J., KLEIMAN, N. S., AMBROSE, J., COHEN, M., RODRIGUEZ, S., PALABRICA, T., HERRMANN, H. C., SUTTON, J. M., WEAVER, W. D., MCKEE, D. B., FITZPATRICK, V. & SAX, F. L. 1996. Randomized, double-blind, placebo-controlled dose-ranging study of tirofiban (MK-383) platelet IIb/IIIa blockade in high risk patients undergoing coronary angioplasty. J Am Coll Cardiol, 27**,** 536-42.

KEREIAKES, D. J., LINCOFF, A. M., MILLER, D. P., TCHENG, J. E., CABOT, C. F., ANDERSON, K. M., WEISMAN, H. F., CALIFF, R. M. & TOPOL, E. J. 1998. Abciximab therapy and unplanned coronary stent deployment: favorable effects on stent use, clinical outcomes, and bleeding complications. EPILOG Trial Investigators. Circulation, 97**,** 857-64.

KEREIAKES, D. J., LORENZ, T., YOUNG, J. J., KUKIELKA, G., MUELLER, M. N., NANNIAZZI-ALAIMO, L. & PHILLIPS, D. R. 2001. Differential effects of citrate versus PPACK anticoagulation on measured platelet inhibition by abciximab, eptifibatide and tirofiban. J Thromb Thrombolysis, 12**,** 123-7.

KIRCHHOFER, D., RIEDERER, M. A. & BAUMGARTNER, H. R. 1997. Specific accumulation of circulating monocytes and polymorphonuclear leukocytes on platelet thrombi in a vascular injury model. Blood, 89**,** 1270-8.

KLING, D., STUCKI, C., KRONENBERG, S., TUERCK, D., RHEAUME, E., TARDIF, J. C., GAUDREAULT, J. & SCHMITT, C. 2013. Pharmacological control of platelet-leukocyte interactions by the human anti-P-selectin antibody inclacumab--preclinical and clinical studies. Thromb Res, 131**,** 401-10.

KLINKHARDT, U., BAUERSACHS, R., ADAMS, J., GRAFF, J., LINDHOFF-LAST, E. & HARDER, S. 2003. Clopidogrel but not aspirin reduces P-selectin expression and formation of platelet-leukocyte aggregates in patients with atherosclerotic vascular disease. Clinical Pharmacology & Therapeutics, 73**,** 232-241.

KLINKHARDT, U. & HARDER, S. 2005. Flow cytometric measurement of platelet-leukocyte aggregates: a possible target to monitor platelet function? Seminars in Thrombosis and Hemostasis, 31**,** 400-3.

KLINKHARDT, U., KIRCHMAIER, C. M., WESTRUP, D., BREDDIN, H. K., MAHNEL, R., GRAFF, J., HILD, M. & HARDER, S. 2000. Differential in vitro effects of the platelet glycoprotein IIb/IIIa inhibitors abixicimab or SR121566A on platelet aggregation, fibrinogen binding and platelet secretory parameters. Thromb Res, 97**,** 201-7.

KOH, T. W., DESOUZA, A. C. & PEPPER, J. R. 2004. The adhesion molecule P-selectin and cardiovascular disease--cardiac surgical implications. Eur Heart J, 25**,** 993.

KOIKE, J., NAGATA, K., KUDO, S., TSUJI, T. & IRIMURA, T. 2000. Density-dependent induction of TNF-alpha release from human monocytes by immobilized P-selectin. FEBS Lett, 477**,** 84-8.

KOLANSKY, D. M., KLUGHERZ, B. D., CURRAN, S. C., HERRMANN, H. C., MAGNESS, K., WILENSKY, R. L. & HIRSHFELD, J. W., JR. 2000. Combination therapy with clopidogrel and aspirin after coronary stenting. Catheter Cardiovasc Interv, 50**,** 276-9.

KONOPKA, A., SPYCHALSKA, J., PIOTROWSKI, W. & STEPINSKA, J. 2009. Influence of Some Cardiovascular Risk Factors on the Expression of Platelet Glycoprotein IIb/IIIa Receptors in Patients with Myocardial Infarction Treated with Antiplatelet Drugs under Primary Percutaneous Coronary Intervention. Molecular Diagnosis & Therapy, 13**,** 375-382.

KRATZER, M. A. 2006. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function: a rebuttal. J Thromb Haemost, 4**,** 1845-6.

KUMAR, A. & CANNON, C. P. 2009. Acute coronary syndromes: Diagnosis and management, part II. Mayo Clin Proc, 84**,** 1021-36.

LAGARDE, M. 1988. Metabolism of fatty acids by platelets and the functions of various metabolites in mediating platelet function. Prog Lipid Res, 27**,** 135-52.

LI, N., GOODALL, A. H. & HJEMDAHL, P. 1997. A sensitive flow cytometric assay for circulating platelet-leucocyte aggregates. Br J Haematol, 99**,** 808-16.

LI, N., HU, H., LINDQVIST, M., WIKSTROM-JONSSON, E., GOODALL, A. H. & HJEMDAHL, P. 2000. Platelet-leukocyte cross talk in whole blood. Arterioscler Thromb Vasc Biol, 20**,** 2702-8.

LINDEN, M. D., BARNARD, M. R., FRELINGER, A. L., MICHELSON, A. D. & PRZYKLENK, K. 2008. Effect of adenosine A2 receptor stimulation on platelet activation-aggregation: differences between canine and human models. Thromb Res, 121**,** 689-98.

LINDEN, M. D., FURMAN, M. I., FRELINGER, A. L., 3RD, FOX, M. L., BARNARD, M. R., LI, Y., PRZYKLENK, K. & MICHELSON, A. D. 2007. Indices of platelet activation and the stability of coronary artery disease. J Thromb Haemost, 5**,** 761-5.

LINDMARK, E., TENNO, T. & SIEGBAHN, A. 2000. Role of platelet P-selectin and CD40 ligand in the induction of monocytic tissue factor expression. Arterioscler Thromb Vasc Biol, 20**,** 2322-8.

LINNEMANN, B., SCHWONBERG, J., MANI, H., PROCHNOW, S. & LINDHOFF-LAST, E. 2008. Standardization of light transmittance aggregometry for monitoring antiplatelet therapy: an adjustment for platelet count is not necessary. J Thromb Haemost, 6**,** 677-83.

LIPPI, G., FRANCHINI, M. & GUIDI, G. C. 2007. Diagnostic approach to inherited bleeding disorders. Clin Chem Lab Med, 45**,** 2-12.

LITOSCH, I. 2002. Novel mechanisms for feedback regulation of phospholipase C-beta activity. IUBMB Life, 54**,** 253-60.

