
UNIVERSITY OF BIRMINGHAM

# LABORATORY INVESTIGATION OF PLATELET FUNCTION IN PATIENTS WITH MILD BLEEDING DISORDERS

by

Rashid Hafidh Rashid Al Ghaithi

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Institute of Inflammation and Ageing
College of Medical and Dental Sciences
University of Birmingham

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# ABSTRACT

Platelets play a crucial role in haemostasis by preventing bleeding at sites of vascular injury. Inherited or acquired platelet defects can impair haemostasis resulting in bleeding symptoms of varying severity ranging from mild to excessive which can be life threatening. Diagnosis of mild platelet-based bleeding disorders is challenging due to the absence of a gold standard technique and their variable bleeding symptoms and bleeding phenotypes observed in healthy individual as well as other haemostatic disorders. Furthermore their bleeding symptoms often only manifest after haemostatic challenge. The work in this thesis built on the previous studies in the genotyping and platelet phenotyping project allowing further characterization of inherited platelet function defects in individuals with mild bleeding disorders. The bleeding symptoms of patients were evaluated using the bleeding assessment tool, and its likelihood in diagnosing platelet function defects was assessed and recorded. Platelet aggregation and secretion in samples from 206 patients were investigated during the course of this thesis and were categorised on the basis of the observed defects. Surprisingly, in over a half of these patients, an ex vivo platelet function defect was not found. The genetic investigation of selected cases using whole exome sequencing identified mutations in number of genes previously known to be critical in platelet biology. However, subsequent functional studies are required to further understand their contribution to the platelet defect observed in these patients. This thesis also focused on evaluation of three other platelet techniques by comparison with lumi-aggregometry to assess their overall potential in detecting platelet function defects. Further studies are still needed to further assess the potential of these techniques before they can be applied in routine clinical diagnosis.

# PUBLICATIONS ARISING FROM THIS WORK

## i) Published manuscripts

a. **Al Ghaithi R**, Drake S, Watson SP, Morgan NV and Harrison P Comparison of multiple electrode aggregometry with lumi-aggregometry for the diagnosis of patients with mild bleeding disorders. Journal of Thrombosis and Haemostasis 2017 **15**(10):2045-2052.

b. Jun Mori, Zoltan Nagy, Giada Di Nunzio, Christopher W. Smith, Mitchel J. Geer, **Rashid Al Ghaithi** et al. Maintenance of platelet homeostasis by the kinase-phosphatase pair Csk-CD148 in mice. *Submitted*

## ii) Published abstracts

a. **R. Al Ghaithi**, J. Mori, L. Hardy, H. Philippou, E. Hethershaw, YA. Senis, NV. Morgan and P. Harrison. Evaluation of the Total Thrombus-formation System (T-TAS): Application to Human and Mouse Blood Analysis. Accepted for poster presentation at International Society on Thrombosis and Haemostasis 2017 Congress (Berlin, Germany). (abstract reference PB 2230)

b. **R. Al Ghaithi**, J. Mori, L. Hardy, H. Philippou, E. Hethershaw, NV. Morgan, YA. Senis and P. Harrison. Evaluation of the Total Thrombus-formation System (T-TAS): Application to Human and Mouse Blood Analysis. Accepted for oral presentation at A Joint Meeting of BSHT / AiP / UK Platelet Group 2016 (Leeds, UK).

c. **Al Ghaithi R**, Drake S, Watson SP, Morgan NV and Harrison P Comparison of multiple electrode aggregometry with lumi-aggregometry for the diagnosis of patients with mild bleeding disorders (abstract published in the platelet journal )

# DEDICATION

I wish to dedicate this work firstly to my parents who brought me up in the best possible manner. Then to my family, my beloved wife Nasra Al Jahdhami whose sacrifice and her unconditional love have greatly contributed to the success of this work, and to my three lovely children, Hafidh, Muthla and Maher who always stand proud for their father’s achievements

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# LIST OF ABBREVIATIONS

| | | |
|---|---|---|
| ACMG | - | American College of Medical Genetics and Genomics |
| ADP | - | Adenosine diphosphate |
| ARC | - | Arthrogryposis-renal dysfunction-cholestasis |
| ATP | - | Adenosine triphosphate |
| AUC | - | Area under the curve |
| BAT | - | Bleeding Assessment Tool |
| BSS | - | Bernard-Soulier syndrome |
| BT | - | Bleeding time |
| CHS | - | Chediak-Higashi syndrome |
| CLEC-2 | - | C-type lectin-like receptor-2 |
| COX1 | - | Cyclooxygenase 1 |
| CPA | - | Cone and platelet analyser |
| CRP | - | Collagen-related peptide |
| DNA | - | Deoxyribonucleic acid |
| GAPP | - | Genotyping and Phenotyping of Platelets |
| GT | - | Glanzmann thrombasthenia |
| GTP | - | Guanosine-5'- triphosphate |
| GP | - | Glycoprotein |
| GPCRs | - | G protein–coupled receptors |
| GDP | - | Guanosine diphosphate |
| GPS | - | Grey platelet syndrome |
| HMW | - | High molecular weight |
| HPO | - | Human Phenotype Ontology |
| HPS | - | Hermansky-Pudlak syndrome |
| Ig | - | Immunoglobulin |
| IPA | - | Impedance platelet aggregometry |
| IPF | - | Immature platelet fraction |
| ISTH | - | International Society on Thrombosis and Haemostasis |
| ITAM | - | Immunotyrosine-based activation motif |
| ITP | - | Immune thrombocytopenic purpura |
| JAMs | - | Junctional adhesion molecules |
| KO | - | Knock out |
| Lumi-LTA | - | Lumi-aggregometry |
| LTA | - | Light transmission aggregometry |
| MAF | - | Minor allele frequency |
| MEA | - | Multiple Electrode Aggregometry |
| MFI | - | Median fluorescence intensity |
| MKs | - | Megakaryocyte |
| MPV | - | Mean platelet volume |
| NGS | - | Next generation sequencing |

| | | |
|---|---|---|
| NO | - | Nitric oxide |
| NPV | - | Negative predictive value |
| OCA | - | Oculocutaneous albinism |
| OCS | - | Open canalicular system |
| OT | - | Occlusion time |
| PCR | - | Polymerase Chain Reaction |
| PFD | - | Platelet function defects |
| PFT | - | Platelet function testing |
| $PGI_2$ | - | Prostacyclin |
| PI3K | - | Phosphoinositide 3-kinases |
| PLCβ | - | Phospholipase C-β |
| POC | - | Point of care |
| PPP | - | Platelet poor plasma |
| PPV | - | Positive predictive value |
| PRP | - | Platelet rich plasma |
| PS | - | Phosphatidylserine |
| PT-VWD | - | Platelet-type von Willebrand disease |
| PTF | - | Platelet thrombus formation |
| QPD | - | Quebec platelet disorder |
| ROC | - | Receiver-operator curve |
| ROTEM | - | Rotational thromboelastometry |
| RPFT | - | Remote platelet function test |
| SIFT | - | Sorting Intolerant from Tolerant |
| SLE | - | Systemic lupus erythematosus |
| SLMA | - | Signalling lymphocyte activation molecules |
| SNV | - | Single nucleotide variant |
| SPD | - | Storage pool disease |
| T-TAS | - | Total Thrombus-formation System |
| TEG | - | Thromboelastography |
| TPO | - | Thrombopoietin |
| TRAP | - | Thrombin receptor activating peptide |
| TTF | - | Total thrombus-formation |
| $TxA_2$ | - | Thromboxane $A_2$ |
| uPA | - | Urokinase plasminogen activator |
| VWD | - | von Willebrand disease |
| VWF | - | von Willebrand factor |
| WES | - | Whole exome sequencing |
| WGS | - | Whole-genome sequencing |
| WT | - | Wild type |
| WTF | - | White thrombus formation |

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# CHAPTER ONE: GENERAL INTRODUCTION

## 1.1Platelet physiology

### 1.1.1 Overview

Platelets are small enucleate blood cells with a diameter of 2-3 µm. They are produced in large numbers in the bone marrow by mature megakaryocytes (MKs) with a production rate of approximately 100 x $10^9$ platelets per day to maintain a normal circulating count of (150–400) × $10^9$ platelets per litre of blood. The lifespan of platelets is estimated to be 7-10 days after which they are eliminated by the reticulo-endothelial system (Ghoshal and Bhattacharyya, 2014).

The history of discovery of platelets is dated as early as 1780 when Hewson described very small undefined particles in the blood (Hewson, 1780). Throughout the 1800s a number of other scientists described platelets with a variety of different names, morphology and diameter. Moreover, understanding of their origin, significance and function had stimulated much debate (Gazzaniga and Ottini, 2001). In 1882 an Italian pathologist Giulio Bizzozero described platelets as the third distinctive cellular element of circulating blood but unrelated to erythrocytes and leukocytes. Remarkably, Bizzozero also demonstrated that platelets were vital for both haemostasis and thrombosis by using microscopy to study their interactions with sites of vessel wall injury within the circulating blood of living animals (Bizzozero, 1882).

### 1.1.2 Platelet formation

The formation of platelets is unique as they arise from the cytoplasmic fragmentation of MKs, the precursor cell reside within the bone marrow (Vainchenker et al., 2013). This process, also known as thrombopoiesis is basically divided into two phases. The initial phase is regulated by

the cytokine thrombopoietin (TPO) where the MK undergoes maturation through the process of endomitosis to amplify its deoxyribonucleic acid (DNA). This is followed by a rapid cytoplasmic expansion phase resulting in a dramatic increase in protein biosynthesis and organelle expansion necessary for mature platelet function and structure (Machlus and Italiano, 2013). The mature MK then undergoes massive reorganization and transformation of the cytoplasm to form elongated pseudopodia known as proplatelets. Platelet organelles are then transported from the cytoplasm of MK to the ends of proplatelets. Individual platelets are then shed from proplatelets directly into the circulation (McCormack et al., 2006).

### 1.1.3 Platelet morphology

Platelets can be structurally divided into distinct functional zones. The peripheral zone comprises the plasma membrane consisting of a phospholipid bilayer which can then be further divided into three areas. 1) The exterior coat which is the site of expression of a wide range of surface receptors that trigger platelet activation, adhesion and aggregation. 2) The unit membrane is the middle layer of peripheral zone and is rich in phospholipid which is important for the interaction with coagulation proteins and 3) The sub-membrane region is an important zone for the platelet signalling and intracellular trafficking (Gresele et al., 2002).

The sol-gel zone is composed of the cytoplasmic matrix with a cytoskeleton that supports the discoid shape of resting platelet. The cytoskeleton also plays essential roles in supporting platelet shape change, pseudopod extension, internal contraction and secretion during platelet activation (White and Krivit, 1967). The open canalicular system (OCS) is another important element in platelet structure. This membrane system plays an essential role in providing entry of external elements into the platelets and also release of platelet granular contents to the

exterior. Furthermore the OCS serves as a reservoir for many platelet receptors (Ghoshal and Bhattacharyya, 2014).

The other important feature of platelet structure is the organelle zone. It has two major storage granules, the alpha and the dense granules. The alpha granules are the most abundant. Each platelet can contain up to 80 alpha granules which make up roughly 10% of its volume. Alpha granules contain over 300 proteins including membrane P-Selectin, von Willebrand factor (VWF) and fibrinogen which are important molecules for adhesion and aggregation (Blair and Flaumenhaft, 2009). The dense granules are the storage sites of non-metabolic elements such adenosine diphosphate (ADP), adenosine triphosphate (ATP), serotonin, polyphosphate and calcium which are secreted during platelet release reaction and play important roles in recruiting other platelets during aggregation (Ruiz et al., 2004). In addition, platelets also have other types of granules called lysosomes which contain hydrolytic enzymes such as acid phosphatase (Gresele et al., 2002). Despite the absence of nuclei, platelets contain mitochondria which are responsible for the cell metabolic and energy requirements (Ghoshal and Bhattacharyya, 2014).

### 1.1.4 Platelet function

The primary function of platelets is to arrest bleeding. When a blood vessel is damaged, platelets are immediately recruited to the site of injury where they accumulate to prevent excesssive blood loss. During this process platelets undergo adhesion, activation, aggregation and provide a surface for efficient thrombin generation (Kitchen et al., 2009). In addtion, platelets are also known to involve in several other physiologic and pathologic processes such as wound healing, thrombosis, inflammation, anti-microbial host defence and tumour growth and metastasis.

### 1.1.4.1Adhesion

Adhesion is generally viewed as the first step of platelet function and it is essential process in response to vascular injury. In normal vessels platelets are marginated towards the vessel wall and the vascular endothelium. However, when vascular injury occurs sub-endothelial matrix components are exposed which initiate platelet adhesion (Ruggeri and Mendolicchio, 2007). The process of adhesion is mediated by various platelet receptors. For example the initial step involves interactions between platelet GPIb-IX-V complexes with the A1 domain of immobilized VWF, Although this binding is insufficient for firm adhesion it does promote transient tethering of platelets especially in areas of the highest levels of shear stress (Savage et al., 1996). For firm adhesion, platelets are required to directly bind to collagen; this process is mediated through the collagen receptors including GPVI and integrin $\alpha_2\beta_1$. The binding of platelets to collagen particularly through GPVI receptor is essential for platelet activation and signal transduction (Massberg et al., 2003).

### 1.1.4.2 Activation and spreading

Following adhesion, platelets undergo activation. The binding of agonists to their specific surface receptors lead to a series of events resulting in increases of the intracytoplasmic concentration of calcium ions released from intracellular stores and calcium influx through the plasma membrane (Rumbaut and Thiagarajan, 2010). As a result, platelets will undergo a number of structural and functional changes that lead to platelet activation.

Upon activation platelets undergo a dramatic reorganisation of the cytoskeleton leading to the transformation from disks to fully spread cells. This process also increases platelet surface area through production of filopodia and lamellipodia. The spreading is important to strengthen the

interactions of the platelet with the surface and with other platelets. It also provides a solid base for thrombus formation (Lee et al., 2012).

### 1.1.4.3 Secretion and aggregation

Aggregation is initiated by activated platelets when they secret soluble agonists such ADP and serotonin from their dense granules and by the generation of thromboxane $A_2$ ($TxA_2$). This recruits additional platelets to form platelet aggregates necessary for the formation of a stable haemostatic plug. The process of aggregation is mediated mainly through the $\alpha_{IIb}\beta_3$ complex, an integrin receptor present on the plasma membrane, the OCS and α-granules (Rumbaut and Thiagarajan, 2010). In resting platelets $\alpha_{IIb}\beta_3$ exists as an inactive form. However, upon platelet activation the molecule becomes active by undergoing a conformational change and can then bind plasma fibrinogen and VWF thus promoting bridging of adjacent platelets together (Varga-Szabo et al., 2008).

### 1.1.4.4 Platelet pro-coagulant activity

Activated platelets are also known to contribute to the overall coagulation process. Firstly they provide a surface for the generation of thrombin through exposure of the procoagulant phospholipid, phosphatidlyserine (PS) from the inner leaflet of the platelet surface, and secondly through the release of additional coagulation factors from the α-granules leading to a local increase in the concentration of coagulation factors. Generation of thrombin therefore occurs at the appropriate site and further reinforces platelet activation and thrombus growth (Ilveskero et al., 2001).

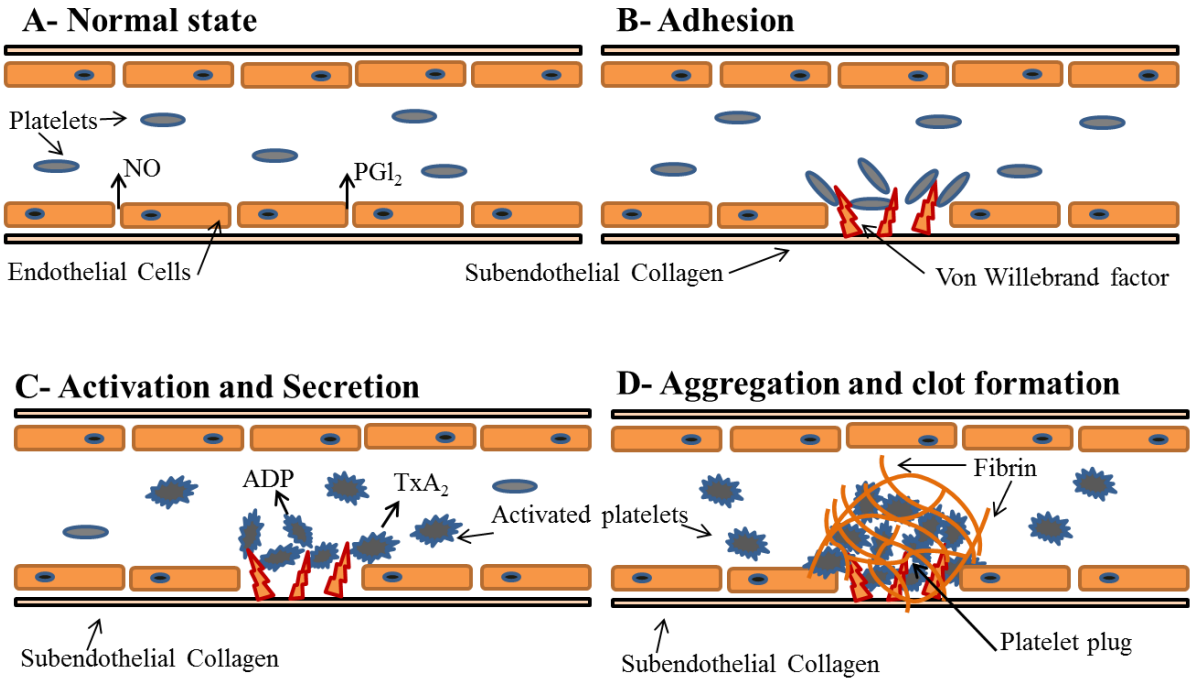



**Figure 1.1** Stages in platelet plug formation. (A) Prior to vascular injury, normal endothelial cells release NO and $PGI_2$ to maintain platelet quiescence (B) Vessel wall injury exposes platelets to the subendothelial matrix to promote platelet adhesion to immobilised VWF and collagen. (C) Activated platelets then further release molecules that mediate platelet recruitment to promote thrombus formation. (D) Development of the platelet plug at the site of injury by procoagulant activity and stabilization with fibrin. ADP, adenosine diphosphate; NO, nitric oxide; $PGI_2$, prostacyclin; $TxA_2$, thromboxane $A_2$;

## 1.2 Platelet receptors, agonists and signalling pathways

Platelet membranes express a wide variety of surface receptors through which platelets interact with and sense their immediate environment. By interacting with specific agonists, surface receptors play an important role in promoting platelet function both in health and disease (Rivera et al., 2009). Major platelet receptors and their corresponding agonists are listed in table 1.1.

| Agonist | Surface Receptor | Effect and physiological role |
|---|---|---|
| ***Adhesion molecules*** | | |
| Collagen | GPVI | Major signalling receptor for collagen |
| | α2β1 | Supports adhesion by collagen |
| Fibrinogen | $\alpha_{IIb}\beta_3$ | Aggregation, spreading and clot retraction |
| Fibronectin | α5β1, $\alpha_{IIb}\beta_3$ | Adhesion through α5β1 |
| Laminin | α6β1 | Adhesion |
| von Willebrand factor | GPIB-IX-V, $\alpha_{IIb}\beta_3$ | Platelet tethering and aggregation |
| ***Amines*** | | |
| Adrenaline | α2 | Positive feedback agonist |
| 5-HT | 5-HT2A | Mediates vasoconstriction, positive feedback agonist |
| ***Cytokines*** | | |
| TPO | c-Mpl | Maturation of megakaryocytes, control of platelet number in circulation |
| ***Immune complexes*** | | |
| Fc portion of antibodies | FcγRIIA | Immune- and bacterial-induced platelet activation |
| ***Lipids*** | | |
| PAF | PAF | Positive feedback agonist |
| Prostacyclin ($PGI_2$) | IP | Inhibition of platelet activation |
| Thromboxane $A_2$ | TP | Major positive feedback agonist |
| Prostaglandin $E_2$ ($PGE_2$) | EP3 | Inhibition of platelet activation |

| Agonist | Surface Receptor | Effect and physiological role |
|---|---|---|
| ***Nucleotides*** | | |
| Adenosine | A2a | Inhibition of platelet activation |
| ADP | $P2Y_1$ | Early role in platelet activation |
| | $P2Y_{12}$ | Major positive feedback receptor |
| ATP | $P2X_1$ | Possible early role in platelet activation |
| ***Proteases*** | | |
| Thrombin | PAR-1 | Coagulation-dependent platelet activation |
| | PAR-4 | |
| ***Surface molecules*** | | |
| CD40 ligand | CD40 and $\alpha_{IIb}\beta_3$ | Interaction with other blood cells, role in immune response and in inflammation |
| P-selectin glycoprotein ligand 1 (PSGL-1) | P-selectin | Interaction with leukocytes |
| ***Tyrosine kinase receptors*** | | |
| Podoplanin/possible other unknown ligand | CLEC-2 | Role in foetal vascular development<br><br>Possible role in platelet activation and in atherosclerosis and cancer progression (?) |
| EphrinB1 | EphA4 and EphB1 | Late events in platelet activation (?) |
| ***Vitamin K-dependent*** | | |
| Gas6 | Sky, Axl and Mer | Supports platelet activation (?) |

**Table 1.1:** Major platelet agonists and their surface receptors. (?) Indicates a putative role/effect. Adapted from (Lordkipanidze et al., 2013).

## Platelet signaling pathways

### 1.2.1 G protein–coupled receptors

G protein–coupled receptors (GPCRs) are the largest class and most diverse family of membrane proteins. They also referred as heptahelical receptors as they are composed of seven-transmembrane domains. They activate heterotrimeric G proteins that consist of α, β, and γ subunits. GPCRs use four classes of α units ($G\alpha_s$ $G\alpha_q$ $G\alpha_i$ and $G\alpha_{12/13}$), each of which promote different signaling effects. Activation of these receptors cause dissociation of guanosine diphosphate (GDP) from the α-subunit and facilitates guanosine-5'- triphosphate (GTP) binding. As a result, the α subunit is dissociated from the βγ subunit complex and can bind to target proteins such as adenylyl cyclase and phospholipase Cβ (Kobilka, 2007).

#### 1.2.1.1 $G_i$ proteins

$G_i$ proteins are highly expressed in platelets, with $G\alpha_{z\ and}$ $G\alpha_{i2}$ being the most abundant of this group. The proteins are so-named for their inhibitory effect on adenylyl cyclases resulting in a decreased level of cAMP. In addition, $G_i$ proteins are also involved in activation of phosphoinositide 3-kinases (PI3K). $P2Y_{12}$ and $\alpha_2$-adrenoceptor are the two major $G_i$-coupled proteins on the platelet. However, neither of these receptors mediates activation in washed platelets alone, and in platelet rich plasma they both require the presence of feedback mediators to fully activate platelets.

$P2Y_{12}$ is an ADP receptor that highly expressed in human platelets and to a smaller extent in other tissue such as neurones. In response to activation, $P2Y_{12}$ downstream signaling amplifies the platelet response induced by ADP and other agonists by inhibiting cAMP synthesis through the Gα subunit and regulating PI3Kinase and Rap1b through Gβγ subunits leading to

sustainable platelet aggregation (Gurbel et al., 2015). Bleeding disorders associated with $P2Y_{12}$ defect will be discussed in Section 1.3.4. $P2Y_{12}$ is also an important therapeutic target for antiplatelet drugs (e.g. Clopidogrel, ticagrelor, prasugrel and cangrelor) that are used in the treatment and prevention of cardiovascular disease (Falcao et al., 2013).

#### 1.2.1.2 $G_q$ proteins

Two forms of the Gq protein has been identified (Gq and G11), however, only Gq is expressed in platelets (Offermanns, 2006). Gq protein coupled receptors activate platelets through regulation of phospholipase C-β (PLCβ) isoforms to generate inositol-1,4,5-trisphosphate and 1,2 diacylglycerol. These second messengers cause calcium mobilization from intracellular stores and activation of protein kinase C (Offermanns et al., 1997, Zhang and Shi, 2016). The receptors in human platelets that are coupled through Gq protein are the $P2Y_1$ ADP receptor, the $TXA_2$ receptor and the two thrombin receptors, PAR-1 and PAR-4.

The $P2Y_1$ receptor is expressed on human platelets in relatively low levels of approximately 150 copies per platelet (Gachet, 2012). When activated by ADP, $P2Y_1$ plays a critical role in synergising with the $P2Y_{12}$ receptor to mediate full aggregation of platelets. No patients have so far been reported with excessive bleeding associated with mutations within the $P2Y_1$ receptor. On the other hand, Leon et al performed studies on mouse platelets and showed that $P2Y_1$ receptor plays an essential role in mediating thrombus formation. This finding suggests that a drug that targeting $P2Y_1$ receptor could prevent thrombotic events without bleeding complications (Leon et al., 1999).

The thromboxane receptor consists of two isoforms, α and β, although the α form is the only isoform known to be expressed on platelets (Habib et al., 1999). The receptor signals via

specific G protein-coupled receptors to mediate the stimulation of phospholipase C and an increase in intracellular concentrations of inositol 1,4,5-triphosphate and diacylglycerol. The formation of inositol 1,4,5-triphosphate results in an increase in the cytosolic concentration of $Ca^{2+}$ whereas release of diacylglycerol leads to PKC activation. Moreover the signalling of thromboxane receptor α stimulates the formation of cAMP (Paul et al., 1999). The physiological ligand for thromboxane receptor is thromboxane A2 ($TxA_2$). It is synthesized by the action of enzyme cyclooxygenase 1 (COX-1). COX-1 converts arachidonic acid released within activated platelets to form $TxA_2$ which acts as a positive feedback mediator in platelet recruitment and aggregation (Caughey et al., 2001). When activated, the thromboxane receptor also synergises with the $P2Y_{12}$ receptor that enables it to play such a critical role as a feedback receptor in the control of platelet activation. Targeting the $TxA_2$ pathway using aspirin has also proven to be an effective method in preventing platelet activation. Aspirin prevents platelet activation by irreversibly inhibiting platelet COX-1 and thus inhibits thrombus formation. As such, aspirin is widely used in the treatment and prevention of cardiovascular disease. Studies on both human (Kamae et al., 2011, Mumford et al., 2010) and animal models (Thomas et al., 1998) showed that defects in the thromboxane receptor are associated with bleeding tendencies.

PAR-1 and PAR-4 are two protease-activated receptors that are also expressed on human platelets. PAR-1 is not expressed on mouse platelets; instead, PAR-3 is expressed in addition to the PAR-4 (Sokolova and Reiser, 2008). PAR receptors are activated by thrombin, a soluble protease that cleaves the N-terminus of these receptors exposing a tethered peptide ligand that mediates receptor activation. Once activated, the thrombin receptors activate Gq and G13 proteins which regulate phospholipase C β and Rho kinase. Being a very powerful agonist,

thrombin plays an essential role in platelet activation; moreover it also provides a feedback via the coagulation system through promotion of the surface expression of negatively charged phospholipids. The thrombin receptors can also be activated using short thrombin receptor activating peptides (TRAPs) that mimic thrombin action without the need for receptor cleavage. TRAP is a useful agonist as it makes it possible to activate platelets in platelet rich plasma (PRP) independent of coagulation (Coughlin, 2000). No patients have so far being identified with mutation in the PAR receptors that are associated with excess bleeding. However, evidence suggests that polymorphisms on PAR receptors could affect platelet responses to thrombin (Muehlschlegel et al., 2012, Patel et al., 2014).

### 1.2.2 Glycoprotein receptors

Platelets express also a wide range of membrane glycoproteins (GP) receptors that play essential role in the activation and aggregation of platelets. Among these are GPIb-IX-V that tethers VWF, the GPVI-FcR-γ chain complex which is the major signalling receptor for collagen and the platelet integrin $\alpha_{IIb}\beta_3$ a receptor for a number of adhesive proteins include fibrinogen and VWF.

GPVI is a membrane glycoprotein receptor of the immunoglobulin (Ig) family which has two extracellular Ig domains. In 1989, Moroi et al were the first to propose GPVI as a major collagen receptor on platelets when they showed that GPVI autoantibody from patient with autoimmune thrombocytopenia caused abnormal platelet aggregation to collagen (Moroi et al., 1989). Seven years later, GPVI became widely recognised as a major signalling receptor for collagen when Gibbins et al. demonstrated the association of GPVI with FcR-γ in the platelet membrane (Gibbins et al., 1996). The FcR-γ chain contains an immunotyrosine-based activation motif (ITAM) that plays an essential role during signalling following clustering of GPVI by collagen

(Gibbins et al., 1998). The expression of GPVI on platelet surface is tightly regulated with around 4,000-6,000 copies per platelet (Best et al., 2003). Collagen interacts with GPVI with relative low affinity; however, the interaction is strengthened by binding to $\alpha_2\beta_1$ in a two step process (Nieswandt and Watson, 2003). GPVI can also be activated specifically without the association with $\alpha_2\beta_1$ using a synthetic Collagen-related peptide (CRP). Moreover, the CRP is able to induce shape change and aggregation even in the absence of ADP and $TxA_2$ (Jarvis et al., 2002). Deficiency of GPVI and FcR-γ is associated with a prolonged bleeding phenotype in mice (Kalia et al., 2008, Kato et al., 2003). Mice injected with GPVI antibody are also protected from lethal thrombogenic injections of adrenaline and collagen (Nieswandt et al., 2001). Bleeding diatheses have also been reported in a number of patients with inherited or acquired defects of GPVI receptor (Moroi et al., 1989, Hermans et al., 2009, Dumont et al., 2009, Boylan et al., 2004).

The GPIb-IX-V complex is a VWF receptor that exclusively expressed on platelets and MK. It is the second most abundant receptor complex on human platelets composed of two subunits of GPIbα, GPIbβ, and GPIX and one subunit of GPV (Li and Emsley, 2013).The GP Ib-IX-V complex plays two essential roles in platelet function; firstly, when it binds to immobilized VWF it mediates tethering of platelets at intermediate and high shear rate and also induces weak signalling leading to activation of the integrin $\alpha_{IIb}\beta_3$. Secondly, it also facilitates high affinity binding and promotes the ability of thrombin to activate platelets (Dormann et al., 2000). Qualitative and quantitative defects of GPIbα, GPIbβ, and GPIX subunits causes Bernard-Soulier syndrome (BSS) a severe bleeding disorder characterized by thrombocytopenia and large platelets (Li and Emsley, 2013). (BSS is described in details in section 1.3.1).

The integrin $\alpha_{IIb}\beta_3$ is a fibrinogen receptor that is exclusively expressed on platelets and MK. It is the most abundant receptor on platelets and consists of α and β subunits (Bussel et al., 2000). In circulating platelets the $\alpha_{IIb}\beta_3$ is maintained in a resting state but transforms into high affinity form (activated) following platelet activation. When activated, $\alpha_{IIb}\beta_3$ binds to a number of adhesive proteins such as fibrinogen and VWF to promote platelet aggregation and thrombus formation (Li et al., 2010). Defects of either subunit of $\alpha_{IIb}\beta_3$ causes Glanzmann thrombasthenia (GT) a bleeding disorder characterized by severe bleeding with a normal platelet count (Nurden and Caen, 1974). (GT is described in details in section 1.3.2).

### 1.2.3 Other platelet receptors

Platelets are found to express a number of other receptors that have been shown to induce platelet activation, although in some cases their physiological role is not understood. Some examples of these are shown below

C-type lectin-like receptor-2 (CLEC-2): is a transmembrane platelet receptor that activates platelet through a hemi-immunoreceptor tyrosine-based activation motif (hemITAM). CLEC2 can be activated by either its physiological ligand podoplanin or by a snake venom rhodocytin (Suzuki-Inoue et al., 2011).

Platelet $P2X_1$ receptor: is a ligand-gated ion channel, when activated by ATP, $P2X_1$ increases intracellular $Ca^{2+}$ levels to mediate platelet activation (Mahaut-Smith et al., 2011).

cMpl: is a receptor for TPO, a cytokine that regulates the differentiation and maturation of MK. Defects within the cMpl receptor cause congenital amegakaryocytic thrombocytopenia (Ihara, 2000). Mutations in JAK2, a cMpl signalling molecule also lead to myeloproliferative disorders (Baxter et al., 2005).

## 1.3 Platelet disorders

Defects within platelets can impair platelet function resulting in bleeding symptoms of varying severity ranging from mild to excessive bleeding which can be life threatening (Watson et al., 2013). Inherited platelet function disorders are catagorised depending on the type of abnormality as described below. An additional list of platelet disorders is summarized in table 1.2.

### 1.3.1 Defects in platelet adhesion

BSS and Platelet-type von Willebrand disease (PT-VWD) are two inherited disorders resulting in impaired platelet interaction with subendothelium. BSS was first described in 1948 by two French haematologists Jean Bernard and Jean Pierre Soulier (Bernard and Soulier, 1948). It is characterized by either complete absence of or a dysfunctional GPIb-IX-V receptor complex resulting in defective adhesion of platelets to the subendothelium (Nurden and Caen, 1975). BSS is a very rare disorder which is transmitted in an autosomal recessive manner. A variety of mutations of the *GPIb-α, GPIb-β,* or *GPIX* genes have been described which is most likely to be responsible for its heterogeneity (Sumitha et al., 2011). The disease classically presents with a severe phenotype characterized by severe bleeding episodes, thrombocytopenia, and very large platelets. The bleeding symptoms of BSS may range from mild to life-threatening, most commonly epistaxis, ecchymosis, cutaneous and gingival bleeding, menorrhagia and gastrointestinal bleeding (Berndt and Andrews, 2011). A number of laboratory tests can be used to identify BSS. Full blood counting reveals macrothrombocytopenia and evaluation of the peripheral blood smears often confirms the presence of large platelets. Platelet aggregation studies will show a defective response to ristocetin which cannot be corrected by addition of normal plasma, which can distinguish BSS from von Willebrand disease (VWD).

Aggregometry will also reveal a slow response with low doses of thrombin but normal aggregation to all other agonists. A confirmatory test is normally performed using flow cytometry to demonstrate a specific deficiency of GPIbα on platelet surface (Pham and Wang, 2007).

PT-VWD or pseudo VWD was first described in 1982 by Weiss and Miller (Weiss et al., 1982, Miller and Castella, 1982). It is a rare autosomal dominant bleeding disorder characterized by gain-of-function mutations within the platelet membrane glycoprotein Ibα (GPIbα) receptor. As a consequence enhanced binding of VWF to GpIb not only causes thrombocytopenia but a loss of high molecular weight (HMW) VWF multimers (Favaloro, 2008). Glycoprotein Ibα which contains the binding domain of VWF is the largest component of the platelet membrane receptor complex GPIb/IX/V, which consists of four subunits (GPIbα, GPIbβ, GPIX, GPV) (Berndt and Andrews, 2011). Mutations such as missense (Gly233Val, Met239Val and Gly233Ser) and various gene deletions in the Glycoprotein Ibα gene that give rise to PT-VWD have been described. Bleeding symptoms of individuals with PT-VWD include mild-to-moderate mucocutaneous bleeding and haemorrhage after surgical procedures. Diagnosis of PT-VWD is challenging because laboratory features such as a prolonged bleeding time and mild macrothrombocytopenia are very similar to those of type 2B VWD (Othman, 2007). Other findings include the presence of platelet clumps on blood smears, enhanced platelet aggregation in response to low dose of ristocetin and loss of HMW VWF multimers in plasma.

### 1.3.2 Defects in platelet aggregation

GT is a rare autosomal recessive bleeding disorder. It is characterized by a deficiency or functional defect in the platelet integrin $\alpha_{IIb}\beta_3$, which mediates platelet aggregation through

interaction with plasma fibrinogen (Nurden, 2006). GT was first described in 1918 by Glanzmann as "hereditary haemorrhagic thrombasthenia" due to a prolonged bleeding time and an isolated, rather than clumped, appearance of platelets on a peripheral blood smear (Glanzmann, 1918). Then in 1956 the disease was reviewed by Braunsteiner and Pakesch who described GT as "platelets of normal size that failed to spread onto a surface and did not support clot retraction" (Braunsteiner and Pakesch, 1956). In 1964, Caen *et al*, established the current diagnostic features of GT including the absence of platelet aggregation (Caen et al., 1966). In a recent study of a large group of patients with GT, Nurden et al. found 78 genetic variants on *ITGA2B* and *ITGB3* genes that associated with GT (Nurden et al., 2015).

### 1.3.3 Defects in platelet secretion

Storage pool disease (SPD) and release defects are heterogeneous group of disorders resulting from a deficiency of platelet granules or a dysfunction in the release of granules upon platelet activation. SPD is associated with a defect of α or δ granules but in rarely both (Bolton-Maggs et al., 2006b).

**Dense granule defects:** Inherited disorders of dense granules are also referred to as δ-storage pool deficiencies. They may occur as isolated disorders or in combination with other genetic conditions. The pathogenesis of SPD is variable associated with bleeding of various severities. SPD can be diagnosed using platelet aggregation, measurement of dense granules content and electron microscopy (Fitch-Tewfik and Flaumenhaft, 2013). The following two conditions have also been described to be associated with dense granule deficiency.

**Hermansky-Pudlak syndrome** (HPS): is a rare heterogeneous group of autosomal recessive disorders. It was first described in Czechoslovakia in 1959 (Hermansky and Pudlak, 1959) but large numbers of patients are now found in Puerto Rico. Eight different mutations associated with HPS have so far been identified in nine human genes. These are HPS1, AP3B1/HPS2, HPS3, HPS4, HPS5, HPS6, HPS7 (Di Pietro and Dell'Angelica, 2005), HPS8 (Morgan et al., 2006) and HPS9 (Wei, 2006). There is a marked phenotype of oculocutaneous albinism associated with a bleeding diathesis and pulmonary disease. In addition, some forms of HPS can cause dysfunction in the lungs, heart, intestine or kidneys (Pierson et al., 2006). The bleeding diatheses in patients with HPS are caused by a lack of dense granules in platelets resulting in impaired platelet aggregation and secretion.

**Chediak-Higashi syndrome (CHS)**: is a rare childhood disorder inherited as autosomal recessive. Patients with CHS are characterized by variable degrees of oculocutaneous albinism due to defects in the melanosomes and a tendency for mild bleeding due to a defect in platelet dense granules and recurrent infection due to impaired neutrophil function (Maaloul et al., 2016). In most cases CHS is identified in early childhood and is fatal if not treated. In human, 63 mutations have so far been descried on CHS1/LYST gene that are involved in lysosomal trafficking (Lozano et al., 2014).

**α granular defects**

α–granule deficiency can be caused by mutations or deletions of specific transcription factors involved in vesicular trafficking resulting in markedly decreased or absent α–granules and their content. Impaired secretion of α–granules may also affect both primary and secondary

haemostasis resulting in a variable severity of bleeding (Blair and Flaumenhaft, 2009). The following conditions have been described to be associated with α–granule deficiencies.

**Grey platelet syndrome (GPS):** It is an inherited disorder initially described in 1971(Raccuglia, 1971). The name GPS comes from the gray appearance of the platelets in the peripheral blood smear as a result of the absence of α-granules and their constituents. Lack of α-granular constituents in GPS is caused by abnormal formation of the granule membranes. Patients with GPS present with bleeding symptoms due to a defect in platelet α-granules, and thrombocytopenia due to a reduction in platelet survival. The key laboratory finding in patients with GPS is the presence of macrothrombocytopenia and appearance of grey platelets on the blood smear. Clot retraction can also be abnormal with variable platelet function (Nurden and Nurden, 2007). The genetic defect causing GPS has only recently been identified where different mutations in the neurobeachin-like 2 (*NBEAL2*) gene were detected in patients with GPS (Albers et al., 2011). Several other causative mutations related to GPS have also been identified within the *NBEAL2* gene that encodes a BEACH domain (Gunay-Aygun et al., 2011, Kahr et al., 2011). In 2007, Tubman et al reported an X-linked pattern of inheritance in GATA1 gene on a family with GPS (Tubman et al., 2007).

**Paris-Trousseau and Jacobsen syndromes:** Paris-Trousseau thrombocytopenia and Jacobsen syndrome are two closely related disorders associated with an increased bleeding tendency, thrombocytopenia, heart defects, skeletal abnormalities and mental retardation. The syndrome is inherited as autosomal dominant/recessive due to a deletion of a variable region on chromosome 11q that including the FLI1 (Jones et al., 1994). Although the two conditions are very similar, reports showed that in contrast to Paris-Trousseau thrombocytopenia, patients with

Jacobsen syndrome present with marked dense body deficiency indicating a storage pool defect (White, 2007).

**Arthrogryposis-renal dysfunction-cholestasis (ARC) syndrome**: is a rare but fatal autosomal recessive inherited disorder associated with arthrogryposis, renal tubular dysfunction and cholestasis. It is normally diagnosed early in life presenting with impaired liver and kidney function, muscular and bone deformities, and bleeding problems. The syndrome is caused by mutations in the *VPS33B* or *VIPAR* gene (Gissen et al., 2006). Peripheral blood smears and electron microscopy are valuable tools for diagnosis of ARC where platelets appear large, pale and agranulated (Kim et al., 2010).

**Quebec platelet disorder (QPD)** is a unique platelet function defect which differs from most platelet disorders. It is associated with a gain of function mutation resulting in enhanced fibrinolysis due to increased expression and storage of urokinase plasminogen activator (uPA) in platelet alpha granules. QPD is autosomal dominant resulting from duplicate mutation of *PLAU* gene (Diamandis et al., 2009). Patients with QPD present with spontaneous haematuria and delayed-onset bleeding, following trauma or dental procedures which responds only to treatment with fibrinolytic inhibitors (McKay et al., 2004). Laboratory tests may often but not always show a reduced platelet count and variable responses to different platelet agonist typically in response to epinephrine (Hayward and Rivard, 2011).

**α, δ – storage pool deficiency:** is described as a rare inherited platelet abnormality where both α and δ granules are deficient. It was first described by Weiss and his colleagues when they found patients with variable deficiency of α and δ granules (Weiss et al., 1979). Patients may present with mild bleeding, bruising and menorrhagia. In most cases platelets are moderately

deficient in α and δ granules. However a severe decrease in both granules has also been reported in four members of the same family (White et al., 2007).

### 1.3.4 Defects in agonist receptors

Interaction between platelet membrane receptors and various agonists promote platelet activation and function. Deficiency or dysfunction of these receptors can also result in impaired platelet function resulting in bleeding of variable severities.

#### Defects in ADP receptors

ADP was the first known aggregating agent and was identified in 1961. Although itself a weak agonist, ADP plays a key role in haemostasis and thrombosis. There are three distinct receptors expressed on human platelets known to interact with adenine nucleotides; $P2Y_1$ and $P2Y_{12}$ bind ADP while $P2X_1$ binds ATP. No human mutation of the $P2Y_1$ has so far been identified. However, a patient with a history of bleeding following surgery and three members of his family were described to be associated with low levels of $P2Y_1$ mRNA suggestive of deficiency in $P2Y_1$ gene transcription (Cattaneo, 2011a). In contrast the pathology associated with platelet $P2Y_{12}$ receptor has been well-characterized. Patients with either congenital deficiency/dysfunction of $P2Y_{12}$ have so far been described. Congenital deficiencies are typically autosomal recessive and were first described in 1992 in a patient with a lifelong history of excessive bleeding. The genetic defect of this patient displayed homozygous base pair deletions in the coding sequence resulting in frameshifts leading to introduction of premature stop codons and a lack of demonstrable $P2Y_{12}$ expression (Cattaneo et al., 1992, Cattaneo et al., 2003). Subsequently, similar patients were later described worldwide (Shiraga et al., 2005, Daly et al., 2009). Platelets from these patients display severely impaired ADP-induced

aggregation, a failure of ADP to inhibit PGE-stimulated adenylyl cyclase and a reduction in the number of binding sites for radiolabelled ADP analogues. As a result patients present with lifelong history of mucosal bleeding, easy bruising, and excessive bleeding following haemostatic challenge.

Patients with heterozygous mutations of $P2Y_{12}$ demonstrate mild bleeding with abnormal aggregation only to low dose ADP and borderline ATP secretion. Hollopeter et al., reported a patient with a heterozygous mutation of $P2Y_{12}$. Although the coding sequence on the second allele appeared to be normal the patient's platelets demonstrate a complete absence of $P2Y_{12}$ receptors (Nurden et al., 1995, Hollopeter et al., 2001).

A congenital bleeding disorder associated with expression of dysfunction $P2Y_{12}$ was also reported in a patient with a lifelong history of easy bruising and excessive blood loss in response to surgery and trauma (Cattaneo et al., 2003). Platelets from this patient display reduced and reversible aggregation in response to 20 μM ADP. Genetic analysis demonstrated a mutation in two different allies (G256A and C265T) resulting in normal expression of $P2Y_{12}$ but with altered functionality (Cattaneo, 2011b). A heterozygous mutation in $P2Y_{12}$ was also detected in a patient diagnosed with type 1 VWD disease. This finding suggests that platelet function defects can also be found in conjunction with other bleeding disorders (Daly et al., 2009). A mutation within the platelet $P2X_1$ receptor was also described in a patient with a severe bleeding disorder. The mutation was caused by a deletion of three nucleotides resulting in the loss of one leucine residue located between residues 351–353 of the P2X channel (Oury et al., 2000).

**Defects in collagen receptor ($\alpha_2\beta_1$)**

Integrin $\alpha_2\beta_1$ is also known as platelet glycoprotein GP) Ia/IIa and is expressed on various types of cells including epithelial cells and platelets. On platelets, the integrin $\alpha_2\beta_1$ functions as a

platelet collagen receptor that initiates the platelet adhesion to collagen and contribute synergistically to platelet function (Jung and Moroi, 2001). Defects in $\alpha_2\beta_1$ have been reported in several unrelated patients with mucocutaneous bleeding symptoms. Platelets from a patient reported by Nieuwenhuis et al failed to aggregate in response to collagen. These platelets only expressed 15-25% of GP Ia and adhesion to subendothelial surfaces was impaired at both low and high shear rates (Nieuwenhuis et al., 1985, Nieuwenhuis et al., 1986). Kehrel et al. reported a defect of both thrombospondin and GP Ia in a patient with a severe lifelong bleeding tendency. Platelets from this patient demonstrate a markedly reduced aggregation in response to collagen (Kehrel et al., 1988). In an animal model, Habart et al also reported that platelets from α2β1 deficient mice fail to respond to collagen but no haemostatic defects were observed (Habart et al., 2013)

**Defects in collagen receptor (GPVI)**

A number of cases of inherited GPVI deficiencies have also been reported. The majority of these patients present with mild mucocutaneous bleeding and are characterised by platelets that fail to respond to collagen stimulation. A non-functional form of GPVI was described in a 13 year old female presenting with ecchymosis and a life long history of bleeding. Her platelets failed to response to collagen, convulxin and the CRP but aggregated normally in response to all standard agonists. Genetic analysis showed a compound heterozygous 16bp deletion and a missense mutation S175N (Hermans et al., 2009). A hereditary disorder in GPVI signalling was also reported on a 60 year old female presenting with a history of excessive bleeding since childhood, a life-threatening post traumatic haemorrhage and impaired immune function. Her platelets showed normal expression of GPVI/FcR$\gamma$ receptors but failed to response to collagen, convulxin and CRP (Dunkley et al., 2007).

In contrast, several patients with acquired dysfunction of GPVI have been reported. Most of these cases are also associated with autoimmune disorders such as systemic lupus erythematosus (SLE) and immune thrombocytopenic purpura (ITP) (Arthur et al., 2007). One patient however with severe deficiency of GPVI was also diagnosed with GPS but a genetic defect of GPVI was not detected (Nurden et al., 2004). A list of platelet disorders is summarized in table 1.2.

| Platelet abnormality | Disease | Inheritance | Defective gene | Laboratory and other findings |
|---|---|---|---|---|
| **Platelet adhesion** | Platelet-type von Willebrand's disease | Autosomal dominant | *GP1BA* (17p13.2) | Thrombocytopenia<br>Diminished or absent large vWF multimers Enhanced ristocetin agglutination (occurs at low concentrations), which is corrected when donor platelets and patient plasma are used in mixing studies |
| | Bernard–Soulier syndrome | Autosomal recessive | *GP9* (3q21.3)<br>*GP1BA* (17p13.2)<br>*GP1BB* (22q11.21) | Thrombocytopenia with increased MPV<br>Anomalies in components of the GPIb–V–IX complex<br>Platelet aggregation: absent ristocetin-induced agglutination |
| **Platelet receptor defects** | $P2Y_{12}$ ADP receptor | Autosomal recessive (mild phenotype in heterozygotes) | *P2Y12* (3q25.1) | Platelet count normal<br>Platelet aggregation: normal $P2Y_1$ receptor-driven responses; shape change and transient aggregation |
| | GPVI collagen receptor | Autosomal recessive | *GP6* (19q13.42) | Platelet count normal<br>Platelet aggregation: absent with GPVI-specific agonists, e.g. convulxin and collagen-related peptide; and marked reduction with collagen |

| | | | | |
|---|---|---|---|---|
| | Thromboxane $A_2$ receptor | Autosomal recessive (mild phenotype in heterozygotes) | *TBXA2R* (19p13.3) | Platelet count normal<br>Platelet aggregation in response to arachidonic acid and U44619 reduced in heterozygotes to and presumed to be absent in homozygotes |
| | GPIIb/IIIa ($\alpha_{IIb}\beta_3$)<br><br>(Glanzmann thrombasthenia) | Autosomal recessive | *ITGA2B* (17q21.32)<br><br>*ITGB3* (17q21.32) | Normal platelet count, size, and morphology. Presents with severe bleeding symptoms in early life<br>Absent platelet aggregation with all agonists; agglutination in response to ristocetin is normal. Flow cytometry with CD41 and CD61 antibodies may show reduced levels of either GPIIb or GPIIIa |
| **Platelet secretion** | Hermansky–Pudlak syndrome | Autosomal recessive | *HPS1 (*10q24.2)<br>*HPS2/AP3B1* (5q14.1)<br><br>*HPS3* (3q24),<br>*HPS4 (22q12.1*)<br>*HPS5* (11p14)<br>*HPS6* (10q24.32)<br>*HPS7/dysbindin* (6p22.3)<br>*HPS8* (19q13.32)<br>*HPS9* (15q21.1) | Platelet count normal<br>Skin and hair hypopigmentation<br>Reduced/absent δ-granules on electron microscopy<br>Lumiaggregometry: reduced/absent ATP release |

| | | | | |
|---|---|---|---|---|
| | Chediak–Higashi syndrome | Autosomal recessive | *CHS1/LYST* (1q42) | Platelet count normal<br>Skin and hair hypopigmentation<br>Immunodeficiency<br>Giant inclusions in granulocytes and their precursors<br>Reduced or irregular α-granules<br>Lumiaggregometry: reduced/absent ATP release |
| | Gray platelet syndrome | Autosomal recessive | *NBEAL2* (3p21.31) | Thrombocytopenia<br>Increased MPV with platelet anisocytosis<br>Platelets grey in colour on blood film<br>Absent α-granules |
| | X-linked dyserythropoietic Anaemia and thrombocytopenia | X-linked | *GATA1* (Xp11.23) | Thrombocytopenia with increased MPV<br>Reduced α-granules<br>Anaemia |
| | Arthrogryposis, renal dysfunction and cholestasis syndrome | Autosomal recessive | *VPS33B* (15q261)<br>*VIPAS39* (14q24.3) | Thrombocytopenia with increased MPV<br>Severe multisystem syndrome, leading to fatal complications very early in life<br>Absent α-granul |
| | Paris–Trousseau/Jacobsen syndrome | Autosomal dominant | *FLI1* (11q24.1-24.3) | Thrombocytopenia with increased MPV<br>Developmental delay and facial abnormalities |
| | Quebec platelet disorder | Autosomal dominant | *PLAU* (10q22.2) | Platelet count at low end of normal range<br>α-Granule protein degradation<br>Increased urokinase-type plasminogen activator storage in platelets |

| | | | | |
|---|---|---|---|---|
| **Platelet cytoskeleton** | MYH9-related disorders (May–Hegglin anomaly, also known as Sebastian/Fechtner/Epstein syndrome) | Autosomal dominant | *MYH9* (22q12-13) | Thrombocytopenia with increased MPV<br>Döhle-like inclusions in neutrophils<br>Nephritis and hearing loss in some forms |
| | Wiskott–Aldrich syndrome/X-linked thrombocytopenia | X-linked | *WAS* (Xp11.23) | Thrombocytopenia with small platelets<br>Immunodeficiency and eczema (in Wiskott–Aldrich syndrome) |
| | Filamin A disorders (periventricular nodular heterotopia/otopalatodigital syndrome) | X-linked | *FLNa* (Xq28) | Thrombocytopenia with raised MPV and abnormal platelet morphology<br>Abnormal distribution of platelet filamin on confocal microscopy |
| | Actin disorders | Autosomal dominant | *ACTIN1* (14q2401) | Thrombocytopenia with raised MPV and platelet anisocytosis<br>Patients either have moderate bleeding tendency or may be asymptomatic |
| **Platelet procoagulant activity** | Scott syndrome | Autosomal recessive | *TMEM16F* (12q12-13.11) | Platelet count normal<br>Anomalies in scramblases translocating negatively charged phospholipids on the plasma membranes<br>Impaired annexin A5 binding with flow cytometry |

| | | | | |
|---|---|---|---|---|
| **Other thrombocytopenias** | Congenital amegakaryocytic thrombocytopenia (CAMT) | Autosomal recessive | *MPL* (1p34) | Severe thrombocytopenia<br>Pancytopenia<br>Absent megakaryocytes in bone marrow<br>Increased plasma thrombopoietin levels |
| | Thrombocytopenia with absent radius syndrome | Autosomal recessive | *MASTL*, *ACBD5*, *ANKRD26* (all 10p12.1) | Severe thrombocytopenia<br>Normal platelet morphology<br>Shortened/absent radii in forearm |
| | THC2 | Autosomal dominant | *MASTL*, *ACBD5*, *ANKRD26* (all 10p12.1) | Mild to moderate thrombocytopenia with normal MPV<br>Platelets deficient in GPIa and α-granules<br>Platelet aggregation normal<br>Possible dysmegakaryopoiesis |
| | Familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML) | Autosomal dominant | *RUNX1* (21q22.12) | Mild thrombocytopenia, with possible raised MPV<br>Abnormal aggregation in response to multiple agonists |
| | GFI1B mutation | Autosomal dominant | GFI1B (single nucleotide insertion in exon 7, c880-881insC – results in frameshift mutation) | Red cell anisopoikilocytosis<br>Moderate thrombocytopenia, raised MPV<br>Decrease in platelet alpha granules<br>Absent aggregation with collagen, +/- other agonists |

**Table 1.2:** Brief reference guide on inherited platelet disorders. Adopted from (Watson et al., 2013). ADP: Adenosine diphosphate; ATP: Adenosine triphosphate; GP: glycoprotein; MPV: mean platelet volume; VWF: von Willebrand factor.