LITWACK, G. 1962. Effect of a pyrimido-pyrimidine derivative on non-specific enzymic stimulation in vivo. Biochim Biophys Acta, 56**,** 593-4.

LORDKIPANIDZE, M., DOVLATOVA, N., LOWE, G. C., WATSON, S. P. & FOX, S. C. 2013. Development of a novel high throughput 96 well plate-based whole blood assay for investigation of platelet function in healthy volunteers and patients with clinically diagnosed bleeding disorders. Journal of Thrombosis and Haemostasis, 11**,** 763-763.

LORDKIPANIDZE, M., PHARAND, C., SCHAMPAERT, E., TURGEON, J., PALISAITIS, D. A. & DIODATI, J. G. 2007. A comparison of six major platelet function tests to determine the prevalence of aspirin resistance in patients with stable coronary artery disease. Eur Heart J, 28**,** 1702-8.

LOSCALZO, J. 2001. Nitric oxide insufficiency, platelet activation, and arterial thrombosis. Circ Res, 88**,** 756-62.

MACEY, M., AZAM, U., MCCARTHY, D., WEBB, L., CHAPMAN, E. S., OKRONGLY, D., ZELMANOVIC, D. & NEWLAND, A. 2002. Evaluation of the anticoagulants EDTA and citrate, theophylline, adenosine, and dipyridamole (CTAD) for assessing platelet activation on the ADVIA 120 hematology system. Clin Chem, 48**,** 891-9.

MADAN, M., BERKOWITZ, S. D., CHRISTIE, D. J., SMIT, A. C., SIGMON, K. N. & TCHENG, J. E. 2002. Determination of platelet aggregation inhibition during percutaneous coronary intervention with the platelet function analyzer PFA-100. Am Heart J, 144**,** 151-8.

MAHAUT-SMITH, M. P., TOLHURST, G. & EVANS, R. J. 2004. Emerging roles for P2X1 receptors in platelet activation. Platelets, 15**,** 131-44.

MAHMUD, E. & ANG, L. 2006. Monitoring antiplatelet therapy during peripheral vascular and coronary interventions. Tech Vasc Interv Radiol, 9**,** 56-63.

MALMSJO, M., ERLINGE, D., HOGESTATT, E. D. & ZYGMUNT, P. M. 1999. Endothelial P2Y receptors induce hyperpolarisation of vascular smooth muscle by release of endothelium-derived hyperpolarising factor. Eur J Pharmacol, 364**,** 169-73.

MANI, H., KIRCHMAYR, K., KLAFFLING, C., SCHINDEWOLF, M., LUXEMBOURG, B., LINNEMANN, B. & LINDHOFF-LAST, E. 2004.

Influence of blood collection techniques on platelet function. Platelets, 15**,** 315-8.

MAREE, A. O., COX, D. & FITZGERALD, D. J. 2007. Drug insight: aspirin resistance-fact or fashion? Nat Clin Pract Cardiovasc Med, 4**,** E1; author reply E2.

MARZOT, F. & PENGO, V. 2009. Prasugrel for the treatment of patients with acute coronary syndrome. Vasc Health Risk Manag, 5**,** 321-4.

MASCELLI, M. A., LANCE, E. T., DAMARAJU, L., WAGNER, C. L., WEISMAN, H. F. & JORDAN, R. E. 1998. Pharmacodynamic profile of short-term abciximab treatment demonstrates prolonged platelet inhibition with gradual recovery from GP IIb/IIIa receptor blockade. Circulation, 97**,** 1680-8.

MASCELLI, M. A., WORLEY, S., VERIABO, N. J., LANCE, E. T., MACK, S., SCHAIBLE, T., WEISMAN, H. F. & JORDAN, R. E. 1997. Rapid assessment of platelet function with a modified whole-blood aggregometer in percutaneous transluminal coronary angioplasty patients receiving anti-GP IIb/IIIa therapy. Circulation, 96**,** 3860-6.

MATETZKY, S., SHENKMAN, B., GUETTA, V., SHECHTER, M., BEINART, R., GOLDENBERG, I., NOVIKOV, I., PRES, H., SAVION, N., VARON, D. & HOD, H. 2004. Clopidogrel resistance is associated with increased risk of recurrent atherothrombotic events in patients with acute myocardial infarction. Circulation, 109**,** 3171-5.

MATZDORFF, A. C., KUHNEL, G., KEMKES-MATTHES, B., PRALLE, H., VOSS, R. & FAREED, J. 2000. Effect of glycoprotein IIb/IIIa inhibitors on

CD62p expression, platelet aggregates, and microparticles in vitro. J Lab Clin Med, 135**,** 247-55.

MAY JA, H. S., FOX SC. 09/05/08. Stabilisation of blood cell conjugates. International patent application number PCT/GB2009/050482, University of Nottingham, priority date 09/05/08.

MENDELSOHN, M. E., O'NEILL, S., GEORGE, D. & LOSCALZO, J. 1990. Inhibition of fibrinogen binding to human platelets by S-nitroso-N-acetylcysteine. J Biol Chem, 265**,** 19028-34.

MERHI, Y., PROVOST, P., GUIDOIN, R. & LATOUR, J. G. 1997. Importance of platelets in neutrophil adhesion and vasoconstriction after deep carotid arterial injury by angioplasty in pigs. Arterioscler Thromb Vasc Biol, 17**,** 1185-91.

MEROLLA, M., NARDI, M. A. & BERGER, J. S. 2011. Centrifugation speed affects light transmission aggregometry. Int J Lab Hematol.

MERTEN, M. & THIAGARAJAN, P. 2000. P-selectin expression on platelets determines size and stability of platelet aggregates. Circulation, 102**,** 1931-6.

MICHELSON, A. D. 1996. Flow cytometry: A clinical test of platelet function. Blood, 87**,** 4925-4936.

MICHELSON, A. D. 2004. Platelet function testing in cardiovascular diseases. Circulation, 110**,** e489-93.

MICHELSON, A. D. 2005. Platelet function testing in cardiovascular diseases. Hematology, 10 Suppl 1**,** 132-7.

MICHELSON, A. D. 2006. Evaluation of platelet function by flow cytometry. Pathophysiol Haemost Thromb, 35**,** 67-82.

MICHELSON, A. D. 2009. New P2Y12 antagonists. Curr Opin Hematol, 16**,** 371-7.

MICHELSON, A. D., BARNARD, M. R., HECHTMAN, H. B., MACGREGOR, H., CONNOLLY, R. J., LOSCALZO, J. & VALERI, C. R. 1996a. In vivo tracking of platelets: circulating degranulated platelets rapidly lose surface P-selectin but continue to circulate and function. Proc Natl Acad Sci U S A, 93**,** 11877-82.

MICHELSON, A. D., BARNARD, M. R., KRUEGER, L. A., FRELINGER, A. L., 3RD & FURMAN, M. I. 2000. Evaluation of platelet function by flow cytometry. Methods, 21**,** 259-70.

MICHELSON, A. D., BARNARD, M. R., KRUEGER, L. A., VALERI, C. R. & FURMAN, M. I. 2001. Circulating monocyte-platelet aggregates are a more sensitive marker of in vivo platelet activation than platelet surface P-selectin: studies in baboons, human coronary intervention, and human acute myocardial infarction. Circulation, 104**,** 1533-7.

MICHELSON, A. D., BENOIT, S. E., FURMAN, M. I., BARNARD, M. R., NURDEN, P. & NURDEN, A. T. 1996b. The platelet surface expression of glycoprotein V is regulated by two independent mechanisms: proteolysis and a reversible cytoskeletal-mediated redistribution to the surface-connected canalicular system. Blood, 87**,** 1396-408.

MICHELSON, A. D., FRELINGER, A. L., 3RD & FURMAN, M. I. 2006. Current options in platelet function testing. Am J Cardiol, 98**,** 4N-10N.

MICHELSON, A. D. & NEWBURGER, P. E. 2007. Platelets and leukocytes: aggregate knowledge. Blood, 110**,** 794-795.

MODICA, A., KARLSSON, F. & MOOE, T. 2007. Platelet aggregation and aspirin non-responsiveness increase when an acute coronary syndrome is complicated by an infection. J Thromb Haemost, 5**,** 507-11.

MODY, M., LAZARUS, A. H., SEMPLE, J. W. & FREEDMAN, J. 1999. Preanalytical requirements for flow cytometric evaluation of platelet activation: choice of anticoagulant. Transfus Med, 9**,** 147-54.

MONCADA, S. & KORBUT, R. 1978. Dipyridamole and other phosphodiesterase inhibitors act as antithrombotic agents by potentiating endogenous prostacyclin. Lancet, 1**,** 1286-9.

MONCADA, S. & VANE, J. R. 1979. Arachidonic acid metabolites and the interactions between platelets and blood-vessel walls. N Engl J Med, 300**,** 1142-7.

MUELLER, M. R., SALAT, A., STANGL, P., MURABITO, M., PULAKI, S., BOEHM, D., KOPPENSTEINER, R., ERGUN, E., MITTLBOECK, M., SCHREINER, W., LOSERT, U. & WOLNER, E. 1997. Variable platelet response to low-dose ASA and the risk of limb deterioration in patients submitted to peripheral arterial angioplasty. Thromb Haemost, 78**,** 1003-7.

MUELLER, T., DIEPLINGER, B., POELZ, W. & HALTMAYER, M. 2009. Utility of the PFA-100 instrument and the novel multiplate analyzer for the

assessment of aspirin and clopidogrel effects on platelet function in patients with cardiovascular disease. Clin Appl Thromb Hemost, 15**,** 652-9.

MULLER, C., BUTTNER, H. J., PETERSEN, J. & ROSKAMM, H. 2000. A randomized comparison of clopidogrel and aspirin versus ticlopidine and aspirin after the placement of coronary-artery stents. Circulation, 101**,** 590-3.

MULLER, T. H., SU, C. A., WEISENBERGER, H., BRICKL, R., NEHMIZ, G. & EISERT, W. G. 1990. Dipyridamole alone or combined with low-dose acetylsalicylic acid inhibits platelet aggregation in human whole blood ex vivo. Br J Clin Pharmacol, 30**,** 179-86.

MUNDELL, S. J., BARTON, J. F., MAYO-MARTIN, M. B., HARDY, A. R. & POOLE, A. W. 2008. Rapid resensitization of purinergic receptor function in human platelets. J Thromb Haemost, 6**,** 1393-404.

MURUGAPPA, S. & KUNAPULI, S. P. 2006. The role of ADP receptors in platelet function. Front Biosci, 11**,** 1977-86.

NAIR, S., GHOSH, K., KULKARNI, B., SHETTY, S. & MOHANTY, D. 2002. Glanzmann's thrombasthenia: updated. Platelets, 13**,** 387-93.

NAWARSKAS, J. J. & CLARK, S. M. 2011. Ticagrelor: a novel reversible oral antiplatelet agent. Cardiol Rev, 19**,** 95-100.

NEUFELD, M., NOWAK-GOTTL, U. & JUNKER, R. 1999. Citrate-theophylline-adenine-dipyridamol buffer is preferable to citrate buffer as an anticoagulant for flow cytometric measurement of platelet activation. Clin Chem, 45**,** 2030-3.

NICHOLSON, N. S., PANZER-KNODLE, S. G., HAAS, N. F., TAITE, B. B., SZALONY, J. A., PAGE, J. D., FEIGEN, L. P., LANSKY, D. M. & SALYERS, A. K. 1998. Assessment of platelet function assays. Am Heart J, 135**,** S170-8.

NIESWANDT, B. & WATSON, S. P. 2003. Platelet-collagen interaction: is GPVI the central receptor? Blood, 102**,** 449-61.

NURDEN, P. 1997. Bidirectional trafficking of membrane glycoproteins following platelet activation in suspension. Thromb Haemost, 78**,** 1305-15.

NYLANDER, S., MATTSSON, C., RAMSTROM, S. & LINDAHL, T. L. 2003. The relative importance of the ADP receptors, P2Y12 and P2Y1, in thrombin-induced platelet activation. Thromb Res, 111**,** 65-73.

O'BRIEN, J. R. 1965. Effects of adenosine diphosphate and adrenaline on mean platelet shape. Nature, 207**,** 306-7.

O'BRIEN, J. R., SHOOBRIDGE, S. M. & FINCH, W. J. 1969. Comparison of the effect of heparin and citrate on platelet aggregation. J Clin Pathol, 22**,** 28-31.

O'DONNELL, C. J., LARSON, M. G., FENG, D., SUTHERLAND, P. A., LINDPAINTNER, K., MYERS, R. H., D'AGOSTINO, R. A., LEVY, D., TOFLER, G. H. & FRAMINGHAM HEART, S. 2001. Genetic and environmental contributions to platelet aggregation: the Framingham heart study. Circulation, 103**,** 3051-6.

OFFERMANNS, S. 2006. Activation of platelet function through G protein-coupled receptors. Circ Res, 99**,** 1293-304.