### 1.4 Diagnosis of Platelet Function disorders

Characterisation of individual platelet function defects (PFD) is crucial for optimal treatment and management as frequent minor bleeding episodes can have a significant impact on quality of life. Diagnosis of severe forms of PFD such as GT and BSS is less challenging because bleeding symptoms are not only usually identified early in life, but the laboratory tests are often straightforward due to the absence of aggregation and ristocetin induced agglutination, respectively, and lack of expression of the glycoproteins by flow cytometry (Daly et al., 2014). In contrast, diagnosis of the milder forms of PFD is complex and challenging for several reasons. Firstly, bleeding symptoms are often not recognised in early age as patients are not usually exposed to severe haemostatic challenges e.g. surgery, injury, and childbirth. In addition, similarities of bleeding patterns are often observed in other haemostatic disorders including type 1 VWD (Watson et al., 2013).

Individuals with inherited PFD normally present with a history of prolonged bleeding during injury, unexplained bruising, excessive mucocutaneous bleeding such as epistaxis and menorrhagia, bleeding after dental extraction and post-partum bleeding (Daly et al., 2014). A proper assessment of this clinical history is therefore very important to exclude acquired platelet disorders or any other haemostatic abnormalities. This is now commonly performed by using the Bleeding Assessment Tool (BAT), a standardised questionnaire which assesses the bleeding symptoms of the patient which are then recorded as a single bleeding score (Rodeghiero et al., 2010b). Additional investigations such as platelet count and morphology with screening tests to evaluate coagulation abnormalities and VWD are also performed to rule out non-platelet related disorders so that more specialized testing can be avoided (Harrison et al., 2011).

### 1.4.1 Phenotyping of platelet function disorders

The history of platelet function testing (PFT) started in 1910 with the development of the bleeding time (BT) by the Duke procedure. The BT was designed to evaluate in vivo primary hemostasis by assessing the platelets ability to form a hemostatic plug by recording the time taken for a small skin wound to stop bleeding. The test was once considered as an important tool used to test platelet function. The use of BT however has dramatically reduced due to lack of sensitivity and poor reproducibility, and it is no longer recommended (Harrison et al., 2011). The in vitro bleeding time or PFA-100 is another screening test which measures high shear dependent platelet function and it is currently used in many laboratories to investigate PFD. Similarly to the BT, the PFA-100 has poor sensitivity and can also be significantly affected by number of factors such as platelet count, haematocrit and VWF levels (Bolton-Maggs et al., 2006a).

Over the years a wide range of platelet function assays have been developed, some of which focus on response to specific agonist and some of which measure a global output of platelet activation. Light transmission aggregometry (LTA) is the most widely used methodology for assessing platelet function disorders. It simply measures the change in light transmission in real time when agonists are added to platelet-rich plasma or washed platelets. The basic principle of LTA has not changed in over 50 years since it was first described by Born in early1960s (Born, 1962), even though modern day aggregometers are much smaller and easy to use (Harrison, 2009). In addition, the secretion of platelet ATP from the dense granules can also be simultaneously monitored using luminescence by addition of luciferin–luciferase reagents during the aggregation process (Dawood et al., 2007, Miller, 1984). An example trace of lumi-aggregometry (lumi-LTA) is shown in figure 1.2. Measuring the secretion of dense granules is

important in diagnosis of HPS and other dense granule secretory and release disorders. However, a defect in ATP secretion cannot discriminate between abnormal granule formation or defects in platelet signalling, so additional investigations are usually necessary for confirmation including e.g. electron microscopy, serotonin uptake and measurement of total platelet ATP/ADP content (Watson et al., 2013). LTA is also time consuming, requires large blood volumes for preparation of PRP, needs to be performed on fresh samples and requires expertise for correct performance and interpretation. Furthermore, platelet responses can also be transiently affected by diet and intake of certain medications which may also inhibit various activation pathways (Daly et al., 2014).

In an attempt to improve platelet function testing and overcome the major drawbacks of LTA, a number of alternative platelet function techniques have been developed in the past few decades. Each places a differing emphasis on the role of platelet aggregation and feedback pathways, which explains the subtle distinction in their results. Impedance platelet aggregometry (IPA) is designed to evaluate platelet function in whole blood to avoid further platelet processing before analysis (Toth et al., 2006). The technique measures the changes in resistance or impedance between two electrodes as platelets adhere and aggregate in response to platelet agonists (Cardinal and Flower, 1980). The use of whole blood also offers an advantage of rapid testing of platelets within physiological conditions without centrifugation and lower blood volume requirements. The use of IPA has regained popularity in the last decade after the development of Multiple Electrode Aggregometry (MEA) and has been widely used for the monitoring of anti-platelet therapy (Lenk and Spannagl, 2013). (MEA will be discussed in detail in chapter five).

A number of platelet function instruments have now become available that are simple to use and can be utilized at patient's bedside as point of care tests to be used as perioperative tools or for monitoring the efficacy of anti-platelet therapies. The experience of these techniques in identifying inherited platelet function defects however is limited (Harrison, 2009). The impact cone and plate(let) analyser (CPA) is a fully automated, simple and rapid to use device that monitors platelet adhesion to a polystyrene plate. The platelet adhesion is dependent on VWF, fibrinogen binding, and the platelet receptors GPIb and integrin $\alpha_{IIb}\beta_3$ (Pakala and Waksman, 2011). Thromboelastography (TEG) and rotational thromboelastometry (ROTEM) measure real time clot formation using an oscillating cup and oscillating pin (respectively) that holds whole blood samples. The assays provide a complete profile of clot formation by measuring fibrin formation, clot development, clot stability and fibrinolysis (Choi et al., 2014). Platelets can also be pre activated using arachidonic acid and ADP, thus, making the techniques theoretically suitable to monitor antiplatelet therapy (Sambu and Curzen, 2011, Lordkipanidze et al., 2013). The drawback of these techniques is their lack of sensitivity and they are time consuming, and their utility is currently limited to the monitoring of surgical haemostasis (Choi et al., 2014). The VerifyNow is a rapid fully automated device which monitors the platelets interaction with the fibrinogen-coated beads in whole blood, resulting in agglutination. Currently, three types of cartridges are available for use with VerifyNow for the monitoring of the effect of aspirin, $P2Y_{12}$ inhibitors and $\alpha_{IIb}\beta_3$ antagonists (Pakala and Waksman, 2011). The Total Thrombus-formation Analysis System (T-TAS) is another whole blood platelet function test that has recently being developed. It is a microchip-based analytical system that evaluates total thrombus formation under flow conditions using two types of microchips with thrombogenic surfaces (Hosokawa et al., 2011). (T-TAS will be discussed in detail in chapter six).

Flow cytometry is now increasingly used in assessing patients with suspected platelet function defects. It is mainly used to measure the levels of glycoproteins and activation markers on the platelet surface. For example, measurement of the levels of glycoprotein receptors GPIb and $\alpha_{IIb}\beta_3$ integrin can confirm or exclude BSS and GT respectively, while measurement of the levels of P-selectin and CD63 can verify platelet activation (Harrison et al., 2011). Flow cytometry is also used to assess platelet procoagulant function by measuring PS expression, which facilitates thrombin generation. Following stimulation with strong agonists such collagen or calcium ionophore, platelets expose PS which can be measured using specialized probes such as Annexin V or lactadherin (Presseizen et al., 2002).

The main advantage of flow cytometry is that is only requires a small volume of whole blood. However, the technique is restricted to specialized laboratories and can only be performed on a fresh blood sample. This drawback can now be potentially overcome by using remote activation and fixing solutions (Platelet Solutions, Nottingham UK) which can activate and then stabilize whole blood samples for up to 9 days (Dovlatova, 2015). Samples are taken and stimulated at point of blood collection, fixed and sent to a central laboratory for analysis. This approach of platelet function disorder diagnosis has recently being evaluated and has demonstrated a good agreement with lumiaggregometry (Dovlatova et al., 2014). However, the test has yet to be evaluated as a potential clinical screening test in clinics. (Platelet solutions will be discussed in more detail in chapter seven).

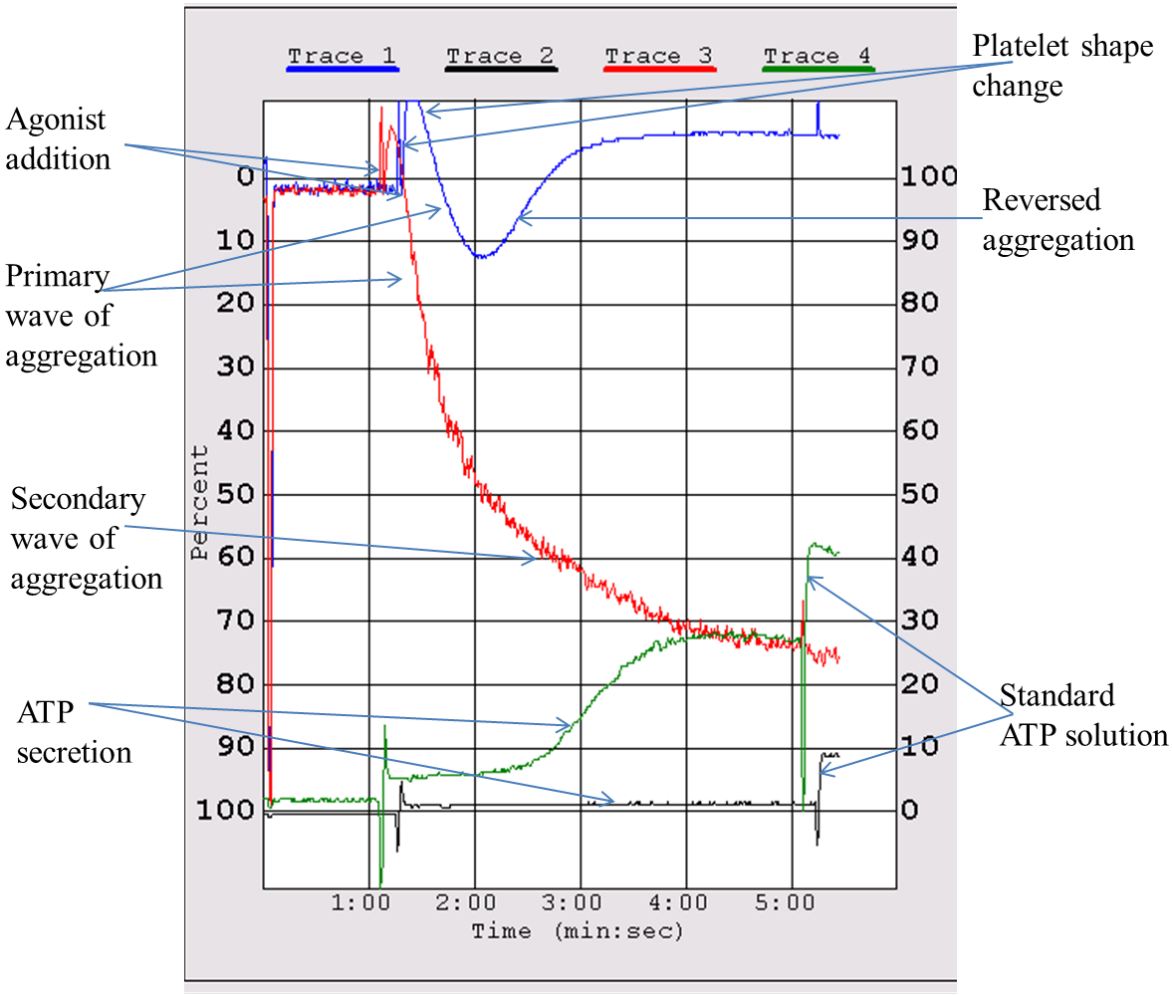



**Figure 1.2:** Example of an aggregation trace obtained with Lumi-LTA. Aggregation traces after addition of a high concentration of ADP (30 µM) from a healthy individual (control) and a patient with a suspected homozygous $P2Y_{12}$ mutation. Control aggregation is shown in red, and the patient aggregation shown in blue. Control ATP secretion is shown in green, patient ATP secretion shown in black. The patient shows reversible aggregation with absent ATP secretion. The ATP standard was added to allow calculation of secretion to normalized platelet count.

### 1.4.2 Genotyping of Platelet Function disorders

Genetic testing has contributed greatly to the understanding of cellular and molecular mechanisms that facilitate the identification and confirmation of heritable disorders. The utility of genetic testing increased after the invention of Sanger sequencing by Edward Sanger (Sanger et al., 1977). The technique was developed in 1975 and was considered the gold standard for genetic testing for more than two decades. Sanger sequencing was utilised within the 13-year-long Human Genome Project (HGP) that was completed in 2003 (Lander et al., 2001, Sachidanandam et al., 2001). Following the HGP, other genetic projects such as the 1000 Genomes Project were designed with goal of providing a valuable resource to the research community on human genetic variation (Bunimov et al., 2013).

Due to the drawbacks of Sanger sequencing related to cost and low throughput, demand for new sequencing methods that are cheaper and faster increased rapidly. This demand led to the development of next-generation sequencing (NGS) that was first introduced in 2005 (Shendure and Ji, 2008). Several NGS platforms are now available which perform high throughput sequencing that allows an entire genome to be sequenced in less than a day. The two most commonly used platforms in research and clinical labs today are the Life Technologies Ion Torrent Personal Genome Machine (PGM) and the illumina MiSeq. Despite that the sequencing technique of each of NGS platform is unique, the basic methodology applied in these two techniques are similar which includes template preparation, sequencing and imaging, and data analysis (Metzker, 2010). The inventions of NGS platforms with high-throughput and low cost made sequencing accessible to more laboratories and has resulted in the rapidly increasing utility of genetic testing in both research and clinical setting.

Two analytic strategies are applied in NGS to identify genetic variants, whole-genome sequencing (WGS) and whole-exome sequencing (WES). Despite that the WGS is the most straightforward and comprehensive strategy, its application is still limited due to the relatively high cost. Moreover, about 85% of disease-related mutations that underlie Mendelian disorders found within the exons only constitute approximately 1% of the human genome (Majewski et al., 2011). As such, the WES approach alone can therefore provide sufficient data to uncover the majority of rare and potential genetic disorders. In 2009, Ng *et al* published the first report of selectively sequencing the whole exome (Ng et al., 2009). In the same year Choi *et al* used WES to correct the diagnosis of a patient who was previously misdiagnosed with Bartter syndrome (Choi et al., 2009). In another case, a patient with oculocutaneous albinism (OCA) and neutropenia was misdiagnosed with HPS type 2. WES however identified two disease causative mutations, one (*SLC45A2*) related to OCA and the other (*G6PC3*) related to neutropenia (Cullinane et al., 2011).

There are several drawbacks of WES that still limits its global application; the most obvious is the inability of WES to assess the impact of non-coding regions since the method is only limited to coding and splice site variants (Rabbani et al., 2014). In addition, the method remains time consuming and the coverage of regions of interest is still limited despite recent improvements. Furthermore, due to the difficulty of WES in identifying repeat mutations and copy number variants, any possible disease-causing variants need to be confirmed and validated by Sanger sequencing, thus making WES less cost effective (Sanger et al., 1977, Majewski et al., 2011).

WGS and WES are becoming increasingly popular and widely used in the diagnosis of PFD for the aim of identifying variants that are responsible for the clinical presentation of patients. However, phenotypic variants among individuals with PFD makes it difficult to generate genotype–phenotype correlations that can elucidate a disease-causing mechanism within

individuals. A number of research groups now use NSG each with different approaches to identify the causes of unexplained bleeding. The Genotyping and Phenotyping of Platelets (GAPP) study run from the University of Birmingham aims to categorise a patient's disease through a combination of extensive platelet phenotyping and WES to determine their genotype (Watson et al., 2010) (The GAPP study is described in details in section 1.5). The BRIDGE Consortium-Bleeding and Platelet Disorders (BPDs) study (B20) takes an alternative approach to identify novel and known variants in a large patient cohort (>1000 donors) by combining WGS and WES with annotation with Human Phenotype Ontology (HPO) terms (https://bridgestudy.medschl.cam.ac.uk/bpd.shtml). Other smaller groups in Italy and USA also use WES in diagnosis of patients with inherited thrombocytopenia.

The first successful study of NGS in patients with PFDs was published in 2011 when Albers CA et al identified *NBEAL2* as the causative gene for GPS (Albers et al., 2011). Several other causative mutations within the *NBEAL2* gene that encodes a BEACH domain have been identified in individuals with GPS (Gunay-Aygun et al., 2011). With a collaboration of several research groups more novel variants have recently being discovered in patients with an unexplained bleeding (Maclachlan et al., 2017b).

The power of NGS has led to major advances in the ability to sequence large amounts of genetic material that facilitate the identification and confirmation of PFD. One of the main challenges arises however, is the analysis of huge amount of data generated by NGS. This often requires expert interpretation of bioinformatics data and comparison to datasets to identify the causal gene and variants among multiple possible variants and link it to disease and function. More over the terminologies that are used by bioinformatics datasets to name the identified variants often lead to confusion. Standards and guidelines have recently been published by the American College of Medical Genetics and Genomics (ACMG) recommending the use of specific

consensus terminology to describe variants identified in possible disease-causing genes (Richards et al., 2015). Another potential challenge is when discovery of incidental or secondary findings of genetic variants that are not related to the disease state in question. The question is whether clinicians should disclose any secondary findings with potential clinical relevance to patients. The ACMG has recently published a statement suggesting that reporting of incidental findings to patients should be proceed with caution in related to ethical and social concern which could require education and genetic counseling (Green et al., 2013).

### 1.5 The Genotyping and Phenotyping of Platelets (GAPP) study

The United Kingdom Genotyping and Phenotyping of Platelets (GAPP) study was established in 2009 with the aim of investigating populations of patients who present with excessive bleeding (Watson et al., 2010). Patients with suspected PFD have been recruited from 28 haemophilia referral centres in the UK which accounts for more than 66% of the overall number of Haemophilia Comprehensive care centres (Figure 1.3). The study uses a combination of platelet phenotyping and gene sequencing approach to identify candidate mutations underlying platelet dysfunction. The ultimate goal is to identify novel gene defects that would provide information on genes and proteins involved in normal platelet physiology and would extend our understanding of disease pathogenesis. These findings may lead us to the development of new methods in diagnosis and treatment strategies that would minimize risk of bleeding. The GAPP study has so far identified several patients with functional disrupting mutations in the $TxA_2$ receptor (Mumford et al., 2010) and the $P2Y_{12}$ ADP receptor (Dawood et al., 2012). Furthermore, a novel mutation in the $P2Y_{12}$ ADP receptor has been reported in a family with type 1 VWD (Daly et al., 2009). More recently, mutations in SLFN14, RUNX1 and FLI1 genes in patients with thrombocytopenia have been identified through the GAPP study (Fletcher et al., 2015, Johnson et al., 2016).

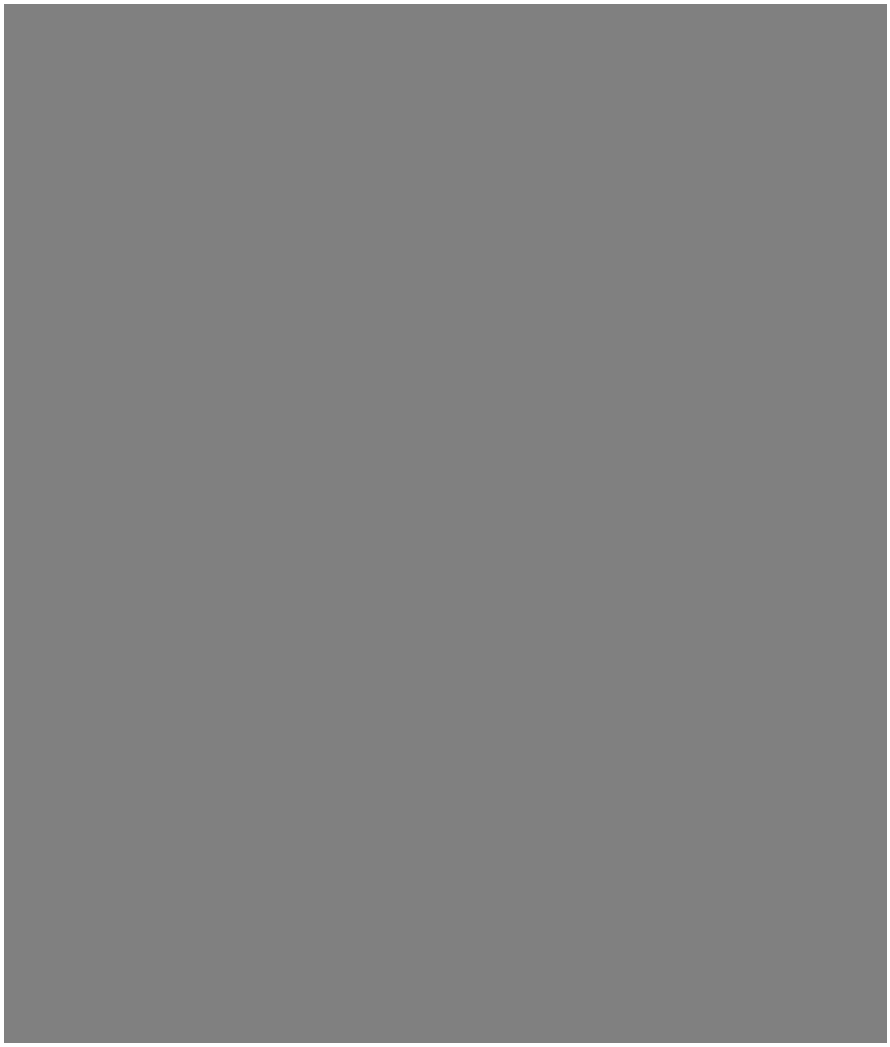


**Figure 1.3** Map showing the UK-GAPP study collaboration network. Blue star shows the coordinating and central laboratory for measuring phenotypes (Birmingham Platelet Group), Blue squares show other central laboratories for measuring phenotypes (Royal Hallamshire Hospital [Sheffield] and Bristol Royal Infirmary), Red pins indicate all referring Haemophilia Comprehensive Care Centres.

## 1.6 Aims of PhD study

1. To identify defects in platelet function using Lumi-LTA in patients who exhibit abnormal bleeding but have normal platelet counts included in the GAPP study.
2. To perform genotyping of selected patients found to have an abnormal phenotype searching for mutations within specific genes involved in platelet function and megakaryopoiesis using Sanger sequencing and whole exome sequencing.
3. To evaluate the ability of MEA in diagnosing patients with mild bleeding disorders by comparison with lumi-LTA
4. To perform a prospective study using a new remote platelet function test (RPFT) as a screening tool for patients with inherited platelet function disorders in comparison with lumi-LTA.
5. To evaluate the utility of the Total Thrombus-formation System (T-TAS) for assessing thrombus formation within human and mouse blood.

# CHAPTER TWO: MATERIALS AND METHODS

## 2.1 Study approval

The UK-GAPP study was approved by the National Research Ethics Service Committee of West Midlands–Edgbaston (REC reference: 06/MRE07/36) and participants gave written informed consent in accordance with the Declaration of Helsinki. This study was registered at www.isrctn.org as #ISRCTN 77951167. The GAPP study is also included in the National Institute of Health Research Non-Malignant Haematology study portfolio (ID9858). The GAPP study approach used in this thesis is summarised in figure 2.1

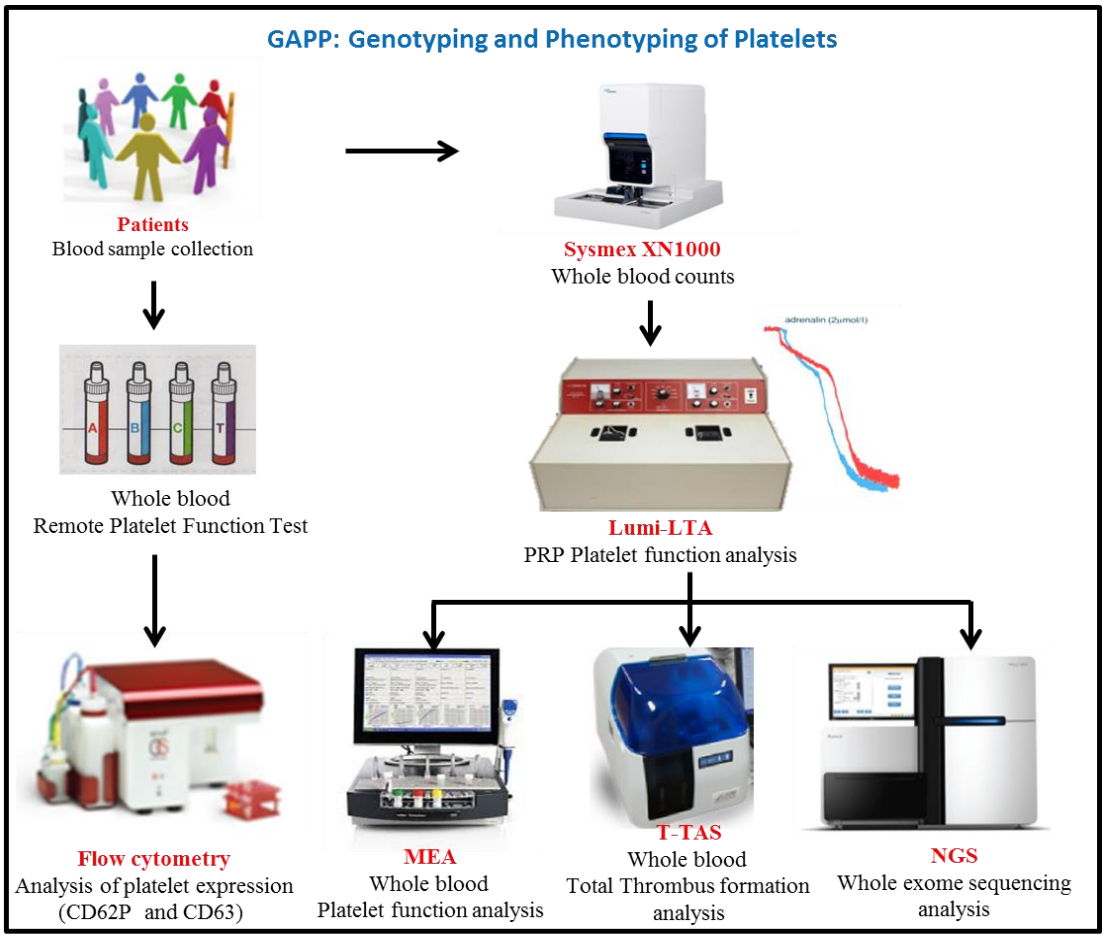



**Figure 2.1** GAPP approach to investigate patients with platelet disorders showing initial sample collection and lumi-LTA study, in addition to examples of further testing methods Modified from (Watson et al., 2010).

## 2.2 Materials

ADP, adrenaline, $PGI_2$, Trisodium citrate, ristocetin and U46619 were purchased from Sigma-Aldrich (Poole, UK). Arachidonic acid was purchased from Cayman Chemical Company (Michigan, USA). The PAR-1 peptide (SFLLRN) was purchased from Severn Biotech (Kidderminster, UK) and Collagen was purchased from Takeda (Linz, Austria), CRP from Dr. Richard Farndale (Cambridge, UK), and Luciferin Luciferase reagent (Chrono-lume) and ATP standard were purchased from Chrono-log Corporation (Havertown, PA, USA). The reagents were dissolved in phosphate-buffered saline (PBS) at PH 7.4 and stored as frozen aliquots at the concentrations shown in supplementary tables (1 and 2), thawed and diluted in PBS when required and kept on ice. Collagen was stored as a concentrated stock at 1 mg/ml as supplied by the manufacturer at 4°C and diluted with the special buffer provided. Platelet solution kits were purchased from Platelet solutions Ltd (Nottingham, UK). Monoclonal mouse anti-human CD61 (Pre CP) antibody and a Mouse IgG1 fluorescence isotype control were purchased from Becton, Dickinson and Company (BD Biosciences, Oxford, UK). Monoclonal mouse anti-human CD63 (FITC) antibody was purchased from Beckman Coulter (High Wycombe, UK) and monoclonal mouse anti-human CD62P (FITC) antibody were purchased from Bio-Rad Company (Watford, UK). AR chip TC0101, PL chip TC0201, CaCTI TR0101and 3.2% sodium citrate were provided by Quadratech Diagnostics Limited (Epsom, UK). 25 μg/ml hirudin blood tubes were purchased from Roche Diagnostics (Munich, Germany).

| *Agonist* | *Stock concentration* | *Final concentrations tested (in cuvette)* | *Source* |
|---|---|---|---|
| **Primary Reagents** | | | |
| **ADP** | 10mM | 3 µM, 10 µM, 30 µM and 100 µM | Sigma-Aldrich A2754 |
| **Adrenaline** | 100mM | 3µM, 10µM and 30µM | Sigma-Aldrich E-4375 |
| **Arachidonic acid** | 15mM | 0.5mM, 1.0mM and 1.5mM | Cayman Chemical 10006607 |
| **Collagen** | 1mg/ml | 1 µg/ml, 3µg/ml and 10 µg/ml | Takeda-Austria |
| **PAR-1 peptide (TRAP-SFLLRN)** | 10mM | 30 µM and 100 µM | Severn Biotech, Kidderminster, UK |
| **Ristocetin** | 50mg/ml | 1.5mg/ml and 2.0mg/ml | Sigma-Aldrich R7752 |
| **Secondary Reagents** | | | |
| **U46619** | 1mM | 1 µM and 3 µM | Cayman Chemical CAY16450 |
| **Collagen-related peptide** | 3mg/ml | 1 µg/ml and 3µg/ml | Dr. Richard Farndale, Cambridge, UK |

**Table 2.1** Panel of agonists and concentrations used to perform Light Transmission Aggregometry within the GAPP study

| *Agonist* | *Stock concentration* | *Final concentrations tested (in cuvette)* | *Source* |
|---|---|---|---|
| **ADP** | 10mM | 2.5µM and 10µM | Sigma-Aldrich A2754 |
| **Arachidonic acid** | 15mM | 0.5mM | Cayman Chemical 10006607 |
| **Collagen** | 1mg/ml | 1 µg/ml and 3µg/ml | Takeda-Austria |
| **PAR-1 peptide (TRAP-SFLLRN)** | 10mM | 100µM | Severn Biotech, Kidderminster, UK |

**Table 2.2** Panel of agonists and concentrations used to perform Platelet Aggregation using Whole Blood Multiplate Aggregometty within the GAPP study

## 2.3 Patient recruitments and blood sample collection

### 2.3.1 Patient recruitment

Patient recruitment processes for this study were either through regional Comprehensive Care Haemophilia centres across the UK or by referring haematologists specialising in bleeding disorders. Patients were recruited following a clinical history and subsequent diagnosis of a suspected platelet based bleeding disorder. Potential candidates were invited to participant dependent upon following specific inclusion and exclusion criteria as set out by the study.

**Inclusion criteria**

a. Aged 0 – 85 years
b. Abnormal bleeding symptoms compatible with an inherited platelet function disorder
c. Results from coagulation factors and VWD assays all within local laboratory reference interval
d. Willing to participate and able to provide informed consent

**Exclusion criteria**

a. Patients with existing diagnosis of Glanzmann thrombasthenia, Bernard-Soulier syndrome, May Hegglin anomaly or Hermansky-Pudlak syndrome
b. Patients taking drugs that are known to influence platelet function, including nonsteroidal anti-inflammatory drugs, aspirin, clopidogrel, dipyridamole, within 7 days of enrolment
c. Patients having undergone a major surgical procedure within 1 month of enrolment
d. Patients with chronic renal failure requiring dialysis
e. Patients with severe anaemia (Haemoglobin < 8g/dl)

Healthy volunteers were aged 18 years or older and considered healthy if they did not have a history of bleeding symptoms or did not require long-term medical therapy. All healthy volunteers denied taking drugs known to influence platelet function and had not donated blood in the previous two weeks

All healthy volunteers and patients or their parents gave written informed consent in accordance with the GAPP project ethical approval (GAPP, ISRCTN 77951167). Bleeding history of adult participants was assessed using the International Society on Thrombosis and Hemostasis bleeding assessment tool (ISTH BAT) (The full questionnaire is included in Appendix 2.). This score can range from 0-56 in women and 0-48 in men, based on a maximal score of 4 in fourteen separate categories assessing different types and severity of bleeding.

### 2.3.2 Blood sample collection

Blood samples (40 ml from adults or 20 ml from children) was obtained from patients and anticoagulated with one tenth volume of 0.109 mol/L buffered trisodium citrate in vacutainer plastic tubes (Becton Dickinson, Plymouth, UK). An additional 2-3ml blood was also anticoagulated within EDTA vacutainer plastic tubes (Becton Dickinson, Plymouth, UK). The same volume of blood was also collected from a healthy control at the same time in each centre. In the majority of cases, blood samples were then transported by courier to Birmingham and assayed within 6 hours from collection.

## 2.4 Blood sample preparation and processing

### 2.4.1 Whole Blood Platelet Counting using the Sysmex XN-1000 Analyser

EDTA blood samples from patients and controls were analysed using the XN-1000 whole blood counter (Sysmex UK, Milton Keynes). The XN-1000 is a state of the art whole blood analyser that is capable of rapidly producing a full blood differential cell count from 88 µl of human blood. The analyser has a set of unique platelet parameters including three platelet counts: platelet impedance (PLT-I), platelet optical (PLT-O) and platelet fluorescence (PLT-F) counts, mean platelet volume (MPV) and the immature platelet fraction (IPF). Platelet counts and MPV were recorded for each patient while IPF were recorded for patients with low platelet counts. The platelet parameters were compared against reference ranges established from 40 healthy normal controls. Daily quality control of the instrument was checked using commercial fixed samples with known cell counts (Xn Check, Sysmex, Milton Keynes, UK.). The analyser was also enrolled into an External Quality control scheme (UKNEQAS, Watford, UK).

### 2.4.2 Preparation of PRP and PPP

Platelet Rich Plasma (PRP) was prepared by centrifuging the citrated blood samples at 200*g* for 20 minutes. PRP was transferred carefully into a separate clean 15 ml falcon tube, capped and stored upright at room temperature. Platelet-poor plasma (PPP) was then prepared by further centrifugation at 1,000*g* for 10 minutes and transferred into a separate clean 15 ml falcon tube, capped and stored upright at room temperature. About 2-3ml of the buffy coat layer was also taken from the same tubes and stored at -80$^{o}$C for DNA analysis.

### 2.4.3 Platelet counting of PRP

An impedance analyser (Coulter Z2 Beckman Coulter, High Wycombe, Bucks, UK) was utilised to count the platelets within the PRP by diluting 5 µl of PRP into 10 ml of fresh isoton II diluent (Beckman Coulter, Bucks, UK). The platelets were counted in triplicate and the mean was calculated and recorded as $x10^8$/ml along with the MPV.

### 2.4.4 Storage of platelets for protein analysis

Patient and control samples were diluted to platelet counts of 3 x $10^8$/ml. 1 µl of 1 µg/mL prostacyclin (Cayman Chemical Company, Michigan, USA) was then added and the samples were centrifuged at 1000 g for 10 minutes. The supernatants were then removed and the pellets re-suspended in 1 ml PBS. 1 µl of 1 µg/mL prostacyclin was again added and the platelets centrifuged at 13,000 rpm for 2 minutes. The supernatant was again removed and the pellets were re-suspended into 600 µl of 10% sample buffer (or 300 µl 20% sample buffer + 300 µl tyrodes buffer) and then boiled at 100˚C for 5 minutes. The samples were then centrifuged for a short time at 5000 rpm and then stored at -80˚C. All samples were coded and dated.

## 2.5 Platelet function assay in whole blood

Whole blood impedance aggregometry was performed on citrated blood using Multiple Electrode aggregometry (MEA) (Multiplate, Roche Diagnostics Munich, Germany) consisting of 5 reaction channels, an integrated computer, and guided automatic pipetting. The tests were performed in disposable ready-to-use test cells with 2 independent sensor units consisting of 2 silver-coated highly conductive copper electrodes. In each test cuvette, mixed whole citrated blood (300 µl) was diluted, 1:1 vol/vol, with 0.9% Saline solution and incubated at 37°C for 3 minutes before addition of 30 µl of each agonist. Platelets were stimulated with ADP (10 and

2.5 µM), PAR-1 peptide (100 µM), Arachidonic acid (0.5 mM) and collagen (1 and 3 µg/mL). During the test platelets adhere and aggregate upon the sensor electrode surfaces resulting in an increase in the electrical resistance (impedance) and the signals arerecorded and plotted as an aggregometry trace. The total aggregation measured with this device is quantified as area under the curve (AUC) or AU*min.

**2.6 Platelet function assay in platelet-rich plasma**

Light transmission aggregometry (LTA) was carried out in all patients and controls within 6 hours of preparation of the PRP, as described previously (Dawood et al., 2007). Aggregation studies were performed by using a dual Chrono-log lumiaggregometer (model 460 VS, Havertown, PA, USA) in 400 µl mini cuvettes and stirred at 1,000 rpm at 37°C. The 100% line was set using autologous platelet-poor plasma (PPP) and the 0% baseline established with native undiluted PRP. After one minute incubation and stirring to set the baseline, platelets were stimulated with either ADP (10, 30, and 100 µM), adrenaline (10, 30, and 100 µM), arachidonic acid (0.5, 1, and 1.5 mM), U46619 (1 and 3 mM), collagen (1 and 3 µg/mL), CRP (1, 3, and 10 µg/mL), PAR-1 peptide (10, 30 and 100 µM), or ristocetin (1.5 and 2 mg/mL). Platelet aggregation was monitored by measuring the change in optical density (or light transmittance) over 5 minutes after addition of the agonists and the maximal percentage of aggregation was recorded. Traces were monitored for longer times if required depending upon the responses observed.

**2.7 Measurement of platelet ATP secretion**

ATP secretion from platelet dense granules was also assessed simultaneously by Lumiaggregometry using the luciferase reagent (Chronolume). 30 µl of Luciferin reagent was

added to the PRP and incubated for 1 minute. Platelets were then stimulated with the agonists listed above and monitored for 5 minutes followed by addition of 4 µl of ATP standard (2 µM) to facilitate internal calibration. Secreted ATP levels were calculated by measuring the maximal amplitude of luminescence during the aggregation to PAR-1 peptide and comparing to the standard. A cut-off point of 0.65 nmol ATP / $1x10^8$ platelets was established from previously studied healthy volunteers as the $5^{th}$ percentile for secretion in response to maximal stimulation with PAR-1 peptide.

### 2.8 Evaluation of platelet function using RPFT

For the RPFT measurements, whole blood samples were collected and processed locally by a trained member of staff at each haemophilia centre. Briefly, sodium citrate blood samples were pre-warmed and stimulated at 37°C for 5 minutes using four vials containing either (A) arachidonic acid and epinephrine (AA 0.5 mM/Epi 100 µM), (B) saline alone to provide a baseline test, (C) adenosine diphosphate with U46619 (ADP 10 µM/U4 1 µM) and (T) thrombin receptor activating peptide (TRAP [SFLLRN] 20 µM). Stimulated samples were then fixed using 1 ml PAMFix (Platelet Solutions Ltd, Nottingham, UK) and transported at room temperature to University of Birmingham by courier and processed within 5 days. In all cases the flow cytometry analysis were performed at the University of Birmingham. 5 µl of the fixed whole blood was incubated with 10 µl of diluted antibodies anti-CD61- PerCP (1/8 dilution) (BD Biosciences, Oxford, UK) and CD62p-FITC (1/20 dilution) (Bio-Rad, Watford, UK) and CD63-FITC (1/20 dilution) (Beckman Coulter, High Wycombe, UK) for 20-30 minutes in the dark at room temperature. After incubation, 200 µl of filtered PBS was added and analyzed within one hour using an Accuri C6 flow cytometer (BD Biosciences, Oxford, UK). For platelet identification a gating was initially setup on the Accuri C6 flow cytometer based on platelet

size (FSC) and expression of CD61 (SSC). 10,000 events were then acquired on each sample and the results of platelet markers expression (p-selectin/CD63) from unstimulated platelets were first quantified to set up the baseline reading. The expression of platelet markers (p-selectin/CD63) after platelet stimulation using specific agonists were then quantified in comparison to the baseline reading and reported as median fluorescence intensity (MFI). Platelets stained with FITC mouse IgG (Bio-Rad, Watford, UK) were used as isotype controls for nonspecific staining.

**2.9 Evaluation of thrombus formation under flow conditions on human blood**

In vitro thrombus formation was analysed on the T-TAS instrument (Zacros, Fujimori Kogyo Co. Ltd., Tokyo, Japan) using two types of microchips, the PL chip (width 40 μm × depth 40 μm) (containing 25 capillary channels coated with type 1 collagen) and the AR chip (width 300 μm, depth 60 or 120 μm, length 15 mm) (consisting of a single capillary channel coated with collagen and thromboplastin). The PL chip was used to analyse platelet thrombus formation (PTF). Briefly, hirudin anticoagulated whole blood (320 μL) was pipetted in the reservoir then perfused at 37°C through the PL chip by a pneumatic pump. After the perfusion of blood was initiated, platelets were then activated by the collagen coated on the capillary. The AR chip was used to analyse white thrombus formation (WTF). Briefly, citrated whole blood (480 μl) was mixed with 20 μl of 0.3 M $CaCl_2$ containing 1.25 mg/mL of Corn Trypsin Inhibitor (CTI) immediately before it was pipetted in the reservoir. The re-calcified blood was then perfused at 37°C through the AR chip by a pneumatic pump. After the perfusion of blood through the capillary was initiated, platelets and the extrinsic coagulation pathway were simultaneously activated by collagen and tissue thromboplastin. To prevent the outlet port from clotting, the blood is mixed with 25 mM EDTA (pH 10.5). The process of thrombus formation in both chips

was monitored by flow pressure changes in the capillary using the pressure sensor located between the pump and the reservoir. As thrombus formation proceeded on the coated surface, the capillary is gradually occluded, increasing the flow pressure. Based on the flow pressure pattern, The following four parameters are used to analyse the results (i) T10 (time to reach 10 kPa) was defined as the onset of thrombus formation and represents the duration (sec) for the flow pressure to increase to 10 kPa from baseline due to partial occlusion of microcapillaries. (ii) OT (occlusion time) was defined as the complete occlusion of the capillary, which coincides with a pressure of 60 and 80 kPa for the PL and AR chips respectively. (iii) T10–60 for PL chip and T10–80 for AR chip were defined as the interval between T10 and OT, representing the rate of thrombus growth; (iv) AUC (area under curve) is an area under the flow pressure curve (under 60/80 kPa) for 10/30 min after the start of assay for the PL and AR chips respectively. AUC is used to quantify a decrease in WTF when OT is not achieved during the time period of assay.

### 2.9.1 Antithrombotic effects of ticagrelor and rivaroxaban under flow conditions

Ticagrelor (10 mM) and Rivaroxaban (10 mM) were kindly provided by University of Leeds (Leeds, UK). Ticagrelor (final concentration, 10 µM) was incubated either in hirudin or citrate anticoagulated blood for 10 min at room temperature. Rivaroxaban (final concentration, 1 µM) was incubated in citrate anticoagulated blood for 3-5 min at room temperature. After incubation blood were perfused through either the PL chip and/or AR chip within the T-TAS instrument as described above.

### 2.9.2 Evaluation of thrombus formation under flow conditions on mouse blood

Mouse blood was tested in the T-TAS in collaboration with the Birmingham platelet signalling group led by Professor Yotis A. Senis. *CSK* and/or *CD148* conditional knockout (KO) mouse

models were generated by crossing *CSK*$^{fl/fl}$ (Schmedt et al., 1998) and/or *CD148*$^{fl/fl}$ (Katsumoto et al., 2013) mice with *Pf4-Cre* (Tiedt et al., 2007) transgenic mice to obtain megakaryocyte/platelet-specific knockout mice. Deletion of *lox*P-flanked genes in megakaryocytes and platelets was achieved by crossing the *Pf4-Cre* mice to obtain animals with homozygous *lox*P-flanked alleles either without or hemizgous for Cre. All mice were previously backcrossed over ten generations to the C57BL/6 background. All procedures were undertaken with United Kingdom Home Office approval in accordance with the Animals (Scientific Procedures) Act of 1986. Blood sampling was performed by puncture of the inferior vena cava in isoflurane-anaesthetized mice and 0.5 and 1.0 mL of blood was collected into tubes containing either 3.2% sodium citrate (9 vol blood to 1 vol citrate) or hirudin (final concentration, 25 μg/ml). Haematological parameters were obtained using ABX Pentra 60 Haematology Analyzer (Horiba ABX, Montpellier, France). Blood samples were perfused through either the PL chip or AR chip on the T-TAS instrument as described above.

### 2.10 Genetic analysis

#### 2.10.1 DNA extraction

Following separation of PPP, the buffy coats from patients' blood samples were collected and stored at -80°C. Genomic DNA was then isolated from the buffy coat samples and purified as recommended by the manufacturer using a Gentra Puregene kit (Qiagen Sciences, Maryland, USA). The concentration of the isolated DNA was measured using the Qubit 3.0 Fluorometer (Fisher Scientific, Loughborough, UK).

### 2.10.2 Polymerase Chain Reaction (PCR)

Prior to gene sequencing the patient's DNA was first amplified by PCR. Oligonucleotide primers used for PCR were designed using the online exon primer tool available on Helmholtz Center Munich institute of human genetic website (http://ihg.helmholtz-muenchen.de/ihg/ExonPrimer.html). The primers were then stored at -20°C at working concentration of 10μM. For each PCR reaction, 20μl of master mix was prepared as shown below:

- 12.5μl RedTaq
- 0.5μl forward primer
- 0.5μl reverse primer
- 6.5μl DNA/RNA free water

The master mix was then vortexed and centrifuged before 20μl was dispensed to a well of a 96-well plate. 100ng of patient DNA was added to the well and mixed by pipetting. One well containing dd$H_2O$ was run in parallel to act as a negative control. The plate was then centrifuged using a plate centrifuge and the PCR reaction was performed on the DNA Engine Tetrad®2 Peltier thermal Cycler (BIO-RAD, California, USA). 30 cycles of PCR were carried out as per standard cycle conditions (denaturing, annealing and hybridisation) as shown below:

- 94°C for 3 minutes
- 94°C for 1 minute
- 60°C for 1 minute
- 72°C for 1 minute
- 72°C for 5 minutes

DNA amplification was verified by electrophoresis using a ethidium stained 1% Agarose TAE gel. 10µl of the PCR product was loaded on the gel next to a 1kb DNA ladder (BioLabs, Ipswich, UK) and run for 30-40 minutes at 100V. To visualize the electrophoresis fragments the gel was imaged under the ultraviolet trans-illuminator system (Syngen, Gene Genius Bio imaging system) using GeneSnap software (Synoptics, GeneSnap running version 6.03.00). The DNA amplification was confirmed by correct sizing and alignment of the bands of the PCR fragments to the DNA ladder.

### 2.10.3 Sanger Sequencing

Preparation of PCR products for Sanger sequencing involved a four-step procedure of microclean, amplification, washing and denaturation. For sequencing, the forward and reverse primers were tested separately, so two sets of PCR product were required for each test

**Microclean:** 2.4µl of each PCR product for each sample was pipetted into two separate wells. 2.4µl of miroCLEAN solution (Microzone, Haywards Heath, UK) was then added to each well and the plate was spun in a Hettich Universal 320 (Hettich,Tuttlingen, Germany) plate centrifuge for 40 minutes at 2200g. To remove the microclean solution, the plate was centrifuged by inverting for 30 seconds at 250g.

**Amplification:** For each PCR reaction, 10µl of master mix was prepared as shown below:

- 0.5µl BigDye reaction Mix (ThermoFisher, Loughborough, UK)
- 2µl BigDye sequencing Buffer
- 2µl forward/reverse primer
- 5.5µl dd$H_2O$

10µl of master mix was added to each well containing the dried PCR product, mixed by pipetting and briefly centrifuged in a plate centrifuge. The plate was then placed on the DNA Engine Tetrad®2 Peltier thermal Cycler (Bio-Rad, California, USA) and 30 cycles of the sequencing were carried out as shown below:

- 96°C for 30 seconds
- 50°C for 15 seconds
- 60°C for 4 minutes

**Washing:** Two steps of ethanol washes were carried out to wash the sequencing reaction products. Firstly, 2 µl of 0.125M EDTA was dispensed into the wells and 30 µl of 100% ethanol was added. The plate was centrifuged at 1100g in a balanced plate centrifuge for 20 minutes and then centrifuged with the plate inverted for 30 seconds at 250 g to remove the washing solution. The second wash involved addition of 90 µl of freshly prepared 70% ethanol followed by 10 minutes spin at 1100 g. The plate was again inverted and centrifuged to remove the ethanol and the plate was allowed to air dry.

**Denaturation:** For denaturation, 10 µl of Hi Di Formamide was first added to each well and pipetted mixed to re-suspend the pellet. The plate was then heated on the PCR thermocycler for 2 minutes at 94°C. To avoid re-annealing of single stranded DNA, the plate was immediately snap chilled on ice.

**Sequencing:** All DNA sequencing runs were carried out by the Functional Genomics laboratory, University of Birmingham, using the ABI 3730 DNA sequencer (Applied Biosystems, California, USA). For data analysis, the Sanger DNA sequencing was viewed using Chromas and/or analysed using MutationSurveyor® DNA Variant Analysis Software

(BioLabs, Ipswich, UK). A variant can be identified by comparing the patient's result with an online reference data within the software.

### 2.10.4 Whole exome sequencing using NGS

Whole exome sequencing of candidate PFD genes was undertaken externally by collaborators in Kings College London headed by Prof Michael Simpson. Genomic DNA was isolated from buffy coat samples and purified as recommended by the manufacturer using a Gentra Puregene kit (Qiagen Sciences, Maryland, USA). <1µg of quantified genomic DNA was sent to the assigned laboratory at ambient room temperature. DNA enrichment of coding regions and intron/exon boundaries was undertaken using the SureSelect Human All Exon 50Mb Kit (Agilent Technologies, UK) following the manufacturer's instructions. Subsequently, DNA Sequencing was performed on an Illumina HiSeq 2500 (Illumina, Chesterford, UK) with 100bp paired-end reads. Sequence reads were aligned based upon the reference genome (hg19) using Novoalign (Novocraft technologies, Selangor, Malaysia). Duplicate reads and reads that mapped to multiple locations in the exome were excluded from further analysis. Small insertions/deletions and single nucleotide variations were identified and filtered for quality using the SAM tools software package and in-house software tools (Li et al., 2009). All calls with a read coverage of <4 were excluded from downstream analysis. The variants were then filtered to determine for novelty by comparison to known variants present within dbSNP139, EVS (evs.gs.washington.edu/EVS/), the 1000 Genomes Project and the results from over 1200 in house control exomes analysed using the same protocol. Variants found with a minor allele frequency (MAF) > 0.01 were excluded and novelty was confirmed by comparison to dbSNP138. A bioinformatics filtering pipeline used to analysis WES data is shown on figure 2.2.

### 2.10.5 Conservation and pathogenicity prediction

The pathogenicity of a candidate variant was predicted using different pathogenicity prediction software to identify a variant that may affect protein function. These included: Polymorphism Phenotyping v2 (PolyPhen-2) which uses two pairs of datasets to predict the pathogenic effect of a variant on protein structure and function (Adzhubei et al., 2010). MutationTaster (Schwarz et al., 2014) which is designed to evaluate the disease potential of an alteration and can predict functional consequences of different classes of sequence variations, including substitutions, insertions, deletions, intronic and synonymous alterations. Sorting Intolerant from Tolerant (SIFT) assesses the effect of a substitution by using sequence homology to predict the effects of all possible substitutions at each position in the protein sequence (Kumar et al., 2009). Protein Variation Effect Analyzer (PROVEAN) uses alignment-based score to predict the damaging effects of single or multiple amino acid substitutions by measuring the change in sequence similarity between the reference and variant versions in comparison to sequence homologs (Choi et al., 2012).

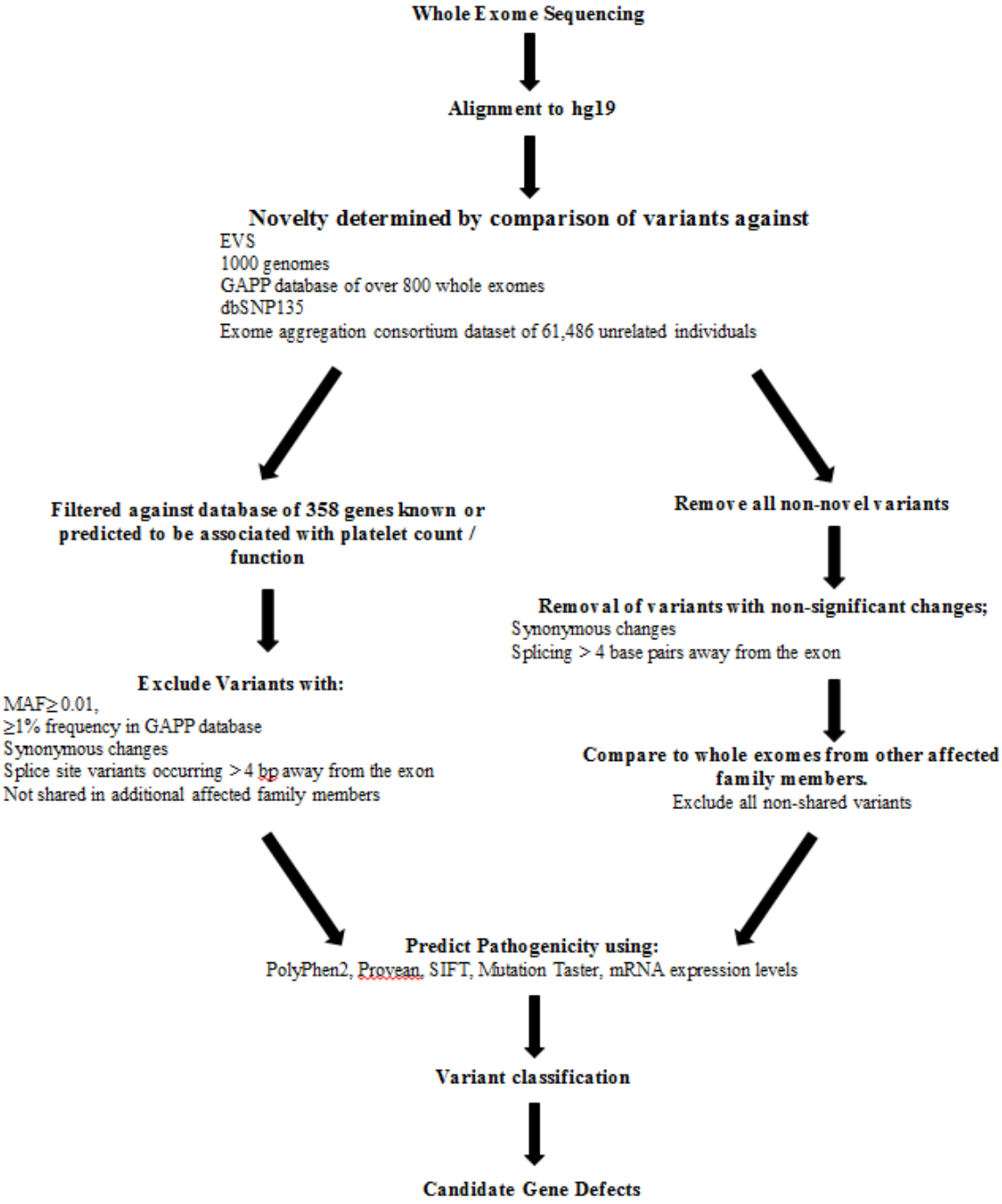



**Figure 2.2**: Bioinformatics pipeline used to analysis WES data. It comprises of two main arms, the left arm which focused upon comparison to a panel of 357 genes that known to affect platelet phenotype, and the right arm focused upon novel variants that are significant and shared within affected family members.