OFFERMANNS, S., LAUGWITZ, K. L., SPICHER, K. & SCHULTZ, G. 1994. G proteins of the G12 family are activated via thromboxane A2 and thrombin receptors in human platelets. Proc Natl Acad Sci U S A, 91**,** 504-8.

OURY, C., TOTH-ZSAMBOKI, E., THYS, C., TYTGAT, J., VERMYLEN, J. & HOYLAERTS, M. F. 2001. The ATP-gated P2X1 ion channel acts as a positive regulator of platelet responses to collagen. Thromb Haemost, 86**,** 1264-71.

PAKALA, R. & WAKSMAN, R. 2011. Currently available methods for platelet function analysis: advantages and disadvantages. Cardiovasc Revasc Med, 12**,** 312-22.

PALABRICA, T., LOBB, R., FURIE, B. C., ARONOVITZ, M., BENJAMIN, C., HSU, Y. M., SAJER, S. A. & FURIE, B. 1992. Leukocyte accumulation promoting fibrin deposition is mediated in vivo by P-selectin on adherent platelets. Nature, 359**,** 848-51.

PANICCIA, R., ANTONUCCI, E., MAGGINI, N., ROMANO, E., GORI, A. M., MARCUCCI, R., PRISCO, D. & ABBATE, R. 2009. Assessment of platelet function on whole blood by multiple electrode aggregometry in high-risk patients with coronary artery disease receiving antiplatelet therapy. Am J Clin Pathol, 131**,** 834-42.

PATRONO, C. & BAIGENT, C. 2009. Low-dose aspirin, coxibs, and other NSAIDS: a clinical mosaic emerges. Mol Interv, 9**,** 31-9.

PATRONO, C. & ROCCA, B. 2007. Drug insight: aspirin resistance--fact or fashion? Nat Clin Pract Cardiovasc Med, 4**,** 42-50.

PEACE, A. J., TEDESCO, A. F., FOLEY, D. P., DICKER, P., BERNDT, M. C. & KENNY, D. 2008. Dual antiplatelet therapy unmasks distinct platelet reactivity in patients with coronary artery disease. J Thromb Haemost, 6**,** 2027-34.

PEARSON, L., THOM, J., ADAMS, M., OOSTRYCK, R., KRUEGER, R., YONG, G. & BAKER, R. 2009. A rapid flow cytometric technique for the detection of platelet-monocyte complexes, activated platelets and platelet-derived microparticles. Int J Lab Hematol, 31**,** 430-9.

PENZ, S. M., REININGER, A. J., TOTH, O., DECKMYN, H., BRANDL, R. & SIESS, W. 2007. Glycoprotein Ib alpha inhibition and ADP receptor antagonists, but not aspirin, reduce platelet thrombus formation in flowing blood exposed to atherosclerotic plaques. Thrombosis and Haemostasis, 97**,** 435-443.

POMMER, M. S., ZHANG, Y., KEERTHI, N., CHEN, D., THOMSON, J. A., MEINHART, C. D. & SOH, H. T. 2008. Dielectrophoretic separation of platelets from diluted whole blood in microfluidic channels. Electrophoresis, 29**,** 1213-8.

PRICE, M. J., BERGER, P. B., TEIRSTEIN, P. S., TANGUAY, J. F., ANGIOLILLO, D. J., SPRIGGS, D., PURI, S., ROBBINS, M., GARRATT, K. N., BERTRAND, O. F., STILLABOWER, M. E., ARAGON, J. R., KANDZARI, D. E., STINIS, C. T., LEE, M. S., MANOUKIAN, S. V., CANNON, C. P., SCHORK, N. J., TOPOL, E. J. & INVESTIGATORS, G. 2011. Standard- vs high-dose clopidogrel based on platelet function testing

after percutaneous coronary intervention: the GRAVITAS randomized trial. JAMA, 305, 1097-105.

RALEVIC, V. & BURNSTOCK, G. 1998. Receptors for purines and pyrimidines. Pharmacol Rev, 50, 413-92.

REBELLO, S. S., HUANG, J., FAUL, J. D. & LUCCHESI, B. R. 2000. Role of extracellular ionized calcium in the in vitro assessment of GPIIb/IIIa receptor antagonists. J Thromb Thrombolysis, 9, 23-8.

REDLICH, H., VICKERS, J., LOSCHE, W., HEPTINSTALL, S., KEHREL, B. & SPANGENBERG, P. 1997. Formation of platelet-leukocyte conjugates in whole blood. Platelets, 8, 419-25.

RINDER, C. S., BOHNERT, J., RINDER, H. M., MITCHELL, J., AULT, K. & HILLMAN, R. 1991a. Platelet activation and aggregation during cardiopulmonary bypass. Anesthesiology, 75, 388-93.

RINDER, H. M., BONAN, J. L., RINDER, C. S., AULT, K. A. & SMITH, B. R. 1991b. Activated and unactivated platelet adhesion to monocytes and neutrophils. Blood, 78, 1760-9.

RODGERS, R. P. & LEVIN, J. 1990. A critical reappraisal of the bleeding time. Semin Thromb Hemost, 16, 1-20.

ROGERS, A. B. & DES PREZ, R. M. 1972. The effect of pH on human platelet aggregation induced by epinephrine and ADP. Proc Soc Exp Biol Med, 139, 1100-3.

ROTH, G. J. & MAJERUS, P. W. 1975. The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein. J Clin Invest, 56**,** 624-32.

ROUSSEL, M., BENARD, C., LY-SUNNARAM, B. & FEST, T. 2010. Refining the white blood cell differential: the first flow cytometry routine application. Cytometry A**,** 77**,** 552-63.

RUGGERI, Z. M. 2002. Platelets in atherothrombosis. Nat Med, 8**,** 1227-34.

SABOOR, M., MOINUDDIN, M. & ILYAS, S. 2013. New horizons in platelets flow cytometry. Malays J Med Sci, 20**,** 62-6.

SANDERSON, H. M., FOX, S. C., ROBBINS, R. A., LOSCHE, W., SPANGENBERG, P. & HEPTINSTALL, S. 1998. Role of GPIIb-IIIa in platelet-monocyte and platelet-neutrophil conjugate formation in whole blood. Platelets, 9**,** 245-250.

SANIABADI, A. R., LOWE, G. D., FORBES, C. D., PRENTICE, C. R. & BARBENEL, J. C. 1983. Platelet aggregation studies in whole human blood. Thromb Res, 30**,** 625-32.

SANIABADI, A. R., LOWE, G. D., MADHOK, R., SPOWART, K., SHAW, B., BARBENEL, J. C. & FORBES, C. D. 1987. Red blood cells mediate spontaneous aggregation of platelets in whole blood. Atherosclerosis, 66**,** 175-80.

SARMA, J., LAAN, C. A., ALAM, S., JHA, A., FOX, K. A. & DRANSFIELD, I. 2002. Increased platelet binding to circulating monocytes in acute coronary syndromes. Circulation, 105**,** 2166-71.

SAVI, P., PEREILLO, J. M., UZABIAGA, M. F., COMBALBERT, J., PICARD, C., MAFFRAND, J. P., PASCAL, M. & HERBERT, J. M. 2000. Identification and biological activity of the active metabolite of clopidogrel. Thromb Haemost, 84**,** 891-6.

SCARBOROUGH, R. M., KLEIMAN, N. S. & PHILLIPS, D. R. 1999. Platelet glycoprotein IIb/IIIa antagonists - What are the relevant issues concerning their pharmacology and clinical use? Circulation, 100**,** 437-444.

SCHAPER, W. 2005. Dipyridamole, an underestimated vascular protective drug. Cardiovasc Drugs Ther, 19**,** 357-63.

SCHARBERT, G., KALB, M., MARSCHALEK, C. & KOZEK-LANGENECKER, S. A. 2006. The effects of test temperature and storage temperature on platelet aggregation: a whole blood in vitro study. Anesth Analg, 102**,** 1280-4.

SCHNEIDER, D. J., TAATJES, D. J. & SOBEL, B. E. 2000. Paradoxical inhibition of fibrinogen binding and potentiation of alpha-granule release by specific types of inhibitors of glycoprotein IIb-IIIa. Cardiovasc Res, 45**,** 437-46.

SCHOLZ, T., ZHAO, L., TEMMLER, U., BATH, P., HEPTINSTALL, S. & LOSCHE, W. 2002. The GPIIb/IIIa antagonist eptifibatide markedly potentiates platelet-leukocyte interaction and tissue factor expression following platelet activation in whole blood in vitro. Platelets, 13**,** 401-6.

SCHULZ, C. & MASSBERG, S. 2012. Platelets in atherosclerosis and thrombosis. Handb Exp Pharmacol**,** 111-33.

SCHWARZ, U. R., GEIGER, J., WALTER, U. & EIGENTHALER, M. 1999. Flow cytometry analysis of intracellular VASP phosphorylation for the assessment of activating and inhibitory signal transduction pathways in human platelets--definition and detection of ticlopidine/clopidogrel effects. Thromb Haemost, 82**,** 1145-52.

SCOTT D. BERKOWITZ, M., ANDREW L. FRELINGER III, PHD, AND ROBERT S. HILLMAN, PHD DURHAM, N.C., AND SAN DIEGO,CALIF. 1998. Progree in point-of-care laboratory testing for assessing platelet function. American Heart Journal**,** s51-s65.

SHATTIL, S. J., CUNNINGHAM, M. & HOXIE, J. A. 1987. Detection of activated platelets in whole blood using activation-dependent monoclonal antibodies and flow cytometry. Blood, 70**,** 307-15.

SHATTIL, S. J., GAO, J. & KASHIWAGI, H. 1997. Not just another pretty face: regulation of platelet function at the cytoplasmic face of integrin alpha IIb beta 3. Thromb Haemost, 78**,** 220-5.

SIESS, W. 1989. Molecular mechanisms of platelet activation. Physiol Rev, 69**,** 58-178.

SILLER-MATULA, J. M., FRANCESCONI, M., DECHANT, C., JILMA, B., MAUER, G., DELLE-KARTH, G., GOUYA, G., RUZICKA, K., PODCZECK-SCHWEIGHHOFER, A. & CHRIST, G. 2012. Personalized antiplatelet treatment after percutaneous coronary intervention: the MADONNA study. European Heart Journal, 33**,** 41-41.

SILLER-MATULA, J. M., FRANCESCONI, M., DECHANT, C., JILMA, B., MAURER, G., DELLE-KARTH, G., GOUYA, G., RUZICKA, K., PODCZECK-SCHWEIGHOFER, A. & CHRIST, G. 2013. Personalized antiplatelet treatment after percutaneous coronary intervention: the MADONNA study. Int J Cardiol, 167**,** 2018-23.

SILVAIN, J., KERNEIS, M., COLLET, J. P., MONTALESCOT, G. & GROUP, A. S. 2014. New insights for low dosing with the new P2Y12 inhibitors. Circ J, 78**,** 2840-2.

SIMON, D. I., CHEN, Z., XU, H., LI, C. Q., DONG, J., MCINTIRE, L. V., BALLANTYNE, C. M., ZHANG, L., FURMAN, M. I., BERNDT, M. C. & LOPEZ, J. A. 2000. Platelet glycoprotein ibalpha is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). J Exp Med, 192**,** 193-204.

SINGH, R. B., MENGI, S. A., XU, Y. J., ARNEJA, A. S. & DHALLA, N. S. 2002. Pathogenesis of atherosclerosis: A multifactorial process. Exp Clin Cardiol, 7**,** 40-53.

SMITH, W. L., GARAVITO, R. M. & DEWITT, D. L. 1996. Prostaglandin endoperoxide H synthases (cyclooxygenases)-1 and -2. J Biol Chem, 271**,** 33157-60.

SNAPP, K. R., DING, H., ATKINS, K., WARNKE, R., LUSCINSKAS, F. W. & KANSAS, G. S. 1998. A novel P-selectin glycoprotein ligand-1 monoclonal antibody recognizes an epitope within the tyrosine sulfate motif of human PSGL-1 and blocks recognition of both P- and L-selectin. Blood, 91**,** 154-64.

SNOEP, J. D., HOVENS, M. M., EIKENBOOM, J. C., VAN DER BOM, J. G. & HUISMAN, M. V. 2007. Association of laboratory-defined aspirin resistance with a higher risk of recurrent cardiovascular events: a systematic review and meta-analysis. Arch Intern Med, 167**,** 1593-9.

SOHN, J. H., KANG, H. A., RAO, K. J., KIM, C. H., CHOI, E. S., CHUNG, B. H. & RHEE, S. K. 2001. Current status of the anticoagulant hirudin: its biotechnological production and clinical practice. Appl Microbiol Biotechnol, 57**,** 606-13.

SPANGENBERG, P., REDLICH, H., BERGMANN, I., LOSCHE, W., GOTZRATH, M. & KEHREL, B. 1993. The platelet glycoprotein IIb/IIIa complex is involved in the adhesion of activated platelets to leukocytes. Thromb Haemost, 70**,** 514-21.

STAFFORD, N. P., PINK, A. E., WHITE, A. E., GLENN, J. R. & HEPTINSTALL, S. 2003. Mechanisms involved in adenosine triphosphate--induced platelet aggregation in whole blood. Arterioscler Thromb Vasc Biol, 23**,** 1928-33.