### 2.11 Statistical analysis

Statistical analysis was carried out using Microsoft Excel 2010, Graph Pad Prism version 7.0 and IBM SPSS statistics version 20, with testing methodologies as specified in figure legends (dependent upon the data being interrogated). P-values of $\leq 0.05$ were considered to be significant.

# CHAPTER THREE: PHENOTYPE OF INDIVIDUALS SUSPECTED WITH MILD BLEEDING DISORDERS USING LIGHT TRANSMISION AGGREGOMETRY

## 3.1 Introduction

Characterisation of individual platelet function defects (PFD) is crucial for optimal treatment and management as frequent minor bleeding episodes can have a significant impact on quality of life. Diagnosis of milder forms of PFDs is complex and challenging due to their variable bleeding phenotype, which often only manifests after haemostaic challenges, and the absence of a gold standard laboratory technique. Moreover, similarities of bleeding patterns are often observed in other haemostatic disorders including type 1 VWD (Watson et al., 2013). As such, a proper assessment of clinical history is therefore very important to exclude acquired platelet disorders or any other haemostatic abnormalities. In 2010, the International Society on Thrombosis and Haemostasis developed a Bleeding Assessment Tool (ISTH BAT), a standardised questionnaire which assesses the recurrent milder bleeding symptoms of patients which can also be applicable for individual of inherited PFDs (Rodeghiero et al., 2010b).

The most widely used methodology for assessing PFD is light transmission aggregometry (LTA). A recent worldwide survey showed that 73.4% of laboratories use this for the diagnosis of inherited PFD (Gresele et al., 2014). The technique simply measures the change in light transmission in real time when agonists are added to platelet-rich plasma or washed platelets. A typical panel of agonists includes ADP, collagen, arachidonic acid, adrenaline, PAR-1 peptide, U46619 and ristocetin (Gresele, 2015). The nature of the response is dependent on the agonist used, its concentration and the role of the feedback agonists ADP and thromboxane $A_2$ ($TxA_2$). During aggregation the platelet response may demonstrate an initial increase in optical density as a result of shape change, followed by a primary, biphasic, sustained or reversible increase in

light transmission (Dawood et al., 2007). An example of a typical aggregation and secretion trace is shown in figure 1.2.

In an attempt to improve platelet function testing, some modern aggregometers (lumi-LTA) can simultaneously monitor the secretion of platelet ATP from the dense granules using luminescence by addition of luciferin–luciferase reagents during the aggregation process (Dawood et al., 2007, Miller, 1984). Measuring the secretion of dense granules is important for diagnosis of HPS and other dense granule secretory and release disorders. However, the availability of this technique is limited to specialized laboratories. The 2014 survey showed that only 40.7% of laboratories have access to lumi-LTA (Gresele et al., 2014).

Although LTA is considered to be the gold standard method for the investigation of PFD, the technique is time consuming, requires large blood volumes for preparation of PRP, needs to be performed on fresh samples and requires expertise for correct performance and interpretation (Daly et al., 2014). Furthermore, platelet responses can also be transiently affected by diet and intake of certain medications which may also inhibit various activation pathways (Daly et al., 2014).

### 3.2 Aim

To test and classify patients with a suspected platelet function defect using lumi-aggregometry (lumi-LTA).

## 3.3 Results

### 3.3.1 Overall patient recruitments

Between September 2014 and April 2017, 318 participants (112 healthy controls and 206 patients) were recruited into the GAPP study from 10 haemophilia centers for evaluation of platelet function disorders (figure 3.1). The healthy control population (n=112) were recruited as per the GAPP study criteria. The age range of the healthy controls was between 18 to 57 years (other data e.g. demographic information is unavailable). Out of 206 patients, 25% (n=51) were males and 75% (n=155) were females (figure 3.2) with a median age of 34, range between 2 to 80 years (figure 3.3). The highest number of patients came from Lincoln (n=72) followed by Nottingham (n=45), Birmingham (n=43) and Canterbury (n=21). Only one patient was recruited from Oxford over the 32 month period. More details are shown in figure 3.4. Abnormal results were detected in 13/21 (69%) of patients from Canterbury, 26/45 (58%) from Nottingham and 21/43 (49%) from Birmingham. Surprisingly, despite the highest number of patients came from Lincoln, only 15/72 (21%) were found to have platelet defects. More details are shown in figure 3.5

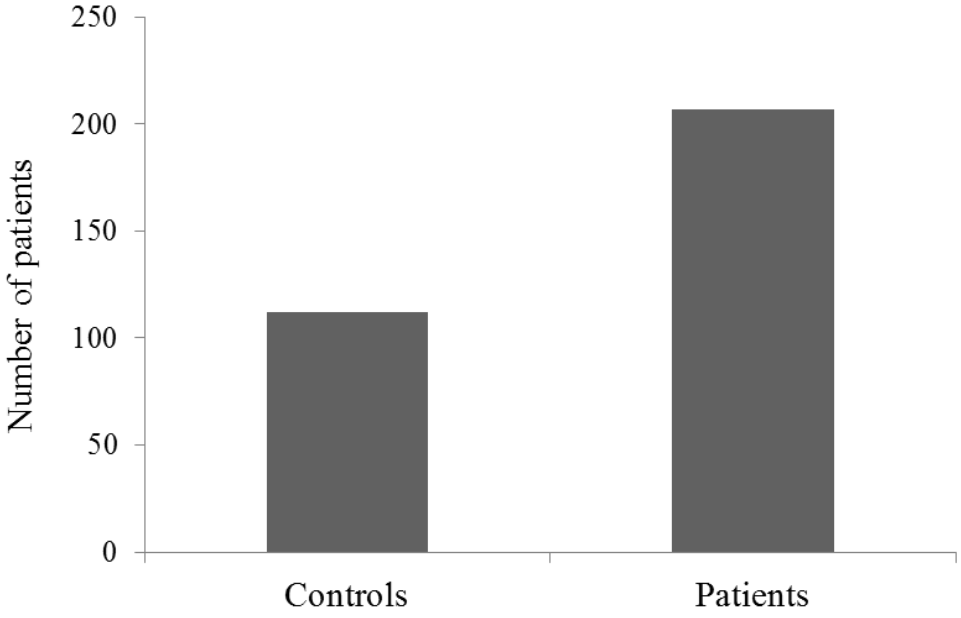



**Figure 3.1** Overall number of participants recruited in the GAPP study between September 2014 and April 2017 showing healthy controls (n=112) and patients (n=206)

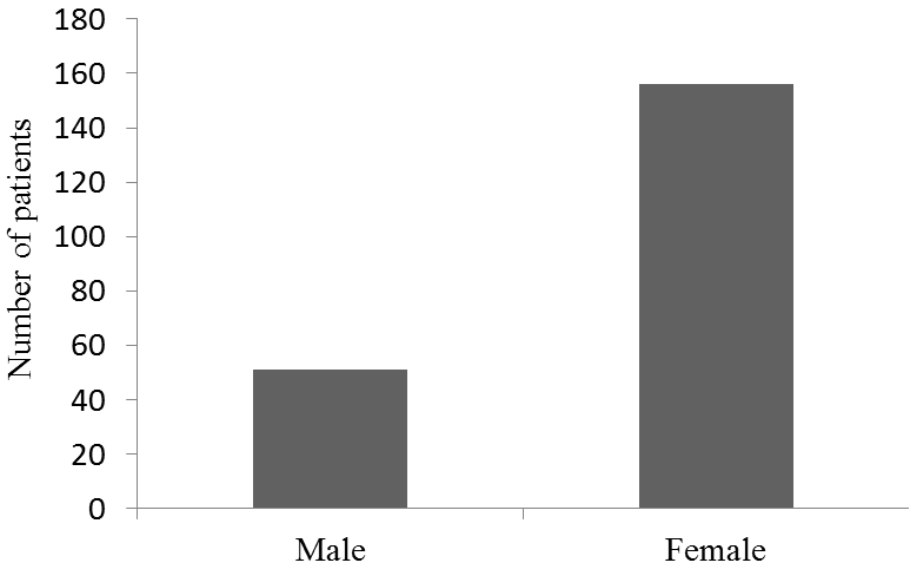



**Figure 3.2** Number of males (n=51(25%) and females (n=155(75%) recruited to the GAPP study

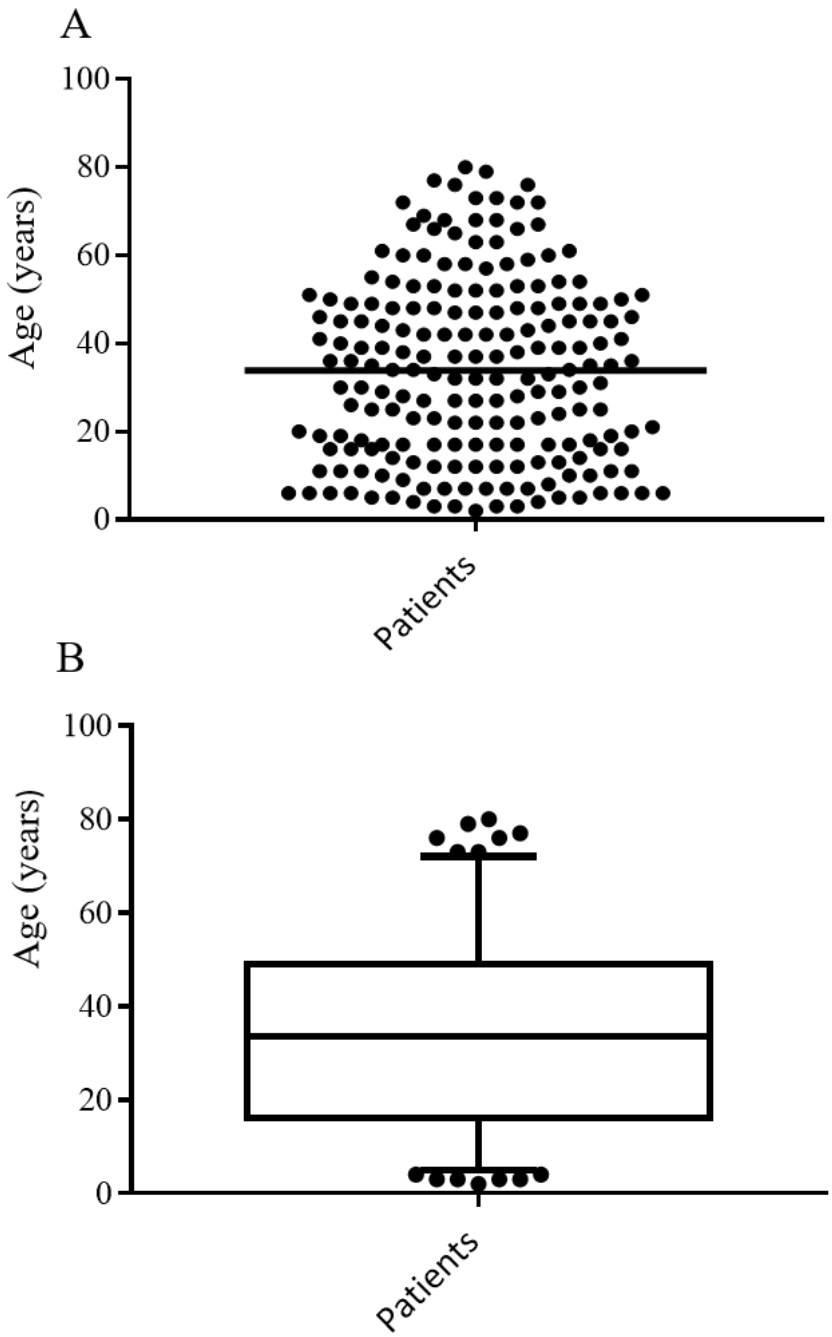



**Figure 3.3** Age distribution amongst patients (n=206) recruited into the GAPP study. For panel A, horizontal bars represent a mean age. For panel B, the box represents median and interquartile range and the whiskers represent 5$^{th}$ and 95$^{th}$ percentiles. Outlying points are represented by dots.

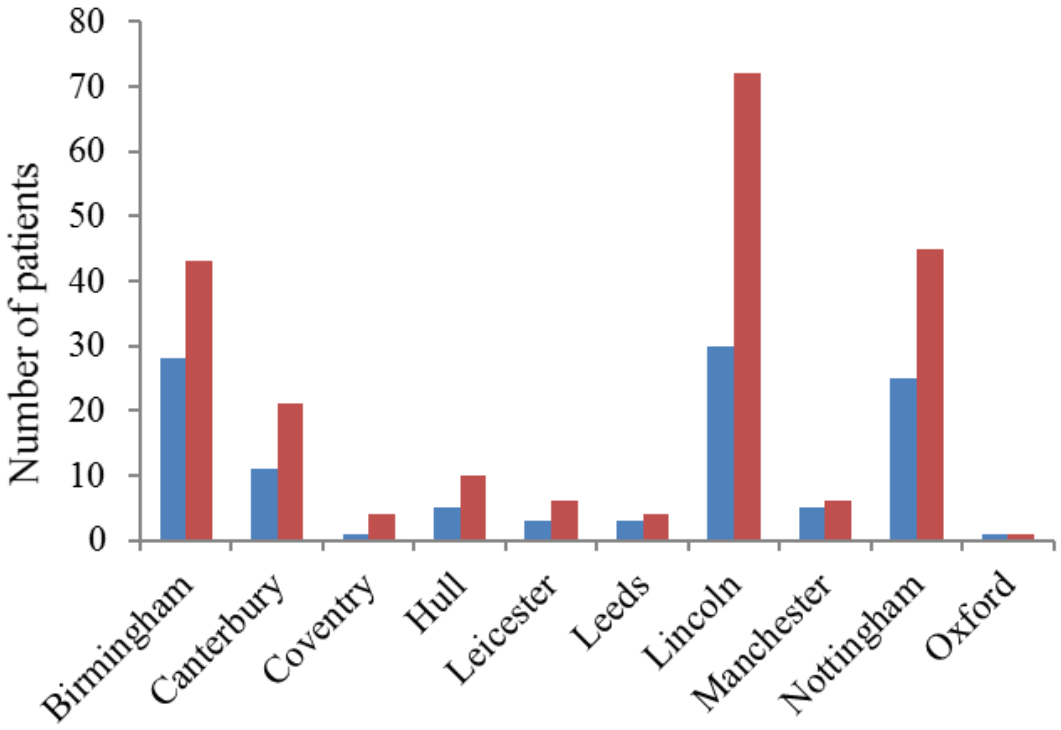



**Figure 3.4** Summary of blood samples recruited to the GAPP study from different haemophilia centres. Healthy controls (blue) and patients (red).

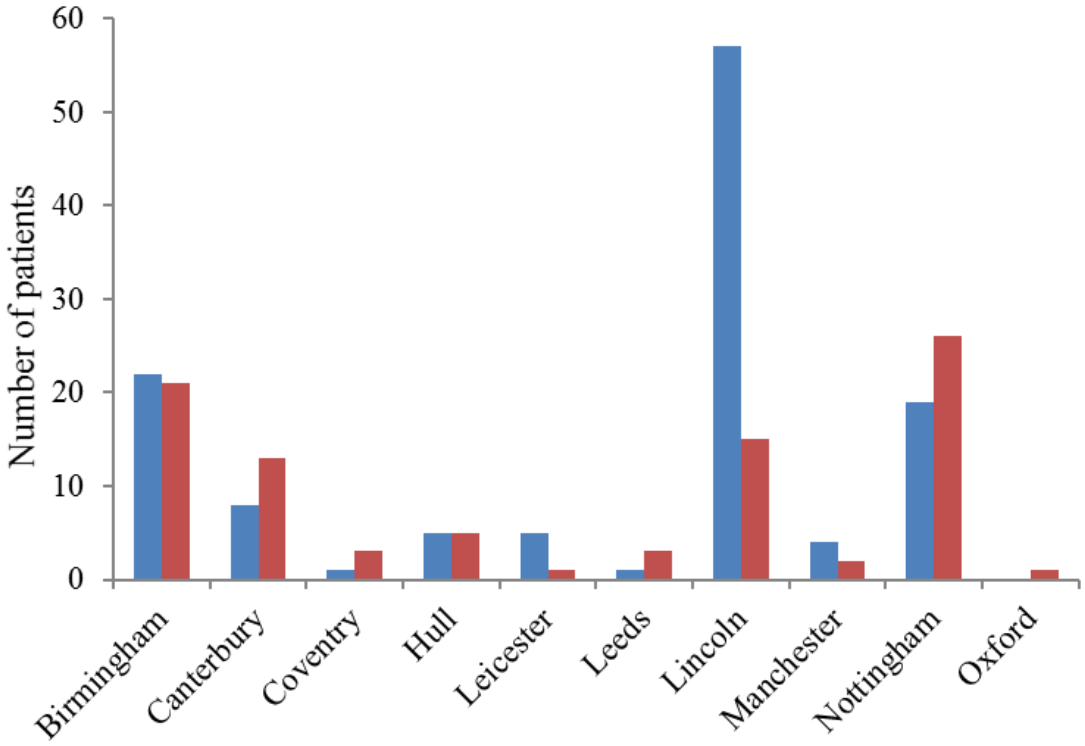



**Figure 3.5** Summary of platelet function results for samples received from each haemophilia centre. Numbers are divided into those with (red) and without (blue) detectable platelet function defects.

### 3.3.2 Platelet parameters and ATP secretion values

The overall platelet parameters and ATP secretion values of healthy controls (n=112) and patients (n=206) are shown in table 3.1. In patients, the values (mean ± SD) of whole blood platelet counts and MPV were 226.0 ± 84 x $10^9$/L and 11.2 ± 1.3fL respectively. The PRP count (mean ± SD) was 3.52 ± 1.58 x $10^8$/ml. ATP secretion values (mean ± SD) were 0.89 ± 0.49 nmol/1 x $10^8$/ml platelets. The patients' platelet counts in whole blood (figure 3.6) and PRP (figure 3.7) were not different compared to that of healthy controls. In contrast, the MPV (figure 3.8) and ATP secretion values (figure 3.9) of patients were significantly different when compared to healthy controls.

| | Healthy controls (mean ± SD) | Patients with normal platelet count (mean ± SD) | Statistical analysis | Healthy controls (mean ± SD) | Patients with thrombocytopenia (mean ± SD) | Statistical analysis |
|---|---|---|---|---|---|---|
| **Platelet count in Whole Blood (x$10^9$/L)** | 225.0 ± 65 | 261± 68 | ns | 225.0 ± 65 | 106 ± 32 | *** |
| **(range)** | (150 - 420) | (150 - 430) | | (150 - 420) | (34 - 147) | |
| **Platelet count in PRP (x $10^8$/ml)** | 3.50 ± 1.29 | 3.9 ± 0.84 | ns | 3.50 ± 1.29 | 1.87 ± 0.77 | *** |
| **(range)** | (1.0 - 8.02) | (1.4 – 10.5) | | (1.0 - 8.02) | (0.8 – 3.92) | |
| **Mean Platelet Volume (fL)** | 11.6 ± 1.1 | 11.0 ± 1.1 | ns | 11.6 ± 1.1 | 12.2 ± 1.8 | ** |
| **(range)** | (8.3 - 14.2) | (8.6 – 13.2) | | (8.3 - 14.2) | (8.9 - 15.1) | |
| **Immature Platelet Fraction (%)** | 6.3 ± 3.3 | N/A | | 6.3 ± 3.3 | 16.9 ± 17.8 | * |
| **(range)** | (1.8 – 12.1) | N/A | | (1.8 – 12.1) | (2.1 - 63.2) | |
| **ATP secretion level (nmol/1x$10^8$ platelets)** | 1.1 ± 0.47 | 0.89 ± 0.42 | *** | 1.1 ± 0.47 | 0.87 ± 0.71 | ** |
| **(range)** | (0.33 - 2.90) | (0.19 – 2.74) | | (0.33 - 2.90) | (0.1 – 1.85) | |

**Table 3.1** Summary of platelet parameters and ATP secretion values obtained from healthy controls (n=112) and patients (n=206): Data are presented as mean, +/- standard deviation and ranges. Statistical analysis performed by the Mann Whitney U test. Significance was compared to healthy controls (***p <0.001, **p<0.01 and *p<0.05).

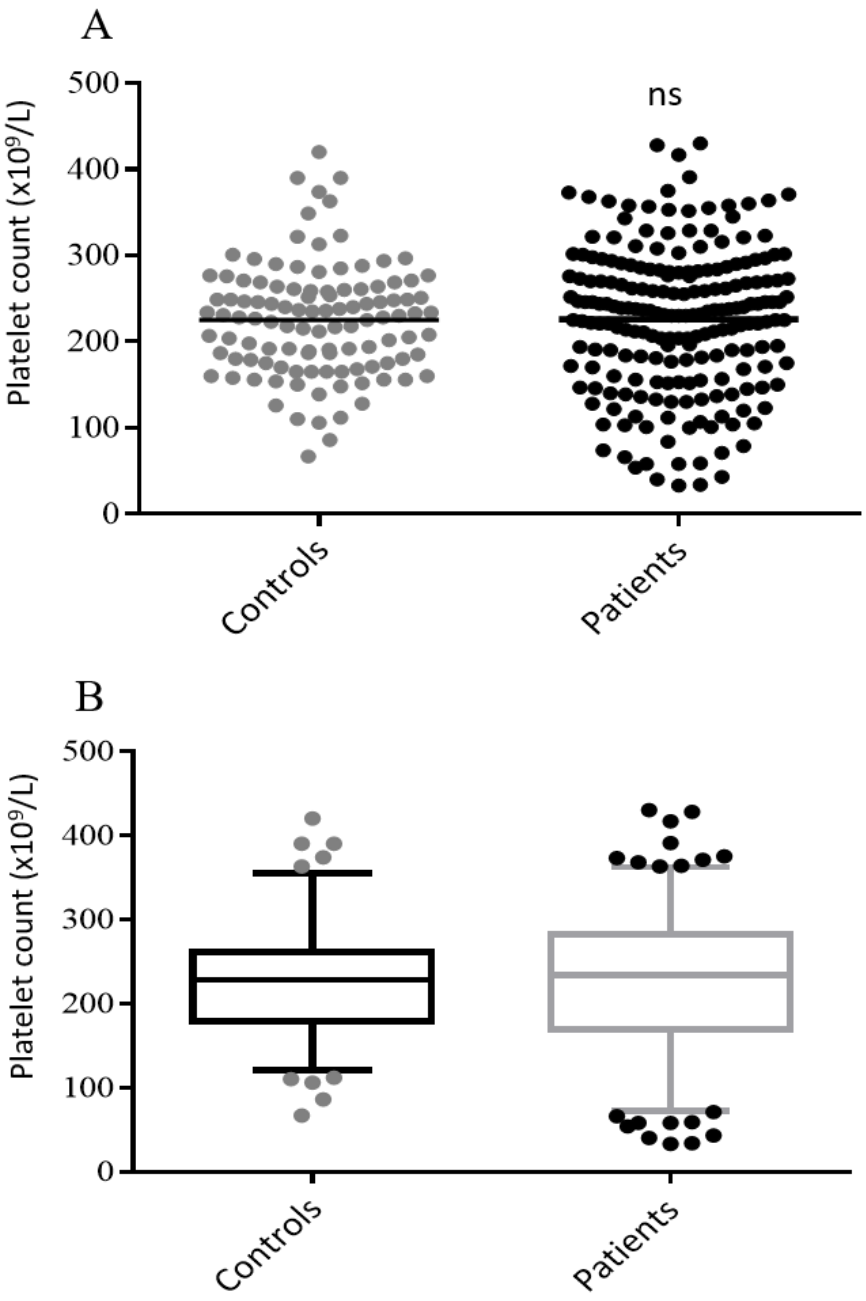



**Figure 3.6** Measurement of platelet counts in whole blood from healthy controls (n=112) and patients (n=206) as analysed by the Sysmex XN-1000. For panel A, horizontal bars represent mean values, statistical analysis performed by the Mann Whitney U test. For panel B, Boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

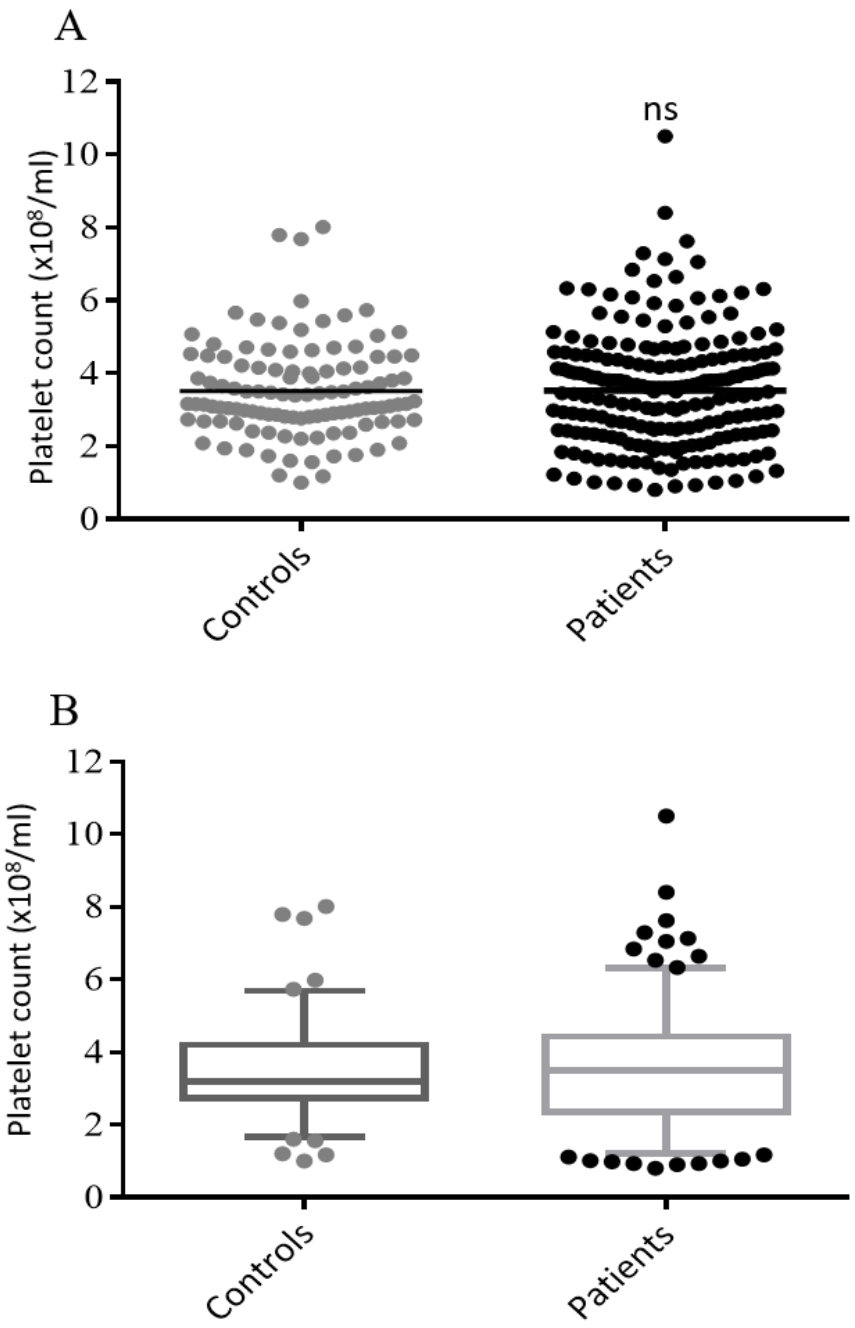



**Figure 3.7** Measurement of platelet counts in PRP from healthy controls (n=112) and patients (n=206) as analysed by the Coulter $Z_2$ analyser. For panel A, horizontal bars represent mean values and statistical analysis performed by the Mann Whitney U test. For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

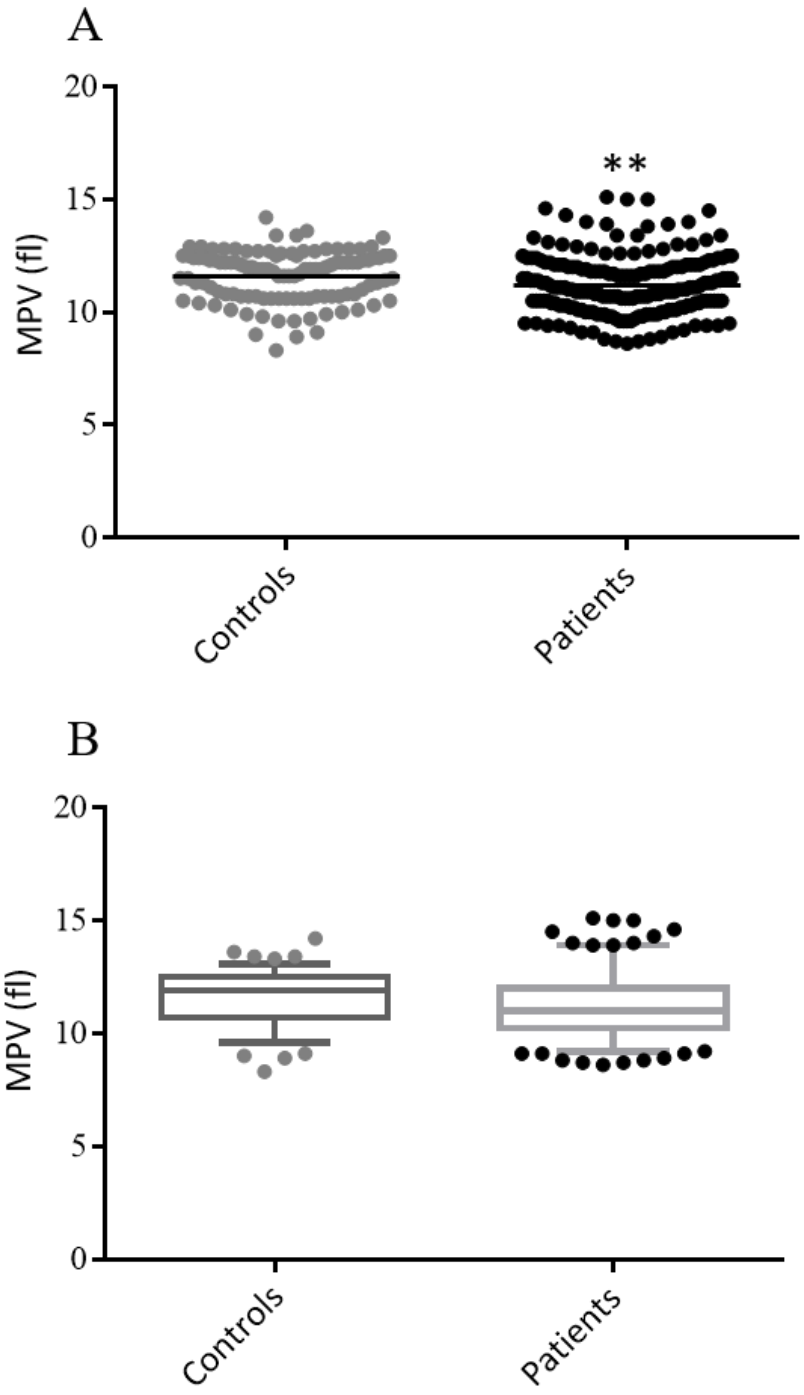



**Figure 3.8** Measurement of MPV values in whole blood from healthy controls (n=112) and patients (n=206) as analysed by the Sysmex XN-1000. For panel A, horizontal bars represent mean values, statistical analysis performed by the Mann Whitney U test. Significance was compared to healthy controls (**$p<0.01$). For panel B, Box represents median and interquartile ranges, whiskers represent $5^{th}$ and $95^{th}$ percentiles and outlying points are represented by dots.

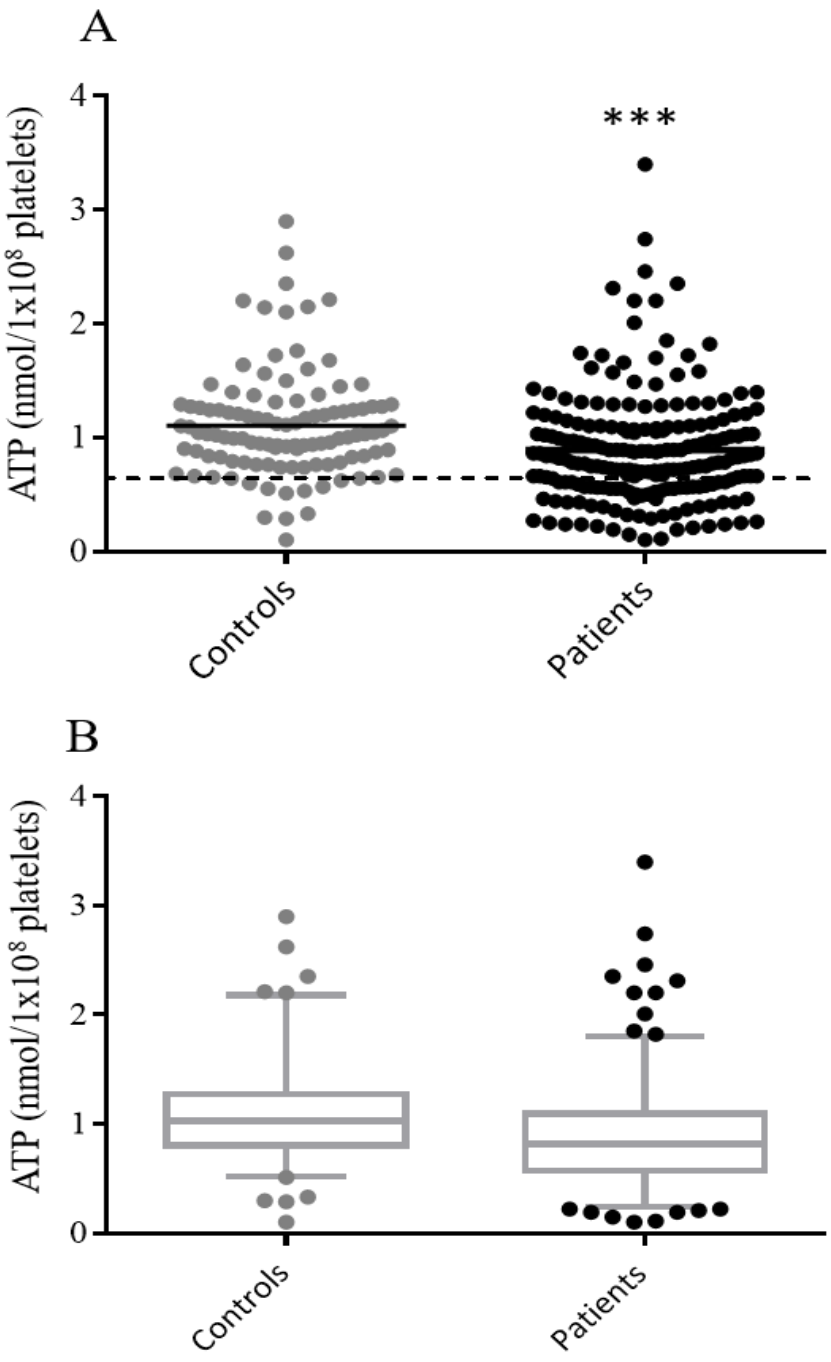



**Figure 3.9** ATP secretion values in PRP in response to 100µM PAR-1 TRAP peptide from healthy controls (n=112) and patients (n=206). For panel A, horizontal bars represent mean values and horizontal dotted line represents the 95$^{th}$ percentile (cut off value) used to determine abnormal results. Statistical analysis performed by the Mann Whitney U test. Significance was compared to healthy controls (***$p<0.001$). For panel B, the Boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

### 3.3.3 Overall aggregation results and platelet defect classification

Out of 206 patients tested, 90(44%) patients gave abnormal responses to one or more agonists, however, a significant proportion of patients (56%) were also found to exhibit normal platelet function (figure 3.10). As shown in figure 3.11B, the functional defects were classified into five main groups (Gi signalling defects, Gq signalling defecst, dense granular secretion defects, cyclooxygenase pathway defects, and other defects) according to their pattern of responses to specific agonists as previously described (Dawood et al., 2012). The distribution of defects amongst 90 patients is shown in figure 3.11A. A high proportion of patients were identified to have dense granular secretion defects (16%), followed by Gi signalling defects (11%). Defects within the cyclooxygenase pathway, Gq signalling, multiple defects and other defects were 5.8%, 3%, 6.2% and 2%, respectively.

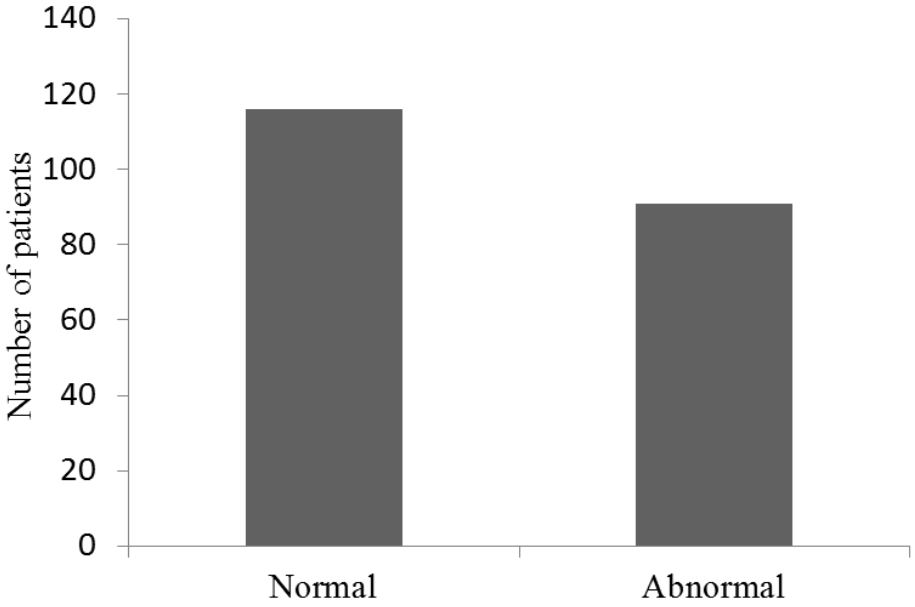



**Figure 3.10** Comparison between overall normal and abnormal lumi-LTA results identified among 206 patients recruited in into the GAPP study for the investigation of mild bleeding disorders.

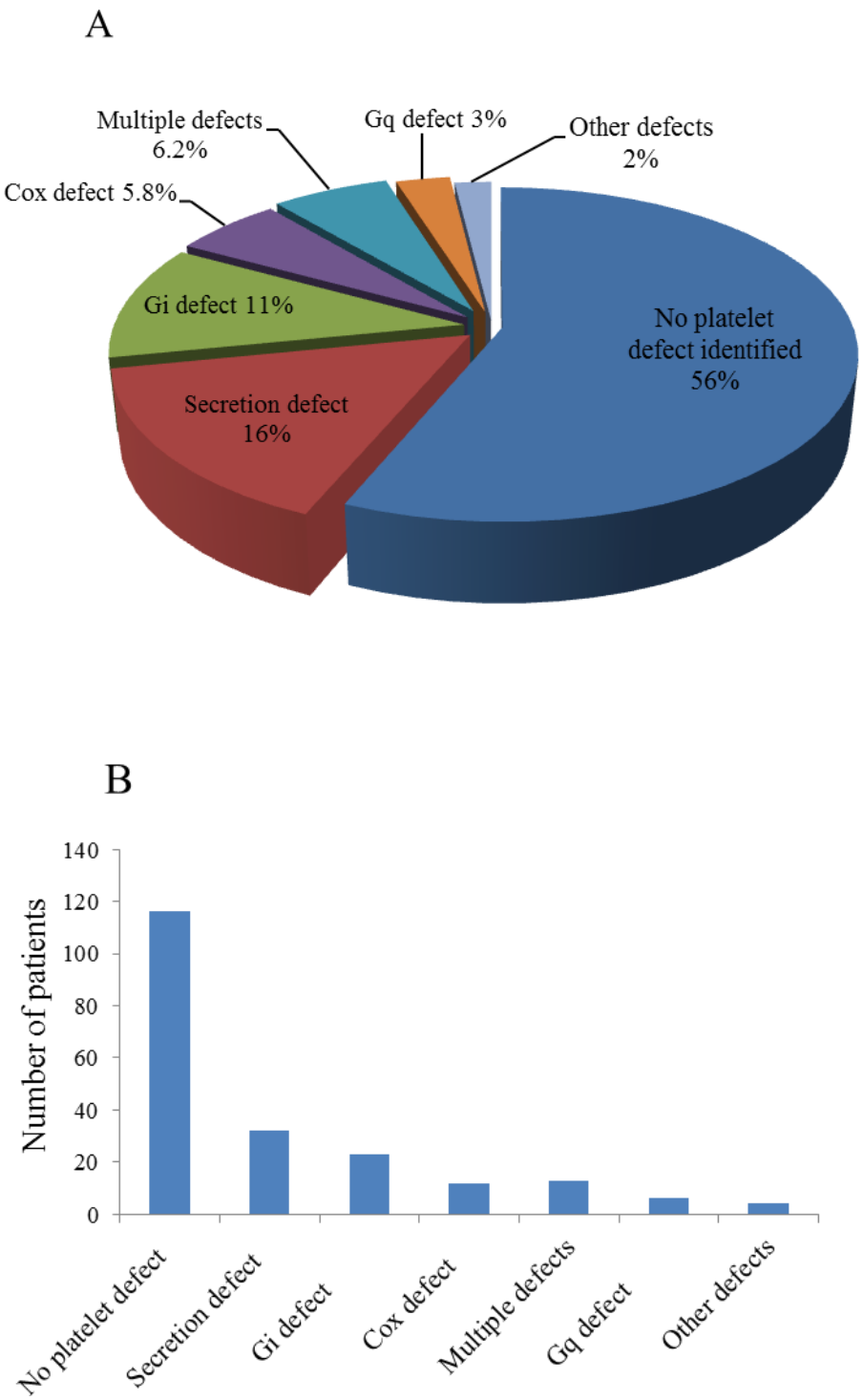



**Figure 3.11** Distribution of platelet function defects among 206 patients recruited into the GAPP study. (A) Pie chart segments showing the percentage of each defect (B) Bar chart showing the absolute number of patients in each defect. Cox = cyclooxygenase, Gi = Gi signalling pathway, Gq= Gq signalling pathway

### 3.3.4 Analysis of patients with thrombocytopenia

Out of 206 patients tested, 39 (19%) were found to have mild to moderate thrombocytopenia as characterized by low platelet counts (<150 $x10^9$/L) in whole blood (Figure 3.12). There was a small but significant difference in age between patients with thrombocytopenia and those with normal platelet counts (figure 3.13). The age (mean ± SD) of patients with thrombocytopenia was 27 ± 22 years and that of patients with normal platelet count was 35 ± 20 years. The proportion of males was higher among patients with thrombocytopenia than those with normal counts (35% and 22% respectively) (figure 3.14). The overall platelet parameters and ATP secretion of patients with thrombocytopenia and those with normal platelet counts are shown in table 3.2. The values (mean ± SD) of whole blood platelet count and MPV were 261 ± 68 x $10^9$/L and 11.0 ± 1.1fL for patients with normal count and 106 ± 32 x $10^9$/L and 12.2 ± 1.8fL for patients with thrombocytopenia, respectively. The PRP count (mean ± SD) was 3.9 ± 0.84 x $10^8$/ml in patients with normal counts and 1.87 ± 0.77 x $10^8$/ml in thrombocytopenia. The platelet counts in whole blood (figure 3.15) and in PRP (figure 3.16) were significant lower in patients with thrombocytopenia than patients with normal platelet count. In contrast, the MPV was higher in patients with thrombocytopenia than patients with normal platelet counts (figure 3.17). ATP secretion values (mean ± SD) were 0.89 ± 0.42 nmol/1 x $10^8$ and 0.87 ± 0.71 nmol/1 x $10^8$ in patients with normal count and in thrombocytopenia respectively (table 3.2).

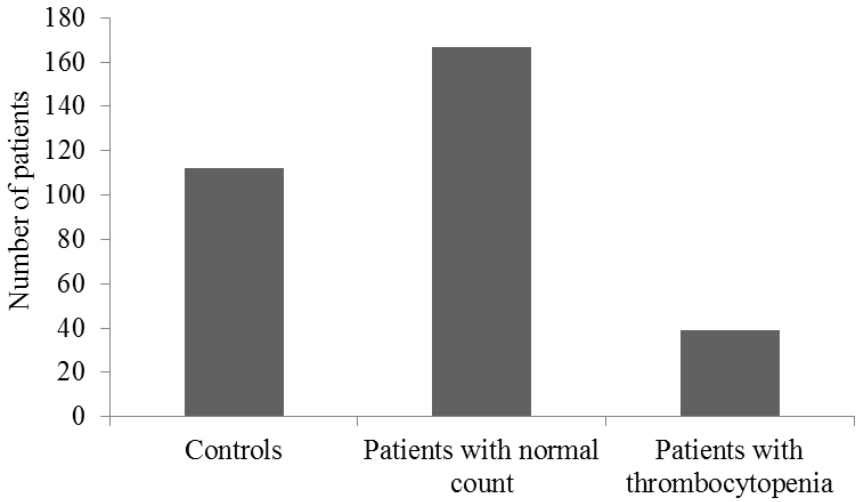



**Figure 3.12** Overall number of participants recruited in the GAPP study between September 2014 and April 2017 showing healthy controls (n=112), patients with normal platelet counts (n=167) and thrombocytopenia (n=39)

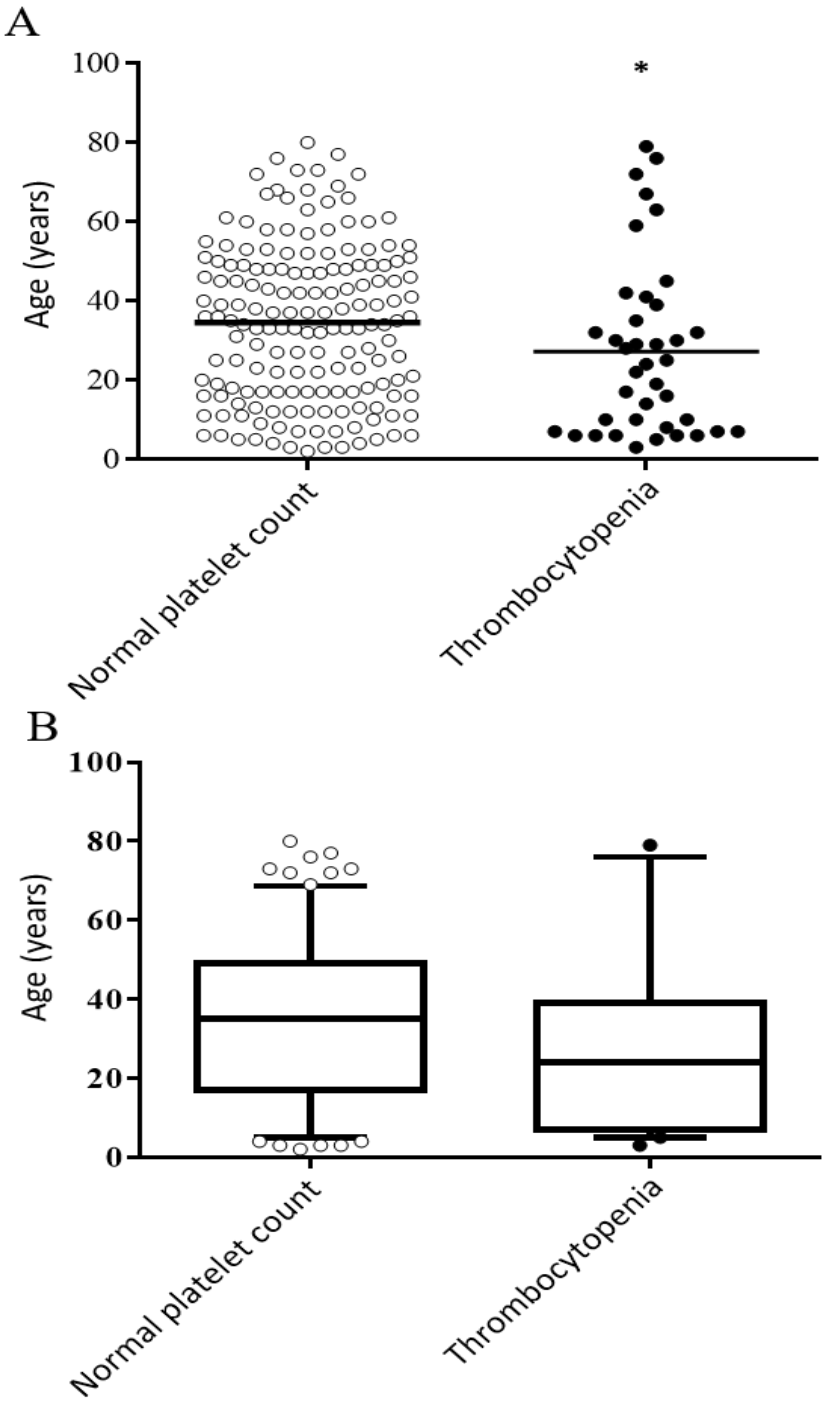



**Figure 3.13** Age distribution amongst patients with normal platelet counts (n=167) and thrombocytopenia (n=39) recruited into the GAPP study. For panel A, horizontal bars represent a mean age. Statistical analysis performed by the Mann Whitney U test. Significance as compared to patients with normal platelet count (*$p<0.05$). For panel B, Boxes represent median and interquartile range, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

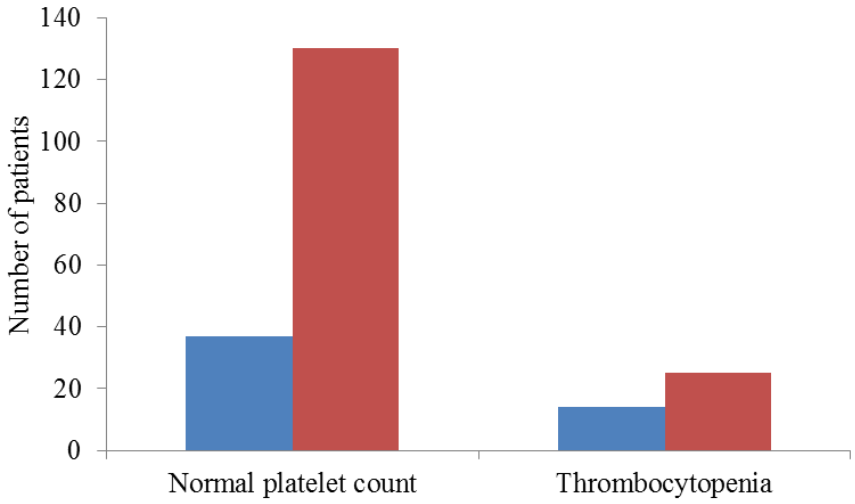



**Figure 3.14** Distribution between male (blue) and female (red) among patients with normal platelet counts and thrombocytopenia.

| | Healthy controls (mean ± SD) | Patients with normal platelet count (mean ± SD) | Patients with thrombocytopenia (mean ± SD) |
|---|---|---|---|
| **Platelet count in Whole Blood ($x10^9$/L)** | 225.0 ± 65 | 261± 68 | 106 ± 32 |
| **(range)** | (150 - 420) | (150 - 430) | (34 - 147) |
| **Platelet count in PRP ($x\ 10^8$/ml)** | 3.50 ± 1.29 | 3.9 ± 0.84 | 1.87 ± 0.77 |
| **(range)** | (1.0 - 8.02) | (1.4 – 10.5) | (0.8 – 3.92) |
| **Mean Platelet Volume (fL)** | 11.6 ± 1.1 | 11.0 ± 1.1 | 12.2 ± 1.8 |
| **(range)** | (8.3 - 14.2) | (8.6 – 13.2) | (8.9 - 15.1) |
| **Immature Platelet Fraction (%)** | 6.3 ± 3.3 | N/A | 16.9 ± 17.8 |
| **(range)** | (1.8 – 12.1) | N/A | (2.1 - 63.2) |
| **ATP secretion level (nmol/$1x10^8$ platelets)** | 1.1 ± 0.47 | 0.89 ± 0.42 | 0.87 ± 0.71 |
| **(range)** | (0.33 - 2.90) | (0.19 – 2.74) | (0.1 – 1.85) |

**Table 3.2** Comparison of platelet parameters and ATP secretion values between patients with normal platelet counts (n=167) and thrombocytopenia (n=39): Data are presented as mean, +/- standard deviation and range. For statistical analysis please referred to specific graph of each parameters

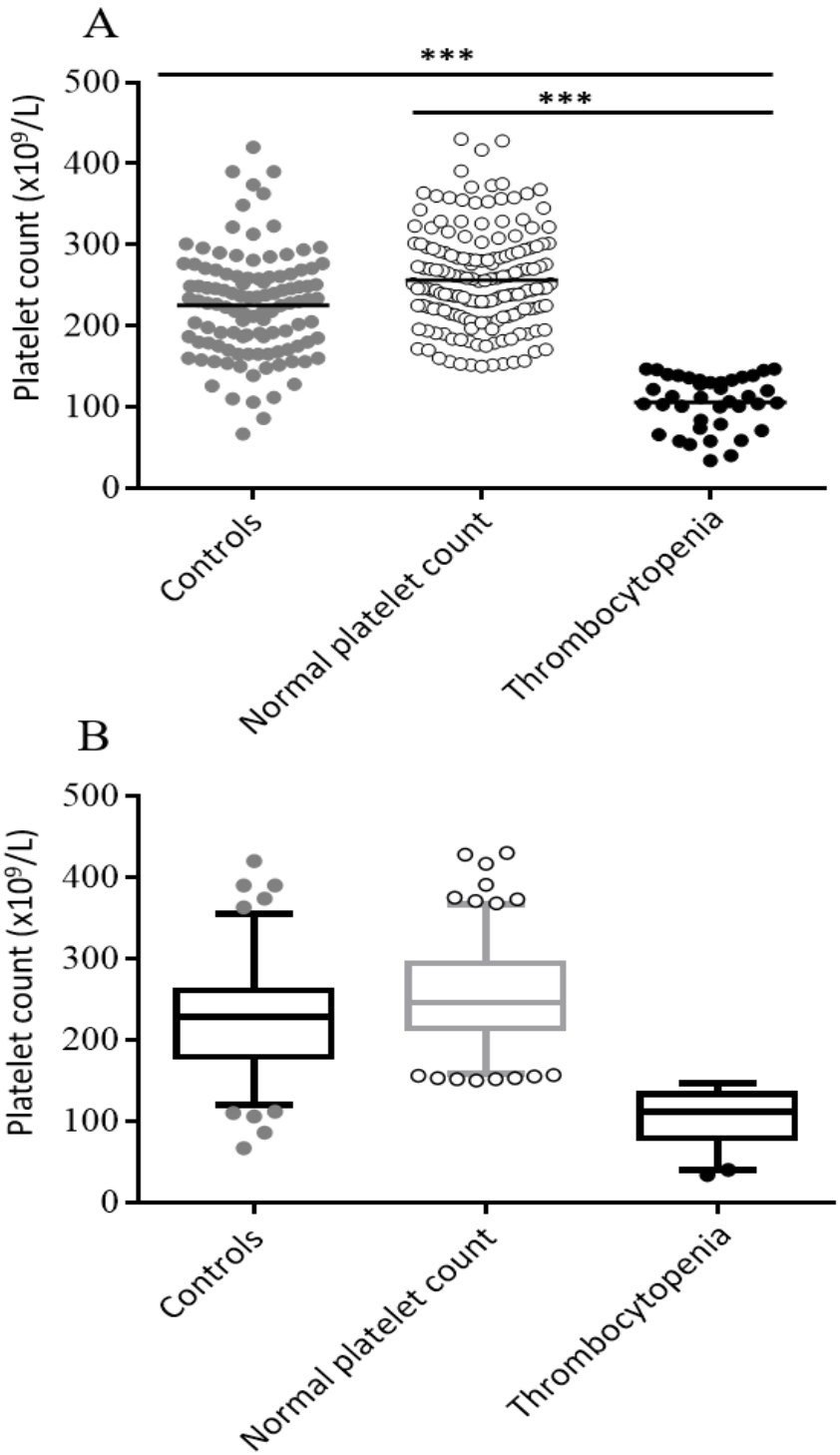



**Figure 3.15** Measurement of platelet counts in whole blood among healthy controls (n=116), patients with normal platelet counts (n=167) and thrombocytopenia (n=39). For panel A, horizontal bars represent mean values. Statistical analysis performed by the Kruskal-Wallis test with Dunn's adjustment for multiple comparisons. Significance as compared to healthy controls (***$p<0.001$). For panel B, Boxes represents median and interquartile range, whiskers represent $5^{th}$ and $95^{th}$ percentiles and outlying points are represented by dots.