STEINHUBL, S. R., BERGER, P. B., MANN, J. T., 3RD, FRY, E. T., DELAGO, A., WILMER, C., TOPOL, E. J. & OBSERVATION, C. I. C. F. T. R. O. E. D. 2002. Early and sustained dual oral antiplatelet therapy following percutaneous coronary intervention: a randomized controlled trial. JAMA, 288**,** 2411-20.

STEINHUBL, S. R., OH, J. J., OESTREICH, J. H., FERRARIS, S., CHARNIGO, R. & AKERS, W. S. 2008. Transitioning patients from cangrelor

to clopidogrel: pharmacodynamic evidence of a competitive effect. Thromb Res, 121**,** 527-34.

STEINHUBL, S. R., TALLEY, J. D., BRADEN, G. A., TCHENG, J. E., CASTERELLA, P. J., MOLITERNO, D. J., NAVETTA, F. I., BERGER, P. B., POPMA, J. J., DANGAS, G., GALLO, R., SANE, D. C., SAUCEDO, J. F., JIA, G., LINCOFF, A. M., THEROUX, P., HOLMES, D. R., TEIRSTEIN, P. S. & KEREIAKES, D. J. 2001. Point-of-care measured platelet inhibition correlates with a reduced risk of an adverse cardiac event after percutaneous coronary intervention: results of the GOLD (AU-Assessing Ultegra) multicenter study. Circulation, 103**,** 2572-8.

STOREY, R. F. 2006. Biology and pharmacology of the platelet P2Y12 receptor. Curr Pharm Des, 12**,** 1255-9.

STOREY, R. F. 2011. Pharmacology and clinical trials of reversibly-binding P2Y12 inhibitors. Thromb Haemost, 105 Suppl 1**,** S75-81.

STOREY, R. F. & HEPTINSTALL, S. 1999. Laboratory investigation of platelet function. Clinical and Laboratory Haematology, 21**,** 317-329.

STOREY, R. F., HUSTED, S., HARRINGTON, R. A., HEPTINSTALL, S., WILCOX, R. G., PETERS, G., WICKENS, M., EMANUELSSON, H., GURBEL, P., GRANDE, P. & CANNON, C. P. 2007. Inhibition of platelet aggregation by AZD6140, a reversible oral P2Y12 receptor antagonist, compared with clopidogrel in patients with acute coronary syndromes. J Am Coll Cardiol, 50**,** 1852-6.

STOREY, R. F., JUDGE, H. M., WILCOX, R. G. & HEPTINSTALL, S. 2002a. Inhibition of ADP-induced P-selectin expression and platelet-leukocyte conjugate formation by clopidogrel and the P2Y12 receptor antagonist AR-C69931MX but not aspirin. Thromb Haemost, 88**,** 488-94.

STOREY, R. F., OLDROYD, K. G. & WILCOX, R. G. 2001. Open multicentre study of the P2T receptor antagonist AR-C69931MX assessing safety, tolerability and activity in patients with acute coronary syndromes. Thromb Haemost, 85**,** 401-7.

STOREY, R. F., SANDERSON, H. M., WHITE, A. E., MAY, J. A., CAMERON, K. E. & HEPTINSTALL, S. 2000a. The central role of the P-2T receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. British Journal of Haematology, 110**,** 925-934.

STOREY, R. F., SANDERSON, H. M., WHITE, A. E., MAY, J. A., CAMERON, K. E. & HEPTINSTALL, S. 2000b. The central role of the P(2T) receptor in amplification of human platelet activation, aggregation, secretion and procoagulant activity. Br J Haematol, 110**,** 925-34.

STOREY, R. F., WILCOX, R. G. & HEPTINSTALL, S. 1998. Differential effects of glycoprotein IIb/IIIa antagonists on platelet microaggregate and macroaggregate formation and effect of anticoagulant on antagonist potency. Implications for assay methodology and comparison of different antagonists. Circulation, 98**,** 1616-21.

STOREY, R. F., WILCOX, R. G. & HEPTINSTALL, S. 2002b. Comparison of the pharmacodynamic effects of the platelet ADP receptor antagonists

clopidogrel and AR-C69931MX in patients with ischaemic heart disease. Platelets, 13**,** 407-13.

STOYKOVA, S., GORANOVA, Y., PANTCHEVA, I., ATANASOV, V., DANCHEV, D. & PETROVA, S. 2013. Hemolytic activity and platelet aggregation inhibitory effect of vipoxin's basic sPLA2 subunit. Interdiscip Toxicol, 6**,** 136-40.

STRAUSS, T., SIDLIK-MUSKATEL, R. & KENET, G. 2011. Developmental hemostasis: primary hemostasis and evaluation of platelet function in neonates. Semin Fetal Neonatal Med, 16**,** 301-4.

SUGIDACHI, A., OGAWA, T., KURIHARA, A., HAGIHARA, K., JAKUBOWSKI, J. A., HASHIMOTO, M., NIITSU, Y. & ASAI, F. 2007. The greater in vivo antiplatelet effects of prasugrel as compared to clopidogrel reflect more efficient generation of its active metabolite with similar antiplatelet activity to that of clopidogrel's active metabolite. J Thromb Haemost, 5**,** 1545-51.

TAKANO, K., ASAZUMA, N., SATOH, K., YATOMI, Y. & OZAKI, Y. 2004. Collagen-induced generation of platelet-derived microparticles in whole blood is dependent on ADP released from red blood cells and calcium ions. Platelets, 15**,** 223-9.

TARDIF, J. C., TANGUAY, J. F., WRIGHT, S. S., DUCHATELLE, V., PETRONI, T., GREGOIRE, J. C., IBRAHIM, R., HEINONEN, T. M., ROBB, S., BERTRAND, O. F., COURNOYER, D., JOHNSON, D., MANN, J., GUERTIN, M. C. & L'ALLIER, P. L. 2013. Effects of the P-selectin

antagonist inclacumab on myocardial damage after percutaneous coronary intervention for non-ST-segment elevation myocardial infarction: results of the SELECT-ACS trial. J Am Coll Cardiol, 61**,** 2048-55.

TAYLOR, M. L., MISSO, N. L., STEWART, G. A. & THOMPSON, P. J. 1992. The effects of varying doses of aspirin on human platelet activation induced by PAF, collagen and arachidonic acid. Br J Clin Pharmacol, 33**,** 25-31.

THEILMEIER, G., LENAERTS, T., REMACLE, C., COLLEN, D., VERMYLEN, J. & HOYLAERTS, M. F. 1999. Circulating activated platelets assist THP-1 monocytoid/endothelial cell interaction under shear stress. Blood, 94**,** 2725-34.

THERAPY, E. I. E. S. O. T. P. I. I. R. W. I. 2000. Novel dosing regimen of eptifibatide in planned coronary stent implantation (ESPRIT): a randomised, placebo-controlled trial. Lancet, 356**,** 2037-44.

THOMAS, M. R., WIJEYERATNE, Y. D., MAY, J. A., JOHNSON, A., HEPTINSTALL, S. & FOX, S. C. 2014. A platelet P-selectin test predicts adverse cardiovascular events in patients with acute coronary syndromes treated with aspirin and clopidogrel. Platelets, 25**,** 612-8.

THORNGREN, M. & GUSTAFSON, A. 1983. Effects of acetylsalicylic acid and dietary intervention on primary hemostasis. Am J Med, 74**,** 66-71.

TIFFANY, M. L. 1983. Technical considerations for platelet aggregation and related problems. Crit Rev Clin Lab Sci, 19**,** 27-69.

TOPOL, E. J., BYZOVA, T. V. & PLOW, E. F. 1999. Platelet GPIIb-IIIa blockers. Lancet, 353**,** 227-31.

TOTANI, L., DELL'ELBA, G., MARTELLI, N., DI SANTO, A., PICCOLI, A., AMORE, C. & EVANGELISTA, V. 2012. Prasugrel inhibits platelet-leukocyte interaction and reduces inflammatory markers in a model of endotoxic shock in the mouse. Thromb Haemost, 107**,** 1130-40.

TOTH, O., CALATZIS, A., PENZ, S., LOSONCZY, H. & SIESS, W. 2006a. Multiple electrode aggregometry: A new device to measure platelet aggregation in whole blood. Thrombosis and Haemostasis, 96**,** 781-788.

TOTH, O., CALATZIS, A., PENZ, S., LOSONCZY, H. & SIESS, W. 2006b. Multiple electrode aggregometry: a new device to measure platelet aggregation in whole blood. Thromb Haemost, 96**,** 781-8.

TRENK, D., STONE, G. W., GAWAZ, M., KASTRATI, A., ANGIOLILLO, D. J., MULLER, U., RICHARDT, G., JAKUBOWSKI, J. A. & NEUMANN, F. J. 2012. A randomized trial of prasugrel versus clopidogrel in patients with high platelet reactivity on clopidogrel after elective percutaneous coronary intervention with implantation of drug-eluting stents: results of the TRIGGER-PCI (Testing Platelet Reactivity In Patients Undergoing Elective Stent Placement on Clopidogrel to Guide Alternative Therapy With Prasugrel) study. J Am Coll Cardiol, 59**,** 2159-64.

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.

TSAO, P. W., FORSYTHE, M. S. & MOUSA, S. A. 1997. Dissociation between the anti-aggregatory & anti-secretory effects of platelet integrin alpha(IIb)beta(3) (GPIIb/IIIa) antagonists, c7E3 and DMP728. Thrombosis Research, 88**,** 137-146.

TSCHOEPE, D., ROESEN, P., ESSER, J., SCHWIPPERT, B., NIEUWENHUIS, H. K., KEHREL, B. & GRIES, F. A. 1991. Large platelets circulate in an activated state in diabetes mellitus. Semin Thromb Hemost, 17**,** 433-8.

TYNNGARD, N., WALLSTEDT, M., SODERGREN, A. L., FAXALV, L. & RAMSTROM, S. 2015. Platelet adhesion changes during storage studied with a novel method using flow cytometry and protein-coated beads. Platelets, 26**,** 177-85.

UENO, M., FERREIRO, J. L. & ANGIOLILLO, D. J. 2010. Update on the clinical development of cangrelor. Expert Rev Cardiovasc Ther, 8**,** 1069-77.

VALGIMIGLI, M., TEBALDI, M., CAMPO, G., GAMBETTI, S., BRISTOT, L., MONTI, M., PARRINELLO, G., FERRARI, R. & INVESTIGATORS, F. P. 2012. Prasugrel versus tirofiban bolus with or without short post-bolus infusion with or without concomitant prasugrel administration in patients with myocardial infarction undergoing coronary stenting: the FABOLUS PRO (Facilitation through Aggrastat By drOpping or shortening Infusion Line in patients with ST-segment elevation myocardial infarction compared to or on

top of PRasugrel given at loading dOse) trial. JACC Cardiovasc Interv, 5**,** 268-77.

VANE, J. R. & BOTTING, R. M. 2003. The mechanism of action of aspirin. Thrombosis Research, 110**,** 255-258.

VELIK-SALCHNER, C., MAIER, S., INNERHOFER, P., STREIF, W., KLINGLER, A., KOLBITSCH, C. & FRIES, D. 2008. Point-of-care whole blood impedance aggregometry versus classical light transmission aggregometry for detecting aspirin and clopidogrel: the results of a pilot study. Anesth Analg, 107**,** 1798-806.

VENTURINELLI, M. L., HOVNAN, A., SOEIRO ADE, M., NICOLAU, J. C., RAMIRES, J. A., D'AMICO, E. A. & SERRANO, C. V., JR. 2006. Platelet activation in different clinical forms of the coronary artery disease (role of P-selectin and others platelet markers in stable and unstable angina). Arq Bras Cardiol, 87**,** 446-50.

VIAL, C., ROLF, M. G., MAHAUT-SMITH, M. P. & EVANS, R. J. 2002. A study of P2X1 receptor function in murine megakaryocytes and human platelets reveals synergy with P2Y receptors. Br J Pharmacol, 135**,** 363-72.

VOSS, R., GREBE, M., PRALLE, H., TILLMANNS, H. & MATZDORFF, A. 2005. Pharmacodynamics of two different dosing regimens of tirofiban in citrate or PPACK anticoagulated blood. Platelets, 16**,** 492-7.

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**,** 907-14.

WANG, L., KARLSSON, L., MOSES, S., HULTGARDH-NILSSON, A., ANDERSSON, M., BORNA, C., GUDBJARTSSON, T., JERN, S. & ERLINGE, D. 2002. P2 receptor expression profiles in human vascular smooth muscle and endothelial cells. J Cardiovasc Pharmacol, 40**,** 841-53.

WEERAKKODY, G. J., JAKUBOWSKI, J. A., BRANDT, J. T., FARID, N. A., PAYNE, C. D., ZHU, J., WARNER, M. R., NAGANUMA, H. & WINTERS, K. J. 2007. Comparison of speed of onset of platelet inhibition after loading doses of clopidogrel versus prasugrel in healthy volunteers and correlation with responder status. Am J Cardiol, 100**,** 331-6.