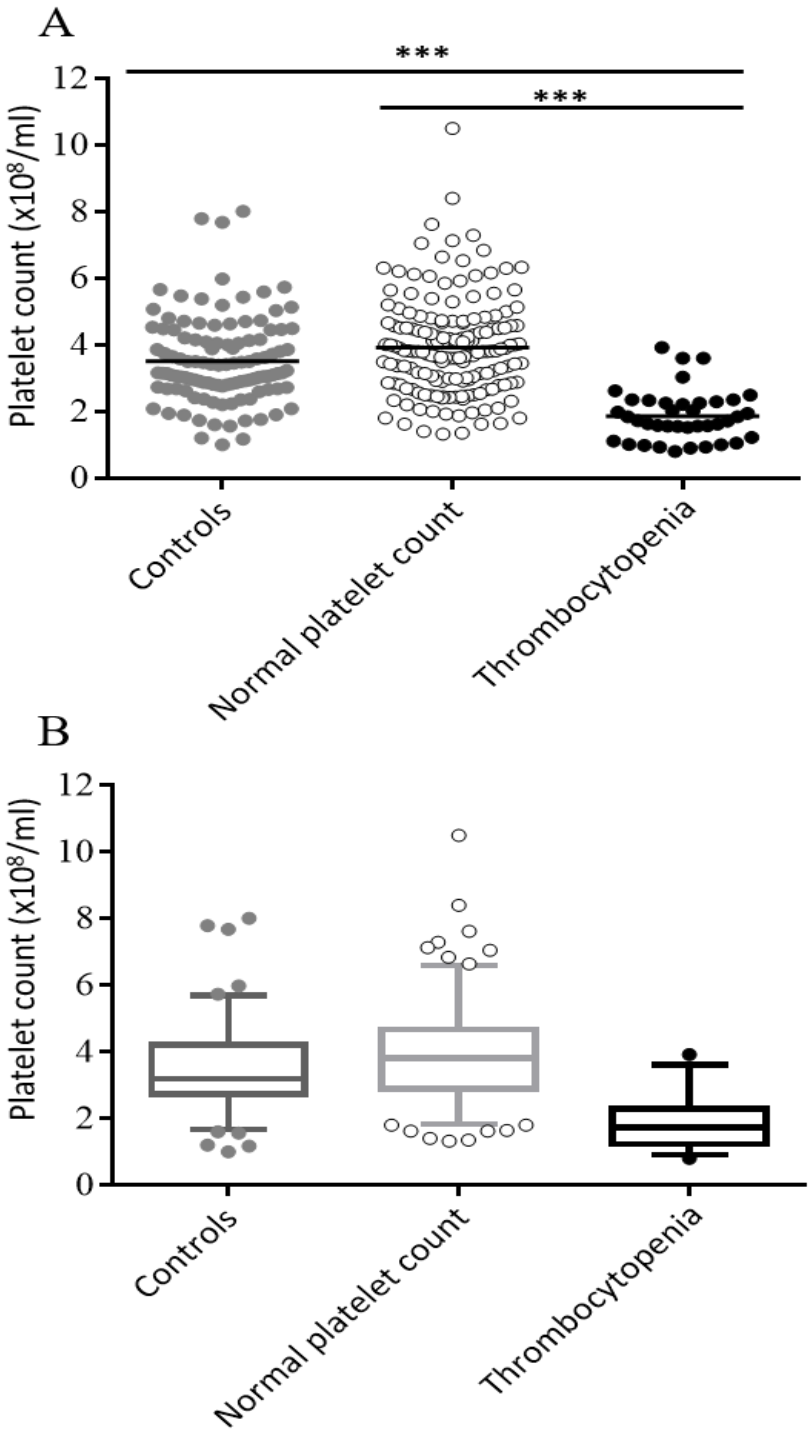



**Figure 3.16** Measurement of PRP platelet counts among healthy controls (n=116), patients with normal platelet counts (n=167) and thrombocytopenia (n=39) as analysed by the Coulter $Z_2$ analyser. For panel A, horizontal bars represent mean values. Statistical analysis performed by the Kruskal-Wallis test with Dunn's adjustment for multiple comparisons. Significance was compared to healthy controls (***$p<0.001$). For panel B, Boxes represent median and interquartile ranges, whiskers represent $5^{th}$ and $95^{th}$ percentiles and outlying points are represented by dots.

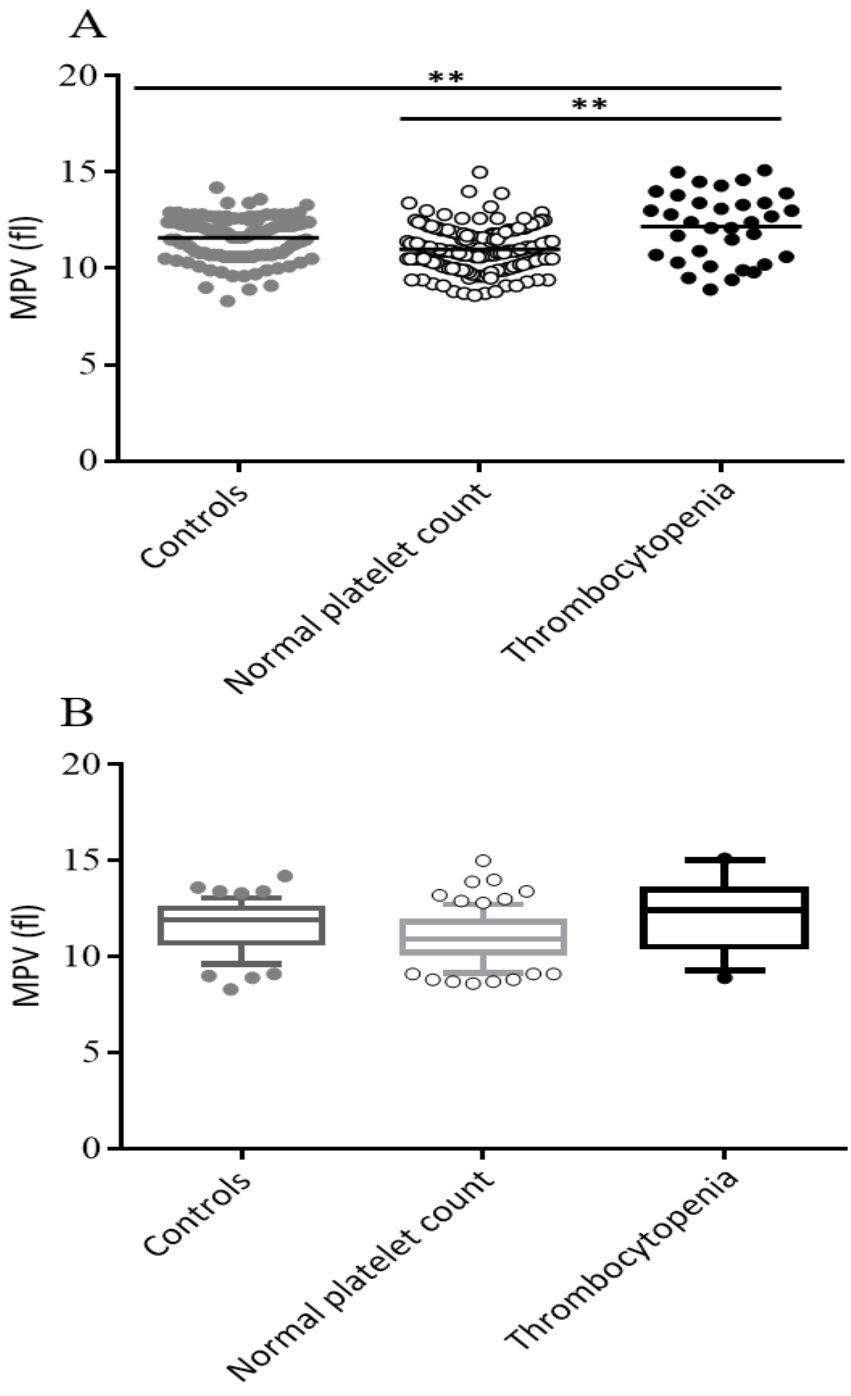



**Figure 3.17** Measurement of MPV in whole blood among healthy controls (n=116), patients with normal platelet counts (n=167) and thrombocytopenia (n=39). For panel A, horizontal bars represent mean values. Statistical analysis performed by the Kruskal-Wallis test with Dunn's adjustment for multiple comparisons (**p<0.01). For panel B, Boxes represent median and interquartile range, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

75% of patients with thrombocytopenia were also found to have secondary defects in platelet function. In contrast, only 36% of patients with normal platelet counts were found to have a platelet function defect (figure 3.18). Interestingly, dense granular secretion defects were the most common functional defects (58%) among patients with thrombocytopenia compared to normal platelet counts (33%) (figure 3.19). Surprisingly, however, there was no significant different in ATP secretion levels between the two patient groups (figure 3.20).

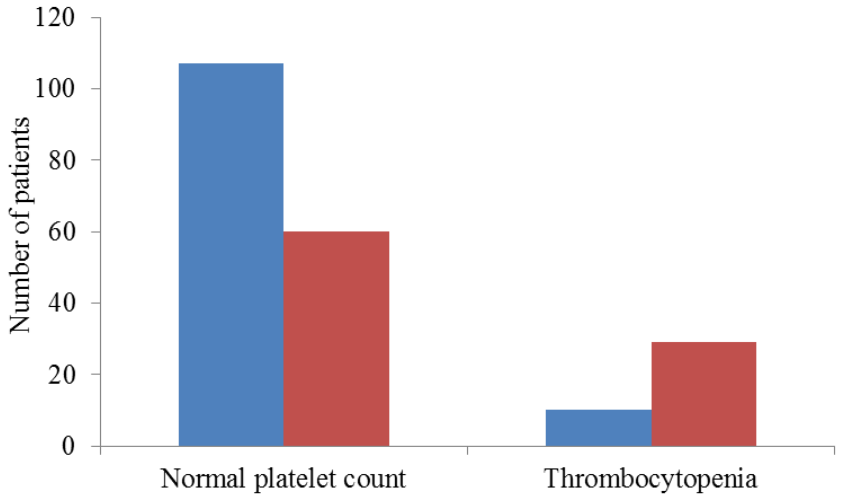



**Figure 3.18** Overall comparison between the number of samples found to have platelet defects (red) in patients with normal platelet counts (n=167) and thrombocytopenia (n=39).

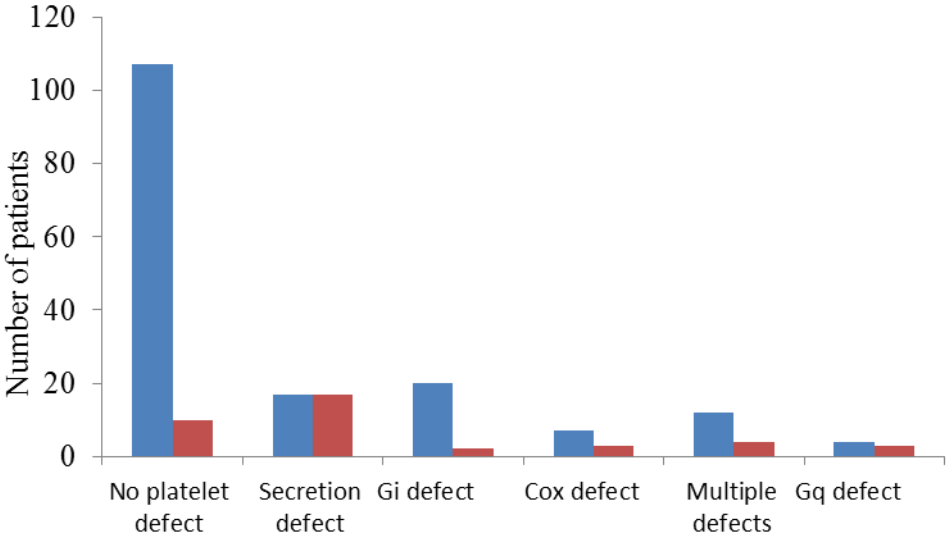



**Figure 3.19** Classification of platelet function defects among patients with normal platelet counts (blue) and patients with thrombocytopenia (red). Cox = cyclooxygenase, Gi = Gi signalling pathway, Gq= Gq signalling pathway

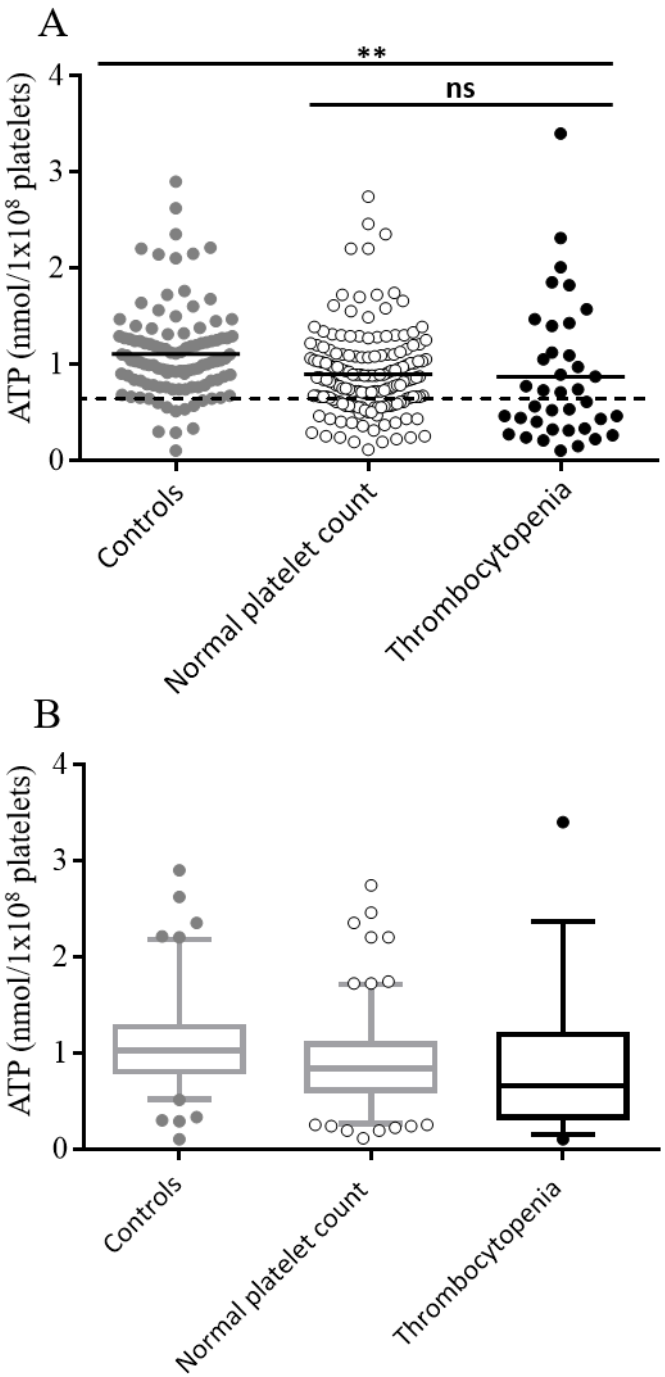



**Figure 3.20** Measurement of ATP secretion values among healthy controls (n=116), patients with normal platelet counts (n=167) and thrombocytopenia (n=39) as determined by lumi-LTA measuring ATP secretion in response to 100μM PAR-1 TRAP stimulation. For panel A, horizontal bars represent mean values. Statistical analysis performed by the Kruskal-Wallis test with Dunn's adjustment for multiple comparisons (**$p<0.01$). For panel B, Boxes represent median and interquartile ranges, whiskers represent $5^{th}$ and $95^{th}$ percentiles, and outlying points are represented by dots.

### 3.3.5 Assessment of ISTH Bleeding score among patients with normal platelet counts

The ISTH BAT was used for assessment of the bleeding history in 122 of patients with normal platelet counts. The age of patients (median ± SD) was 40±17 and the majority were female (84%). I also included the BAT score of 21 healthy controls that were previously assessed within the GAPP study (Lowe et al., 2013). A platelet function defect was identified by lumi-LTA in 32% of patients. The BAT score (median ± SD) of patients was significantly higher than that of healthy controls (11 ± 5.7 and 0.6 ± 1.5 respectively) (figure 3.21). Similarly, female patients had higher BAT scores than males (12 ± 6 and 8 ± 5 respectively) (figure 3.22). However, there was no significant difference between the BAT score of patients with no detectable platelet defect and those with platelet defects detected by lumi-LTA (figure 3.21). Furthermore no association was found between the BAT score and the type of platelet defect detected by lumi-LTA (figure 3.23). Surprisingly, the BAT score of patients with thrombocytopenia (10 ± 5) were similar to that of patients with normal platelet counts (11 ± 6) (figure 3.24).

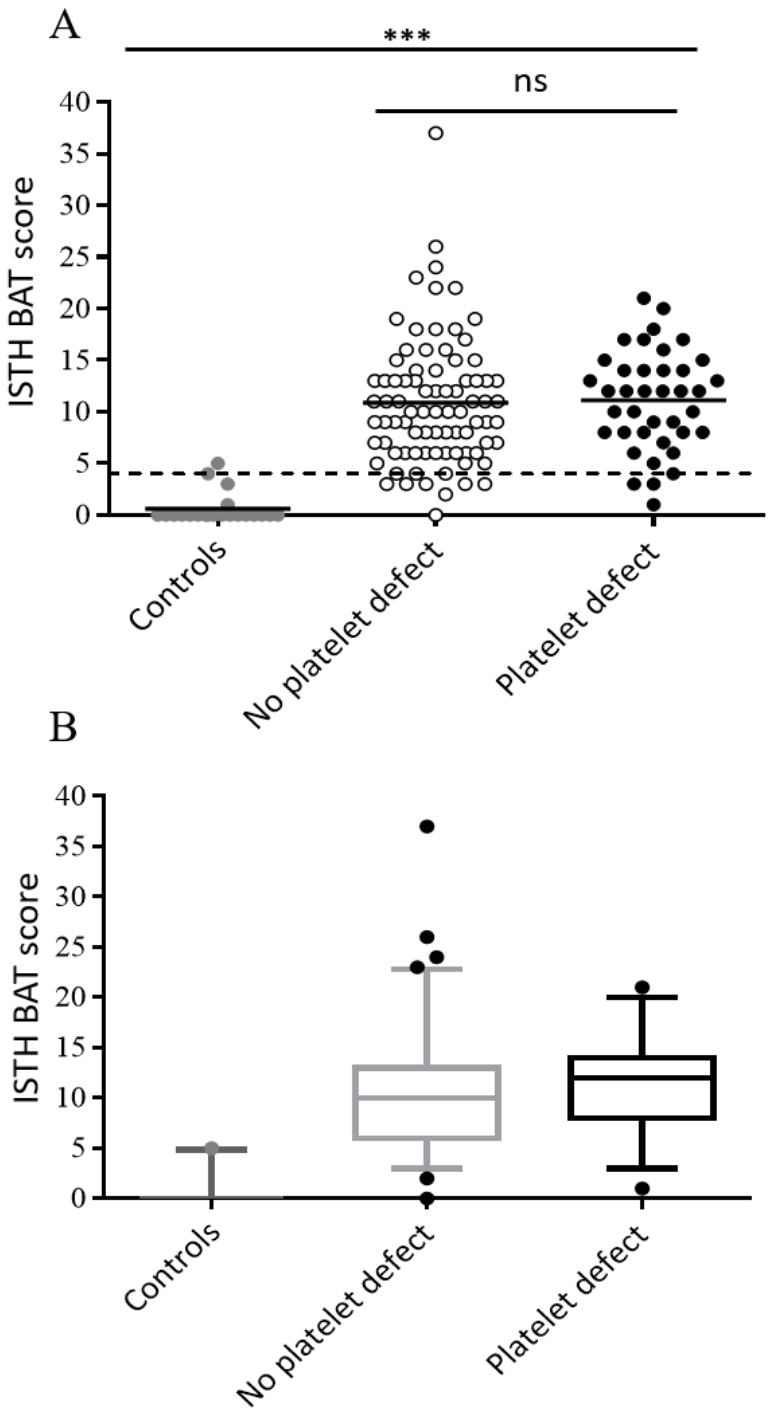



**Figure 3.21** Association between the ISTH BAT score and the presence of a platelet function defect on lumi-LTA. For panel A, horizontal bars represent a mean values, horizontal dotted line represents 95$^{th}$ percentile (score of 4) calculated from healthy controls. Statistical analysis performed by the non-parametric Kruskal-Wallis test, with Dunn's adjustment for multiple comparisons (***$p<0.001$). For panel B, Boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

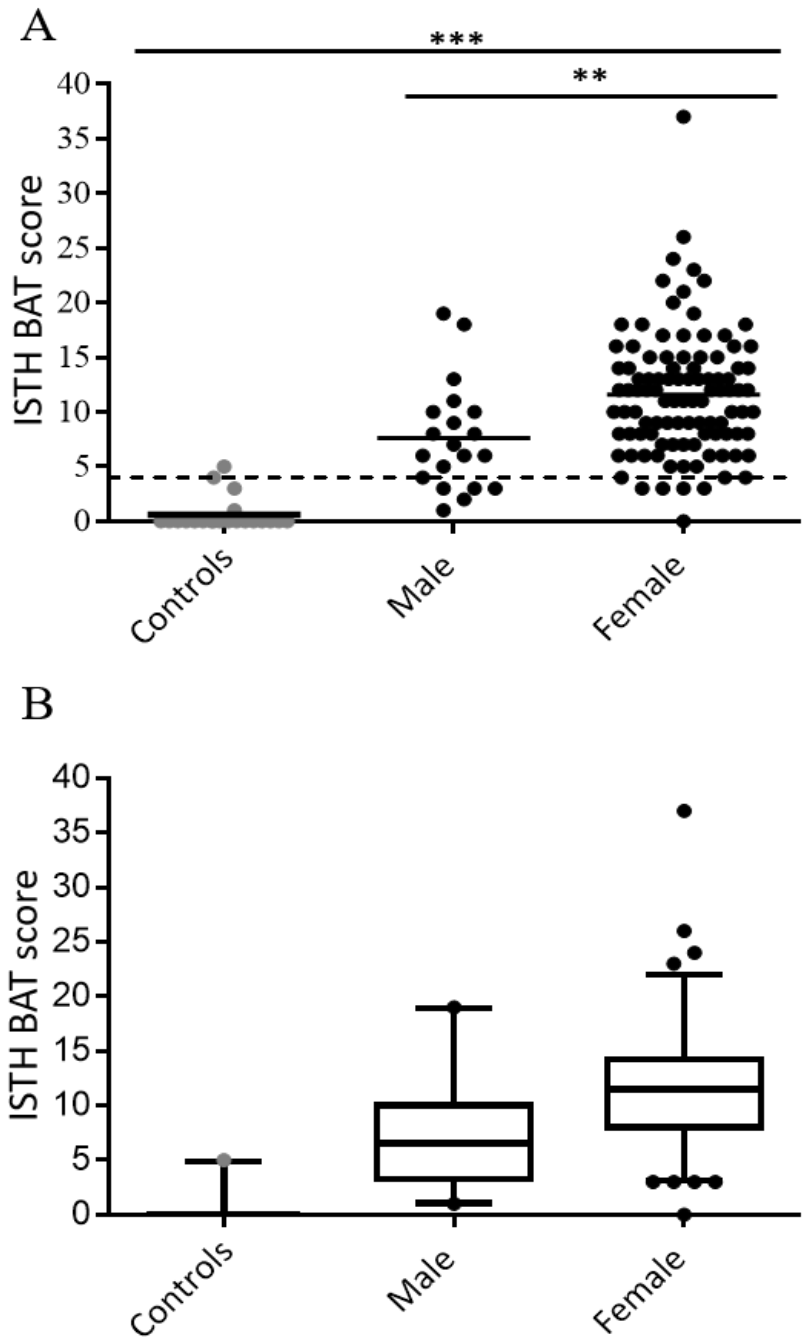



**Figure 3.22** Comparison of ISTH BAT scores between male and female patients. For panel A, horizontal bars represent a mean values, horizontal dotted line represents 95$^{th}$ percentile (score of 4) calculated from healthy controls. Statistical analysis performed by the non-parametric Kruskal-Wallis test, with Dunn's adjustment for multiple comparisons (***$p<0.001$ and **$p<0.01$). For panel B, Boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

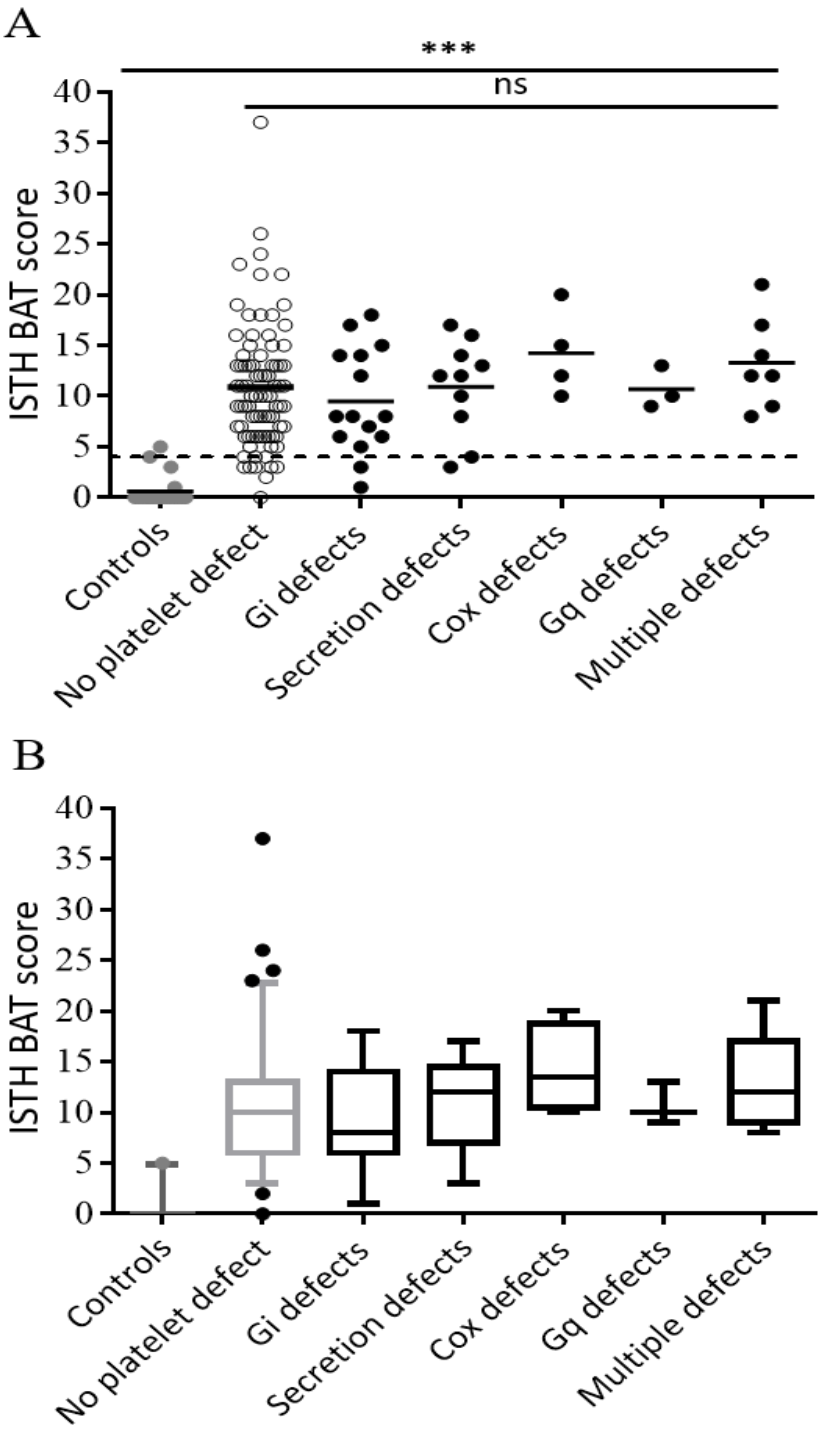



**Figure 3.23** Association between the ISTH BAT scores and the type of platelet function defect on lumi-LTA. For panel A, horizontal bars represent a mean values, horizontal dotted line represents 95$^{th}$ percentile (score of 4) calculated from healthy controls. Statistical analysis performed by the non-parametric Kruskal-Wallis test, with Dunn's adjustment for multiple comparisons (***$p<0.001$). For panel B, Boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

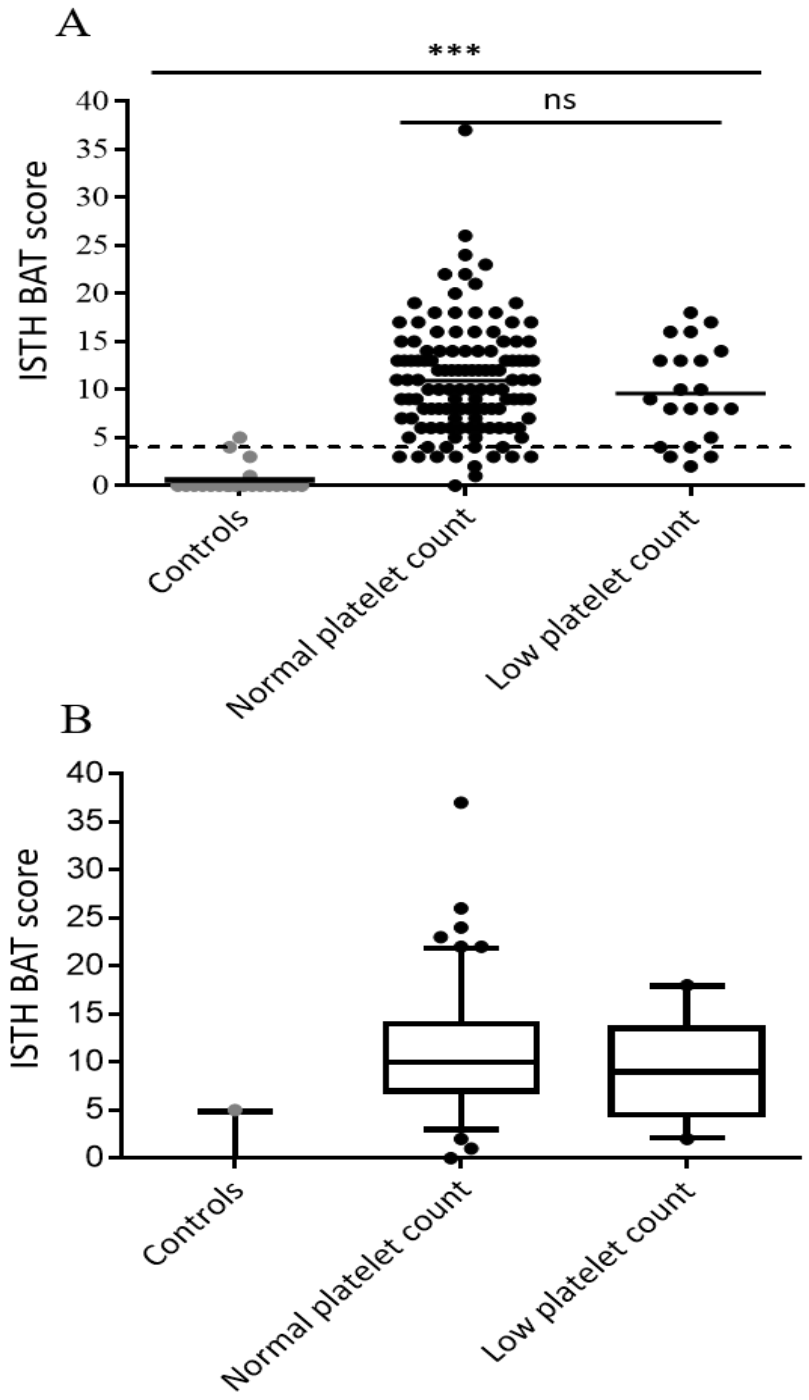



**Figure 3.24** Comparison of ISTH BAT scores between patients with normal counts and thrombocytopenia. For panel A, horizontal bars represent a mean values, horizontal dotted line represents 95$^{th}$ percentile (score of 4) calculated from healthy controls (n=21). Statistical analysis performed by the non-parametric Kruskal-Wallis test, with Dunn's adjustment for multiple comparisons (***$p<0.001$). For panel B, Boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

A receiver-operator curve (ROC) analyse was used to investigate the ability of the ISTH BAT score to discriminate between patients with and without a demonstrable platelet function defect by lumi-LTA (figure 3.25). The area under the curve (AUC) was 0.54 (95% CI 0.43 to 0.65) thus indicating a lack of discriminative ability. Furthermore a comparison study was also performed to analyse the ability of the ISTH BAT score to predict the presence of a platelet defect on lumi-LTA. A score of 11 was used as a cut off value for the ISTH BAT as this presented the $50^{th}$ centile of the ISTH BAT score in all patients. The agreement between the ISTH BAT score and the presence/absence of platelet defect by lumi-LTA is shown in table 3.3. Overall, there was a concordance in 53% of patients, with a positive predictive value of only 35%. This finding demonstrates a lack of association between the high ISTH BAT score and the likelihood of having a platelet defect.

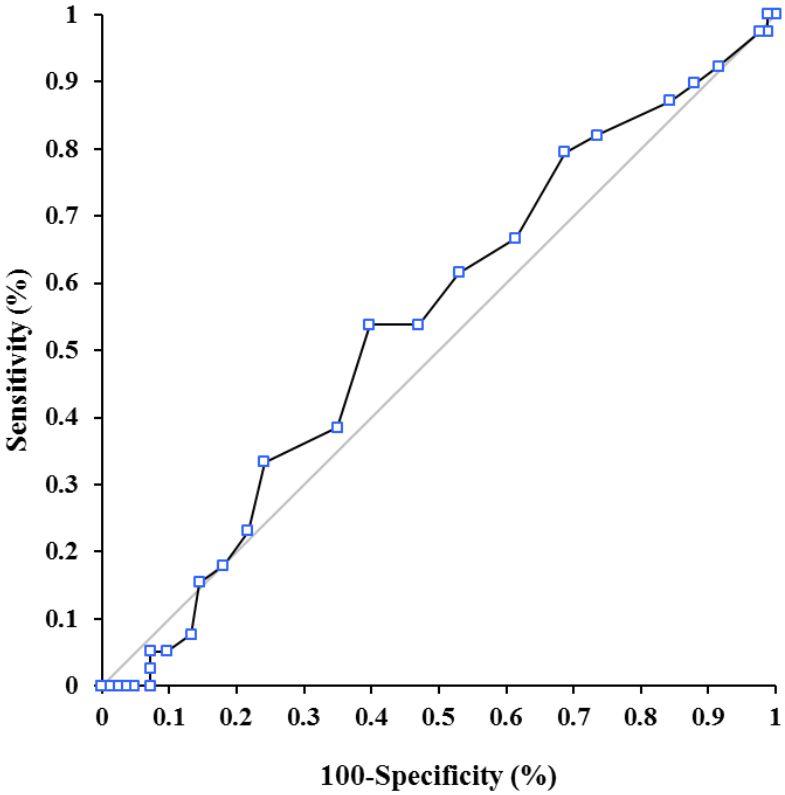



**Figure 3.25** ROC analysis for the presence of a platelet function defect by lumi-LTA and the ISTH BAT score. Area under the curve: 0.54(95% CI 0.43 to 0.65).

| | Lumi-LTA (Abnormal) | Lumi-LTA (Normal) | |
|---|---|---|---|
| BAT score (≥11) | 21 | 39 | PPV=35% |
| BAT score (<11) | 18 | 44 | NPV=71% |
| | Sensitivity=54% | Specificity=53% | |

**Table 3.3** A comparison study to analyse the ISTH BAT score of 11 and over in predicting the presence of a platelet defect by lumi-LTA. ISTH BAT score of 11 represents the 50$^{th}$ centile in all patients studied. Sensitivity, specificity, positive predictive value and negative predictive value are shown.

### 3.3.6 Investigation of families with a history of excessive bleeding

10 families with a history of excessive bleeding were recruited into the GAPP study for the investigation of potential platelet function defects. Families were categorised into three groups based on their platelet count in whole blood. (1) Family with normal platelet counts, (2) family with thrombocytopenia and (3) family with variable platelet counts (only families with normal platelet counts are fully analysed including NSG). All family members had platelet aggregation performed by lumi-LTA. Overall demographic characteristic and summary of platelet phenotyping of all ten families are shown in figure 3.26 (families with normal platelet count and with thrombocytopenia) and figure 3.27 (families with variable platelet count). The two families with normal platelet counts will be discussed in more detail in the results chapter four.

A

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| I | I | 53 | F | 225 | 9.8 | 4.8 | 0.67 | Gi defect | 14 |
| | II | 53 | M | 171 | 10.5 | 2.98 | 1.07 | Gi defect | 1 |
| | III | 31 | F | 247 | 10 | 3.82 | 0.94 | No defect | 5 |
| | IV | 27 | M | 430 | 9.1 | 7.13 | 0.84 | No defect | 7 |

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| III | I | 45 | F | 244 | 11.2 | 4.52 | 0.52 | Cox + Secretion | 17 |
| | II | 23 | M | 428 | 9.1 | 6.53 | 0.39 | Secretion defect | 10 |
| | III | 20 | F | 345 | 10.2 | 5.39 | 0.59 | Cox + Secretion | 12 |
| | IV | 17 | M | 329 | 10.9 | 3.43 | 0.25 | Secretion defect | 3 |

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| X | I | 9 | F | 329 | 12.1 | 3.88 | 1.39 | No defect | - |
| | II | 11 | M | 246 | 11.3 | 2.4 | 1.08 | No defect | - |
| | III | 33 | F | 259 | 11.7 | 2.45 | 1.55 | No defect | - |
| | IV | 31 | M | 196 | 12.6 | 1.96 | 1.09 | Gi defect | - |

B

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| V | I | 10 | F | 71 | 0/53.8 | 1.11 | 2.31 | No defect | - |
| | II | 7 | M | 54 | 0/63.2 | 0.8 | 1.4 | No defect | - |

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| IX | I | 7 | F | 34 | 10.3 | 1 | 0.1 | Secretion defect | - |
| | II | 16 | F | 58 | 9.8 | 2.35 | 0.27 | Secretion defect | - |

**Figure 3.26** Demographic characteristics and summary of platelet phenotyping of family members indicating the patients' age, gender, platelet parameters, ATP secretion values, type of platelet defect identified and the ISTH BAT score. For panel A family with normal platelet count and panel B families with thrombocytopenia.

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| II | I | 21 | F | 190 | 12.8 | 2.31 | 0.37 | Gi+ | - |
| | II | 24 | F | 84 | 12.8 | 0.9 | 0.44 | Gi | - |
| | III | 14 | F | 273 | 11.2 | 3.77 | 0.47 | No defect | - |
| | IV | 3 | M | 368 | 8.7 | 4.58 | 0.24 | Gi+ | - |

| Family | Patient | Age | Gender | Platelet count ($X10^9$/L) | MPV (fL) | PRP count ($X10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| VI | I | 47 | F | 230 | 10.7 | 4.33 | 0.86 | No defect | - |
| | II | 14 | F | 133 | 11.7 | 2.49 | 0.77 | secondary defect | - |
| | III | 7 | M | 231 | 10.4 | 3.15 | 0.89 | secondary defect | - |

| Family | Patient | Age | Gender | Platelet count ($X10^9$/L) | MPV (fL) | PRP count ($X10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| IV | I | 79 | F | 105 | 14.5 | 1.22 | 0.73 | No defect | 9 |
| | II | 53 | F | 172 | 13.9 | 2.93 | 1.03 | No defect | 7 |
| | III | 30 | F | 103 | 15 | 1.72 | 0.21 | Gq defect | 8 |
| | IV | 35 | F | 107 | 15.1 | 1.56 | 0.26 | Gq defect | 4 |

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| VII | I | 30 | M | 59 | 13.0 | 0.93 | 0.1 | Secretion defect | 8 |
| | II | 52 | M | 183 | 12 | 2.51 | 0.66 | No defect | 2 |

| Family | Patient | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP level | Platelet defect identified | BAT score |
|---|---|---|---|---|---|---|---|---|---|
| VIII | I | 7 | F | 287 | 8.8 | 6.06 | 1.01 | No defect | - |
| | II | 73 | F | 150 | 10.5 | 4.52 | 0.5 | Secretion defect | - |

**Figure 3.27** Demographics and summary of platelet phenotyping of family members with various platelet counts indicating the patients' age, gender, platelet parameters, ATP secretion values, type of platelet defect identified and the ISTH BAT score.

### 3.3.7 Case presentations from patients with various categories of platelet defects

#### 3.3.7.1 Index case No.1 - A patient with a Gi signalling defect

A 17 year old girl with a bleeding history includes epistaxis, bruising, bleeding from minor wounds, oral cavity bleeding and menorrhagia since menarche. Her whole blood platelet count, MPV, PRP count and ATP secretion level were normal (214 x $10^9$/L, 10.5 fL, 2.96 x $10^8$/ml and 1.10 nmol ATP/1x$10^8$ platelets, respectively) and the aggregation traces are shown in figure 3.28. The platelet abnormality was characterized by transient aggregation to ADP and absent primary wave of aggregation to adrenaline and impaired ATP secretion to both agonists. A reduced response to collagen is also consistent with impairment of the feedback role of ADP. A robust response to low dose arachidonic acid helps to exclude a defect in the $TxA_2$ pathway. The level of ATP secretion was within the normal range, indicating that the participant did not have a secretion defect.

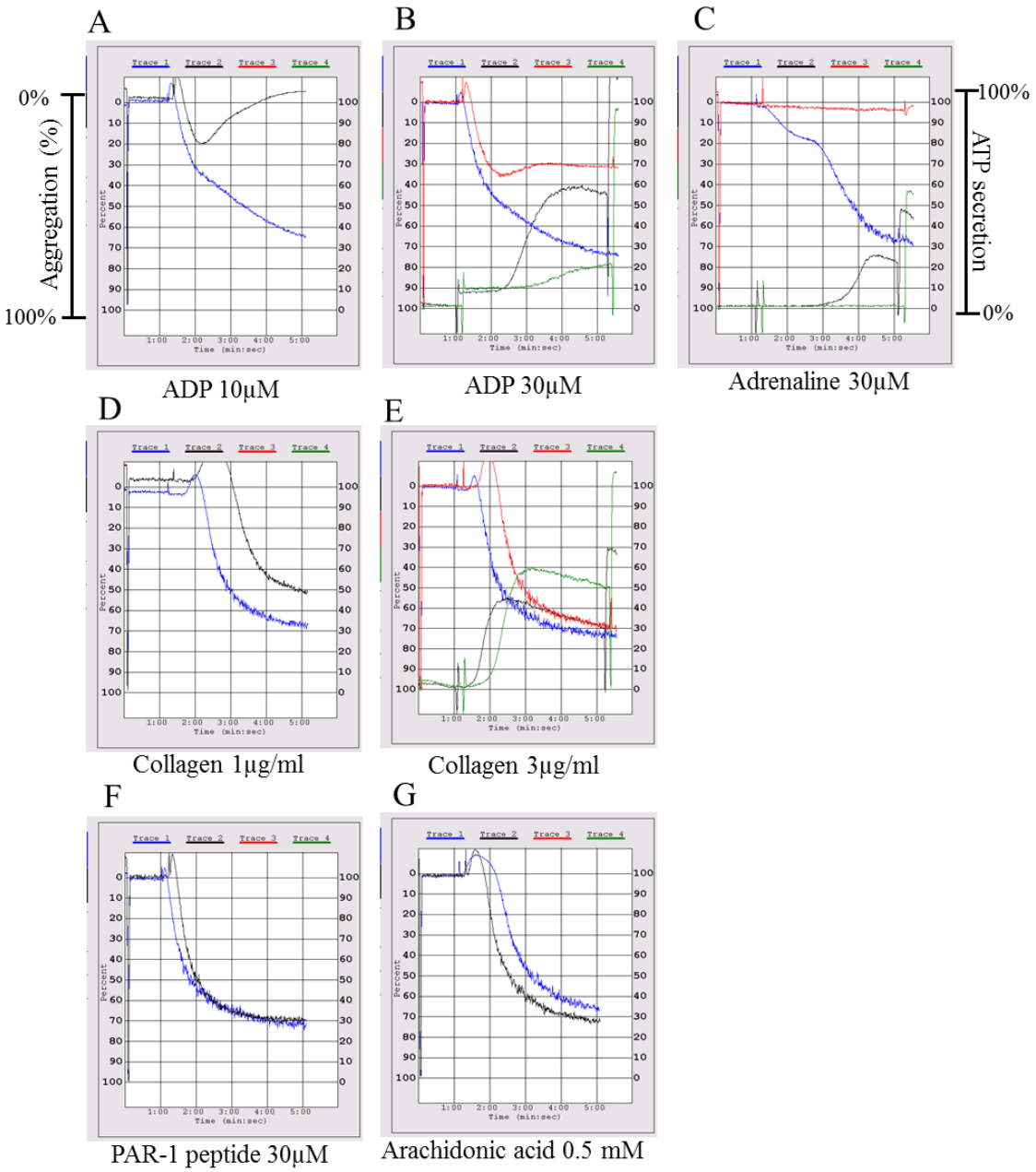



**Figure 3.28** Representative traces from a patient with Gi signalling defect. Left Y axes show percentage aggregation and right Y axes show ATP secretion levels. For panels A, D, F and G control aggregation is shown in blue and patient aggregation is shown in black. For panels B, C and E control aggregation is shown in blue and patient aggregation is shown in red, control secretion is shown in black and patients secretion is shown in green. Reversible aggreagtion is seen in response to low and high doses of ADP, with no secondary wave of aggregation with high dose of adrenaline suggesting a lost of the Gi signalling. Reduced aggregation is seen with a low dose of collagen, although this normalises at a higher concetration. Response to low doses of arachidonic acid and PAR-1 peptide are normal. Normalised secretion to PAR-1 peptide is within the normal range at 1.10nmol ATP / $1x10^8$ platelets ($5^{th}$ centile in healthy controls =0.68 nmol ATP / $1x10^8$ platelets)

### 3.3.7.2 Index case No.2 - A patient with cyclooxygenase pathway defet

A 42 year old lady presented with a bleeding history of epistaxis, bruising and bleeding from minor wounds, muscle haematomas, oral cavity bleeding, bleeding after tooth extractions, bleeding after surgical operation and menorrhagia. All of her platelet parameters were normal including platelet count in whole blood (239 x $10^9$/L), MPV (11.5fL), PRP count (3.36 x $10^8$/ml) and ATP secretion level (0.71/0.68 nmol ATP/1x$10^8$ platelets) with the aggregation traces shown in figure 3.29. A platelet abnormality was characterized by a marked defect in aggregation and secretion in response to arachidonic acid. The normal aggregation in response to U46619 however, indicated a defect in conversion of arachidonic acid to $TxA_2$ because the response to U46619 is insensitive to cyclooxygenase blockade. A reduced response to low dose of collagen is consistent with impairment of the feedback role of $TxA_2$. Normal sustained responses to ADP exclude a defect in the ADP pathway. The level of ATP secretion was within the normal range, indicating that there was no secretion defect.

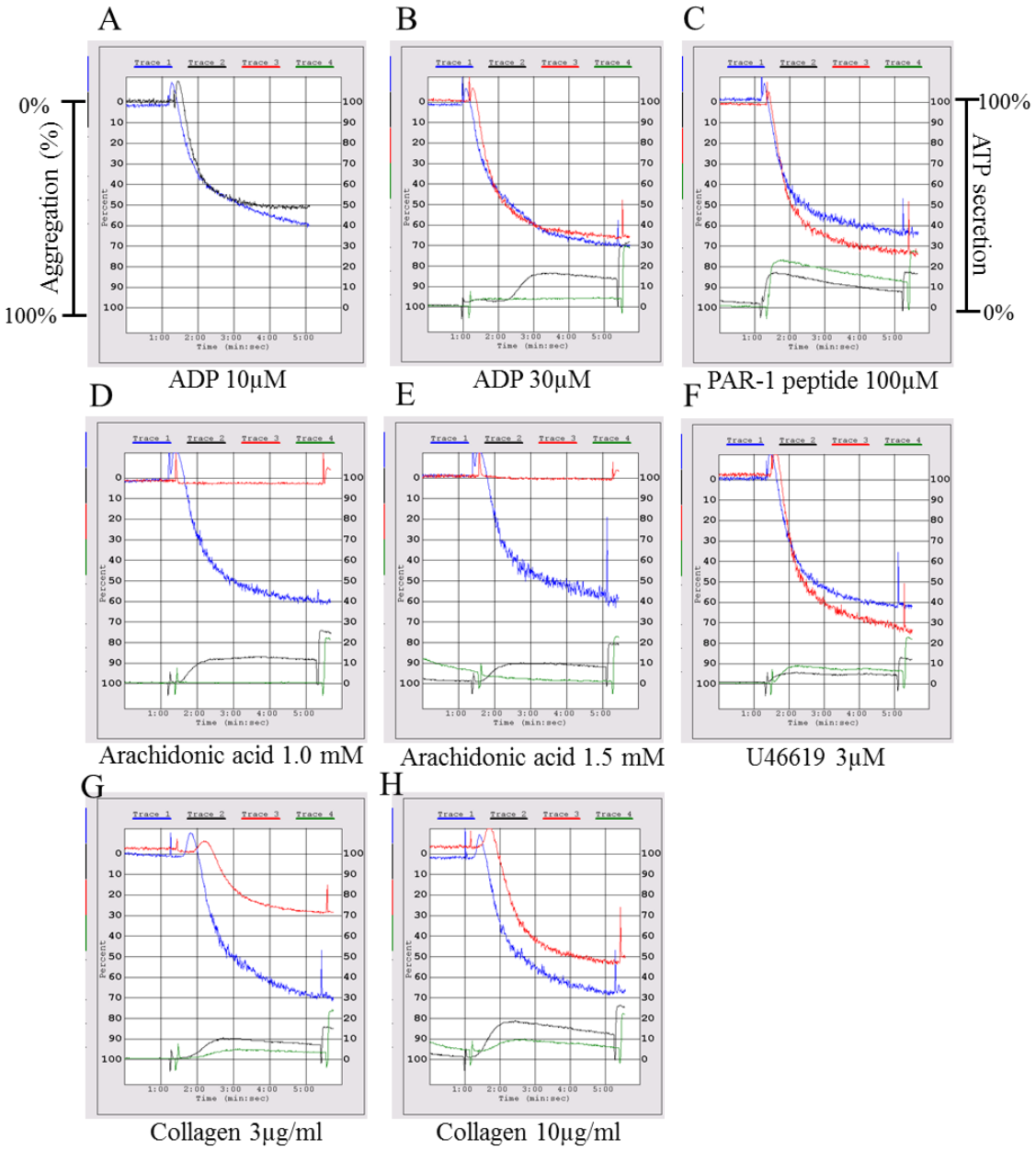



**Figure 3.29** Representative traces from a patient with a potential cyclooxygenase pathway defect. Left Y axes show percentage aggregation and right Y axes show ATP secretion levels. For panel A control aggregation is shown in blue and patient aggregation is shown in black. For panels B, C, D, E, F, G and H control aggregation is shown in blue and patient aggregation is shown in red, control secretion is shown in black and patient secretion is shown in green. No aggreagtion is seen in response to low and high doses of arachidonic acid. Response to U46619 is normal suggesting that the thromboxane receptor is normal. Reduced aggregation is seen with low dose of collagen, although this normalises at a higher concetration. Responses to low and high doses of ADP and to PAR-1 peptide are normal. Normalised secretion to PAR-1 peptide is within the normal range at 0.71nmol ATP / $1x10^8$ platelets ($5^{th}$ centile in healthy controls =0.68nmol ATP / $1x10^8$ platelets)

### 3.3.7.3 Index case No.3 - A patient with a possible dual platelet defect (cyclooxygenase pathway and Gi signalling)

A 47 year old male presented with a clinical history of gum bleeding after dental cleaning which lasted for five days. His WB platelet count, MPV and PRP count were with normal ranges (262 x $10^9$/L, 8.7fL and 4.72 x $10^8$/ml, respectively). His ATP secretion value (0.66 nmol ATP/1x$10^8$ platelets) was borderline and aggregation traces are shown in figure 3.30. A transient aggregation to ADP and absent primary wave of aggregation to adrenaline as well as an impaired ATP secretion to both agonists indicated a defect in Gi signalling pathway. There was also a marked defect in aggregation and secretion to arachidonic acid even at the higher concentration, however, a normal aggregation response to U46619 indicated normal receptor function. A marked defect in response to collagen is consistent with impairment of the feedback mediators as collagen response is markedly dependent on the feedback actions of ADP and $TxA_2$.

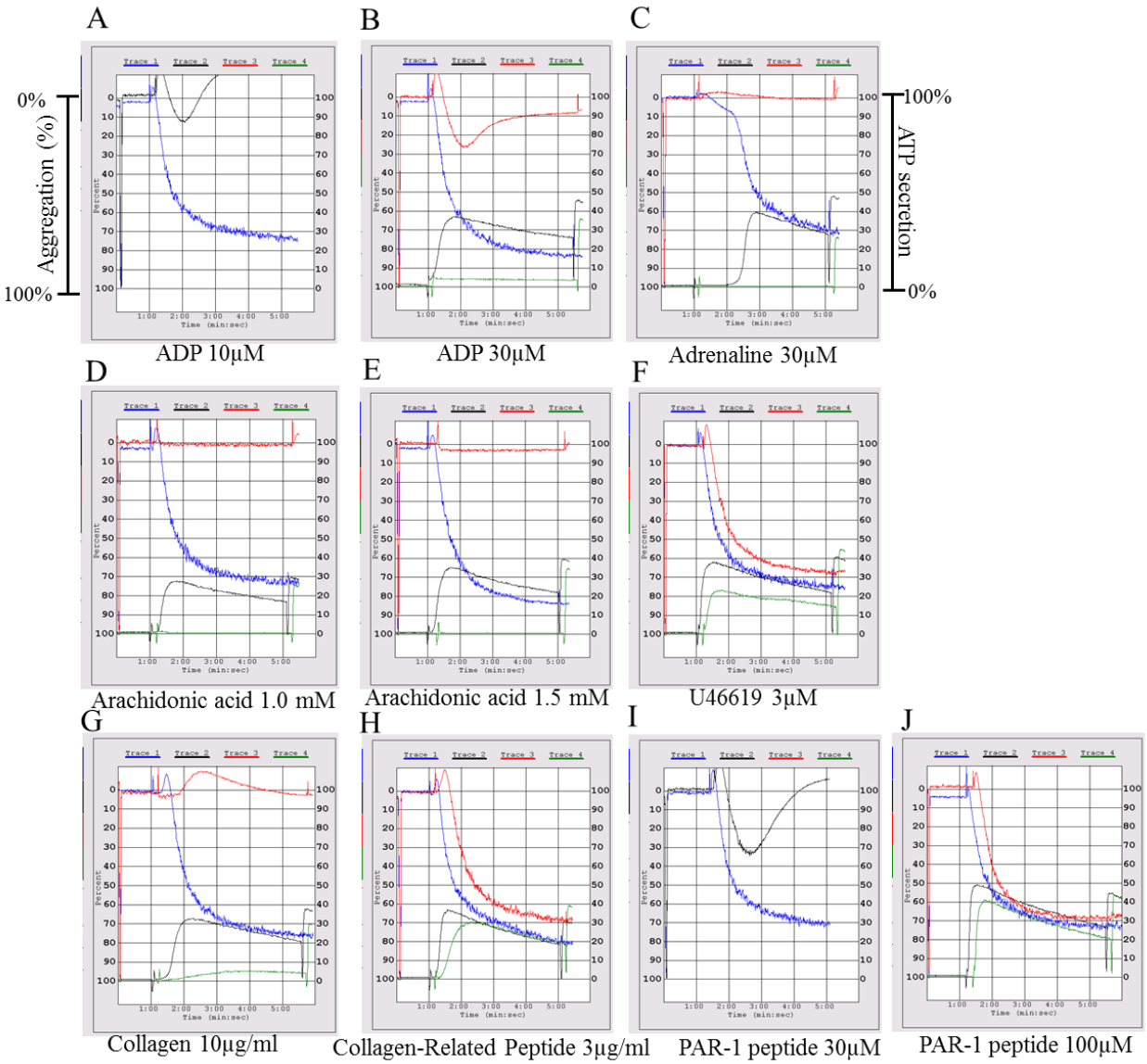



**Figure 3.30** Representative traces from a patient with a possible dual defect (cyclooxygenase pathway and Gi signalling defects). Left Y axes show percentage aggregation and right Y axes show ATP secretion levels. For panels A and I control aggregation is shown in blue and patient aggregation is shown in black. For panels B, C, D, E, F, G, H and J control aggregation is shown in blue and patient aggregation is shown in red, control secretion is shown in black and patient secretion is shown in green. Reversed aggregation is seen in response to low and high doses of ADP, with no secondary wave of aggregation with a high dose of adrenaline suggeting a lost of the Gi signalling. No aggregation is seen in response to low and high doses of arachidonic acid. Response to U46619 is normal suggesting normal thromboxane receptor function. Only shape change is seen in response to a high dose of collagen. Response to CRP is normal suggesting a normal function of the GPVI collagen receptor. Reversable aggregation is seen in response to low PAR-1 peptide although this normalises at a higher concetration. Normalised secretion to PAR-1 peptide is borderline range at 0.66 nmol ATP / $1x10^8$ platelets ($5^{th}$ centile in healthy controls =0.68 nmol ATP / $1x10^8$ platelets)

### 3.3.7.4 Index case No.4 - A patient with a suspected dense granule secretion defect

A 5 year old boy presented with history of excessive bleeding and mild thrombocytopenia. His platelet count in whole blood was 139 x $10^9$/L but the PRP count was 2.02 x $10^8$/ml which was within the accepted range (≥1.00 x $10^8$/ml) for aggregation study. MPV was within normal range at 12.1fL. The ATP secretion level was markedly reduced (0.22 nmol ATP/1x$10^8$ platelets) and aggregation traces are shown in figure 3.31. A platelet abnormality was characterized by decrease in ATP secretion in response to all agonists even though maximal aggregation was sustained at high concentrations. A reduction in aggregation to low concentrations of almost all platelets agonists is consistent with impairment of the feedback role of ADP and $TxA_2$. The participant was confirmed to have a dense granular secretion defect with low ATP secretion.

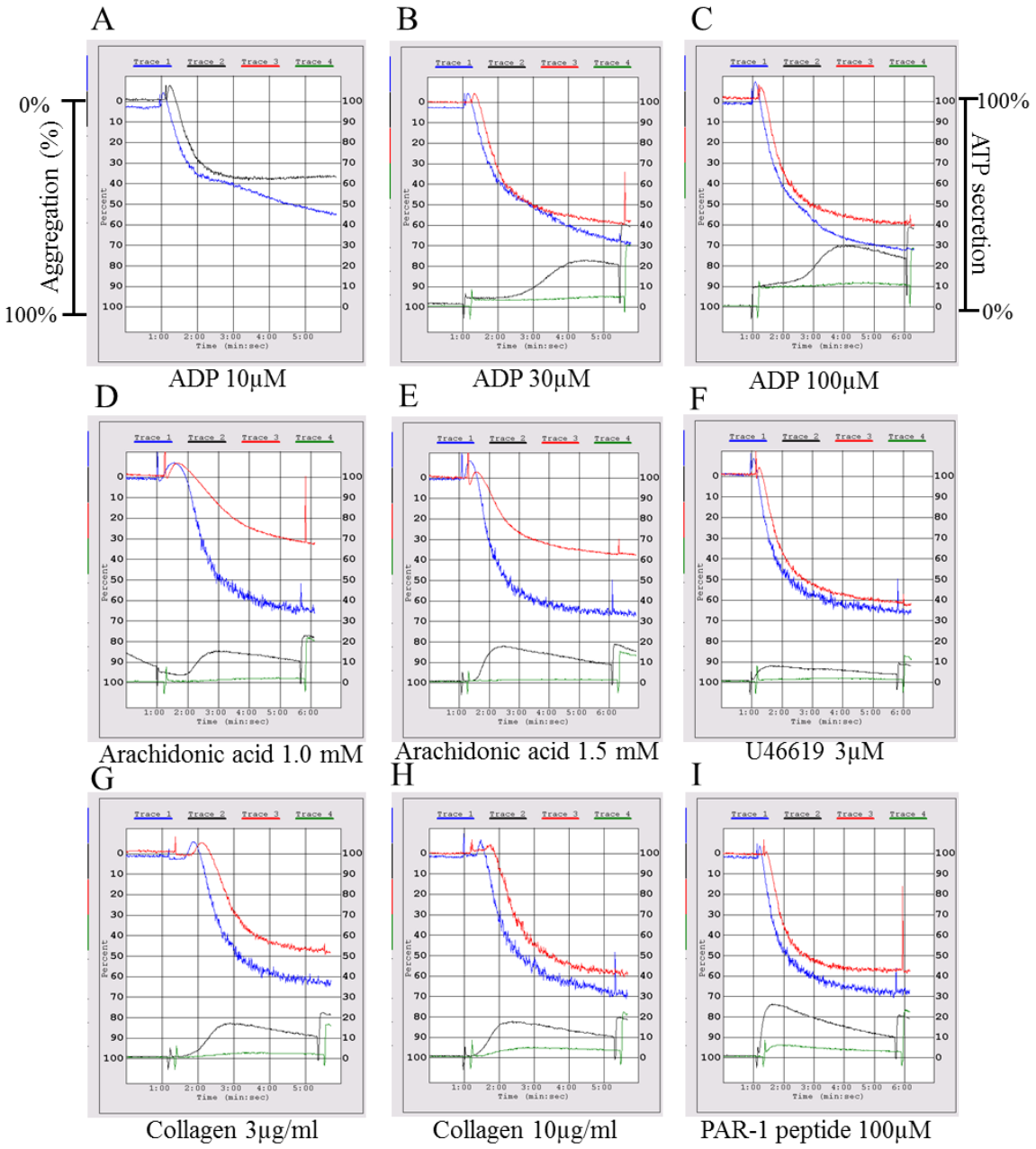



**Figure 3.31** Representative traces from a patient with a potential dense granular secretion defect. Left Y axes show percentage aggregation and right Y axes show ATP secretion levels. For panel A control aggregation is shown in blue and patient aggregation is shown in black. For panels B, C, D, E, F, G, H and I control aggregation is shown in blue and patient aggregation is shown in red, control secretion is shown in black and patient secretion is shown in green. Aggregation responses to low doses of ADP and collagen were reduced, although this normalised at higher concentrations. reduced aggregation is also seen in response to low and high doses of arachidonic acid, but full response is seen to U46619. Response to PAR-1 peptide is normal. There is a reduced secretion level in response to all agonists. Secretion to PAR-1 TRAP peptide is markedly reduced at 0.22 nmol ATP / $1x10^8$ platelets ($5^{th}$ centile in healthy controls =0.68nmol ATP / $1x10^8$ platelets

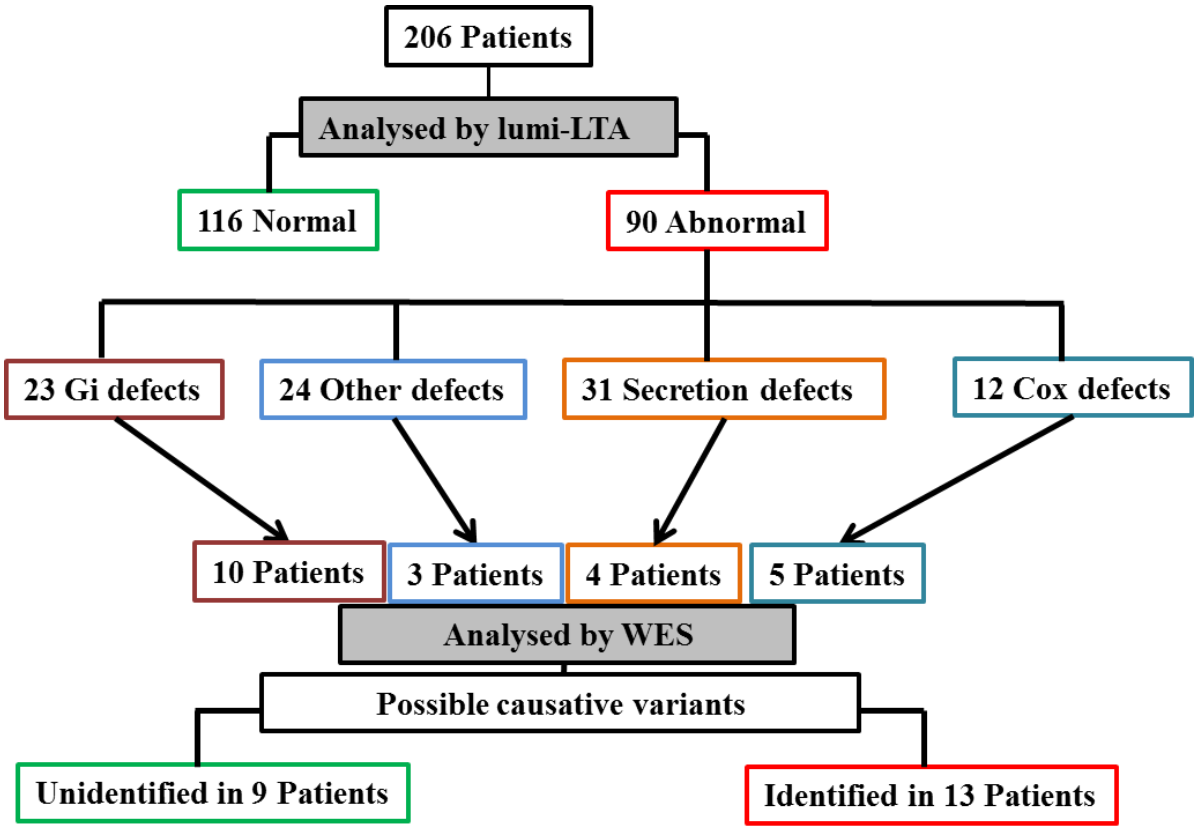



**Figure 3.32** Summary of the overall 206 patients tested by lumi-LTA during the study period presenting the number of normal, abnormal and their four groups of classification according the phenotypic pattern of response. WES was further analyzed on 22 selected cases with genetic defects identified in 13 patients.

### 3.4 Discussion

This chapter reveals the overall recruitment, patients' characteristics and platelet phenotyping by lumi-LTA. The cohort includes a total of 318 participants (112 healthy controls and 206 patients) recruited into the GAPP study from 10 haemophilia centres across the UK. The healthy control population recruited in this cohort were adults with an age range of 18 to 57 years. However, due to the GAPP study criteria and ethical issues, other details and additional demographic information from healthy controls group were not provided. The number of patients recruited from each centre varies with larger numbers of patients referred from Lincoln, Nottingham, Birmingham and Canterbury. It might be possible that due to the geographical location, some of these centres had access to more patients than others. On the other hand, the number of defects identified from each center also varied considerably and were not consistent to the number of patients recruited. This might explain the importance of taking a proper clinical history and applying an appropriate screening test to exclude non platelet disorders.

Patient age varied considerably among all consenting patients, this is probably due to the inclusion criterial of <85 years of age and enrolment of patients from both paediatric and adults haemophilia centres. However, the average age of patients included in this cohort is significantly into adulthood at 33 years. On the other hand, female patients were by far more than male accounts for 75% of all participants. Both, the variation in age towards the adulthood and gender to towards female are in line with previous studies within the GAPP project (Dawood et al., 2012, Lowe et al., 2013, Johnson et al., 2016). This is because, individuals with mild bleeding disorders may not be recognised during their childhood as they have not yet experienced severe haemostatic challenges such as trauma or a major surgical procedure. Similarly, women with bleeding disorders are also more readily identified than men because of menorrhagia and increased potential for bleeding after child birth (Watson et al., 2010). It has

been reported that approximately 5 – 10% of women do seek medical attention at some point during their reproductive life due to prolonged menorrhagia and that about 15% of them do have underlying bleeding diatheses (Rydz and James, 2012).

Analysis of platelet parameters (whole blood count, PRP count and MPV values) are important while diagnosing patients with PFDs. Firstly, they provide a practical guidance as approach of investigating patients with normal count within the GAPP study is different to that of patients with thrombocytopenia. Secondly, platelet parameters along with aggregation trends are essential for result interpretation as some form of platelet defects are associated with low platelet counts of either normal size or macrothrombocytopenia.

Abnormal lumi-LTA results were defined based upon the cut-off values that were calculated as $5^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response. However, applying of the $5^{th}$ percentile cut-off values may resulted in miss diagnosis on a small degree of patients due to an overlap in platelet responsiveness between patients and healthy population. This small degree of overlapping however has also been reported in other laboratory methods (Quiroga et al., 2007).

A qualitative defect in platelet function was observed in 44% of patients (90/206) that were tested by lumi-LTA. This is slightly lower than a previously reported higher frequency of 58% platelet defects within the early stages of the GAPP study (Dawood et al., 2012). The platelet defects reported here were categorised into four main groups (dense granular defects, Gi and Gq signalling defects, and cyclooxygenase pathway defects) based on the aggregation patterns as previously described (Dawood et al., 2012). However, the current cohort identified more patients with dense granular defects than Gi signalling defects (16% and 11% respectively). Although, an accurate classification of an inherited platelet function defect does not necessary change patient treatment and haemostatic management, the correct identification of platelet

function defect is important to minimise bleeding risk and ensures the appropriate family counselling. A number of reasons could be considered for the excessive bleeding in 56% of patients in whom a platelet defect was not identified. For example, the testing undertaken within the GAPP study are not comprehensive enough to detect all platelet defects such as alpha granular function and platelet procoagulant activity (e.g. Scott syndrome). Patients may also possibly have abnormalities in another part of haemostatic system that are not easily tested e.g. structural defects in collagen, vessel wall abnormalities and abnormal fibrinolysis. One example within the GAPP study is the recently reported genetic defect in the thrombomodulin gene in family members with excessive bleeding but normal platelet function (Maclachlan et al., 2017a).

In this cohort, 19% (39/206) of patients were also identified to have a low platelet count (<150 $x10^9$/L) in their whole blood. The GAPP study has a different approach for patients with thrombocytopenia and normally they are recruited into another arm of the GAPP project (Johnson et al., 2016). However, if the PRP count of these patients is within the accepted analytical range (≥1.0 $x10^8$/ml), the phenotyping are still performed by lumi-LTA. Patient's characteristics showed that the proportion of males in patients with thrombocytopenia was slightly higher than those with normal platelet count. Furthermore, the average age was younger in patients with thrombocytopenia than those with normal platelet count. This is of no surprise as the patients with thrombocytopenia can be equally identified in all age and both gender due to their low platelet count. It was also noted that the proportion of patients detected to have platelet functional defect was significant higher among patients with thrombocytopenia than those with normal count. In addition the majority of these were found to have dense granules defect.

In this cohort of patients, the ISTH BAT score was recorded on 122/167 of patients with excessive bleeding but normal platelet counts (Rodeghiero et al., 2010b). Analysis of the association between the ISTH BAT score and platelet function defects was performed in these patients. It was found that the high ISTH BAT score could not predict the presence of platelet function defect among patients with excessive bleeding. Furthermore, there was no association found between the BAT score and the sub-type of platelet defect detected by lumi-LTA. Similar findings were previously reported within the GAPP study (Lowe et al., 2013). Interestingly, the negative predictive value in this study was higher than that previously reported (71% and 54% respectively). This is more likely due to the increase number of patients found to have no platelet defect. Surprisingly, there was no increase in the ISTH BAT score among patients with thrombocytopenia despite their low platelet count and the presence of secondary functional defects. However, the number of patients with thrombocytopenia in this group was small (n=21).

4 index cases discussed in this chapter were selected to represent the 3 major diagnostic groups of platelet function defects (Gi signalling, $TxA_2$ pathway and dense granule secretion) based on the patterns of response. This also demonstrated the importance of using a standard panel of agonists at a range of different concentrations alongside measurement of ATP secretion level for correct interpretation and classification. For example, a Gi signalling defect in case No. 1 was diagnosed based on defect in aggregation and secretion in response to the 2 Gi-coupled heterotrimeric receptors agonists ADP and adrenaline. A key diagnostic feature of Gi defects is the presence of transient aggregation to ADP with a reduced or absent primary wave and no secondary wave of aggregation to adrenaline (Dawood et al., 2012). In contrast, in case No. 2 an individual with $TxA_2$ pathway defect exhibited a marked defect in aggregation and secretion in response to arachidonic acid but normal response to U46619. Because the U46619 is a $TxA_2$-

mimetic agonist it is insensitive to cyclooxygenase blockade, and therefore a normal response indicates normal functioning of the $TxA_2$ receptor. A normal level of ATP secretion in this patient also excluded a secretion defect. In case No. 4 the participant was diagnosed to have a defect in secretion because of the reduced level of ATP secretion. In patients with secretion defect a decrease in ATP secretion is normally observed in response to all agonists (Dawood et al., 2012). They also may exhibit a reduction in aggregation to low concentrations of most platelet agonists in particular to collagen due to impairment of the feedback role of ADP and ATP.

In summary, this chapter demonstrates that lumi-LTA using a streamlined panel of key platelet agonists and specified concentrations can reliably diagnose the majority of PFDs. In many cases the patterns of response were consistent with a specified defect. In some cases however, the interpretation remained unclear mainly due to the feedback effects of ADP and thromboxane A2 ($TxA_2$) and by the overlap with the response of healthy volunteers. Furthermore, the phenotyping of platelets described in this chapter have also been used to direct genotyping of selective cases for the purpose of identifying a causative gene defect that may contribute to bleeding diathesis of these patients. The genetic analyses of selective cases will be discussed in the next chapter.

# CHAPTER FOUR: GENOTYPING OF PATIENTS IDENTIFIED TO HAVE A PLATELET DEFECT

## 4.1 Introduction

Genetic testing has long been used to facilitate the identification and confirmation of heritable disorders. This approach however has progressed rapidly since the invention of Sanger sequencing and has led to major advances in the ability to sequence large amounts of genetic material due to high-throughput sequencing techniques and the decrease in cost (Rabbani et al., 2014, Rizzo and Buck, 2012). In the diagnosis of PFD, genetic testing is widely applied for the aim of identifying variants that are responsible for the clinical presentation of patients and possible screening of other family members. So far, genetic testing has helped in identifying causative mutations behind a number of previously phenotypically well classified disorders such as grey platelet syndrome (GPS) (Albers et al., 2011) and the discovery of novel causative genes not previously implicated in disease (Johnson et al., 2016).

In the GAPP study a combination of a platelet phenotyping and gene sequencing approach is used to identify candidate mutations underlying platelet dysfunction (Watson et al., 2010). This approach has so far identified several patients with functional disrupting mutations in the $TxA_2$ receptor (Mumford et al., 2010) and the $P2Y_{12}$ ADP receptor (Dawood et al., 2012). Furthermore, a novel mutation in the $P2Y_{12}$ ADP receptor has been reported in a family with type 1 VWD (Daly et al., 2009). Other mutations in *SLFN14*, *RUNX1* and *FLI1* have been identified in patients with thrombocytopenia (Fletcher et al., 2015, Johnson et al., 2016). More recently, a genetic variant in thrombomodulin (*THBD*) has been identified in a family with an unexplained bleeding (Maclachlan et al., 2017b).

## 4.2 Aim

This chapter aims to perform genotyping (using WES and Sanger sequencing) of selected patients found to have an abnormal phenotype upon platelet function testing to try to uncover a genetic variant which is likely to be causative of disease

## 4.3 Results

### 4.3.1 Overall patients' demographics and platelet parameters

Genetic analysis was performed on 25 patients with platelet function defects including 2 extended families (9 male and 15 female) with a median age of 33 years (range 4-53). The overall patients' demographic, platelet parameters and Lumi-LTA finding are shown in table 4.1. Phenotyping of one of the patients (P10) was done at a local referral centre and so no details are available. All patients had a normal platelet count and two (P8 and P9) had increased MPV (14fl and 13.8fL, respectively). ISTH BAT score varied between patients (range 4-20). All 19 index cases and 4 family members presented with abnormal platelet function (table 4.1).

WES was performed on genomic DNA. Overall, WES revealed between 26,000 and 36,000 variants (including single nucleotide variants, splice variants, frameshifts and small scale insertions/deletions) in the DNA from each patient. The variants were first subjected to filtering using a variant frequency cut off value of ≤0.01. Any variant with a minor allele frequency (MAF) of >0.01 or synonymous variants not known to change the amino acid were excluded. On average, 3396 variants with a MAF of ≤0.01 excluding synonymous variants were noted per individual. The variants from each individual were then compared to a database of 357 known platelet related genes (the list is outlined on appendix 3). An average of 44 (range, 14-58) variants were observed per individual, and of these variants about 7 (range, 2-10) variants were deemed plausible candidates.

A total of 89 variants within 71 genes, were identified among the 19 index cases presented with a platelet function defect, of which 90% (80/89) were missense alterations and 23 were previously unreported. In addition to the missense variants, 3 potential splicing, and two each

of frameshift, stopgain and nonframeshift variations were identified. All the variants identified in this cohort were inherited in a heterozygous fashion.

| Patient's code | Age | Gender | Platelet count ($x10^9$/L) | MPV (fL) | PRP count ($x10^8$/ml) | ATP secretion (nmol/$1x10^8$ platelet) | Phenotype defect | ISTH BAT score |
|---|---|---|---|---|---|---|---|---|
| P2 | 48 | F | 181 | 10.9 | 3.23 | 0.75 | Gi defect | 7 |
| P4 | 33 | F | 310 | 10 | 3.5 | 0.43 | Gi defect | 8 |
| P5 | 16 | M | 153 | 11.3 | 2.67 | 0.75 | Gi defect | 8 |
| P7 | 32 | M | 209 | 10.7 | 4.26 | 0.88 | Gi defect | 18 |
| P8 | 37 | F | 230 | 14 | 2.9 | 1.02 | Gi defect | 12 |
| P10 | NA | NA | NA | NA | NA | NA | Gi defect | NA |
| P11 | 17 | F | 214 | 10.5 | 2.96 | 1.1 | Gi defect | 6 |
| P12 | 4 | M | 303 | 9.6 | 2.91 | 0.89 | Gi defect | N/A |
| P13 | 37 | F | 244 | 9.6 | 6.08 | 0.57 | Gi defect | N/A |
| P3 | 47 | M | 262 | 8.7 | 4.72 | 0.61 | Gi + Cox defect | N/A |
| P6 | 37 | F | 179 | 11.1 | 3.8 | 0.72 | Gi + Cox defect | 8 |
| P9 | 5 | F | 273 | 13.4 | 1.87 | 0.19 | PAR-1 receptor defect | N/A |
| P14 | 32 | F | 292 | 9.7 | 8.4 | 0.61 | Cox defect | N/A |
| P15 | 51 | F | 204 | 12.2 | 7.62 | 0.55 | Cox defect | 20 |
| P16 | 27 | M | 168 | 10.8 | 2.33 | 0.5 | Cox defect | N/A |
| P17 | 49 | F | 237 | 11.6 | 4.66 | 1.16 | Cox defect | 10 |
| P18 | 34 | F | 207 | 12.1 | 3.3 | 0.97 | Cox defect | 12 |
| P1:F1:I | 55 | F | 225 | 9.8 | 4.8 | 0.67 | Gi defect | 14 |
| F1:II | 53 | M | 171 | 10.5 | 2.98 | 1.07 | Gi defect | 1 |
| F1:III | 31 | F | 247 | 10 | 3.82 | 0.94 | No defect | 5 |
| F1:IV | 27 | M | 430 | 9.1 | 7.13 | 0.84 | No defect | 7 |
| P19:F2:I | 45 | F | 244 | 11.2 | 4.52 | 0.52 | Cox + Secretion defect | 17 |
| F2:II | 23 | M | 428 | 9.1 | 6.53 | 0.39 | Secretion defect | 10 |
| F2:III | 20 | F | 345 | 10.2 | 5.39 | 0.59 | Cox + Secretion defect | 12 |
| F2:IV | 17 | M | 329 | 10.9 | 3.43 | 0.25 | Secretion defect | 3 |

**Table 4.1** Summary of 25 patients that were analysed by genetic test, the table displays patient demographic, platelet parameters, ISTH BAT score and Lumi-LTA finding: Data are presented as an absolute number.

### 4.3.2 Variants found in patients suspected to have Gi defects

Genetic testing was analysed on 10 index cases and one family member that presented with a Gi defect by lumi-LTA (table 4.1). Prior to WES, Sanger sequencing was initially analysed on 4 cases (P1:F1:I, P8, P11 and P13) searching for a mutation on the $P2Y_{12}$ receptor gene, however, no abnormal variant was identified. The results of WES of 9 index cases are presented in the table 4.2. The results of one of the index cases (P1:F1:I) and his related family members will be discussed in section 4.3.6 of this chapter. Of 9 of the index cases (table 4.2) two patients were presented with two variants in genes known to be involved in Gi signalling pathway. In patient P2, a missense variant (c.169C>T, p.Arg57Trp) causing single nucleotide variant (SNV) in the *PRKACG* gene is a novel variant and was not found in all databases searched. In patient P10, a missense variant (c.424G>T, p.Val142Phe) causing an SNV in the *PLA2G4C* gene. The variant (rs00115932) was previously found in other databases with a MAF of 0.0089. Both of these variants were predicted to be damaging by 5 *in silico* prediction softwares which include Mutation Taster, PolyPhen-2 HumDiv, PolyPhen-2 HumVar, Provean Score and Sift Score.

5 out of 9 patients (P4, P7, P10, P12 and P13) were identified with variants in genes (*JMJD1C, VWF, VPS16 and VPS41*) known to be involved in both Gi signalling pathway and platelet secretion (table 4.2) (Leo C V et al 2015). Interestingly, two of these patients (P4 and P13) presented with low ATP secretion in addition to their Gi defect (table 4.1). All of the five variants (JMJD1C; c.3634A>T, VWF; c.817C>T, VWF; c.4751A>G, VPS16; c.1037G>C and VPS41; c.622C>T) were missense variant causing SNVs and four of which (c.3634A>T, c.817C>T, c.4751A>G, and c.622C>T) were previously found in other databases with a MAF of 0.000704, 2.50E-05, 0.002942 and 0.0001568 respectively. Two variants within the *VWF* gene were identified in two different patients. One of the variants in patient P7 occurred within

VWF; c.817C>T, p.Arg273Trp with MAF of 2.50E-05 and the other in patient P10 occurred within VWF; c.4751A>G, p.Tyr1584Cys with a MAF of 0.002942. The variant (c.1037G>C, p.Cys346Ser) within the *VPS16* gene identified in P12 was the only novel variant and was not found in all databases. All of these variants were predicted to be disease causing by using Mutation Taster and damaging by at least three of other prediction software (PolyPhen-2 HumDiv, PolyPhen-2 HumVar, Provean Score or Sift Score).

Seven other variants were identified within 6 genes (*VPS33B, LYST, VPS4B, TRPM7, MYH13* and *ANKRD26*) known to be involved in secretion (Leo et al., 2015). Three of these (*TRPM7, MYH13* and *ANKRD26*) were noted in a single patient (P13). One variant within *ANKRD26* was found in this patient which introduced a premature stop codon at the point of variation (c.105C>G, p.Tyr35*). This variant was previously found in other databases with a MAF of 0.0002. The other variant within the *TRPM7* gene, a missense variant (c.2033C>T, p.Ala678Val) is a novel variant and was not found in all databases. The third variant is within the *MYH13* gene consisting of a missense variant (c.5101C>G, p.Arg1701Gly). It was previously found in other databases with a MAF of 0.002563. Interestingly, the ATP secretion value of this patient was reduced, suggesting that one or more of these genes could be the cause of her bleeding symptoms. 2 out of 7 variants were identified in P11, one of which (c.2806C>A, p.Pro936Thr) in *LYST* was novel and the other (c.40C>G, p.Leu14Val) in *VPS4B* was previously found with a MAF of 0.001519. The last two variants in *VPS33B* (c.1453G>A, p.Val485Ile) and *LYST* (c.8913T>G, p.Asn2971Lys) were observed in two patients (P2 and P12, respectively). Both variants were consisting of a missense change and were previously found in other databases with a MAF of 0.0000825 and 0.002159 respectively. All of the 7 variants were predicted to be disease causing by Mutation Taster and damaging by at least two

of other prediction software (PolyPhen-2 HumDiv, PolyPhen-2 HumVar, Provean Score or Sift Score). However, all the other 3 patients (P2, P11 and P12) gave normal ATP secretion value.

Results-Genotyping of patients identified to have a platelet defect

| Patient code | Gene | Genomic variation | Protein effect | Variation type | Prevalence | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P2** | PRKACG | c.169C>T | p.Arg57Trp | Missense | Novel | Polymorphism | -0.413 | 0.929 | Damaging | Damaging | Deleterious | Damaging | PM2, PP2, PP3 | Uncertain significance |
| | VPS33B | c.1453G>A | p.Val485Ile | Missense | 8.25E-05 | Disease causing | 3.416 | 0.998 | Benign | Benign | Neutral | Tolerated | BP4, PP2,PP4 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P4** | JMJD1C | c.3634A>T | p.Thr1212Ser | Missense | 0.000704 | Disease causing | 1.559 | 1 | Damaging | Damaging | Neutral | Damaging | PP3, PP2, PP4 | Uncertain significance |
| | MYH9 | c.4198C>T | p.Arg1400Trp | Missense | 0.0009562 | Disease causing | 2.369 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P5** | CD36 | c.806C>T | p.Ser269Phe | Missense | 5.77E-05 | Disease causing | 3.573 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | MUC16 | c.33044C>A | p.Thr11015Asn | Missense | 4.14E-05 | Polymorphism | 0.797 | 0.005 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P7** | VWF | c.817C>T | p.Arg273Trp | Missense | 2.50E-05 | Disease causing | 1.424 | 0.995 | Damaging | Damaging | Deleterious | Damaging | PP3,PP2, PP4 | Uncertain significance |
| | EXOC1 | c.2009G>A | p.Gly670Glu | Missense | 0.005692 | Disease causing | 5.878 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P8** | DIAPH3 | c.1051C>A | p.Pro351Thr | Missense | 0.003671 | Disease causing | 3.079 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | FGR | c.692A>T | p.Asp231Val | Missense | 0.0003615 | Disease causing | 2.953 | 1 | Benign | Benign | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P10** | VWF | c.4751A>G | p.Tyr1584Cys | Missense | 0.002942 | Polymorphism | 2.199 | 0.063 | Damaging | Damaging | Neutral | Damaging | PP3, PP2, PP4 | Uncertain significance |
| | PPP1R12A | c.2041C>T | p.Arg681Cys | Missense | 2.50E-05 | Disease causing | 4.908 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | ADCY7 | c.1317C>A | p.Asp439Glu | Missense | 0.001 | Disease causing | 2.253 | 1 | Damaging | Damaging | Neutral | Tolerated | PP3, PP2 | Uncertain significance |
| | PLA2G4C | c.424G>T | p.Val142Phe | Missense | 0.0089 | Polymorphism | -0.116 | 0.001 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2, PP4 | Uncertain significance |

| Patient code | Gene | Genomic variation | Protein effect | Variantion type | Prevelance | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P11** | PLCB2 | c.497C>T | p.Pro166Leu | Missense | Novel | Disease causing | 5.271 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | LYST | c.2806C>A | p.Pro936Thr | Missense | Novel | Disease causing | 4.308 | 1 | Benign | Benign | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | STX11 | c.799G>A | p.Val267Met | Missense | 0.00562 | Disease causing | 0.956 | 0.753 | Damaging | Damaging | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| | GRK6 | c.772G>A | p.Ala258Thr | Missense | 0.00222 | Disease causing | 4.485 | 1 | Damaging | Benign | Deleterious | Tolerated | PP3, PP2 | Uncertain significance |
| | VPS4B | c.40C>G | p.Leu14Val | Missense | 0.001519 | Disease causing | 6.28 | 1 | Damaging | Damaging | Neutral | Tolerated | PP3, PP2 | Uncertain significance |
| | FLNA | c.1286C>T | p.Thr429Met | Missense | 0.01528 | Polymorphism | 3.622 | 0.996 | Damaging | Benign | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | PTPRJ | c.83_85del | p.28_29del | nonframeshift deletion | Novel | Disease causing | -0.331 | 0.002 | NA | NA | NA | NA | PM2, PP3 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P12** | ADCY3 | c.2950G>C | p.Ala984Pro | Missense | Novel | Disease causing | 6.069 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | NBEAL2 | c.6631G>A | p.Asp2211Asn | Missense | Novel | Disease causing | 5.515 | 0.997 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | VPS16 | c.1037G>C | p.Cys346Ser | Missense | Novel | Disease causing | 2.063 | 1 | Damaging | Damaging | Deleterious | Tolerated | PM2, PP3, PP2, PP4 | Uncertain significance |
| | BMP4 | c.676C>T | p.Arg226Trp | Missense | 0.0003957 | Disease causing | 2.088 | 0.996 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | LYST | c.8913T>G | p.Asn2971Lys | Missense | 0.002159 | Disease causing | 0.757 | 1 | Damaging | Damaging | Neutral | Tolerated | PP3, PP2, PP4 | Uncertain significance |
| | KIAA2018 | c.5972G>A | p.Arg1991His | Missense | 1.69E-05 | Disease causing | 4.275 | 1 | NA | NA | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P13** | EPHA4 | c.2233C>T | p.Arg745Cys | Missense | Novel | Disease causing | 2.403 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | TRPM7 | c.1684C>G | p.His562Asp | Missense | Novel | Disease causing | 5.33 | 1 | Benign | Benign | Deleterious | Tolerated | PM2, PP3, PP2, PP4 | Uncertain significance |
| | GRK6 | c.772G>A | p.Ala258Thr | Missense | 0.00222 | Disease causing | 4.485 | 1 | Damaging | Benign | Deleterious | Tolerated | PP3, PP2 | Uncertain significance |
| | PDE3A | c.1376G>A | p.Arg459Gln | Missense | 0.001566 | Disease causing | 5.711 | 1 | Damaging | Damaging | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| | VPS41 | c.622C>T | p.Arg208Trp | Missense | 0.0001568 | Disease causing | 3.297 | 1 | Damaging | Damaging | Neutral | Tolerated | PP3, PP2, PP4 | Uncertain significance |
| | MYH13 | c.5101C>G | p.Arg1701Gly | Missense | 0.002563 | Polymorphism | 0.105 | 0.997 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | ANKRD26 | c.105C>G | p.Tyr35X | stopgain SNV | 0.0002 | Disease causing | 0.082 | 0 | NA | NA | NA | NA | PM4, PP3 | Uncertain significance |

**Table 4.2** Summary of pathogenicity prediction and variant classification of the variants identifed in patients with Gi defects. The table dispays the nucleotide changes and effect of the resulting protein, the type of variantion and the prevelance among the population, PhyloP and PhastCons measure the prediction and probability of conservation at each individual base, Mutationtaster, SIFT, Provean and PolyPhen-2 are used to predict the pathogenicity a mutation. The ACMG provides supporting evidence for variants classification.

### 4.3.3 Variants found in two patients with dual defects and one patient suspected with a defect on the PAR-1 receptor.

WES analysis of three patients (P3, P6 and P9) is outlined in table 4.3. The platelet function test of P3 and P6 revealed a dual defect in Gi signalling and cyclooxygenase pathways (table 4.1). The WES of these two patients identified a total of 14 significant variants in known platelet related genes. Patient P3 presented with 6 variants (table 4.3), two of which are within the genes (*VWF* and *PLCB3*) known to be involved in both Gi signalling and secretion, and one variant in *RAI1* known to be a secretion related gene. Interestingly, the ATP secretion level of this patient was reduced (table 4.1). All three variants consisted of missense changes (c.6187C>T, p.Pro2063Ser, c.2558C>A, p.Pro853His and c.5590G>A, p.Gly1864Arg, respectively) and they were previously found in other databases with a MAF of (0.01273, 0.002786 and 3.32E-05, respectively). P6 presented with 8 variants, one variant is within *PTPRC* which is known to be involved in both Gi signalling and secretion. This variant was a missense change (c.2293C>T, p.His765Tyr), and is a novel variant not found in all databases screened. Two other variants in P6 were within *MYH13* and *ANKRD26* which are known to be involved in platelet secretion. However, the ATP secretion level of the patient was normal (table 4.1). The platelet function test of P9 suggested a defect on the PAR-1 receptor. However, the analysis of the PAR-1 receptor gene by Sanger sequencing did not identified any variant. WES identified four variations (table 4.3), two of which were within the genes *(MPL* and *STX2*) known to be involved in platelet secretion. Interestingly, the ATP secretion value of this patient was reduced (table 4.1). Both variants were missense changes. The variant (c.1652C>T, p.Pro551Leu) in the *MPL* gene was a novel variant, and the variant (c.845T>C, p.Leu282Pro) in *STX2* was previously found in other databases with a MAF of 0.000651.

| Patient code | Gene | Genomic variation | protein effect | Variation type | Prevalence | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P3** | VWF | c.6187C>T | p.Pro2063Ser | Missense | 0.01273 | Disease causing | 4.244 | 0.993 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2, PP4 | Uncertain significance |
| | SLC9A3R2 | c.691G>A | p.Glu231Lys | Missense | 0.0002446 | Disease causing | 3.678 | 0.997 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | ARHGAP32 | c.1192C>T | p.Arg398Cys | Missense | 0.0004292 | Disease causing | 2.913 | 1 | Damaging | Benign | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | NBEAL2 | c.5866G>A | p.Val1956Met | Missense | 3.32E-05 | Disease causing | 3.959 | 1 | Damaging | Damaging | Neutral | Tolerated | PP3, PP2 | Uncertain significance |
| | RAI1 | c.5590G>A | p.Gly1864Arg | Missense | 3.32E-05 | Disease causing | 5.643 | 1 | Damaging | Damaging | Neutral | Tolerated | PP3, PP2 | Uncertain significance |
| | PLCB3 | c.2558C>A | p.Pro853His | Missense | 0.002786 | Disease causing | 2.062 | 0.893 | Benign | Benign | Neutral | Damaging | BP4, PP2, PP4 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P6** | PTPRC | c.2293C>T | p.His765Tyr | Missense | Novel | Disease causing | 5.804 | 0.989 | Damaging | Damaging | Deleterious | Tolerated | PM2, PP3, PP2, PP4 | Uncertain significance |
| | BCOR | c.4663C>T | p.Arg1555Cys | Missense | 0.0008151 | Disease causing | 3.662 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | MLPH | c.415C>T | p.Arg139Trp | Missense | 0.01146 | Disease causing | 1.549 | 0.995 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | EXOC1 | c.86C>G | p.Ala29Gly | Missense | 0.001528 | Disease causing | 5.676 | 1 | Damaging | Damaging | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| | NBEAL2 | c.2231C>T | p.Pro744Leu | Missense | 0.002126 | Disease causing | 1.85 | 0.56 | Damaging | Damaging | Deleterious | Tolerated | PP3, PP2 | Uncertain significance |
| | MYH13 | c.5786G>T | p.Arg1929Leu | Missense | 0.0005866 | Disease causing | 2.648 | 1 | Benign | Benign | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | CD36 | c.186C>G | p.Tyr62X | stopgain SNV | 0.0001239 | Disease causing | 1.216 | 1 | NA | NA | NA | NA | PM4, PP3 | Uncertain significance |
| | ANKRD26 | c.3652-4T>C | NA | splicing | 0.01 | Disease causing | -0.004 | 0.035 | NA | NA | NA | NA | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P9** | CSK | c.979G>A | p.Val327Met | Missense | Novel | Disease causing | 0.795 | 0.992 | Benign | Benign | Neutral | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | MPL | c.1652C>T | p.Pro551Leu | Missense | Novel | Disease causing | 0.535 | 0.998 | Benign | Benign | Neutral | Tolerated | PM2, BP4, PP2PP4 | Uncertain significance |
| | PLCB2 | c.356C>T | p.Ala119Val | Missense | 0.0028 | Disease causing | 1.693 | 0.973 | Damaging | Benign | Neutral | Tolerated | PP3, PP2 | Uncertain significance |
| | STX2 | c.845T>C | p.Leu282Pro | Missense | 0.0006511 | Disease causing | 1.193 | 1 | Benign | Benign | Deleterious | Damaging | PP3, PP2, PP4 | Uncertain significance |

**Table 4.3** Summary of pathogenicity prediction and variant classification of the variants identifed in two patients with dual defects and one patient with PAR-1 receptor defect. The table dispays the nucleotide changes and effect of the resulting protein, the type of variantion and the prevelance among the population, PhyloP and PhastCons measure the prediction and probability of conservation at each individual base, Mutationtaster, SIFT, Provean and PolyPhen-2 are used to predict the pathogenicity of a mutation. The ACMG provides supporting evidence for variants classification.

### 4.3.4 Variants found in patients suspected to have a cyclooxygenase pathway defect

A total of 25 variants were identified by WES analysis in 5 patients suspected with cyclooxygenase pathway defects (table 4.4). Overall, no variant was noted in genes related to the thromboxane receptor or cyclooxygenase 1 enzyme in any of the 5 patients analysed. Interestingly however, 5 variants observed in 3 of the patients (P14, P17 and P18) were found in genes (*MYLK*, *SELP*, *CTTN* and *ITGA2B*), and have previously been reported to be associated in platelet function in individuals with aspirin resistance (Voora et al., 2013). Two of these variants were found in patient P14 (*MYLK* and *SELP*), both missense changes (c.3338C>T, p.Ser1113Phe and c.2180G>A, p.Gly727Glu, respectively) and were previously found in other databases with a MAF of (0.00001648 and 0.001213, respectively). Two other missense variants were noted in P17; one variant (c.1019T>G, p.Val340Gly) in *CTTN* and the second variant (c.439C>G, p.Leu147Val) in *ITGA2B*, both of which were previously found in other databases with a MAF of (0.003746 and 0.009465, respectively). The fifth variant was found in patient P18, a missense change (c.2503A>G, p.Ser835Gly) in *ITGA2B* and was previously found in other databases with a MAF of 0.00004954. All of these variants were predicted to be disease causing and damaging by at least two of prediction software including Mutation Taster.

On the other hand, there are 12 additional variants presented in these 5 patients which are found in genes known to be involved in either the Gi signalling pathway, secretion pathway or in both. In P15, three variants were observed in genes known to be involved in both Gi signalling and secretion. Interestingly, the ATP secretion value of this patient (P15) was moderately reduced (table 4.1). Two of these variants are within *PTGIR* and *LTBP1*, a frameshift causing deletion (c.48delG, p.Gly17* and c.514delC, p.Arg172*, respectively), both of which are novel

variants and were not found in all databases searched. The third variant is in *VPS41*, consisting of a missense change (c.1832G>A, p.Arg611His) and was previously found in other databases with a MAF of (0.001055). Two other variants observed in P15 are within two genes (*ADRA2B* and *PDE5A*) known to be involved in Gi signalling. Both of these variants were missense changes (c.1126G>A, p.Val376Ile and c.2207T>C, p.Ile736Thr, respectively) and were previously found in other databases with a MAF of 0.002181 and 0.001228, respectively.

In P16, two variants were observed in genes (*STXBP2* and *ANKRD12*) known to be involved in secretion and one variant in VWF known to be involved in both Gi signalling and secretion. Interestingly, the ATP secretion value of this patient was also reduced. All of these three variants are missense changes (c.115C>T, p.Arg39Cys, c.971C>T, p.Pro324Leu and c.1078G>A, p.Gly360Ser, respectively). However, only the variant c.115C>T, p.Arg39Cys was a novel variant and was not found in all databases.

Finally, two patients (P17 and P18) were identified with variants in genes known to be involved in platelet secretion (*DNAH11* and *MYH13*, respectively). Both patients however, had normal ATP secretion values (table 4.1). Both of these variants were missense changes (c.9011G>A, p.Arg3004Gln and c.5354G>A, p.Arg1785Gln) and were previously found in other databases with a MAF of 0.01094 and 0.00009884 respectively.

Results-Genotyping of patients identified to have a platelet defect

| Patient code | Gene | Genomic variation | protein effect | Variation type | Prevalence | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P14** | MYLK | c.3338C>T | p.Ser1113Phe | Missense | 0.00001648 | Disease causing | 1.605 | 0.99 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | WAS | c.1088G>A | p.Gly363Asp | Missense | Novel | Disease causing | 1.707 | 0.995 | Damaging | Benign | NA | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | SELP | c.2180G>A | p.Gly727Glu | Missense | 0.001213 | Disease causing | 0.964 | 0.874 | Benign | Benign | Neutral | Tolorated | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P15** | PTGIR | c.48delG | p.Gly17X | Frameshift deletion | Novel | Disease causing | -1.136 | 0.058 | NA | NA | NA | NA | PM2, PM4, PP3, | Uncertain significance |
| | LTBP1 | c.514delC | p.Arg172X | Frameshift deletion | Novel | Disease causing | 3.844 | 1 | NA | NA | NA | NA | PM2, PM4, PP3, | Uncertain significance |
| | ADRA2B | c.1126G>A | p.Val376Ile | Missense | 0.002181 | Disease causing | 3.83 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | VPS41 | c.1832G>A | p.Arg611His | Missense | 0.001055 | Disease causing | 3.72 | 1 | Damaging | Damaging | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| | SNAP23 | c.204G>C | p.Glu68Asp | Missense | 0.0002804 | Disease causing | 0.442 | 1 | Damaging | Damaging | Deleterious | Tolerated | PP3, PP2 | Uncertain significance |
| | PDE5A | c.2207T>C | p.Ile736Thr | Missense | 0.001228 | Disease causing | 4.924 | 1 | Damaging | Damaging | Deleterious | Tolerated | PP3, PP2 | Uncertain significance |
| | GRAP2 | c.919T>C | p.Ser307Pro | Missense | 0.000008247 | Disease causing | 1.124 | 0.416 | Damaging | Benign | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | EXOC1 | c.1519G>A | p.Asp507Asn | Missense | 0.000008264 | Disease causing | 5.64 | 1 | Damaging | Benign | Deleterious | Tolerated | PP3, PP2 | Uncertain significance |
| | GNB3 | c.23G>A | p.Arg8His | Missense | 0.00008435 | Disease causing | 1.234 | 0.996 | Damaging | Benign | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **P16** | VWF | c.1078G>A | p.Gly360Ser | Missense | 0.0001405 | Disease causing | 3.703 | 0.947 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | STXBP2 | c.115C>T | p.Arg39Cys | Missense | Novel | Disease causing | 1.224 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | MYO18B | c.7345C>T | p.Arg2449Trp | Missense | 0.004853 | Disease causing | 1.022 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | ANKRD12 | c.971C>T | p.Pro324Leu | Missense | 0.00009444 | Disease causing | 3.993 | 1 | Damaging | Damaging | Neutral | Damaging | PP3, PP2 | Uncertain significance |

| Patient code | Gene | Genomic variation | protein effect | Variation type | Prevalence | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **P17** | VPS18 | c.2866G>A | p.Asp956Asn | Missense | 0.0003347 | Disease causing | 5.898 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | CTTN | c.1019T>G | p.Val340Gly | Missense | 0.003746 | Disease causing | 4.329 | 0.992 | Benign | Benign | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | DNAH11 | c.9011G>A | p.Arg3004Gln | Missense | 0.01094 | Disease causing | 5.797 | 1 | N/A | N/A | Deleterious | N/A | PP3, PP2 | Uncertain significance |
| | ADAMTS13 | c.262G>C | p.Val88Leu | Missense | Novel | Disease causing | 3.199 | 0.991 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | SLFN14 | c.2309A>T | p.Tyr770Phe | Missense | 0.01322 | Polymorphism | 0.647 | 0.556 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | ITGA2B | c.439C>G | p.Leu147Val | Missense | 0.009465 | Disease causing | 0.692 | 0.018 | Benign | Benign | Neutral | Tolerated | PP3, PP2 | Uncertain significance |
| **Patient code** | | | | | | | | | | | | | | |
| **18** | MYH13 | c.5354G>A | p.Arg1785Gln | Missense | 0.00009884 | Disease causing | 5.583 | 1 | Damaging | Damaging | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| | ITGA2B | c.2503A>G | p.Ser835Gly | Missense | 0.00004954 | Disease causing | 4.144 | 1 | Benign | Benign | Neutral | Damaging | PP3, PP2 | Uncertain significance |
| | MUC2 | c.2227C>T | p.Arg743Trp | Missense | 0.0005496 | NA | NA | NA | NA | NA | Deleterious | NA | PP3, PP2 | Uncertain significance |

**Table 4.4** Summary of pathogenicity prediction and variant classification of the variants identifed in patients with cyclooxygenase pathway defects. The table dispays the nucleotide changes and effect of the resulting protein, the type of variantion and the prevelance among the population, PhyloP and PhastCons measure the prediction and probability of conservation at each individual base, Mutationtaster, SIFT, Provean and PolyPhen-2 are used to predict the pathogenicity of a mutation. The ACMG provides supporting evidences for variants classification.

### 4.3.5 Defect in genes that were identified in multiple patients

A number of gene variants were noted in more than one patient (table 4.5). Candidates were within *VWF* and identified in four patients, two of which (P7, P10) presented with Gi defect, one (P3) with a defect in Gi and cyclooxygenase pathway, and one (P16) with defect in cyclooxygenase pathway. ATP secretion values of patient P3 and P16 were also reduced. All of these 4 variants were missense changes and were previously reported in other databases (table 4.5). Defects in *MYH13* were also found in four different index cases (P1:F1:I, P6, P13 and P18). *MYH13* is a platelet secretion gene, but only one patient (P13) had low ATP secretion values. All of the variants were missense changes and were previously reported in other databases (table 4.5).