WEYRICH, A. S., MCINTYRE, T. M., MCEVER, R. P., PRESCOTT, S. M. & ZIMMERMAN, G. A. 1995. Monocyte tethering by P-selectin regulates monocyte chemotactic protein-1 and tumor necrosis factor-alpha secretion. Signal integration and NF-kappa B translocation. J Clin Invest, 95**,** 2297-303.

WHISS, P. A. & ANDERSSON, R. G. 2002. Divalent cations and the protein surface co-ordinate the intensity of human platelet adhesion and P-selectin surface expression. Blood Coagul Fibrinolysis, 13**,** 407-16.

WHITE, J. G., KRUMWIEDE, M. D. & ESCOLAR, G. 1999. EDTA induced changes in platelet structure and function: influence on particle uptake. Platelets, 10**,** 327-37.

WIJEYERATNE, Y. D. & HEPTINSTALL, S. 2011. Anti-platelet therapy: ADP receptor antagonists. Br J Clin Pharmacol, 72**,** 647-57.

WILLIAMS, P. & CAMARA, M. 2009. Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules. Curr Opin Microbiol, 12**,** 182-91.

WIVIOTT, S. D., TRENK, D., FRELINGER, A. L., O'DONOGHUE, M., NEUMANN, F. J., MICHELSON, A. D., ANGIOLILLO, D. J., HOD, H., MONTALESCOT, G., MILLER, D. L., JAKUBOWSKI, J. A., CAIRNS, R., MURPHY, S. A., MCCABE, C. H., ANTMAN, E. M., BRAUNWALD, E. & INVESTIGATORS, P.-T. 2007. Prasugrel compared with high loading- and maintenance-dose clopidogrel in patients with planned percutaneous coronary intervention: the Prasugrel in Comparison to Clopidogrel for Inhibition of Platelet Activation and Aggregation-Thrombolysis in Myocardial Infarction 44 trial. Circulation, 116**,** 2923-32.

WOODHAMS, B., GIRARDOT, O., BLANCO, M. J., COLESSE, G. & GOURMELIN, Y. 2001. Stability of coagulation proteins in frozen plasma. Blood Coagul Fibrinolysis, 12**,** 229-36.

WOOLLARD, K. J. 2005. Soluble bio-markers in vascular disease: much more than gauges of disease? Clin Exp Pharmacol Physiol, 32**,** 233-40.

XAVIER, R. G., WHITE, A. E., FOX, S. C., WILCOX, R. G. & HEPTINSTALL, S. 2007. Enhanced platelet aggregation and activation under conditions of hypothermia. Thromb Haemost, 98**,** 1266-75.

XIAO, Z. & THEROUX, P. 2004. Clopidogrel inhibits platelet-leukocyte interactions and thrombin receptor agonist peptide-induced platelet activation in patients with an acute coronary syndrome. J Am Coll Cardiol, 43**,** 1982-8.

YAMAMOTO, H., TAKAHASHI, K., WATANABE, H., YOSHIKAWA, Y., SHIRAKAWA, R., HIGASHI, T., KAWATO, M., IKEDA, T., TABUCHI, A., MORIMOTO, T., KITA, T. & HORIUCHI, H. 2008. Evaluation of the antiplatelet effects of cilostazol, a phosphodiesterase 3 inhibitor, by VASP phosphorylation and platelet aggregation. Circ J, 72**,** 1844-51.

YAMAMOTO, N., GRECO, N. J., BARNARD, M. R., TANOUE, K., YAMAZAKI, H., JAMIESON, G. A. & MICHELSON, A. D. 1991. Glycoprotein Ib (GPIb)-dependent and GPIb-independent pathways of thrombin-induced platelet activation. Blood, 77**,** 1740-8.

YANG, D., CHEN, H., KOUPENOVA, M., CARROLL, S. H., ELIADES, A., FREEDMAN, J. E., TOSELLI, P. & RAVID, K. 2010. A new role for the A2b adenosine receptor in regulating platelet function. J Thromb Haemost, 8**,** 817-27.

YANG, J., HIRATA, T., CROCE, K., MERRILL-SKOLOFF, G., TCHERNYCHEV, B., WILLIAMS, E., FLAUMENHAFT, R., FURIE, B. C. & FURIE, B. 1999. Targeted gene disruption demonstrates that P-selectin glycoprotein ligand 1 (PSGL-1) is required for P-selectin-mediated but not E-selectin-mediated neutrophil rolling and migration. J Exp Med, 190**,** 1769-82.

YIP, C., IGNJATOVIC, V., ATTARD, C., MONAGLE, P. & LINDEN, M. D. 2013. First report of elevated monocyte-platelet aggregates in healthy children. PLoS One, 8**,** e67416.

YUSUF, S., ZHAO, F., MEHTA, S. R., CHROLAVICIUS, S., TOGNONI, G., FOX, K. K. & CLOPIDOGREL IN UNSTABLE ANGINA TO PREVENT

RECURRENT EVENTS TRIAL, I. 2001. Effects of clopidogrel in addition to aspirin in patients with acute coronary syndromes without ST-segment elevation. N Engl J Med, 345**,** 494-502.

ZAGLI, G., PATACCHINI, R., TREVISANI, M., ABBATE, R., CINOTTI, S., GENSINI, G. F., MASOTTI, G. & GEPPETTI, P. 2007. Hydrogen sulfide inhibits human platelet aggregation. Eur J Pharmacol, 559**,** 65-8.

ZHAO, L., BATH, P. & HEPTINSTALL, S. 2001. Effects of combining three different antiplatelet agents on platelets and leukocytes in whole blood in vitro. Br J Pharmacol, 134**,** 353-8.

ZHAO, L., BATH, P. M., FOX, S., MAY, J., JUDGE, H., LOSCHE, W. & HEPTINSTALL, S. 2003a. The effects of GPIIb-IIIa antagonists and a combination of three other antiplatelet agents on platelet-leukocyte interactions. Curr Med Res Opin, 19**,** 178-86.

ZHAO, L., BATH, P. M., MAY, J., LOSCHE, W. & HEPTINSTALL, S. 2003b. P-selectin, tissue factor and CD40 ligand expression on platelet-leucocyte conjugates in the presence of a GPIIb/IIIa antagonist. Platelets, 14**,** 473-80.

ZHAO, L., BATH, P. M. W., FOX, S., MAY, J., JUDGE, H., LOSCHE, W. & HEPTINSTALL, S. 2003c. The effects of GPIIb-IIIa antagonists and a combination of three other antiplatelet agents on platelet-leukocyte interactions. Current Medical Research and Opinion, 19**,** 178-186.