Variants in *NBEAL2* were identified in three patients (P3, P6 and P12). All of the three variants were missense changes (c.5866G>A, p.Val1956Met, c.2231C>T, p.Pro744Leu and c.6631G>A, p.Asp2211Asn, respectively) and one, found in patient P12 was novel. *NBEAL2* is a gene involved in platelet secretion however only patient P3 was presented with reduced ATP secretion (table 4.5). *LYST* is another platelet related secretion gene and was found in two patients (P11 and P12), both of which however presented with a normal ATP secretion value. Both variants (c.2806C>A, p.Pro936Thr and c.8913T>G, p.Asn2971Lys) were missense changes and one in patient P11 was novel. Two candidates in *VPS41* were noted in two patients (P13 and P15). Both variants (c.622C>T, p.Arg208Trp and c.1832G>A, p.Arg611His) were missense changes and were previously reported in other databases. *VPS41* is a member of the VPS family genes known to function in intracellular trafficking and cell secretion. Interestingly, both of patients identified with *VPS41* variations have reduced ATP secretion values (table 4.5). *ANKRD26* is associated with thrombocytopenia but variants were found in two patients (P6 and

P13) with a normal platelet count. Similarly, *ITGA2B* is associated with GT but it was identified in two patients (P17 and P18) with defects in cyclooxygenase pathways. This indicated that both genes (*ANKRD26* and *ITGA2B*) are unlikely to contribute to the bleeding diathesis observed in these patients.

| Gene | Patient code | Phenotype | Variation type | Prevelance | Genomic variation | Protein effect |
|---|---|---|---|---|---|---|
| VWF | P3 | Gi + Cox + Low ATP | Missense | 0.01273 | c.6187C>T | p.Pro2063Ser |
| | P7 | Gi | Missense | 2.50E-05 | c.817C>T | p.Arg273Trp |
| | P10 | Gi | Missense | 0.002942 | c.4751A>G | p.Tyr1584Cys |
| | P16 | Cox + Low ATP | Missense | 0.0001405 | c.1078G>A | p.Gly360Ser |
| MYH13 | P1:F1:I | Gi | Missense | 1.68E-05 | c.827C>G | p.Thr276Arg |
| | P6 | Gi + Cox | Missense | 0.0005866 | c.5786G>T | p.Arg1929Leu |
| | P13 | Gi + Low ATP | Missense | 0.002563 | c.5101C>G | p.Arg1701Gly |
| | P18 | Cox | Missense | 0.00009884 | c.5354G>A | p.Arg1785Gln |
| NBEAL2 | P3 | Gi + Cox + Low ATP | Missense | 3.32E-05 | c.5866G>A | p.Val1956Met |
| | P6 | Gi + Cox | Missense | 0.002126 | c.2231C>T | p.Pro744Leu |
| | P12 | Gi | Missense | Novel | c.6631G>A | p.Asp2211Asn |
| EXOC1 | P6 | Gi + Cox | Missense | 0.001528 | c.86C>G | p.Ala29Gly |
| | P7 | Gi | Missense | 0.005692 | c.2009G>A | p.Gly670Glu |
| | P15 | Cox + Low ATP | Missense | 0.000008264 | c.1519G>A | p.Asp507Asn |
| STXBP2 | P1:F1:I | Gi | Missense | Novel | c.841G>A | p.Gly281Arg |
| | P16 | Cox + Low ATP | Missense | Novel | c.115C>T | p.Arg39Cys |
| PLCB3 | P1:F1:I | Gi | Missense | Novel | c.1197G>C | p.Lys399Asn |
| | P3 | Gi + Cox + Low ATP | Missense | 0.002786 | c.2558C>A | p.Pro853His |
| CD36 | P5 | Gi | Missense | 5.77E-05 | c.806C>T | p.Ser269Phe |
| | P6 | Gi + Cox | Stopgain SNV | 0.0001239 | c.186C>G | p.Tyr62X |
| ANKRD26 | P6 | Gi + Cox | Splicing | 0.01 | c.3652-4T>C | NA |
| | P13 | Gi + Low ATP | Stopgain SNV | 0.0002 | c.105C>G | p.Tyr35X |
| PLCB2 | P9 | PAR-1 + Low ATP | Missense | 0.0028 | c.356C>T | p.Ala119Val |
| | P11 | Gi | Missense | Novel | c.497C>T | p.Pro166Leu |
| LYST | P11 | Gi | Missense | Novel | c.2806C>A | p.Pro936Thr |
| | P12 | Gi | Missense | 0.002159 | c.8913T>G | p.Asn2971Lys |
| GRK6 | P11 | Gi | Missense | 0.00222 | c.772G>A | p.Ala258Thr |
| | P13 | Gi + Low ATP | Missense | 0.00222 | c.772G>A | p.Ala258Thr |
| VPS41 | P13 | Gi + Low ATP | Missense | 0.0001568 | c.622C>T | p.Arg208Trp |
| | P15 | Cox +Low ATP | Missense | 0.001055 | c.1832G>A | p.Arg611His |
| ITGA2B | P17 | Cox | Missense | 0.009465 | c.439C>G | p.Leu147Val |
| | P18 | Cox | Missense | 0.00004954 | c.2503A>G | p.Ser835Gly |

**Table 4.5** Summary of defects in genes that were identified in more than one patient. The table dispays the list of genes idenified and patients involved, platelet phenotypes, the type of gene variation, the prevelance among population, code nucleotide changes and effect of the resulting protein.

### 4.3.6 Genetic analysis of an index case presented with a Gi signalling defect and her family members

The index case (P1:F1:I), a 55 year old female was investigated for a platelet function defect and was identified to have an abnormal platelet function defect in response to all concentrations of ADP. Her initial sequencing analysis did not identify any abnormal variant in the *$P2Y_{12}$* receptor gene. Her family members, a brother (F1:II) a daughter (F1:III) and a son (F1:IV) were later recruited and investigated for a platelet function defect. The clinical manifestations presented among family members vary including epistaxis, cutaneous bleeding, oral cavity bleeding, gum bleeding and menorrhagia (in female). The ISTH BAT score, overall patients' demographic, platelet parameters and Lumi-LTA findings are shown in table 4.1. A defect in platelet function was only identified in the index case (P1:F1:I) and her brother (F1:II), both of whom presented with a Gi signalling defect. ATP secretion values were normal in all family members (table 4.1). The family pedigree is shown in figure 4.1.

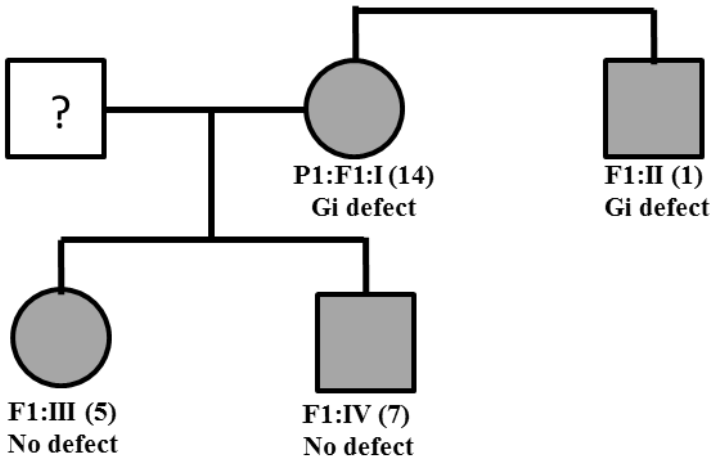



**Figure 4.1** Pedigree from family one investigated for bleeding disorders. Males are represented by squares and females by circles. Individuals with bleeding manifestation are shaded and their ISTH BAT sore are in the brackets. Question mark denotes that the candidate was not tested.

Only the index case (P1:F1:I) of family one was analysed by WES which identified 10 significant variants (table 4.6A). One variant was found in *TTC37* which is known to be involved in Gi signalling. It is a splice site variant (c.4053+3A>G) located within 3bp of the intron-exon boundary. The variant is novel and it was predicted to be a disease causing by Mutation Taster. Two other variants presented in this patient are found in genes (*PLCB3* and *APC*) known to be involved in both Gi signalling and platelet secretion. Both of these variants are missense changes (c.1197G>C, p.Lys399Asn and c.3332T>C, p.Leu1111Ser, respectively), the former variant is novel and was not found in any databases. None of the 4 patients in this family however were found to have a low ATP secretion value. Three additional variants identified in the index case were found in genes (*STXBP, MYH13* and *HOOK3*) known to be involved in platelet secretion. Two of these variants are missense changes (*STXBP*, c.1197G>C and *MYH13*, c.827C>G) and one (*HOOK3* c.1945-2delA) consisting of a splice site deletion located within 2bp of the intron-exon boundary. All of the three variants are novel and were not found in all databases searched.

Sanger sequencing of selected variants (*P2RX1, PLCB3, APC* and *TTC37*) were analysed in all family members, firstly to remove potentially false-positive derived results from WES and to confirm the presence of the variant in other family members. These four variants were selected based on the abnormal platelet function results suggesting a Gi signalling defect in two of the family members (the index case and F1:II). The list of three variants identified by Sanger sequencing in each family member is presented in table 4.6B.

A

| Patient code | Gene | Genomic variation | protein effect | Variation type | Prevelance | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P1:F1:I | STXBP2 | c.841G>A | p.Gly281Arg | Missense | Novel | Disease causing | 5.14 | 0.998 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2 | Uncertain significance |
| | PLCB3 | c.1197G>C | p.Lys399Asn | Missense | Novel | Disease causing | 1.905 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2, PP4 | Uncertain significance |
| | P2RX1 | c.101G>A | p.Arg34Gln | Missense | Novel | Disease causing | 4.685 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2,PP1 | Uncertain significance |
| | MYH13 | c.827C>G | p.Thr276Arg | Missense | 1.68E-05 | Disease causing | 5.267 | 1 | Damaging | Damaging | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | STXBP5L | c.1135G>A | p.Val379Met | Missense | 0.005711 | Disease causing | 5.053 | 0.996 | Damaging | Damaging | Benign | Damaging | PP3, PP2 | Uncertain significance |
| | CHD3 | c.1616C>A | p.Pro539His | Missense | 8.24E-06 | Disease causing | 4.003 | 0.845 | Benign | Benign | Deleterious | Damaging | PP3, PP2 | Uncertain significance |
| | APC | c.3332T>C | p.Leu1111Ser | Missense | 0.001617 | Disease causing | 2.431 | 1 | Benign | Benign | Benign | Damaging | BP4, PP2, PP4 | Uncertain significance |
| | HOOK3 | c.1945-2A>- | NA | Splicing | Novel | Disease causing | 4.752 | 1 | NA | NA | NA | NA | PP3 | Uncertain significance |
| | TTC37 | c.4053+3A>G | NA | Splicing | Novel | Disease causing | 2.554 | 0.955 | NA | NA | NA | NA | PM2, PP3, PP4 | Uncertain significance |
| | MNX1 | c.502_504del | p.168_168del | nonframeshift deletion | Novel | Disease causing | 1.146 | 0.994 | NA | NA | NA | NA | PM2, PP3 | Uncertain significance |

B

| Patient code | Gene | Genomic variation | protein effect | Variation type | Prevalence | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P1:F1:I | P2RX1 | c.101G>A | p.Arg34Gln | Missense | Novel | Disease causing | 4.685 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2,PP1 | Uncertain significance |
| | PLCB3 | c.1197G>C | p.Lys399Asn | Missense | Novel | Disease causing | 1.905 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2, PP4 | Uncertain significance |
| | APC | c.3332T>C | p.Leu1111Ser | Missense | 0.001617 | Disease causing | 2.431 | 1 | Benign | Benign | Benign | Damaging | BP4, PP2, PP4 | Uncertain significance |
| F1:II | APC | c.3332T>C | p.Leu1111Ser | Missense | 0.001617 | Disease causing | 2.431 | 1 | Benign | Benign | Benign | Damaging | BP4, PP2, PP4 | Uncertain significance |
| F1:III | P2RX1 | c.101G>A | p.Arg34Gln | Missense | Novel | Disease causing | 4.685 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2,PP1 | Uncertain significance |
| F1:IV | P2RX1 | c.101G>A | p.Arg34Gln | Missense | Novel | Disease causing | 4.685 | 1 | Damaging | Damaging | Deleterious | Damaging | PM2, PP3, PP2,PP1 | Uncertain significance |
| | APC | c.3332T>C | p.Leu1111Ser | Missense | 0.001617 | Disease causing | 2.431 | 1 | Benign | Benign | Benign | Damaging | BP4, PP2, PP4 | Uncertain significance |

**Table 4.6** Summary of pathogenicity prediction and variant classification of the family one. (A) Variants identifed by WES in index case and (B) Variants identifed by Sanger sequencing in other family members. The tables dispay the nucleotide changes and effect of the resulting protein, the type of variation and the prevalance among the population, PhyloP and PhastCons measure the prediction and probability of conservation at each individual base, Mutationtaster, SIFT, Provean and PolyPhen-2 are used to predict the pathogenicity of a mutation. The ACMG provides supporting evidences for variants classification.

Sanger sequencing confirmed the presence of all three variants in the index case (figure 4.2). None of the variants identified here however, were consistent with the platelet phenotype observed in this family. The variant c.101G>A in *P2RX1* was also identified in F1:III and F1:IV, both of which with normal platelet function (figure 4.2A). The variant c.3332T>C in *APC* was also identified in another two family members (F1:II and F1:IV) one of which (F1:IV) however has normal platelet function (figure 4.2B). In contrast, the variant c.1197G>C in *PLCB3* was only observed in the index case and not in F1:II, both of which however presented with Gi signalling defects (figure 4.2C).

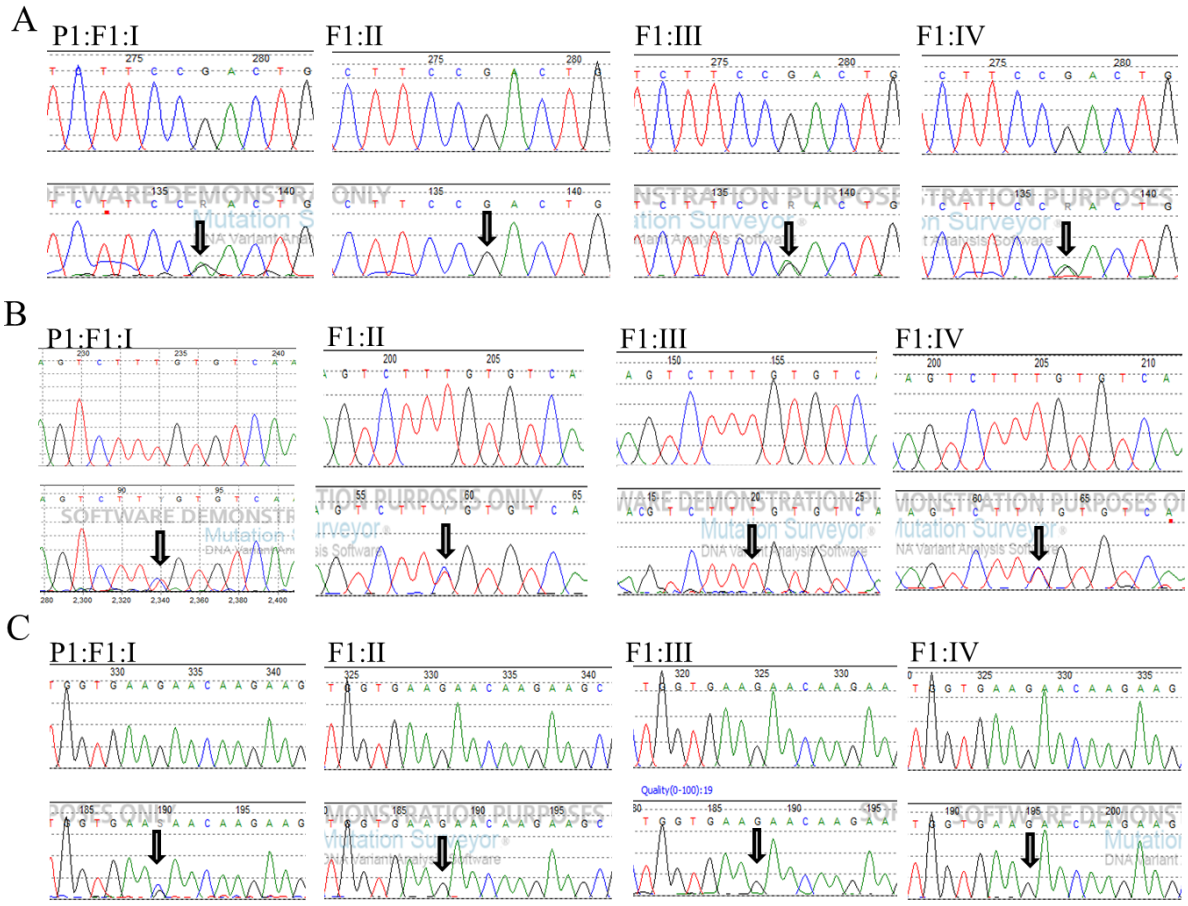



**Figure 4.2** Electropherograms of sanger analysis from four members of the family one showing the nucleotide change in comparison with a control. (A) The variant c.101G>A in *P2RX1* gene identified in three family members (P1:F1:I, F1:III and F1:IV), (B) the variant c.3332T>C in APC gene identified in three family members (P1:F1:I, F1:II and F1:IV) and (C) the variant c.1197G>C in *PLCB3* gene identified only on the index case (P1:F1:I), arrows indicate a point of nucleotide change.

### 4.3.7 Genetic analysis of a family presenting with a platelet secretion defect

Four family members (P19:F2:I, F2:II, F2:III and F2:IV) were investigated in family 2. The family pedigree for all members is shown in figure 4.3. The index case (P19:F2:I) is a 45 year old mother with an ISTH BAT score of 17 (table 4.1). Clinical manifestations including bleeding from minor wounds, cutaneous and oral cavity bleeding were shared among all four family members in addition to menorrhagia in female members. Platelet function tests presented with low ATP secretion value in all family members in addition to cyclooxygenase like defect in two members (P19:F2:I and F2:III). WES analysis revealed an average of 36,200 variants per individual. However, only two variants were identified to be significant (table 4.7). Only one of the two variants was noted to be shared by all four members (table 4.7). Interestingly, the variant was found in *VPS39* which is known to be associated with platelet secretion. The variant is a missense change (c.1723G>T, p.Asp575Tyr) and was previously found in other databases with a MAF of 0.003381. The second variant was only shared by two family members (F2:II and F2:IV), a missense change (c.622G>C, p.Glu208Gln) in *SH2B3* and previously found in other databases with a MAF of 0.0009879.

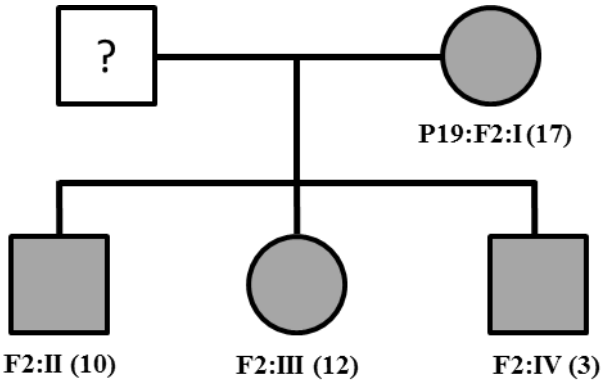
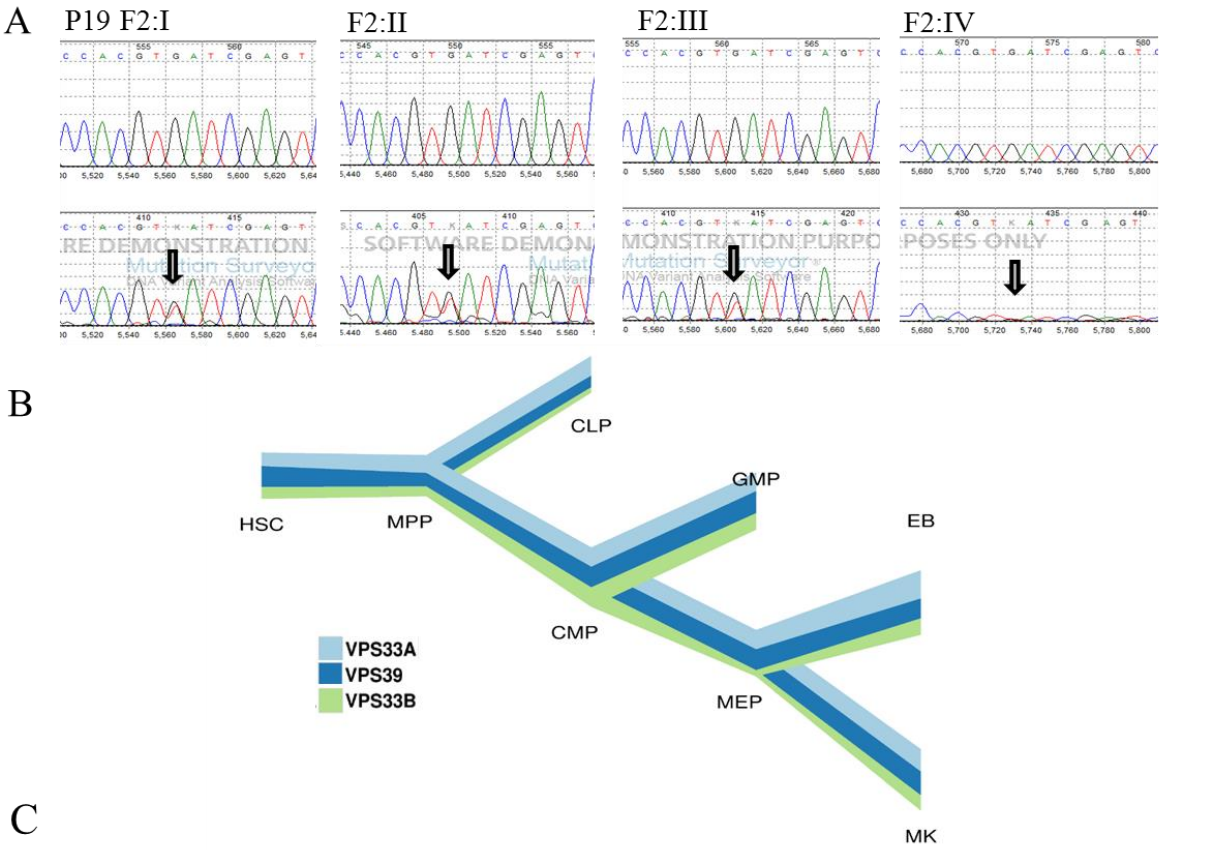



**Figure 4.3** Pedigree from family 2 presenting the inheritance of VPS39 mutation. Males are represented by squares and females by circles. Affected individuals are shaded. Question mark denotes that the candidate was not tested. The ISTH BAT sore are in the brackets.

| Patient code | Gene | Genomic variation | protein effect | Variation type | Prevalence | Mutation Taster | PhyloP | PhastCons | PolyPhen-2 HumDiv | PolyPhen-2 HumVar | Provean Score | Sift Score | ACMG | Classification |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| P19:F2:I | VPS39 | c.1723G>T | p.Asp575Tyr | Missense | 0.003381 | Disease causing | 2.355 | 0.994 | NA | NA | Deleterious | Damaging | BP4,PP2,PP1, PP4 | Uncertain significance |
| F2:II | VPS39 | c.1723G>T | p.Asp575Tyr | Missense | 0.003381 | Disease causing | 2.355 | 0.994 | NA | NA | Deleterious | Damaging | BP4,PP2,PP1, PP4 | Uncertain significance |
| | SH2B3 | c.622G>C | p.Glu208Gln | Missense | 0.0009879 | Disease causing | 2.721 | 0.887 | NA | NA | Deleterious | Damaging | BP4,PP2,PP1 | Uncertain significance |
| F2:III | VPS39 | c.1723G>T | p.Asp575Tyr | Missense | 0.003381 | Disease causing | 2.355 | 0.994 | NA | NA | Deleterious | Damaging | BP4,PP2,PP1, PP4 | Uncertain significance |
| F2:IV | VPS39 | c.1723G>T | p.Asp575Tyr | Missense | 0.003381 | Disease causing | 2.355 | 0.994 | NA | NA | Deleterious | Damaging | BP4,PP2,PP1, PP4 | Uncertain significance |
| | SH2B3 | c.622G>C | p.Glu208Gln | Missense | 0.0009879 | Disease causing | 2.721 | 0.887 | NA | NA | Deleterious | Damaging | BP4,PP2,PP1 | Uncertain significance |

**Table 4.7** Summary of pathogenicity prediction and variant classification of the variants identifed in family two with secretion defect. The table dispays the nucleotide changes and effect of the resulting protein, the type of variantion and the prevelance among the population, PhyloP and PhastCons measure the prediction and probability of conservation at each individual base, Mutationtaster, SIFT, Provean and PolyPhen-2 are used to predict the pathogenicity of a mutation. The ACMG provides supporting evidences for variant classification.

Sanger sequencing of *SH2B3* and *VPS39* was analysed for all four members of family two in order to remove potentially false-positive derived results from WES and to confirm the presence of these variants within the family members. The c.1723G>T is the only candidate noted to be shared by all four members. Interestingly, the variant is found in *VPS39* which is predicted to be a platelet secretion gene. Analysis using the Blueprint epigenome data (Chen et al., 2014) revealed a full expression of *VPS39* throughout the haematopoietic progenitors leading to megakaryocytes (figure 4.4B). Furthermore, the variant is located at highly conserved genetic site in many of species (figure 4.4C), suggesting that a mutation at this point is more likely to affect the function of resulted protein.




| | | | | |
|---|---|---|---|---|
| Humans | 546 | YSVWVLRDFPEDGLKIFTEDLPEV | ESLPRDRVLGFLIENFKGLAIPYLE | 594 |
| Chimpanzee | 546 | YSVWVLRDFPEDGLKIFTEDLPEV | ESLPRDRVLGFLIENFKGLAIPYLE | 594 |
| Rhesus macaque | 557 | YSVWVLRDFPEDGLKIFTEDLPEV | ESLPRDRVLGFLVENFKGLAVPYLE | 605 |
| Wolf | 546 | YSVWVLRDFPEDGLKIFTEDLPEV | ESLPRDRVLCFLVENFKGLAIPYLE | 594 |
| Cow | 546 | YSVWVLRDFPEDGLKIFTEDLPEV | ESLPRDRVLGFLVENFKGLAIPYLE | 594 |
| Mouse | 557 | YSVWVLRDFPEDGLKIFTEDLPEV | ESLPRDRVLNFLIENFKALAIPYLE | 605 |
| Rat | 546 | YSIWVLRDFPEDGLKIFTEDLPEV | ESLPRDRVLNFLIENFKALAIPYLE | 594 |
| Chicken | 546 | YSVWVLRDFPEDGLKIFTEDLPEV | EALPRDEVLSFLIENFKSLAIPYLE | 594 |
| Zebrafish | 545 | FSPWVLKICPEDGLKIFTEDLTEV | ETLPRDQVLNFLKEGFKELAIPYLE | 593 |

**Figure 4.4** Analysis of *VPS39* gene mutation found in all four members of family 2. (A) Representative sanger electropherograms confirming the VPS39 variant repoterd by WES to be presenet in all family bembers, arrows indicate a poin of nucleotide change. (B) Expression of *VPS39* gene throughout the progenitors leading to megakaryocytes as determined using Blueprint epigenome data (HSC–Haematopoietic stem cell, MPP–Mulitpotent progenitor, CMP–Common myeloid progenitor, MEP–Myelo-erythroid progenitor, EB–Erythroblast, and MK–Megakaryocyte). (B) Conservation sight of the *VPS39* mutation in higher order species.

## 4.4 Discussion

The work in this chapter presents genetic findings analysed in a selected group of patients and some family members recruited into the GAPP study. The patients were investigated using the GAPP approach which combines platelet phenotyping with whole exome sequencing. All selected index cases included in this cohort presented with platelet function defects and a normal platelet count (table 4.1). The cohort mainly focused on individuals with a defect in Gi signalling and cyclooxygenase pathways as these are the two major feedback pathways underlying platelet activation, however one candidate suspected with a defect on PAR-1 receptor and a family with secretion defect were also included.

To exclude variants that are unlikely to contribute to the phenotype observed in the patients and to identify a potential causative gene defect following WES, a combination of strategies were applied. Firstly, the data was filtered by excluding synonymous variants not known to change the amino acid, and restricting the analysis of variants with a MAF of less than 0.01. The variants were then compared to a database of 357 known platelet related genes and were predicted to be deleterious using a selection of bioinformatic tools. A further reduction of variants was also achieved in some index cases by comparing candidate variants within related family members.

WES has identified candidate genetic variations that could explain the platelet function defect in 59% (13/22) of the cases. The majority of variants identified in this cohort were missense. This was not of surprise as missense variants are most common due to relative impact on the expressed protein. In general, missense variants are tolerated by retaining expression and partial function of the affected allele.

23 (26%) candidate genes identified in this cohort were consistence with patients' phenotypes. Four of the variants within genes (*TTC37, PRKACG, PLA2G4C* and *PTPRC*) are known to be associated with Gi signalling (Leo et al., 2015). The variants were observed in four patients, all of which presented with a defect in Gi signalling. The variant in *PRKACG* was novel and the one in *TTC37* was a splicing defect causing a loss of the donor site. Nine other variants within genes (*PTGIR, LTBP1, TRPM7, MYH13, RAI1, MPL, STX2, STXBP2* and *ANKRD12*) known to be associated with platelet secretion defects (Leo et al., 2015) were found in 5 patients presenting with low ATP values. 3 out of these 5 patients were presented with two missense variants each, one of which was novel. P15 however, showed two frameshift variants (*PTGIR* and *LTBP1*) both of which were novel. Two other genes (*JMJD1C* and *PLCB3*) previously reported in patients with defect Gi signalling and platelet secretion defects (Leo et al., 2015) were noted in two patients (P4 and P3). Interestingly, both patients are presented with a defect in Gi signalling and reduction in ATP secretion.

Defects in the *VWF* gene is responsible for VWD, however, a number of studies have previously suggested a possible genetic link associated between VWD and platelet function defects (Goodeve et al., 2007, James et al., 2007). Several genetic variants in genes associated in platelet function including two with $P2RY_{12}$ gene have been reported in patients diagnosed with VWD (Stockley et al., 2015, Daly et al., 2009). Recently, genetic variations in *VWF* gene have been observed in patients with Gi signalling and platelet secretion defects (Leo et al., 2015). In this study, candidates within the *VWF* gene were identified in four patients (table 4.5). Interestingly, two of these four patients (P7 and P10) presented with Gi signalling defects, one patient (P16) with a reduced ATP value and one patient (P3) with both defects. Two of these four *VWF* candidates genes mutations identified in this cohort (Pro2063Ser and Tyr1584Cys) were previously reported in patients with type 1 VWD (Kasatkar et al., 2013, Daidone et al.,

2017). The harmful consequences of the former mutation (Pro2063Ser) however, remains controversial (Hampshire and Goodeve, 2014).

Two missense variants in *VPS41* were noted in two patients (P13 and P15), both of which presented with a reduced ATP value. VPS proteins are known to function in intracellular trafficking and therefore a defect in this gene can result in secretion defects (Balderhaar and Ungermann, 2013). Recently, a defect in the *VPS41* gene was identified in patients with a secretion defect (Leo et al., 2015). As both of the patients described are presented with reduced ATP values, it is possible that the defect in *VPS41* identified here could have contributed to their bleeding diathesis. Functional confirmation however will still be required to confirm this.

WES detected 5 genes in 12 patients that were not consistence with patients' phenotypes. *MYH13* has previously been noted in patients with secretion defects (Leo et al., 2015). In this study, variants with *MYH13* were identified in four cases, three of which however presented with normal ATP values (table 4.5). The *NBEAL2* protein is known to be involved in vesicular trafficking and so is critical for the development of α-granules (Rensing-Ehl et al., 2015). Defects in *NBEAL2* have been reported to be associated with GPS (Gunay-Aygun et al., 2011). In this cohort, candidate variations in *NBEAL2* were found in three patients, none of which presented with clinical features of GPS. One of these four patients (P6) was analysed by flow cytometry as a part of RPFT and was found to have normal expression of P-selectin in response to number of agonists. Defect in *LYST* is also known to be associated with CHS (Sepulveda et al., 2015). In this study, two variants within *LYST* were identified in two index cases, both of which have no clinical features of CHS and their ATP values were normal. Interestingly, one of the patients (P12) presented with a defect in both *NBEAL2* and *LYST* (table 4.5). *ANKRD26* is a gene responsible for THC2 which is associated with thrombocytopenia and haematologic

malignancies (Noris et al., 2013). Two variants in *ANKRD26* were identified in two cases. One of the variants was a premature stop codon and it was detected in a patient (P13) presenting with a low ATP value. Both of the two patients however have normal platelet counts, therefore these variants are unlikely to be the cause of their bleeding symptoms. Two patients were identified with variants in *ITGA2B*, a gene known to be associated with GT (Nurden et al., 2011). Platelet phenotyping of these two patients however were not consistence with GT indicating that variants are unlikely to be the cause of bleeding diathesis.

Individual with an 'aspirin-like' defect have been diagnosed in laboratories for almost 50 years now. Many of them were found with a defect in platelet cyclooxygenase (Rao, 2003) and some with thromboxane synthase deficiency (Rolf et al., 2009). However, only few mutations in thromboxane pathway have so far been reported (Hirata et al., 1994, Adler et al., 2008, Mumford et al., 2010). In this study, WES was analysed on 7 patients presented with a defect in cyclooxygenase pathway. However, no causative mutation was noted in genes related to thromboxane receptor or the cyclooxygenase 1 enzyme in any of the 7 patients. Interestingly however, 5 variants observed in 3 of the patients (P14, P17 and P18) were found in genes (*MYLK*, *SELP*, *CTTN* and *ITGA2B*) previously reported to be associated in platelet function in individuals with aspirin resistance (Voora et al., 2013). These variants may or may not have contributed to the aspirin-like' defect observed in these patients, but further work would be needed to determine this.

Two families were investigated in this cohort. Family one is a complex case of four related individuals. A mother (index case) and her two siblings all of which were presented with bleeding diathesis and ISTH BAT score of 5 and above (Figure 4.1). Surprisingly, only the mother was presented with abnormal platelet phenotype. In contrast, the brother of the index

case has almost no bleeding symptoms (ISTH BAT score of 1). However, his platelet function analysis presented with a Gi signalling defect similar to his sister (table 4.1). Four genes (*$P2RX_1$*, *APC*, *PLCB3* and *TTC37*) were identified in P1:F1:I to be consistence with her phenotype and were further investigated in all four family members using Sanger sequencing.

*APC* and *PLCB3* have previously been reported in patients with a defect in Gi signalling (Leo et al., 2015). In this family, the variant in *APC* was also detected in F1:IV who has no platelet defect suggesting that the variant is unlikely to contribute to the platelet phenotype observed in other two family members. On the other hand, the *PLCB3* gene was identified only in P1:F1:I. Absence of this variant in F1:II who also presented with platelet defect has ruled out the likelihood of this variant to be the cause of the abnormal platelet function observed. $P2X_1$ is an ATP receptor when binds to its ligand and evokes $Ca^{2+}$ influx to contribute to platelet function (Mahaut-Smith et al., 2011). A mutation within the platelet *$P2RX_1$* has been reported in a patient with a severe bleeding and abnormal platelet function (Oury et al., 2000). In this family the variant on *$P2RX_1$* gene was observed in three family members, two of which have normal platelet function. Furthermore, the variant was absent on the family member F1:II who also presented with abnormal platelet function indicating that the variant is unlikely to cause the platelet defect in this family. The *TTC37* is the causative gene of Trichohepatoenteric syndrome (THES) characterised by life-threatening diarrhea in infancy, immunodeficiency, liver disease, trichorrhexis nodosa, facial dysmorphism, hypopigmentation and cardiac defects (Hartley et al., 2010). The *TTC37* gene has also been reported in individuals with defect in Gi signalling (Leo et al., 2015). This raises the possibility that, if the *TTC37* variant is proven to be present in both (P1:F1:I and F1:II) it could then explain the abnormal platelet function observed in this family. However, further functional work would still be required to confirm this.

Family two is an interesting case of four affected related individuals of a mother and her three siblings all of which presented with bleeding diathesis and abnormal platelet function. The phenotypic presentation however, varies between the family members. All four present with a secretion defect, but the ATP values in patients F2:II and F2:IV was markedly reduced compared to the index case (P19:F2:I) and patient F2:III. In contrast, the index case and patient F2:III were identified with an additional defect in the cyclooxygenase pathway that was not observed in the other two members (table 4.1). WES identified only one variant within the *VPS39* gene that was present in all 4 individuals which was also confirmed by Sanger sequencing (figure 4.4A). No variant was noted to be common in both, the index case and F2:III, which could explain their additional defect on cyclooxygenase pathway observed. In contrast, one variant in *SH2B3* was identified by WES to be present only in F2:II and F2:IV.

The molecular function of the rare *VPS39* gene variant observed in this family is currently unknown. However, it has been reported that in human cells the gene is involved for the delivery of endocytosed cargo to enzymatically active lysosomes (Pols et al., 2013). Furthermore, the other VPS family genes including *VPS33B* is known to be a genetic cause of ARC syndrome and is related to an alpha granule secretion defect (Urban et al., 2012). Interestingly, the *VPS39* gene is highly expressed in hematopoietic cells. Furthermore, the amino acid position of this gene variant is found to be highly conserved suggesting a possible role for this region in regulating *VPS39* expression that could be disrupted by the mutation. Overall, it highly suggestive that the variant found in *VPS39* gene could be the cause of the clinical presentation observed in this family. It is also possible that, the presence of other genetic variants may have contributed to the variation of platelet phenotype among family members. Functional confirmation of the variant c.1723G>T in *VPS39* would be of interest to determine whether this variant is the true contributor of the phenotype observed in this family.

In summary, WES in this study has identified the possible genetic variant candidates in 10 (53%) patients presented with bleeding symptoms and abnormal platelets function. Further work however is still needed to confirm the real contribution of these variants to the phenotype observed in these patients.

### 4.4.1 Limitation and suggestions

Genetic analysis of this study focused only to those genes known to be platelet related genes. It is therefore suggestive in the future to reanalyse the novel genes not previously known to affect platelet function for the aim of identifying the genetic mutation of the remaining 47% of patients, most importantly the family one which includes family members with both normal and abnormal phenotypes. It will also be useful to include family members to further reduce the number of possible variants identified within the patients.

# CHAPTER FIVE: COMPARISON OF MULTIPLE ELECTRODE AGGREGOMETRY WITH LUMI-AGGREGOMETRY FOR THE DIAGNOSIS OF PATIENTS WITH MILD BLEEDING DISORDERS

## 5.1 Introduction

The Multiple Electrode Aggregometry (MEA) is an impedance aggregometer that has been developed for the rapid assessment of platelet function in whole blood. The device is equipped with 5 channels that allow simultaneous analysis of platelet responses using different agonists. MEA evaluates platelet responses in disposable ready-to-use test cuvettes each containing two pairs of electrodes, thus enabling a duplicate measurement. During the test, activated platelets adhere onto the electrodes resulting in changes to the electrical resistance which is continuously monitored for 6 minutes (Toth et al., 2006). The test results are assessed using the two independent sensors in the test cell, calculated and reported as area under the curve (AUC). A typical normal aggregation curve and defined endpoint parameters are shown in figure 5.1. Although MEA can also measure the kinetic changes in aggregation over time including the parameters of lag time, slope and area under the curve and maximal aggregation, the traces are not as detailed as LTA which not only measures these but also includes shape change, primary (reversible) and secondary aggregation responses. In addition, MEA does not measure released ATP in parallel with aggregation.

As a whole blood method, MEA has an advantage of assessing platelet function in more physiological conditions and avoids the variables associated with the preparation of PRP. In addition, this approach is faster, more convenient and is less technically demanding. Furthermore, the use of whole blood requires only small volumes making it possible to be used as point of care test and for evaluation of platelet function in young children.

The use of MEA has gained popularity in the last decade and has been widely established for the monitoring of anti-platelet therapy. A number of studies have demonstrated the utility of this method in assessing inhibition of platelet function in individuals taking antiplatelet agents e.g. aspirin and clopidogrel (Paniccia et al., 2009, Velik-Salchner et al., 2008). In assessing acquired haemorrhagic conditions, MEA has been found to be able to predict the risk of bleeding and requirement of blood product transfusion in patients before and after cardiac surgery (Rahe-Meyer et al., 2008, Rahe-Meyer et al., 2009) and to detect impaired hemostasis in patients after cardiopulmonary bypass surgery (Mengistu et al., 2009, Steinlechner et al., 2009). Morel-Kopp et al. reported a high sensitivity of MEA in identifying patients with heparin-induced thrombocytopenia (Morel-Kopp et al., 2010). Studies on inherited bleeding disorders have demonstrated the utility of MEA in identifying patients with von Willebrand disease (Valarche et al., 2011) and its potential for the diagnosis of severe platelet disorders (Awidi et al., 2009, Albanyan et al., 2015). The use of MEA for the diagnosis of patients with inherited platelet disorders however has not been fully investigated.

**5.2 Aim**

To evaluate the ability of MEA in diagnosing patients with mild bleeding disorders by comparison with light transmission lumi-aggregometry (Lumi-LTA).

This work was carried out in conjunction with Sian Drake.

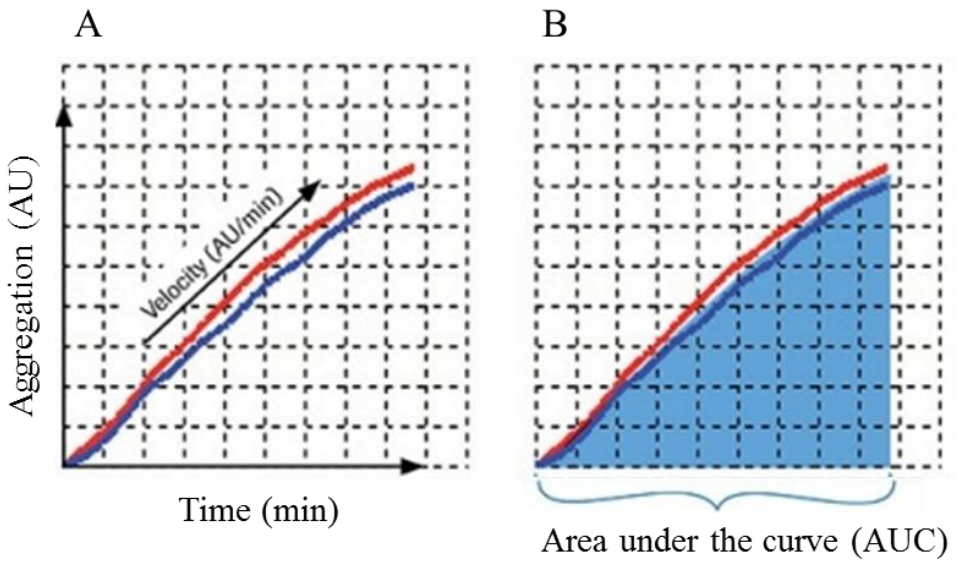



**Figure 5.1** MEA typical normal aggregation curves and the analytical parameters collected (the red and blue lines are the results from the two independent sensors in the test cell). A) The increase of impedance is measured as arbitrary aggregation units (AU) and plotted against time. B) The AUC expressing the overall platelet activity is calculated from the total height of the mean aggregation result against the aggregation slope (Toth et al., 2006).

## 5.3 Results

### 5.3.1 Overall demographics and platelet parameters of patients and healthy controls

We analysed a total of 109 patients (21 male and 88 female) with a median age 33 (range 3-73) and 40 healthy adult volunteers (18 - 57) recruited into the GAPP study and compared MEA results with lumi-LTA. The overall platelet parameters and ATP secretion value of patients and healthy controls are shown in table 5.1.On healthy control group, the values (mean ± SD) of whole blood platelet count and mean platelet volume (MPV) were 234.5 ± 54 x $10^9$/L and 11.7 ± 1.1fL respectively. The PRP count (mean ± SD) was 3.27 ± 1.25 x $10^8$/L with a mean ATP secretion value (mean ± SD) of 1.10 ± 0.33nmol/1 x $10^9$/L platelet. On patients, the values (mean ± SD) of whole blood platelet count and mean platelet volume (MPV) were 237.9 ± 52.5 x $10^9$/L and 11.3 ± 1.2fL respectively. The PRP count (mean ± SD) was 3.61 ± 0.95 x $10^8$/L with a mean ATP secretion value (mean ± SD) of 1.02 ± 0.43 nmol/1 x $10^9$/L platelet. The cut off values for MEA (table 5.2) obtained from healthy controls (n=40) were calculated as the $5^{th}$ percentile of responses of normal of ADP 10 µM, collagen 1µg/ml, collagen 3 µg/ml, PAR-1 100 µM and arachidonic acid 0.5mM were 19.1, 21, 35.1, 42.3 and 19.7 (AUC), respectively.

| | Healthy controls (mean ± SD) | Patients with normal platelet count (mean ± SD) | Statistical analysis | Healthy controls (mean ± SD) | Patients with thrombocytopenia (mean ± SD) | Statistical analysis |
|---|---|---|---|---|---|---|
| **Platelet count in Whole Blood ($x10^9$/L)** | 234.5 ± 54 | 236.2 ± 65 | ns | 234.5 ± 54 | 124.5 ± 35 | ** |
| **(range)** | (150 - 363) | (150 - 430) | | (150 - 363) | (95 - 147) | |
| **Platelet count in PRP (x $10^8$/ml)** | 3.27 ± 1.25 | 3.32 ± 1.04 | ns | 3.27 ± 1.25 | 2.20 ± 0.87 | ** |
| **(range)** | (1.2 - 7.68) | (1.4 – 10.5) | | (1.2 - 7.68) | (0.9 – 3.64) | |
| **Mean Platelet Volume (fL)** | 11.7 ± 1.1 | 11.1 ± 1.1 | ns | 11.7 ± 1.1 | 12.1 ± 1.7 | * |
| **(range)** | (8.3 - 13.4) | (8.6 – 13.2) | | (8.3 - 13.4) | (8.8 - 14.9) | |
| **ATP secretion level (nmol/1x$10^8$ platelets)** | 1.1 ± 0.33 | 1.09 ± 0.42 | * | 1.1 ± 0.33 | 0.98 ± 0.69 | * |
| **(range)** | (0.64 - 2.21) | (0.19 – 2.74) | | (0.64 - 2.21) | (0.45 – 2.02) | |

**Table 5.1** Summary of platelet parameters and ATP secretion values obtained from healthy controls (n=40) and patients (n=109): Data are presented as mean, +/- standard deviation and range. Statistical analysis performed by the Mann Whitney U test. Significance was compared to healthy controls (**$p<0.01$ and *$p<0.05$).

| MEA (AUC) | ADP (10µM) | Collagen (1µg/ml) | Collagen (3µg/ml) | PAR-1 (100µM) | Arachidonic acid (0.5mM) |
|---|---|---|---|---|---|
| **Mean ± SD** | 49.1 ± 15 | 56 ± 17.5 | 69.1 ± 17 | 75.5 ± 16.6 | 46.7 ± 13.5 |
| **Cut off value** | 19.1 | 21 | 35.1 | 42.3 | 19.7 |

**Table 5.2** Summary of MEA results with various agonists (mean and SD) obtained from whole blood from healthy controls (n=40). The cut-off values were calculated as the 5$^{th}$ percentile of the corresponding measurement in healthy control.

### 5.3.2 Correlation between platelet counts and aggregation results by MEA and lumi-LTA

We analyzed the correlation between aggregation results and platelet counts obtained from healthy controls (n=40). With MEA we found a significant but weak correlation between whole blood platelet counts and aggregation results in response to ADP 10 µM ($r$=0.321, $p$ = 0.0434) but not with PAR-1 100 µM ($r$= 0.1725, $p$ = 0.2871), arachidonic acid (r = 0.3631, p = 0.1266), collagen 1 µg/ml ($r$ = 0.1452 $p$ = 0.3714) and collagen 3 µg/ml ($r$ = 0.2068 $p$ = 0.2004) (figure 5.2). In contrast, with lumi-LTA, a weak correlation between the PRP counts and aggregation was only found in response to collagen 3 µg/ml ($r$ = 0.366, $p$ = 0.0202) but not with ADP 10 µM ($r$ = 0.1576, $p$ = 0.3313), PAR-1 100 ($r$ = 0.1607, $p$= 0.3219), arachidonic acid 0.5 mM (r = 0.3428 $p$ = 0.1508) and collagen 1 µg/ml ($r$ = 0.1919 $p$ = 0.2354) (figure 5.3).

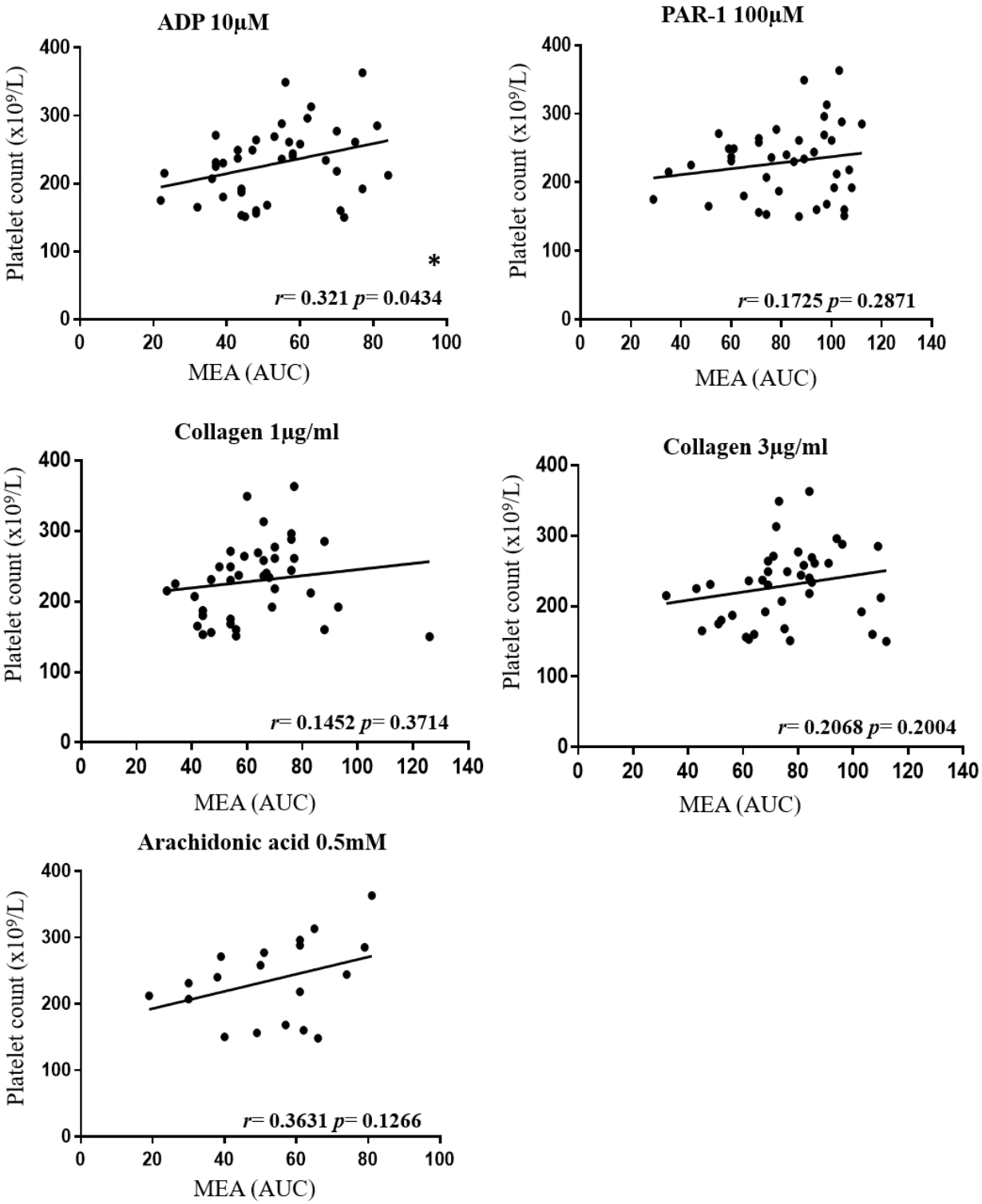



**Figure 5.2** Correlation between MEA (AUC) and whole blood platelet counts (x$10^9$/L) in samples from healthy controls (n=40) after stimulation with ADP 10 µM, Collagen 3 µg/ml PAR-1 activating peptide 100 µM and Arachidonic Acid 0.5 mM. Correlation analysis performed on Graph Pad Prism version 7.0 software using linear regression. r = correlation coefficient, p = probability value and *$p$<0.05

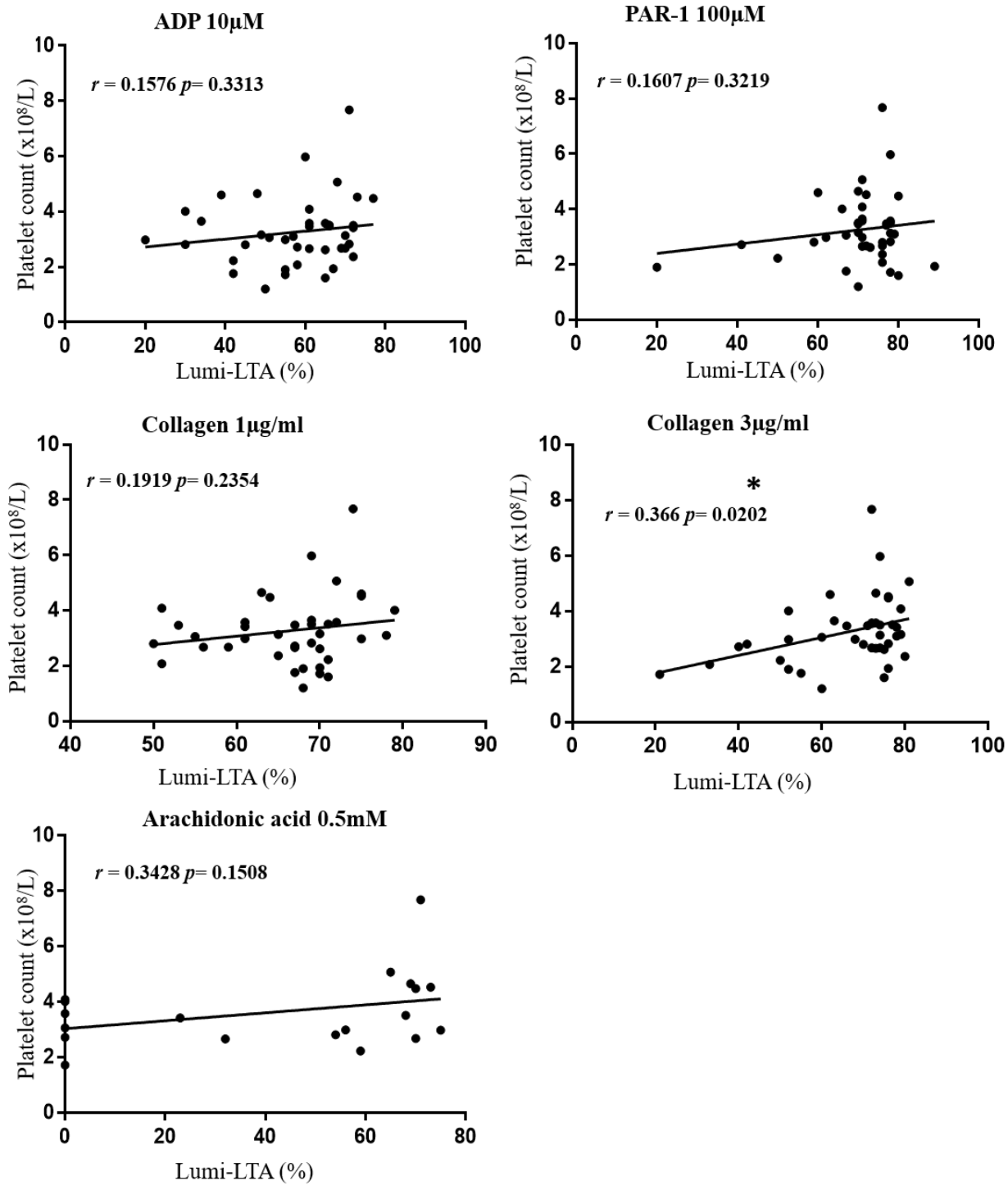



**Figure 5.3** Correlation between Lumi-LTA (aggregation %) and PRP counts (x$10^8$/L) in samples from healthy controls (n=40) after stimulation with ADP 10 µM, Collagen 3 µg/ml PAR-1 activating peptide 100 µM and Arachidonic Acid 0.5 mM. Correlation analysis performed on GraphPad Prism version 7.0 software using linear regression. r = correlation coefficient, p = probability value and *$p$<0.05

### 5.3.3 Overall comparison between MEA and Lumi-LTA

Overall results of 109 patients tested are shown on figure 5.4. 54 (49%) patients gave abnormal responses by lumi-LTA to one or more agonists as defined using previous criteria (Dawood et al., 2012). In contrast, only 16 (15%) patients were shown to have abnormal responses to one or more agonists by MEA, as defined using the cut off values obtained from healthy controls (table 5.2). The overall agreement between the two instruments is shown in table 5.3. 65/109 samples gave identical results by both tests, with the majority (52) giving normal responses. In contrast, there was disagreement in 44/109 samples. MEA gave normal results in 41 patients that were abnormal by lumi-LTA. However, 3 samples were abnormal by MEA but normal by lumi-LTA.

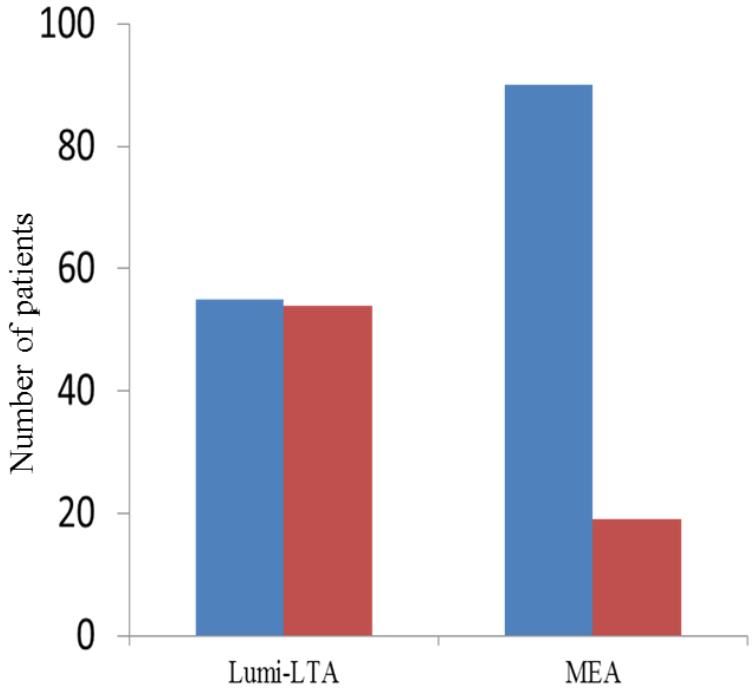



**Figure 5.4** Summary of overall results from patients (n=109) with normal (blue) and abnormal (red) results detected by Lumi-LTA or MEA. Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by MEA) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown in table 5.2

| | **Lumi-LTA (Abnormal)** | **Lumi-LTA (Normal)** | |
|---|---|---|---|
| **MEA (Abnormal)** | 13 | 3 | PPV=81% |
| **MEA (Normal)** | 41 | 52 | NPV=56% |
| | Sensitivity=24% | Specificity=95% | |

**Table 5.3** Analysis of the overall agreement between MEA and lumi-LTA in patient samples (n=109) displaying sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

### 5.3.4-Analysis and identification of patients with various platelet function defects

Platelet function defects identified by LTA were classified into 4 main groups (Gi defect, secretion defects, COX-like defect and unclassified defects) according to their pattern of responses to specific agonists as previously described (Dawood et al., 2012). As shown in figure 5.5, MEA only detected 4 out of 17 patients with Gi defects and 2 out of 20 with secretion defects. MEA only detected 4/7 patients with a COX-like defect and 0/4 patients with unclassified defects by LTA. Finally MEA gave abnormal responses to 3 samples out of 6 with multiple platelet defects. When functional defects were classified based upon identical concentrations/panel of agonists (with no ATP measurements included) used by both tests, this also confirmed that MEA was unable to detect some abnormalities detected by LTA (16 (15%) versus 41(38%) (figure 5.6). Overall kappa statistics demonstrated only a fair agreement between MEA and lumi-LTA in response to ADP (k = 0.26, CI = 0.11 to 0.40) and collagen (k = 0.28, CI = 0.05 to 0.52) and a poor agreement in response to PAR-1 ( k = 0.07, Cl = -0.17 to 0.32) (figure 5.7).

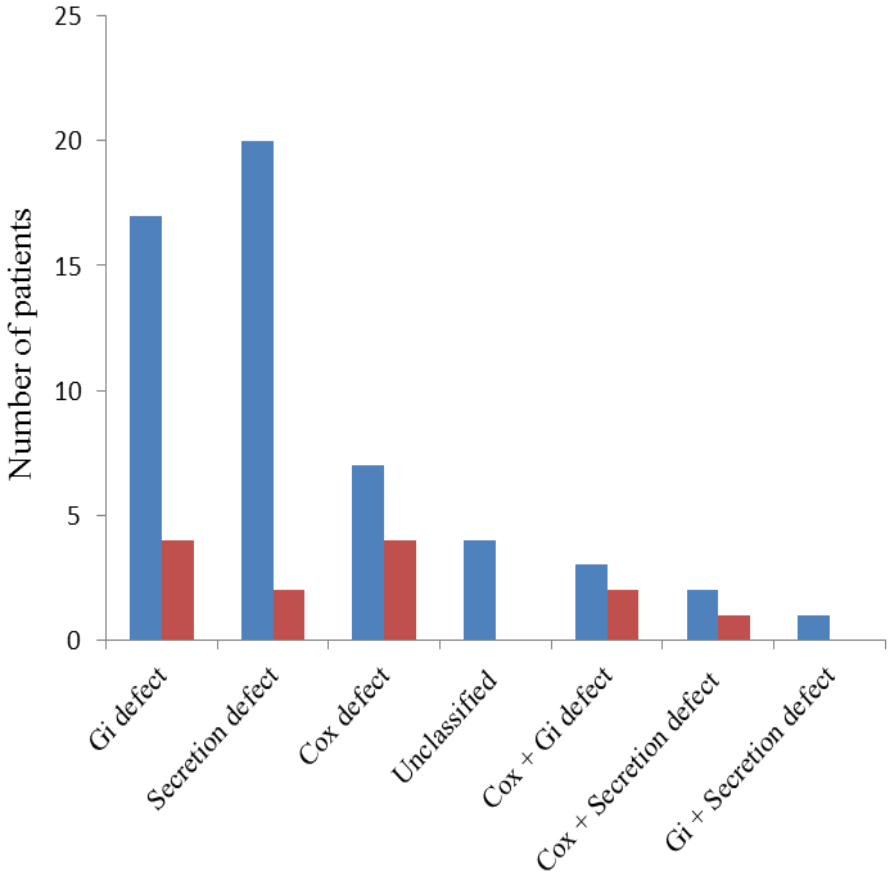



**Figure 5.5** Classification of platelet function defects on patient samples detected by lumi-LTA (blue) and MEA (red). Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by MEA) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown in table 5.2

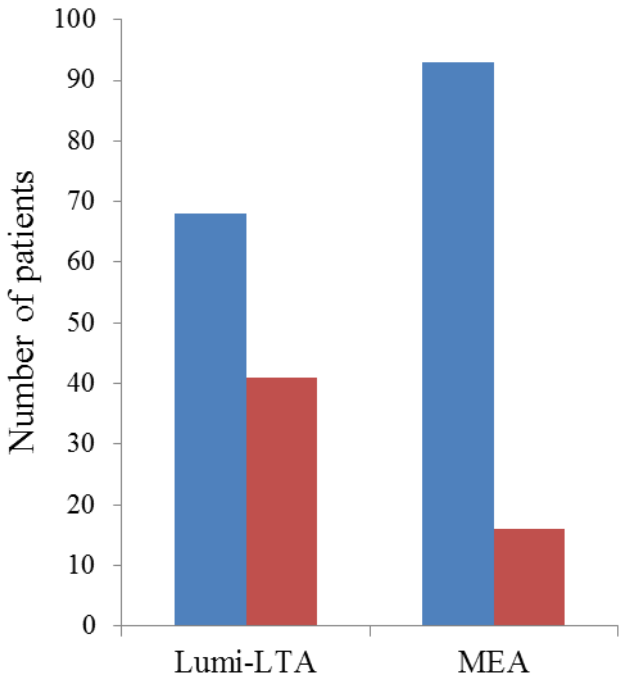



**Figure 5.6** Summary of results from patients (n=109) with normal (blue) and abnormal (red) result detected by Lumi-LTA and MEA using an identical panel and concentration of agonists (with no ATP measurements included). Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by MEA) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown in table 5.2

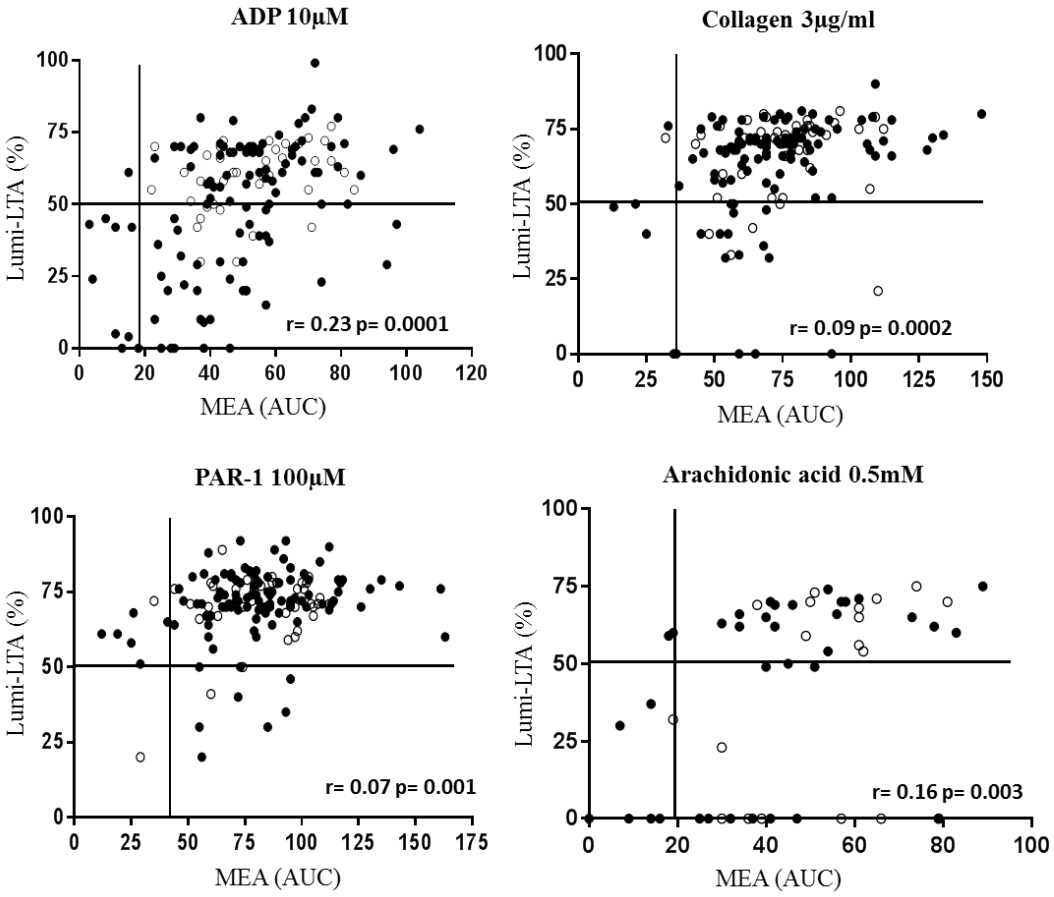



**Figure 5.7** Correlations between MEA (AUC) and lumi-LTA (%) in samples from healthy controls (n=40, open circles) and patients (n=109, closed circles) after stimulation with, ADP 10µM, Collagen 3µg/ml, PAR-1 peptide 100µM and arachidonic acid 0.5 mM (healthy controls= 19 and patients =36). The vertical black lines indicate the cut-off values of MEA determined as the 5$^{th}$ percentile of the normal range established in healthy subjects. The horizontal black lines indicate cut-off values of lumi-LTA previously established in the GAPP program using samples from healthy controls. Correlation analysis performed on Graph Pad Prism version 7.0 software using linear regression, r = correlation coefficient, p = probability value. Value were considered significant when $p$ <0.05.

### 5.3.5-Analysis of MEA sensitivity in response to specific agonists

Further analysis of the response of patients to individual agonists is summarised in figure 5.8. MEA detected an abnormality within 12 patients in response to 10 µM ADP, the majority of which (11) were also abnormal on lumi-LTA. In contrast, lumi-LTA using the same dose of ADP detected an abnormality within 45 patients, 33 of which gave normal responses by MEA. In response to 3 µg/ml collagen, 5 patients were abnormal by MEA, 1 of these however gave a normal response on lumi-LTA. 19 patients gave abnormal responses to 3 µg/ml collagen by lumi-LTA but 15 of these were normal by MEA. With 100 µM PAR-1 peptide, 6 patients were abnormal on MEA compared to 9 patients by lumi-LTA. Surprisingly, only one patient with an abnormal response to 100 µM PAR-1 peptide was detected by both tests. Finally, with arachidonic acid (0.5mM), MEA and lumi-LTA detected 8 and 15 abnormalities respectively.

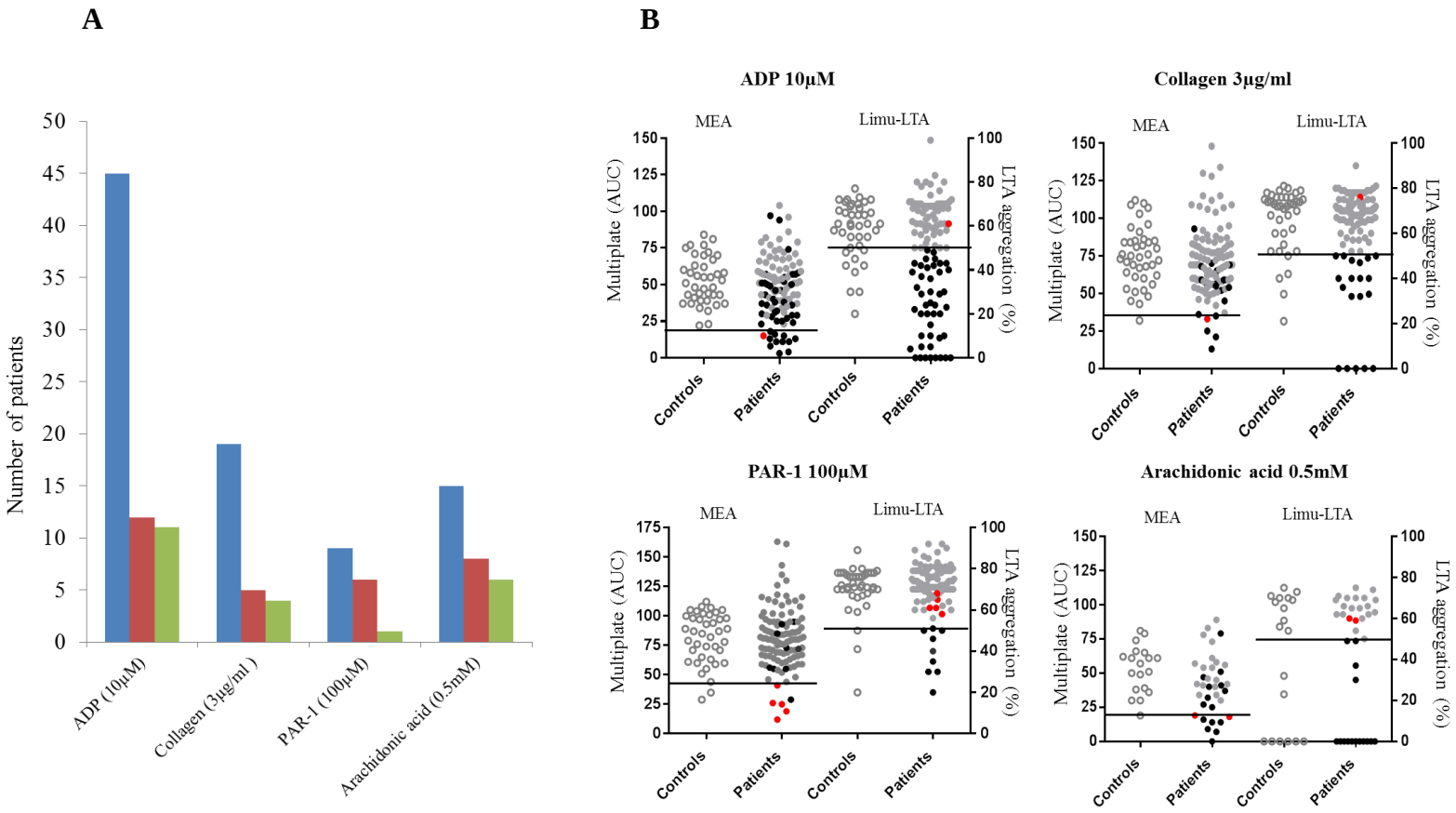



**Figure 5.8** Summary of platelet function results assessed by MEA (AUC) and lumi-LTA (%) after stimulation with, ADP 10 µM, Collagen 3 µg/ml, PAR-1 peptide 100 µM and Arachidonic Acid 0.5 mM. A) Number of patients with abnormal responses to specific agonists detected by lumi-LTA (blue), MEA (red) and both (green). B) Aggregation responses of samples from healthy controls (n=40) and patients (n=109). The horizontal black lines indicate cut-offs determined as 5$^{th}$ percentile of the normal range from healthy controls. In the patient groups: black circles indicate individuals with an abnormality detected by lumi-LTA, and red circles indicate an abnormality detected only by MEA.

### 5.3.6 Analysis of patients with low ATP secretion levels and thrombocytopenia

In 20 patients with secretion defects characterized by reduced ATP levels (table 5.4), the lumi-LTA and MEA aggregation responses were abnormal in only 8 and 3 patients respectively (figure 5.9A). MEA is therefore less sensitive than lumi-LTA at detecting secretion defects which is not surprising in that MEA does not provide a direct readout of secretion. On the other hand, in 12 (11%) patients with mild thrombocytopenia as characterized by low platelet count ($<150\ x10^9/L$) in whole blood (table 5.4), abnormal aggregation responses were detected by MEA on 6/12 patients and lumi-LTA on 8/12 patients (figure 5.9B).

| | No of patients | MEA | Lumi-LTA |
|---|---|---|---|
| Overall results (normal:abnormal)(%) | 109 | 93:16 (85:15) | 55:54 (51:49) |
| Patients with thrombocytopenia (%) | 12 | 6 (50) | 8 (67) |
| Patients with reduced ATP secretion (%) | 20 | 3 (15) | 8 (40) |

**Table 5.4** Comparison between MEA and lumi-LTA on overall results, patients with thrombocytopenia and patients with reduced ATP secretion by lumi-LTA, the data are presented as absolute number and percentage

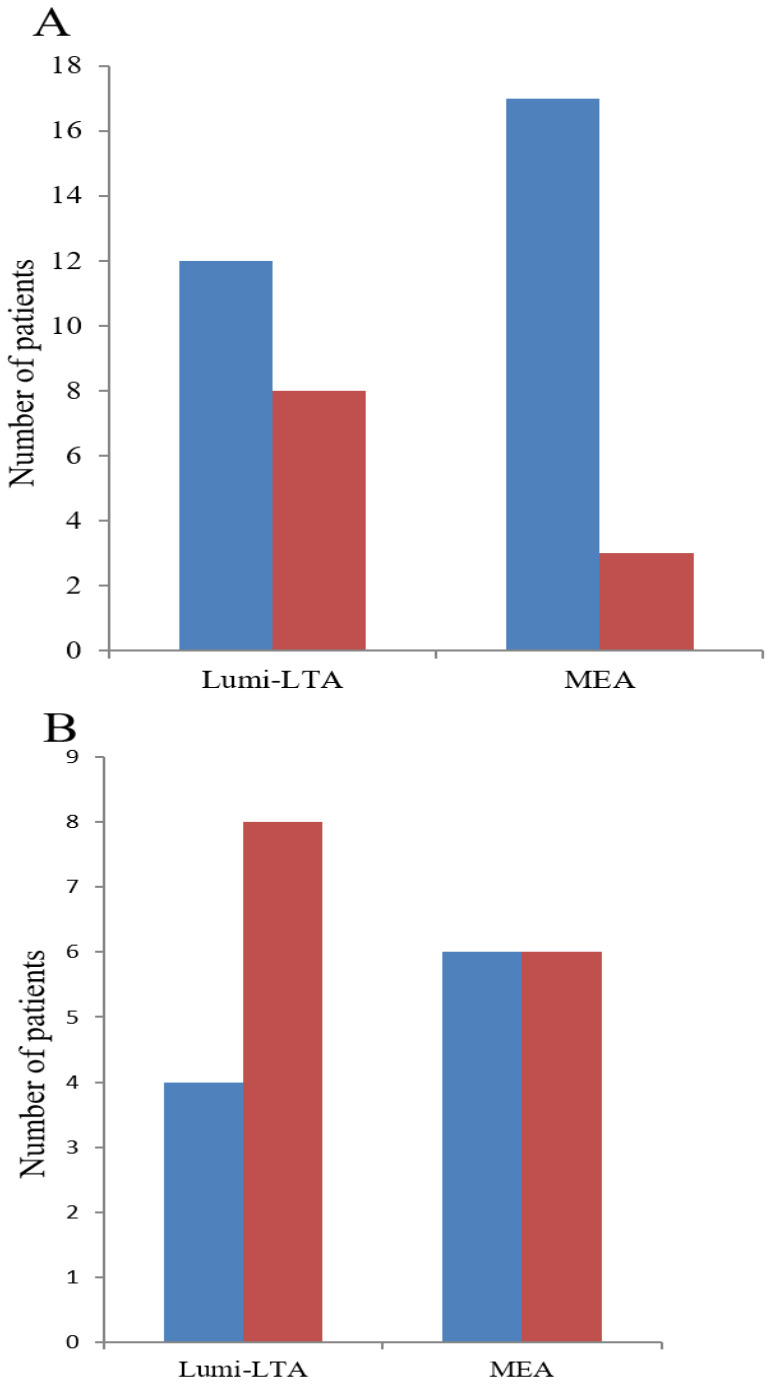



**Figure 5.9** Number of patients with normal (blue) and abnormal (red) aggregation responses detected by lumi-LTA and MEA from patients with (A) low ATP secretion values and (B) thrombocytopenia. Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by MEA) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown in table 5.2

## 5.4 Discussion

The Multiple Electrode Aggregometer (MEA) is an impedance aggregometer used for performing platelet aggregation in whole blood. This approach offers the advantage of assessing platelet function in more physiological conditions and also avoids the variables associated with the preparation of PRP (Toth et al., 2006). MEA is popular for measuring antiplatelet therapy (Paniccia et al., 2015, Velik-Salchner et al., 2008) and has been shown to be useful for detecting severe PFDs (Awidi et al., 2009, Albanyan et al., 2015). However, the use of MEA for the potential diagnosis of patients with inherited platelet disorders has yet to be fully investigated. Here I assessed the potential utility of MEA for detecting mild PFDs.

The MEA manufacturer recommends the use of hirudin as anticoagulant of choice to maintain physiological calcium levels (Toth et al., 2006). Studies however, have shown that different anticoagulants can affect platelet function activities (Wallen et al., 1997). As such, in this study we only used citrated blood for both techniques in order to have a similar comparison with identical levels of free calcium. Furthermore, a study using citrated blood from healthy individuals showed no significant difference between MEA and LTA even at higher citrate concentrations (Seyfert et al., 2007). Another crucial factor when testing platelet activity particularly on whole blood aggregometry is the storage time between blood collection and sample processing. Studies have shown that in citrated whole blood, platelet function activity remain stable up to 4 hours from blood collection (Seyfert et al., 2007, Kaiser et al., 2012). As such, to avoid variation of results due to time delays, MEA was always tested during PRP/PPP preparation for lumi-LTA to ensure that the analysis was always completed rapidly within 4 hours (Seyfert et al., 2007, Kaiser et al., 2012).

As MEA dilutes the whole blood 1:1 with saline before testing it was first thought to investigate the influence of platelet counts (within normal count) on both MEA and lumi-LTA results. With

MEA a linear increase in aggregation was only observed with ADP but not collagen, PAR-1 or arachidonic acid (figure 5.2). In contrast with lumi-LTA this was only observed with collagen but not ADP, PAR-1 or arachidonic acid (figure 5.3). Previous studies with LTA have found no correlation between platelet count and aggregation results (Dawood et al., 2007, Seyfert et al., 2007, Cattaneo et al., 2007, Femia et al., 2013). Seyfert *et al*. also found a significant correlation between platelet count and platelet aggregation on MEA induced by ADP and collagen, but not by arachidonic acid (Seyfert et al., 2007). Mengistu et al. and Stissing et al. also demonstrated the influence of platelet count on whole blood aggregometry towards low platelet concentrations (Mengistu et al., 2009, Stissing et al., 2011). Two more studies also reported correlations between platelet count and extent of platelet aggregation on MEA (Femia et al., 2013, Hanke et al., 2010). However, the latter results are difficult to interpret as the counts were adjusted either by addition of PPP (Hanke et al., 2010) which is known to inhibit platelet aggregation (Cattaneo et al., 2007), or with dilution with Tyrode's buffer (Femia et al., 2013).

This study is the first comprehensive comparison of the potential utility of MEA for detecting platelet function defects in patients with mild bleeding disorders. The overall patient results (figure 5.4/table 5.3) showed a moderate agreement between MEA and lumi-LTA with the diagnosis being concordant in 60% of cases. As many of these gave normal responses, the MEA demonstrated a negative predictive value of 56%. In healthy individuals there was good agreement between MEA and lumi-LTA with no difference in response between identical concentrations of the agonists ADP, collagen, PAR-1 activating peptide and arachidonic acid. This findings support a recent study by Seyfert *et al.*, demonstrating equivalent results of MEA and lumi-LTA in healthy samples (Seyfert et al., 2007). In patients' samples however, we observed significant differences between the two techniques in response to all agonists. We

observed a fair agreement between MEA and lumi-LTA when overall results were analysed using kappa statistics (figure 5.7).

In identification of individuals with platelet function defects we found that MEA detected more patients with COX-1 like defects than those with Gi and secretion defects (figure 5.5). This suggests that MEA is more sensitive in response to defects in the thromboxane pathway. However, the lack of sensitivity of MEA in detecting Gi defects may also be related to the higher ADP concentration (10 µM) used in this study which is slightly higher than the standard recommended concentration (6.5 µM) (Toth et al., 2006). Interestingly, we have also observed that MEA showed high consistency in detecting abnormalities in patients with dual defects. This findings support the earlier reports suggest that MEA is reliable in detecting more severe forms of platelet defects such as GT (Awidi et al., 2009, Albanyan et al., 2015).

In response to specific agonists we demonstrated reduced sensitivity of MEA to detect platelet function defects using all four classes of agonists (figure 5.8A). In particular there was a marked decreased sensitivity with ADP, Collagen and arachidonic acid when compared to lumi-LTA. A high degree of disagreement between the two techniques was also observed in response to PAR-1 activating peptide. Out of 14 abnormal only one (7%) was detected by both techniques (figure 5.8B).

Measurement of secreted ATP levels is an additional but important tool in identifying patients with platelet secretion defects (Cattaneo, 2009). Moreover, it is well known that individuals with reduced dense granule secretion often demonstrate normal aggregation responses (Dawood et al., 2007). In this study MEA and lumi-LTA gave normal responses on 17(85%) and 12(60%) patients respectively that were found to have low secreted ATP levels (figure 5.9A). This is probably unsurprising given that MEA does not directly measure ATP secretion and the aggregation responses are probably less sensitive at detecting the feedback loop from

released ATP/ADP from the dense granules. In related to patients with thrombocytopenia, MEA has previously been reported to be unreliable in diagnosing samples with low platelet counts (Femia et al., 2013). In this study however, MPA gave normal responses on 6 (50%) of patients with thrombocytopenia. Surprisingly 3 of these have also found to have a functional defect by LTA (figure 5.9B). In summary, MEA demonstrates a lack of sensitivity in identifying patients with mild bleeding associated with abnormal platelet function and platelet secretion defects. More studies are required to further evaluate the role for MEA in the diagnosis of bleeding disorders.

# CHAPTER SIX: EVALUATION OF THE TOTAL THROMBUS-FORMATION SYSTEM (T-TAS): APPLICATION TO HUMAN AND MOUSE BLOOD ANALYSIS

## 6.1 Introduction

The Total Thrombus-formation Analysis System (T-TAS) is a flow-chamber system designed to evaluate the growth of total thrombus-formation (TTF) under variable flow conditions (Yamaguchi et al., 2013). The device is equipped with a pneumatic pump, a flow pressure sensor, a video microscope and microchips with different thrombogenic surfaces. Two types of disposable ready-to-use microchips are available, a platelet (PL) chip coated with collagen for assessing platelet thrombus formation and an atherome (AR) chip coated with collagen and tissue factor for assessing white thrombus formation mediated by the activation of coagulation and platelets (Hosokawa et al., 2011, Hosokawa et al., 2012).

To evaluate TTF, T-TAS continuously monitors flow pressure within the capillary using a sensor that tracks pressure changes in the flow path. When hirudinated blood or re-calcified citrated whole blood is perfused through the analytical path of (PL and AR chips respectively) the thrombogenic surface of microchip initiates the thrombus formation. This causes an increase of flow pressure inside the system and gradually occludes the capillary (Hosokawa et al., 2011). The formation of thrombus is analysed using a dual monitoring system, a video microscope that continuously records and display the results as video image, and a pressure sensor that monitors flow pressure changes and displays the results as wave forms and digital parameters (Hosokawa et al., 2012). A typical normal flow pressure curve and defined endpoint parameters are shown in figure 6.1

As a flow based whole blood method, T-TAS has the advantage in evaluating thrombus formation in more physiological but variable flow conditions. In addition, the use of whole

blood only requires small volumes making it possible to be used in young children and studying thrombus formation in small animal models. Furthermore the use of microchip coated with collagen and tissue factor offer the potential advantage of simultaneous assessment of both platelet and hemostasis defects.

The T-TAS was initially designed to monitor the effectiveness of antithrombotic agents. A number of studies have reported its potential utility in assessment of anti-platelet drugs e.g. aspirin, clopidogrel, and PAR-1 and PAR-4 antagonists (Hosokawa et al., 2011, Hosokawa et al., 2013, Hosokawa et al., 2014a) and anti-thrombotic agents e.g. direct thrombin and factor Xa inhibitors (Hosokawa et al., 2014b). T-TAS has also demonstrated high sensitivity in detecting coagulation bleeding disorders such as haemophilia (Ogawa et al., 2012), von Willebrand disease (Ogiwara et al., 2015) and platelet function defects such as storage pool disease (Minami et al., 2015). In assessing acquired haemorrhagic conditions, the T-TAS has been found to be able to predict the risk of bleeding in atrial fibrillation patients undergoing catheter ablation (Ito et al., 2016). Furthermore, T-TAS has also been demonstrated to be a useful tool in the study of thrombus formation in animal models such as micro miniature pigs (Miura et al., 2013) and mice (Ogawa et al., 2012, Ono et al., 2012).

Three mouse models (CSK, CD148 and CSK/CD148) were generated by Senis platelet signalling group in the University of Birmingham to study the regulation of Src family kinase (SFK) on platelet activity. CSK is a member of family kinases function as a negative regulator of SFKs to inhibit platelet activation (Okada, 2012). In contrast, CD148 is a protein tyrosine phosphatase receptor which plays essential role as a positive regulator of platelet activation and thrombosis (Senis et al., 2009). The deletion of either CSK or CD148, or both together in mice resulted in various platelet defects including a reduction in platelet reactivity to collagen (manuscript in preparation).

## 6.2 Aim

To evaluate the potential utility of the T-TAS for assessing thrombus formation in healthy individuals, blood spiked with antithrombotic agents, in patients with suspected platelet function defects and in genetically modified or wild type (WT) mice.

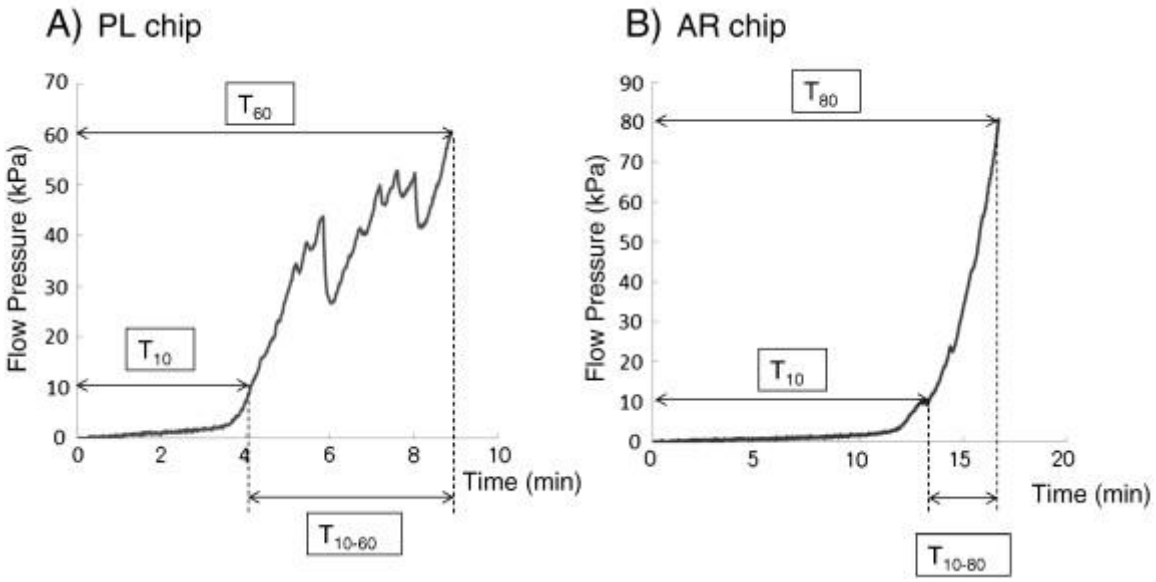



**Figure 6.1** T-TAS typical normal flow pressure curves and the analytical parameters collected for quantification. A) PL chip. $T_{10}$: time from baseline pressure to reach 10 kPa (PTF starting time). $T_{60}$: time to reach 60 kPa (occlusion time). $T_{10\text{-}60}$: $T_{60}$ minus $T_{10}$ (PTF growth rates). $AUC_{10}$: area under the flow pressure curve for 10 min. B) AR chip. $T_{10}$: time from baseline pressure to reach 10 kPa. $T_{80}$: time to reach 80 kPa. $T_{10\text{-}80}$: $T_{80}$ minus $T_{10}$. $AUC_{30}$: area under the flow pressure curve for 30 min (Yamaguchi et al., 2013).

## 6.3 Results

### 6.3.1 Measurement of T-TAS in healthy subjects

22 healthy subjects (10 males and 12 females; mean age 31 ± 7 years) were investigated. Overall, the T-TAS shows some variation in thrombus formation among healthy subjects on both chips (figure 6.2A and 6.3A). Within the PL chip (figure 6.2B and table 6.1), the onset ($T_{10}$) value (mean) and occlusion time ($T_{60}$) at a shear rate of 1000 $s^{-1}$ were greater than at 1500/2000 $s^{-1}$ ($T_{10}$= 03:30, 03:02, 03:29 and $T_{60}$= 08:10, 06:27, 06:42 respectively). In contrast, the $AUC_{10}$ value (mean) was reduced at a shear rate of 1000 $s^{-1}$ than at 1500/2000 $s^{-1}$ (260, 317 and 301 respectively). Furthermore, the thrombus growth rates ($T_{10-60}$) in the PL chip (mean) decreased as the shear rate increased (04:40, 03:25 and 03:12 respectively) (figure 6.2B and table 6.1). A shear dependent decrease was also observed on the occlusion times ($T_{80}$) and thrombus growth rates ($T_{10-80}$) on the AR chip ($T_{80}$=12:1 and 11:30; $T_{10-80}$= 4:36 and 2:21 respectively) (figure 6.3B and table 6.2). However, no significant different of the $AUC_{30}$ values (mean) was observed between low and high shear values (1593 and 1591 respectively). Surprisingly, the onset ($T_{10}$) value (mean) on AR chip was greater at high shear (09:19) than at the low shear (08:16) (figure 6.3B and table 6.2).

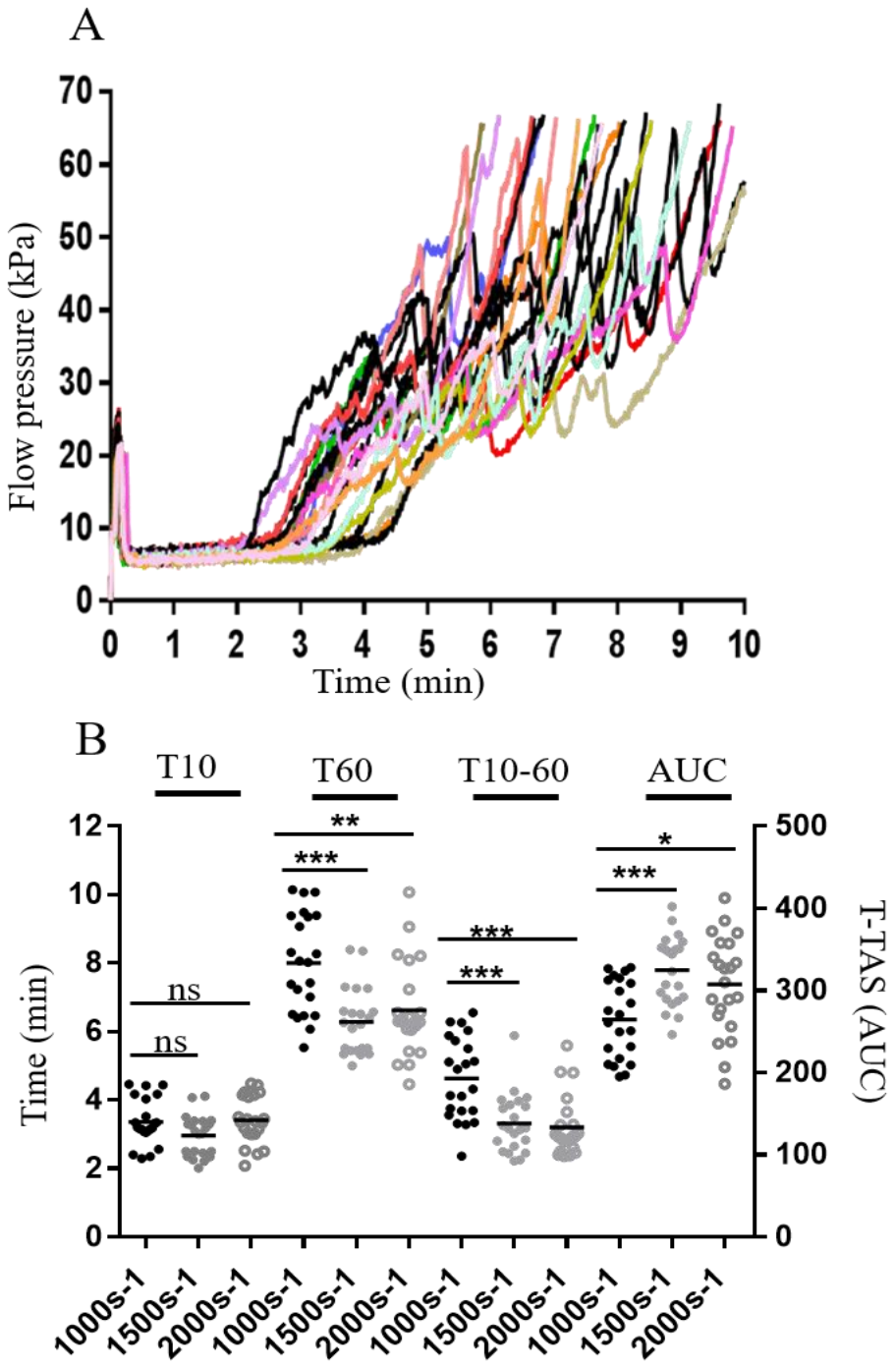



**Figure 6.2** Measurements of blood from healthy controls (n=22) using the PL chip. For panel A, flow pressure curves of all individuals measured at low shear rate (1000 $s^{-1}$). For panel B, distribution of the T-TAS measurements from three different shear rates (bars represent mean values). Statistical analysis performed by the one-way ANOVA test with Sidak's adjustment for multiple comparisons (***p <0.001, **p<0.01 and *p<0.05).

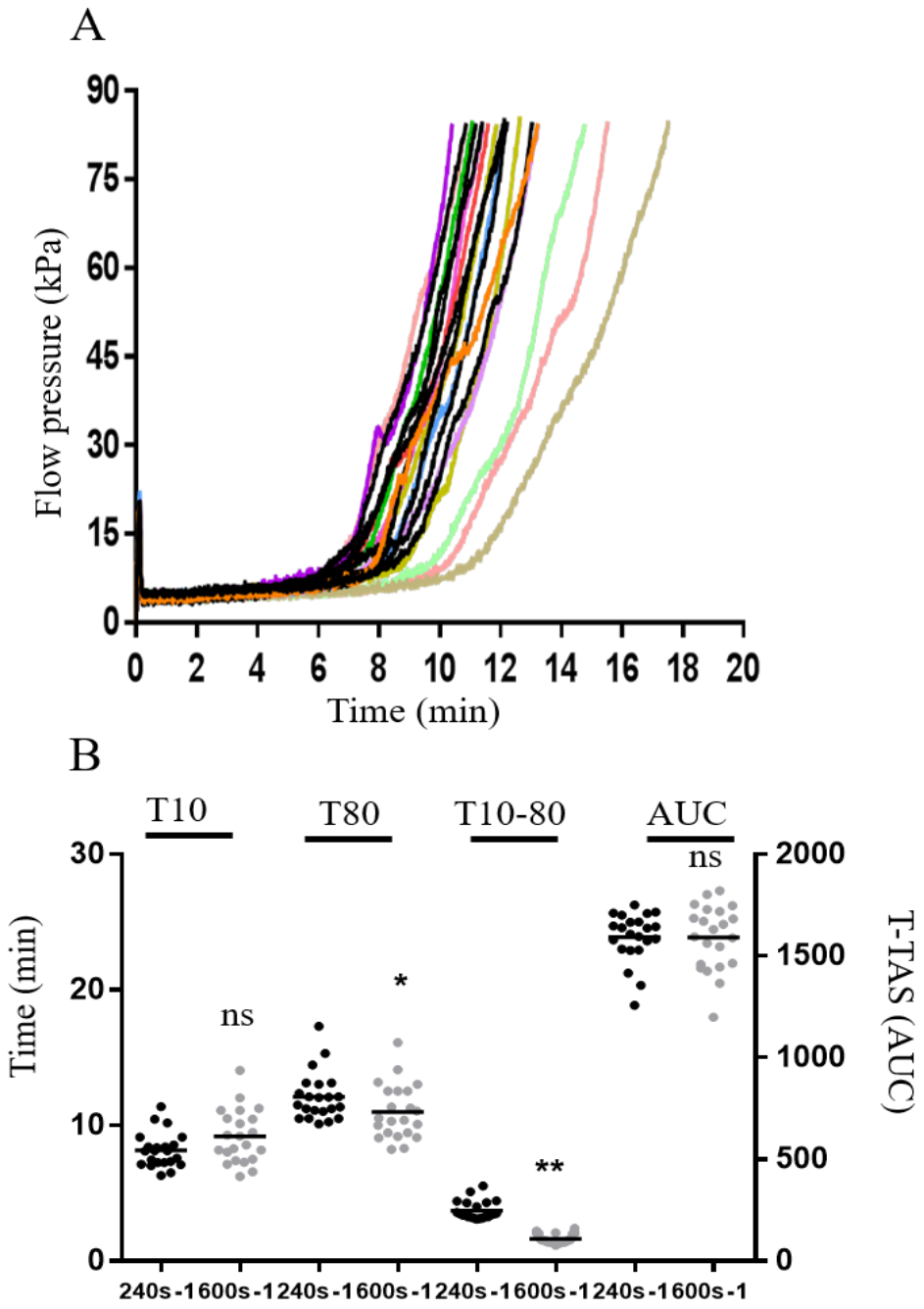



**Figure 6.3** Measurements of blood from healthy controls (n=22) using the AR chip. For panel A, flow pressure curves of individuals measured at low shear rate (240 $s^{-1}$). For panel B, distribution of the T-TAS measurements from two different shear rates (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between 240 $s^{-1}$ and 600 $s^{-1}$ in each parameter (**p<0.01 and *p<0.05).

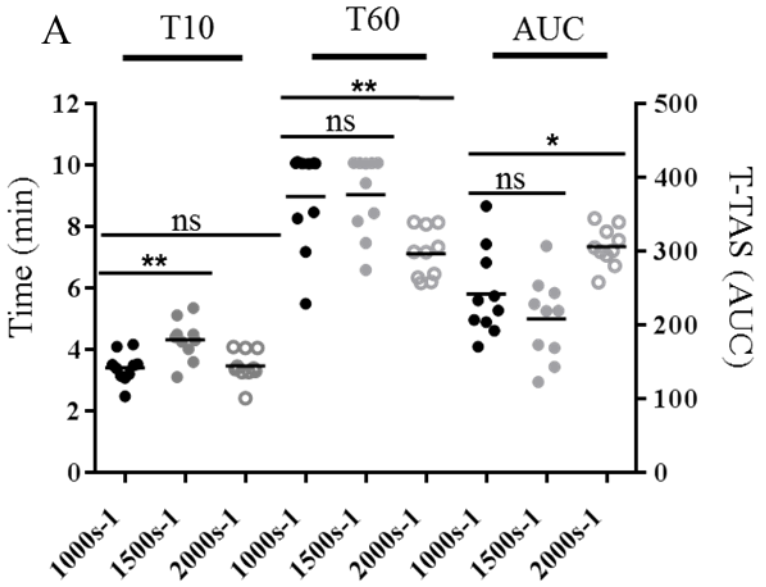
| PL chip | 1000 $s^{-1}$ (Mean ± SD) | 1500 $s^{-1}$ (Mean ± SD) | 2000 $s^{-1}$ (Mean ± SD) |
|---|---|---|---|
| $T_{10}$ (min) | 03:30 ± 0:45 | 03:02 ± 00:34 | 03:29 ± 00:45 |
| $T_{60}$ (min) | 08:10 ± 01:25 | 06:27 ± 00:53 | 06:42 ± 01:21 |
| $T_{10-60}$ (min) | 04:40 ± 01:11 | 03:25 ± 00:43 | 03:12 ± 00:48 |
| $AUC_{10}$ (AUC) | 260 ± 51.7 | 317 ± 55.4 | 301 ± 66.2 |

**Table 6.1** T-TAS measurements on PL chip using three different shear rates in samples obtained from healthy controls (n=22). Data presented as mean and SD.

| AR chip | 240 $s^{-1}$ (Mean ± SD) | 600 $s^{-1}$ (Mean ± SD) |
|---|---|---|
| $T_{10}$ (min) | 8:16 ± 1.28 | 9:19 ± 2:0 |
| $T_{80}$ (min) | 12:1 ± 2:17 | 11:30 ± 2:0 |
| $T_{10-80}$ (min) | 4:36 ± 1:11 | 2:21 ± 0:29 |
| $AUC_{30}$ (AUC) | 1593 ± 122 | 1591 ± 158 |

**Table 6.2** T-TAS measurements on AR chip using two different shear rates in samples obtained from healthy controls (n=22). Data presented as mean and SD.

Intra-assay coefficients of variation (CV) were calculated from 10 runs of a single sample obtained from one healthy subject. Overall, the difference of CV between shear rates was more significant on PL chip (figure 6.4) compared to that of AR chip (figure 6.5). On PL chip the CV of $T_{60,}$ $T_{10\text{-}60}$ and $AUC_{10}$ were smaller at 2000 $s^{-1}$ (20000 $s^{-1}$= 11.4%, 26.4% and 26.9% respectively) than at 1000/1500 $s^{-1}$ (1000 $s^{-1}$= 17.9%, 24.4% and 24.4% ; 15000 $s^{-1}$= 14.3%, 26.4% respectively), whereas the CV of $T_{10}$ were almost the same at all three share rates (14.4%, 15.3% 14.7% respectively) (table 6.3). The statistical analysis of AUC values obtained from the first and last 3 replicates from each set showed no significant different, suggesting that, the time delay of sample process was not the cause of the high CV values observed (figure 6.4 B). In AR chip however, the CV of all four parameters were low at 600 $s^{-1}$ than that at 240 $s^{-1}$ ($T_{10}$ 7.9%, $T_{80}$ 6.8%, $T_{10\text{-}80}$ 20.2%, $AUC_{30}$ 5.0% and $T_{10}$ 8.9%, $T_{80}$ 8.6%, $T_{10\text{-}80}$ 22.9%, $AUC_{30}$ 5.1% respectively) (table 6.4).




B

| Sample process order | AUC (1000s$^{-1}$) | AUC (1500s$^{-1}$) | AUC (2000s$^{-1}$) |
|---|---|---|---|
| 1 | 309.7 | 228.7 | 305 |
| 2 | 284.9 | 307.4 | 327 |
| 3 | 361.5 | 243.7 | 301 |
| 4 | 233.5 | 253.6 | 294.6 |
| 5 | 204.1 | 219.5 | 258.2 |
| 6 | 170.7 | 218.9 | 299.5 |
| 7 | 239.2 | 173.2 | 315.3 |
| 8 | 192.3 | 168.9 | 339.7 |
| 9 | 219.7 | 122.5 | 344.8 |
| 10 | 207 | 143 | 280.7 |
| Statistical analysis | ns | ns | ns |

**Figure 6.4 Analysis of repeat measurements of a single sample obtained from a healthy subject on the PL chip (n=10). Panel A, distribution of the overall T-TAS measurements on the PL chip at different shear rates (bars represent mean values), and Panel B, AUC obtained from repeat measurements base on sample process order. Statistical analysis performed by the Mann Whitney U test. Significance as compared between the first and last 3 replicates from each set of parameter (**p<0.01 and *p<0.05).**

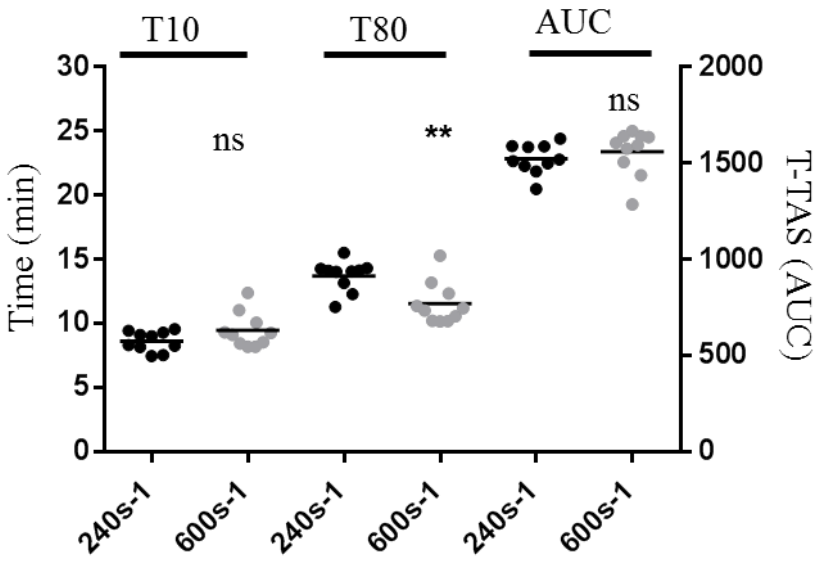



**Figure 6.5** Distribution of repeat measurements of the same sample from a single healthy control on the AR chip (n=10). (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between 240 $s^{-1}$ and 600 $s^{-1}$ in each parameter (**p<0.01).

| PL chip | 1000 $s^{-1}$ | 1500 $s^{-1}$ | 2000 $s^{-1}$ |
|---|---|---|---|
| $T_{10}$ (CV%) | 14.4% | 15.3% | 14.7% |
| $T_{60}$ (CV%) | 17.9% | 14.3% | 11.4% |
| $T_{10-60}$ (CV%) | 24.4% | 26.4% | 19.4% |
| $AUC_{10}$ (CV%) | 24.4% | 26.9% | 8.6% |

**Table 6.3** Intra-assay coefficients of variation (CV) in PL chip measurements in a single sample obtained from a healthy control (n=10).

| AR chip | $240s^{-1}$ | $600s^{-1}$ |
|---|---|---|
| $T_{10}$ (CV%) | 8.9% | 7.9% |
| $T_{80}$ (CV%) | 8.6% | 6.8% |
| $T_{10-80}$ (CV%) | 22.9% | 20.2% |
| $AUC_{30}$ (CV%) | 5.1% | 5.0% |

**Table 6.4** Intra-assay coefficients of variation (CV) in AR chip measurements in a single sample obtained from a healthy control (n=10).

### 6.3.2 Measurement of blood from healthy subjects spiked with antithrombotic drugs

No thrombus formation was observed in the PL chip with both low and high shear rates when blood was pre-treated with ticagrelor (10 μM) (figure 6.6). Within the AR chip, thrombus formation was not affected by addition of ticagrelor (figure 6.7). In contrast, thrombus formation was observed with blood pre-treated with rivaroxaban (1 μM) but varies between subjects when perfused through the AR chip at lower shear rates (figure 6.8).

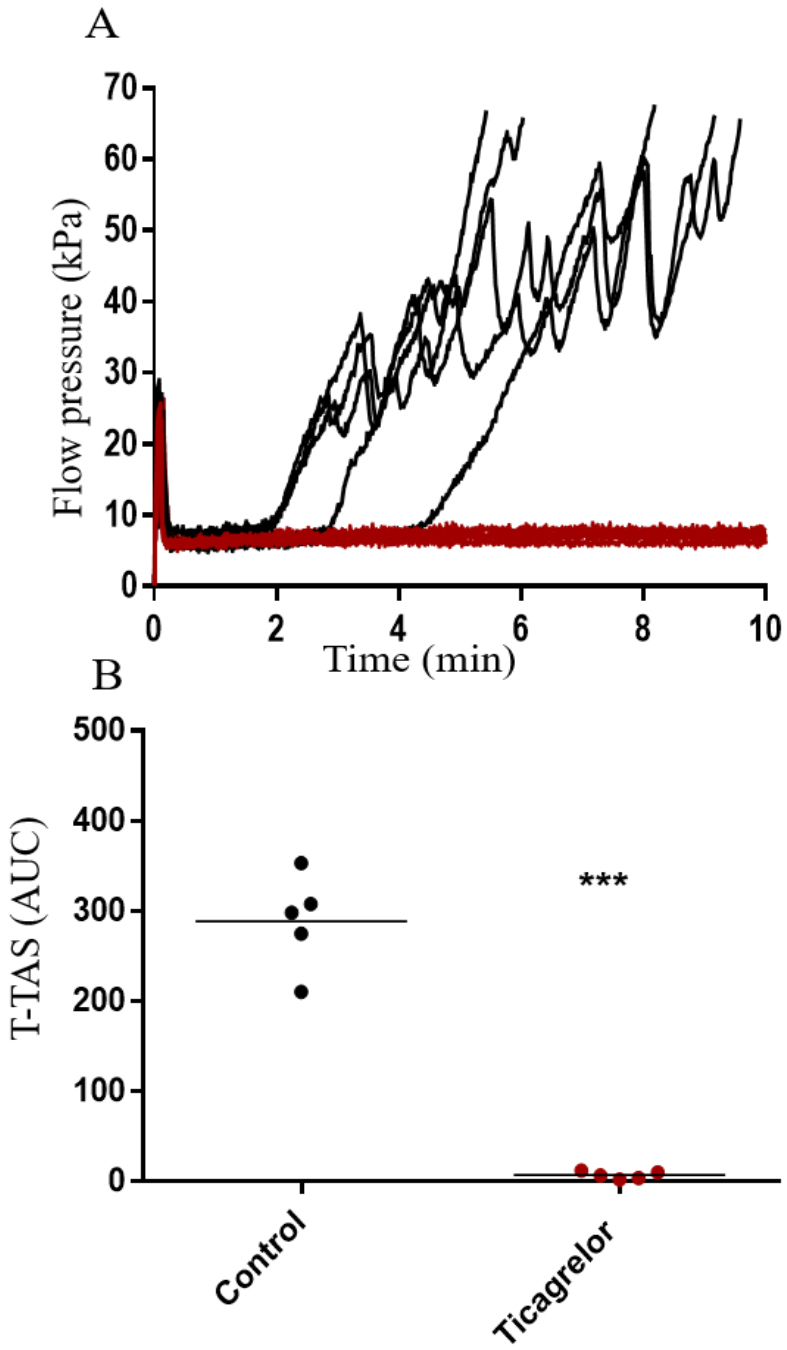



**Figure 6.6** Effects of the antiplatelet agent ticagrelor on PL chip measurements. Panel A, flow pressure curves and panel B, AUC measurements of blood from healthy controls (n=5) in the presence (red) and absence (black) of ticagrelor (10 μM). (bars represent the mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared to control (***$p<0.001$)

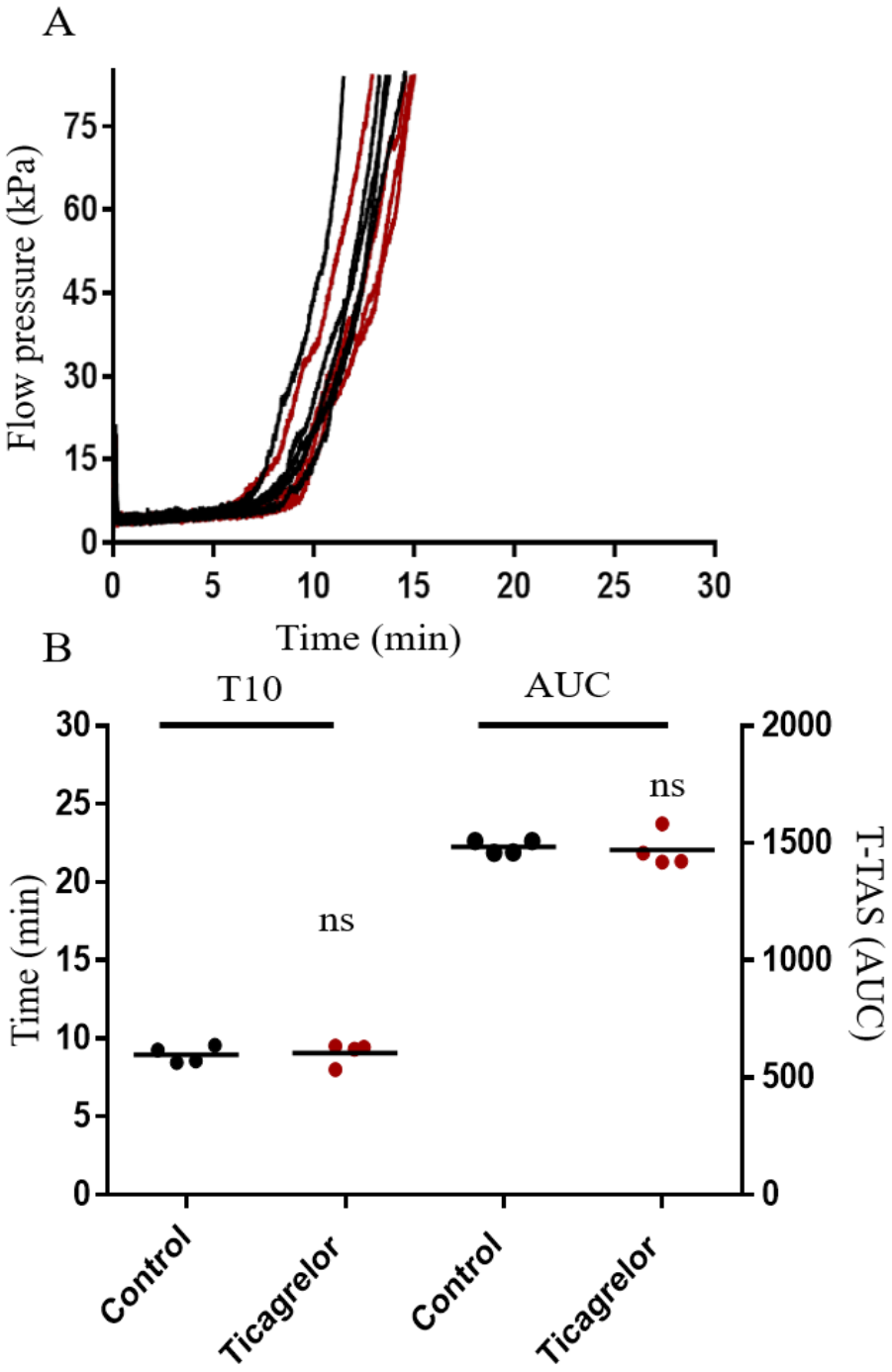



**Figure 6.7** Effects of the antiplatelet agent ticagrelor on AR chip measurements. Panel A, flow pressure curves and panel B, $T_{10}$ and AUC measurements of blood from healthy controls (n=4) in the presence (red) and absence (black) of ticagrelor (10 μM). (bars represent the mean values). Statistical analysis performed by the Mann Whitney U test.

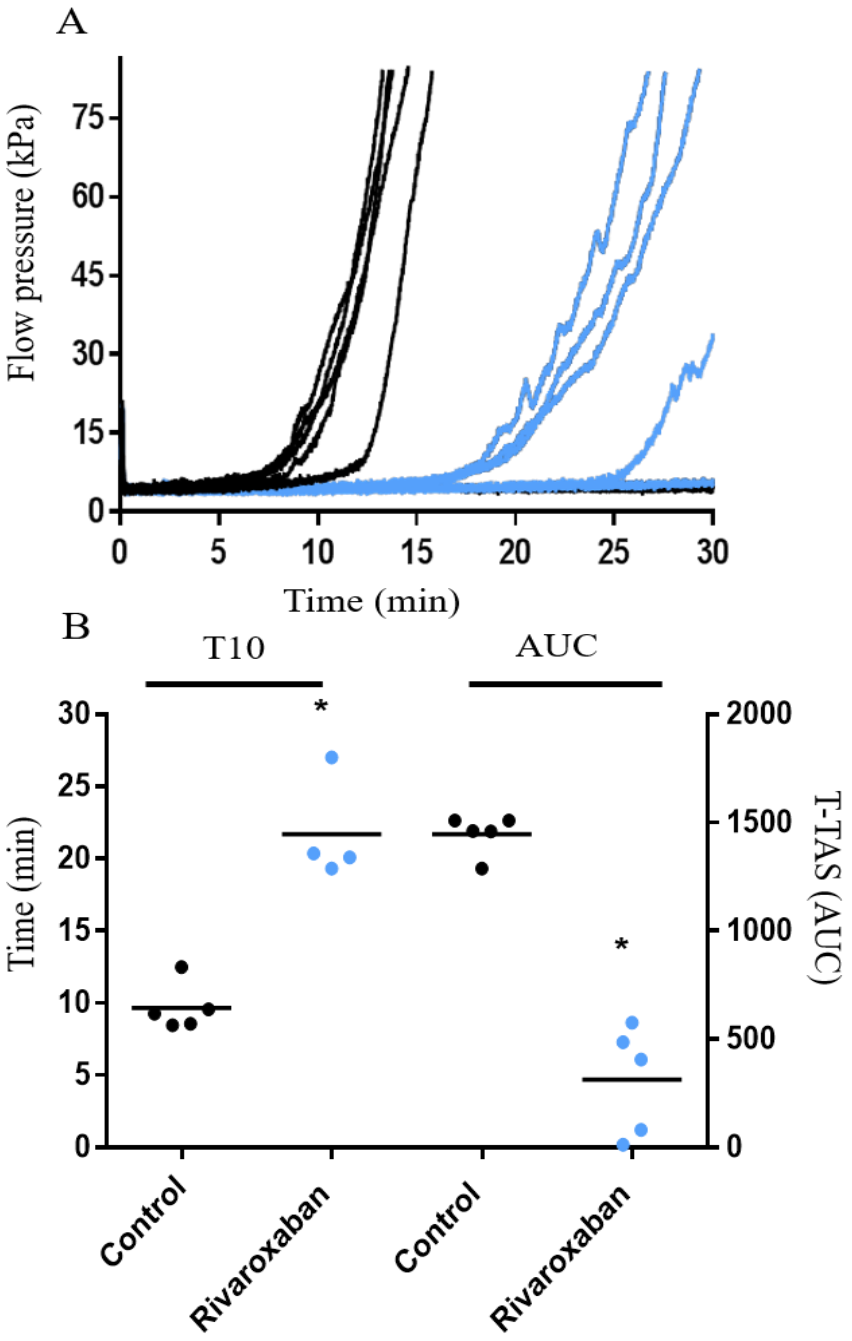



**Figure 6.8** Effects of rivaroxaban on AR chip measurements. Panel A, flow pressure curves and panel B, $T_{10}$ and AUC measurements of blood from healthy controls (n=5) in the presence (blue) and absence (black) of rivaroxaban (1 μM). (bars represent the mean values).Statistical analysis performed by the Mann Whitney U test. Significance as compared to control in each parameter (*$p<0.05$).

### 6.3.3 Measurement of T-TAS on samples from patients recruited into the GAPP study with suspected platelet function defects.

We analysed a total of 30 patients (8 male and 22 female) with a median age 35 (range 7-73) recruited into the GAPP study and compared T-TAS (PL chip) results with lumi-LTA. All patients had normal platelet counts ($261 \pm 68 \times 10^9$/L). The waveform of each individual patients is presented in figure 6.9A and distribution of the T-TAS measurements in healthy controls (n=22) and in patients (n=30) are presented in figure 6.9B. Overall, no significant difference was observed between healthy controls and patient group with three parameters ($T_{10}$, $T_{60}$ and $AUC_{10}$). With the thrombus growth ($T_{10\text{-}60}$) measurements however, a small but significant difference was observed (figure 6.9B). Of 30 patients tested (figure 6.10), 10 (33.3%) patients gave abnormal responses by lumi-LTA to one or more agonists as defined using previous criteria (Dawood et al., 2012). In contrast, 8 (27%) patients were detected to have abnormal thrombus formation by T-TAS, as defined using the cut off values obtained from healthy controls (table 6.1). The overall agreement between the two instruments is shown in table 6.5. 26/30 samples gave identical results by both tests, with the majority (19) giving normal responses whereas 7 samples gave abnormal results by both tests. In contrast, there was disagreement in 4/30 samples. T-TAS detected an abnormality in 1 patient that was normal by lumi-LTA (figure 6.11), whereas 3 samples were normal by T-TAS but abnormal by lumi-LTA (figure 6.12). In this study we used $AUC_{10}$ and the $T_{10}$ parameters to identify a defect on T-TAS. 7/8 (88%) samples with an abnormality detected by T-TAS have low values of $AUC_{10}$. One patient however, had normal $AUC_{10}$ value but a delayed $T_{10}$ parameter. Interestingly, the patient was also found to have a defect by lumi-LTA.

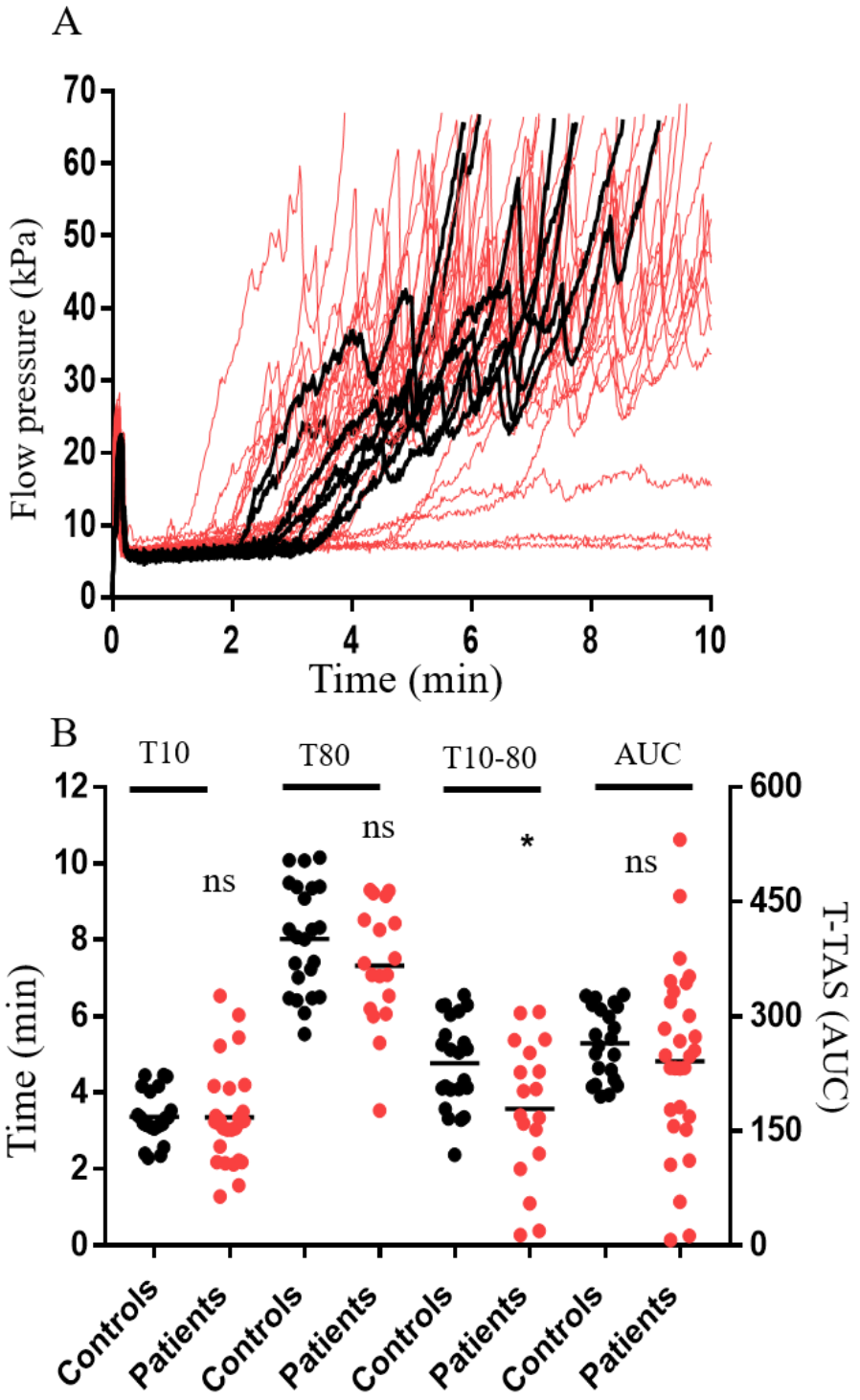



**Figure 6.9** Measurement of thrombus formation with whole blood from healthy controls (n=22) and patients (n=30) in the PL chip (1000 $s^{-1}$). Panel A, Flow pressure curves observed in healthy controls (black) and patients (red). Panel B, Distribution of the $T_{10}$ and $AUC_{10}$ measurements obtained from healthy controls (n=22) and patients (n=30) (bars represent the mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared to control in each parameter (*p<0.05).

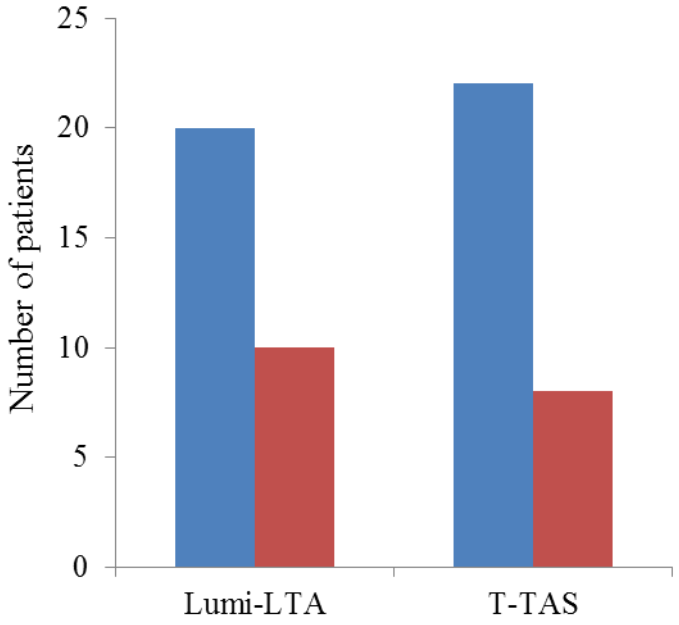



**Figure 6.10** Summary of overall patients results (n=30) analysed by lumi-LTA and T-TAS comparing between normal (blue) and abnormal (red). Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by T-TAS) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy individuals as shown in table 6.1 and 6.2

| | Lumi-LTA (Abnormal) | Lumi-LTA (Normal) | |
|---|---|---|---|
| T-TAS (Abnormal) | 7 | 1 | PPV= 88% |
| T-TAS (Normal) | 3 | 19 | NPV= 86% |
| | Sensitivity = 70% | Specificity = 95% | |

**Table 6.5** Analysis of the agreement between the T-TAS and lumi-LTA in patient samples (n=30) displaying sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

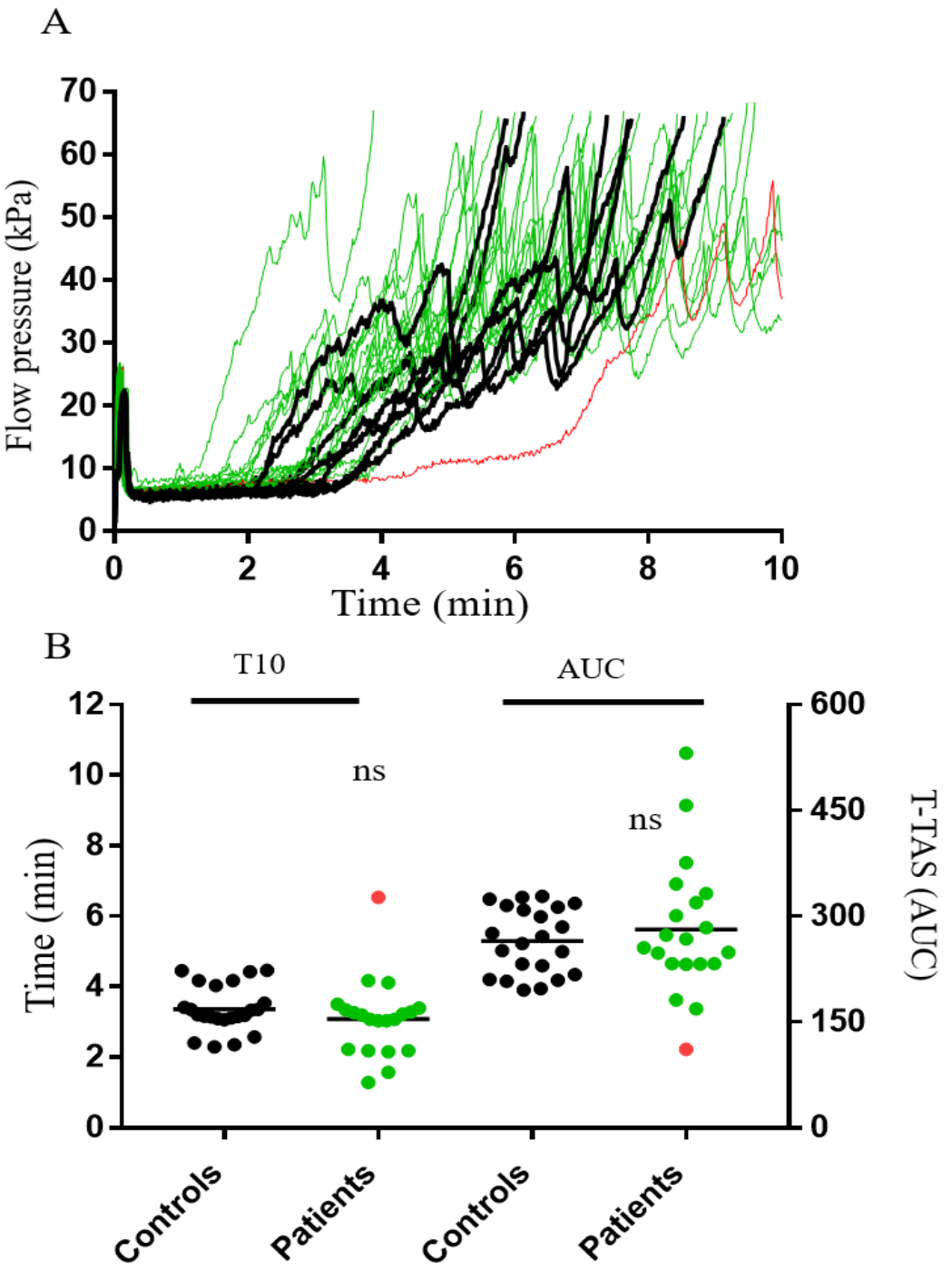



**Figure 6.11** Measurements of thrombus formation in the PL-chip (1000 $s^{-1}$) within samples from patients (n=20) found to have no detectable platelet defects by Lumi-LTA. Panel A, flow pressure curves observed in healthy controls (black), patients with normal (green) and abnormal results respectively (red) by T-TAS (green). Panel B, distribution of the T10 and AUC measurements obtained from healthy controls (n=22) and patients (n=20) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test.

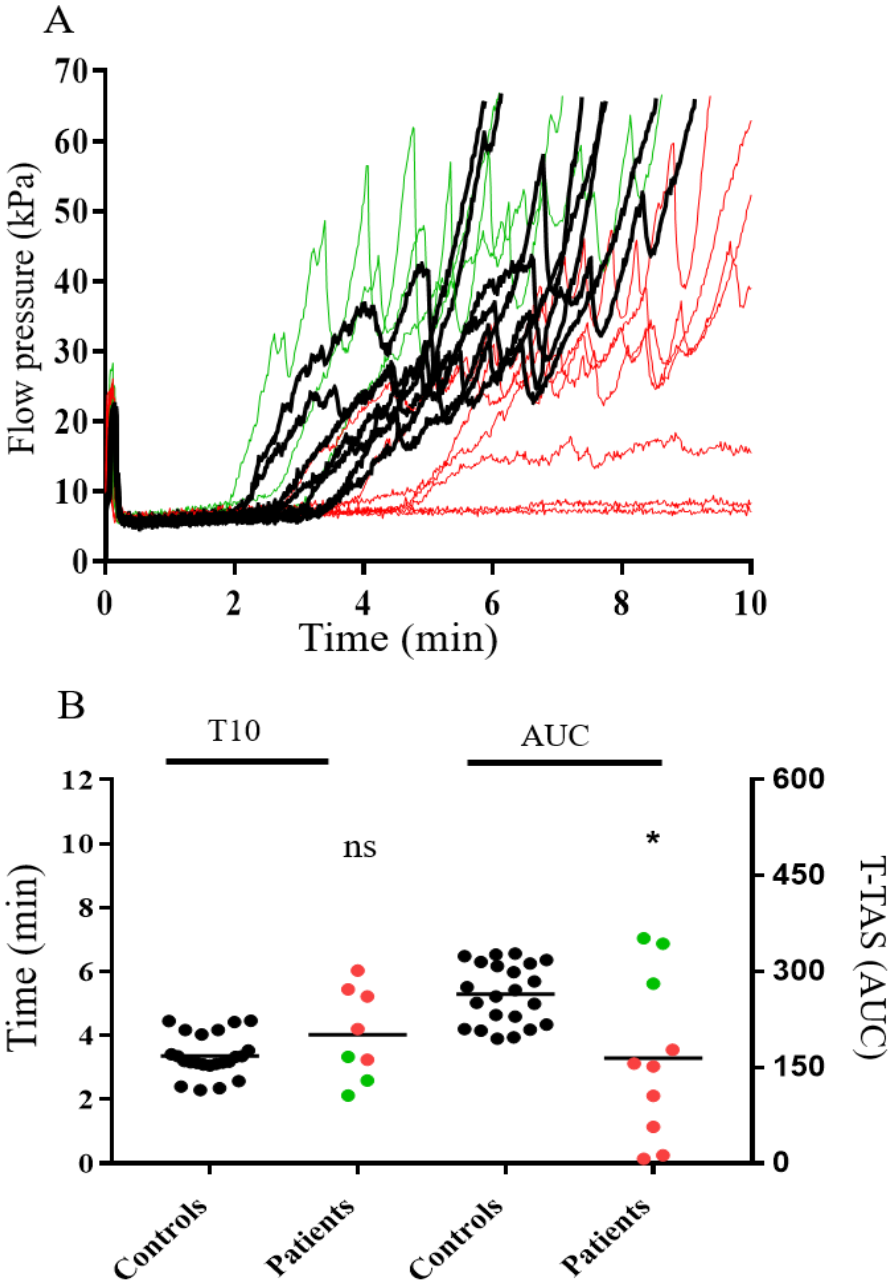



**Figure 6.12** Measurement of thrombus formation in the PL-chip (1000 $s^{-1}$) within samples from patients with platelet defects (n = 10) as defined by lumi-LTA. Panel A, flow pressure curves observed in healthy controls (black), patients with normal T-TAS (green) and patients with abnormal T-TAS (red). Panel B, distribution of the T10 and AUC measurements obtained from healthy controls (n=22) and patients (n=10) (bars represent mean values). Statistical analysis performed by Mann Whitney U test. Significance as compared to control each parameter (*p<0.05).

### 6.3.4 Analysis and identification of patients with various platelet function defects

Functional defects identified by lumi-LTA were classified into 4 main groups (Gi defects, secretion defects, COX-like defect and multiple defects) according to their pattern of responses to specific agonists as previously described (Dawood et al., 2012). As shown in figure 6.13, T-TAS detected all patients classified by lumi-LTA to have either COX-like defect or secretion defects (1 and 2 patients respectively). In contrast, T-TAS only detected 1/3 patients with Gi defects but 3 out of 4 with multiple defects.

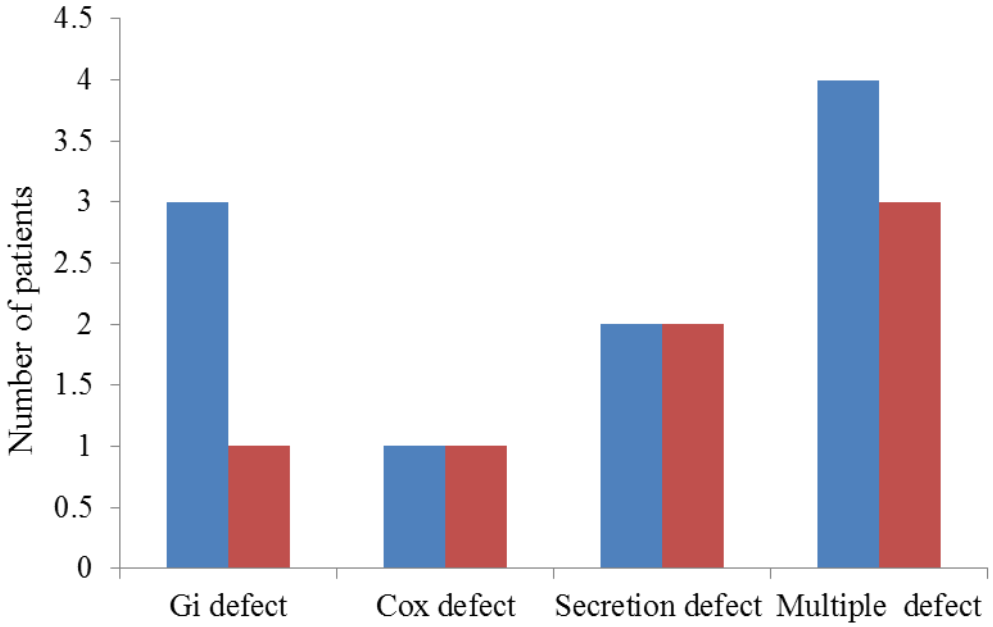



**Figure 6.13** Classification of platelet function defects among patients detected by lumi-LTA (blue) and T-TAS (red). Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by T-TAS) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy individuals as shown in table 6.1 and 6.2

### 6.3.5 Measurement of T-TAS in sample from GAPP patients with thrombocytopenia recruited with suspected platelet function defects

We additionally analysed 7 patients found to have mild to marked thrombocytopenia as characterized by low whole blood platelet counts (<150 $x10^9$/L) , but normal PRP counts when tested by LTA. The whole blood platelet counts (mean ± SD) were 77 ± 34 ($x10^9$/L). The waveform of each individual patient is presented in figure 6.14A. $AUC_{10}$ parameters (figure 6.14B) showed significant differences between healthy controls (n=22) and patients (n=7). All 7 patients tested exhibited abnormal thrombus formation within the T-TAS (figure 6.15). Interestingly, 4/7 patients were also detected by lumi-LTA to have platelet function defects (3 secretion and 1 Cox-like defects) in additional to their thrombocytopenia.

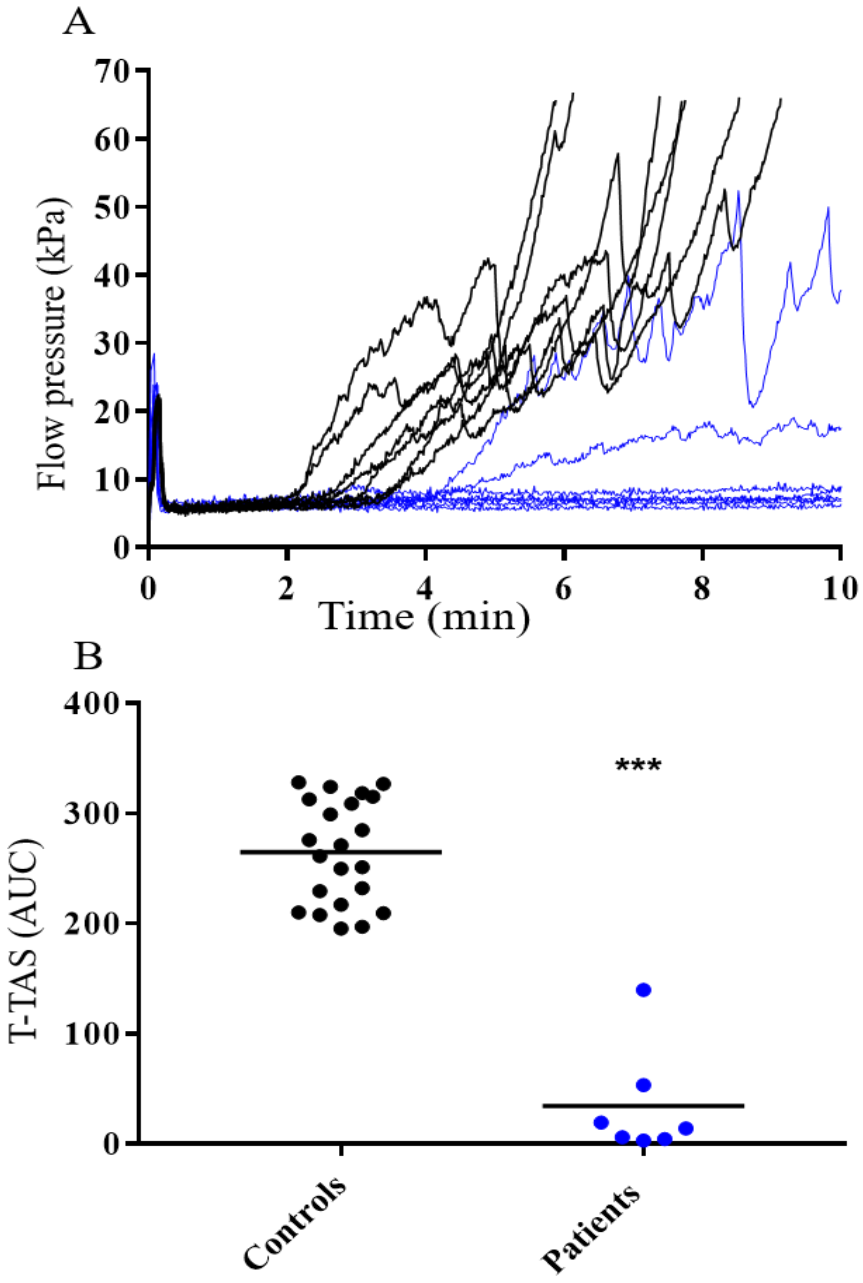



**Figure 6.14** Measurement of thrombus formation in the PL-chip (1000 $s^{-1}$) within samples from patients (n=7) with thrombocytopenia. Panel A, flow pressure curves observed in healthy controls (black) and patients with thrombocytopenia (blue). Panel B, distribution of the AUC measurements obtained from normal controls (n=22) and patients (n=7) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared to control in each parameter (***p<0.001).

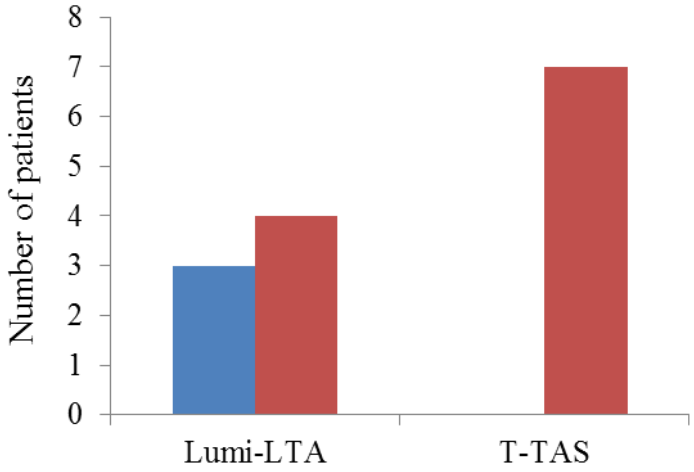



**Figure 6.15** Summary of an overall comparison between normal (blue) and abnormal (red) results detected by lumi-LTA and T-TAS from patients (n=7) with thrombocytopenia. Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by T-TAS) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy individuals as shown in table 6.1 and 6.2

### 6.3.6 Measurement of T-TAS in samples from wild type mice

Blood samples from wild type (WT) mice (n=4) were tested within both PL chips (1000 $s^{-1}$ and 2000 $s^{-1}$) and AR chips (240 $s^{-1}$). Within the PL chip (figure 6.16), shear enhanced thrombus formation was observed with all parameters; however, any differences in measurements between shear rates was only significant with the $AUC_{10}$ parameter. For example, the values (mean) at 1000 $s^{-1}$ ($T_{10}$ = 02:02, $T_{60}$ = 04:40 and $T_{10-60}$ =02:38) were greater than at 2000 $s^{-1}$ ($T_{10}$ = 01:16, $T_{60}$ = 03:26 and $T_{10-60}$ = 02:09). Consequently, the $AUC_{10}$ at 1000 $s^{-1}$ was shorter than at 2000 $s^{-1}$ (405.5 and 463.6 respectively) (table 6.6). The values (mean) of thrombus formation within the AR chip (240$s^{-1}$) are shown in table 6.6. For example, the mean values of $T_{10,}$ $T_{80,}$ $T_{10-80}$ and $AUC_{30}$ were 02:52, 06:41, 03:48 and 2048.98 respectively.

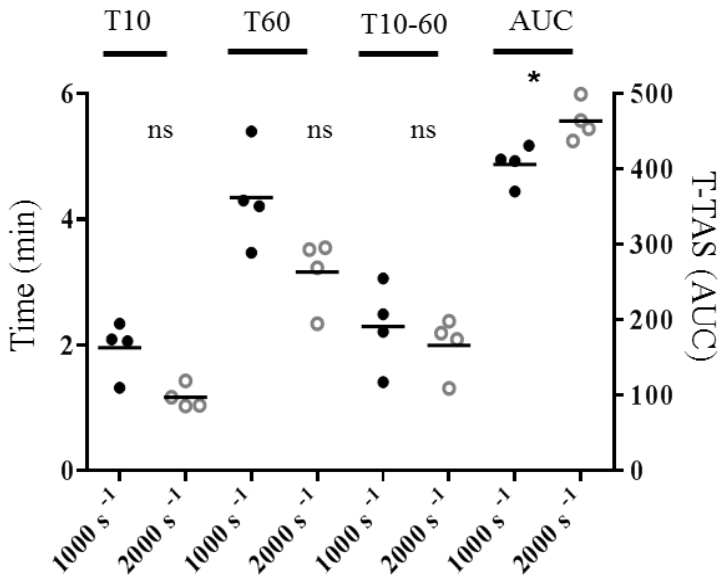



**Figure 6.16** Distribution of thrombus formation values ($T_{10}$, $T_{60}$, $T_{10\text{-}60}$ and $AUC_{10}$) in blood from WT mice (n=4) tested in the PL-chip (1000 $s^{-1}$ and 2000 $s^{-1}$) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between 1000 $s^{-1}$ and 2000 $s^{-1}$ in each parameter (*$p<0.05$).

| PL chip | 1000 $s^{-1}$ (Mean ± SD) | 2000 $s^{-1}$ (Mean ± SD) | AR chip | 240 $s^{-1}$ (Mean ± SD) |
|---|---|---|---|---|
| $T_{10}$ (min) | 02:02 ± 00:23 | 01:16 ± 00:18 | $T_{10}$ (min) | 02:52 ± 00:41 |
| $T_{60}$ (min) | 04:40 ± 00:43 | 03:26 ± 00:37 | $T_{80}$ (min) | 06:41 ± 02:37 |
| $T_{10-60}$ (min) | 02:38 ± 00:38 | 02:09 ± 00:28 | $T_{10-80}$ (min) | 03:48 ± 02:03 |
| $AUC_{10}$ (AUC) | 405.5 ± 22.17 | 463.6 ± 26.34 | $AUC_{30}$ (AUC) | 2048.98 ± 105.4 |

**Table 6.6** T-TAS measurements in samples from WT mice (n=4) performed on both PL and AR chips using different shear rates. Data presented as mean and SD.

### 6.3.7 Comparison of T-TAS measurements between blood samples from WT mice and human

We compared the difference of thrombus formation between WT mice (n=5) and humans (n=22). The whole blood platelet count and MPV of the WT mice is shown in table 6.7. Overall, thrombus formation in WT mice was more rapid on both chips (figure 6.17A and figure 6.18A) than in humans. With PL chip measurements, the differences between two models were significant with all parameters (figure 6.17B). For example, WT mice values (mean) at 1000 $s^{-1}$ were $T_{10}$ = 02:02, $T_{60}$ = 04:40, $T_{10-60}$ = 02:38 and $AUC_{10}$ = 405.5 (table 6.6) whereas the values in humans (mean) at the same shear were $T_{10}$= 03:30, $T_{60}$= 08:10, $T_{10-60}$ = 04:40 and $AUC_{10}$ = 260 (table 6.1). In contrast, with AR chip measurements the different between two models were only significant with $T_{10}$, $T_{80}$ and $AUC_{30}$ but not with $T_{10-80}$ (figure 6.18B).

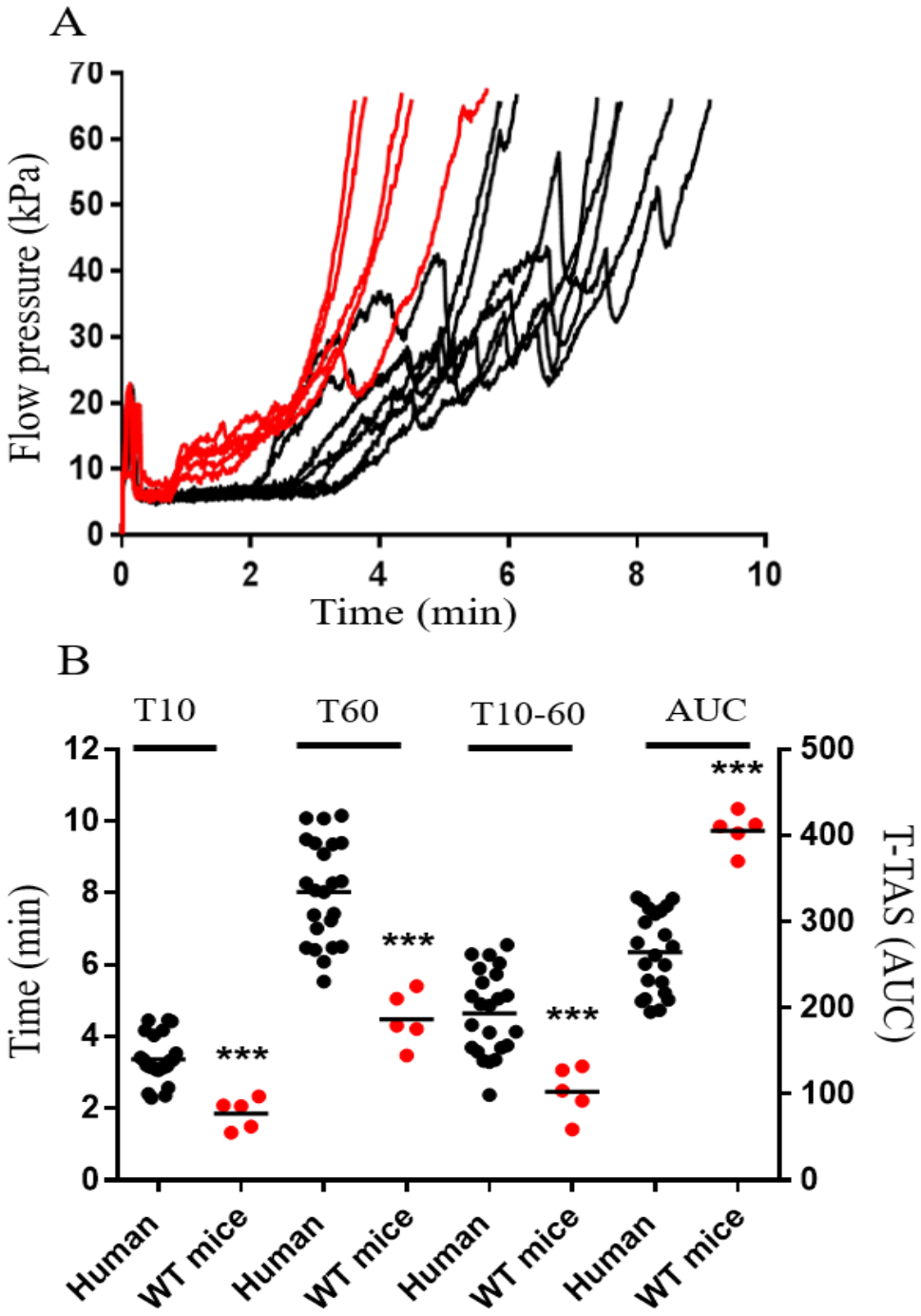



**Figure 6.17** Measurements of thrombus formation within the PL-chip (1000 $s^{-1}$) with blood from humans and WT mice. Panel A, flow pressure curves observed in humans (black) and WT mice (red). Panel B, distribution of the T-TAS measurements on blood from human (n=22) and WT mice (n=5) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and human blood in each parameter (***$p<0.001$).

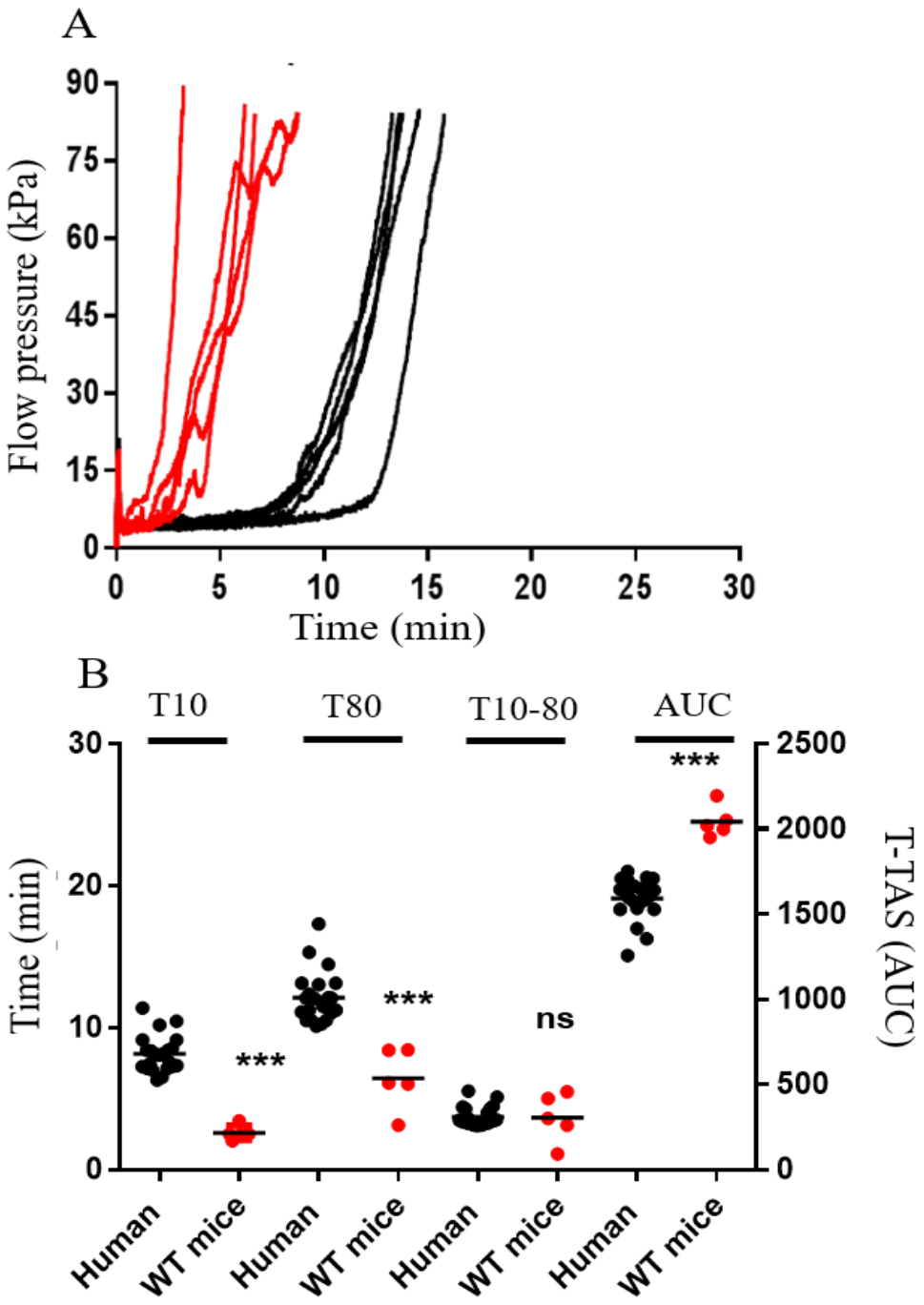



**Figure 6.18** Measurements of thrombus formation within AR-chip (240 $s^{-1}$) with blood from humans and WT mice. Panel A, flow pressure curves observed in humans (black) and WT mice (red). Panel B, distribution of the T-TAS measurements on blood from humans (n=22) and WT mice (n=5) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and human blood in each parameter (***p<0.001).

### 6.3.8 Measurement of T-TAS with blood from genetically modified mice

Three types of genetically modified mice (CD148 KO, CSK KO and Double KO mice) were analysed on T-TAS using both PL and AR chips. Whole blood platelet count and MPV value of all mice is shown in table 6.7. The PL chip detected no thrombus formation in all three types of mice with a significant deference of $AUC_{10}$ parameters in comparison to that of the WT mice (Figures 6.19, 6.21 and 6.23). In contrast, full thrombus formation of all mouse models was observed on AR chip (Figures 6.20A, 6.22A and 6.24A). However, the $T_{10}$ and $AUC_{30}$ parameters of all three models were significant different when compared to the WT. In addition, the $T_{80}$ parameters of CSK KO and DKO were also different than that of the WT. Interestingly, the rate of thrombus growth ($T_{10-80}$) of all mouse models was similar to that of WT mice.

| | Wild type | CSK KO | CD148 KO | DKO |
|---|---|---|---|---|
| Platelet count (Median ± SD) | 839 ± 166 (x$10^6$/ml) | 296 ± 250 (x$10^6$/ml) | 797 ± 192 (x$10^6$/ml) | 858 ± 395 (x$10^6$/ml) |
| MPV (Median ± SD) | 5.55 ± 0.76 (fl) | 9.95 ± 2.7 (fl) | 5.6 ± 0.91(fl) | 6.7 ± 1.6 (fl) |

**Table 6.7** Measurements of whole blood platelet count and MPV in samples from WT mice (n=22), CSK knock out mice (n=12), CD148 knock out mice (n=12) and double (CSK + CD148) knock out mice (n=15). Data presented as median and SD.

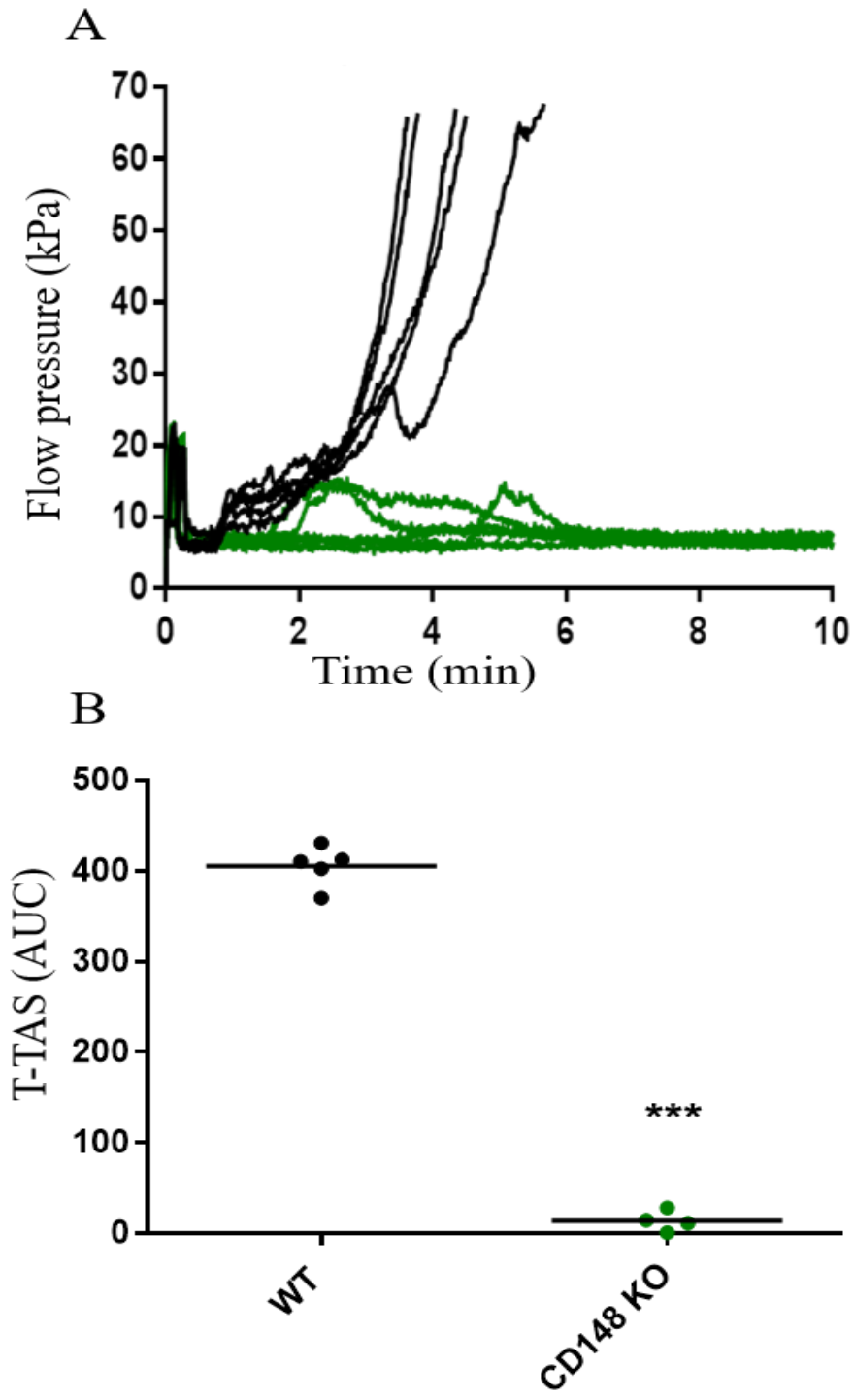



**Figure 6.19** Measurements of thrombus formation in blood from WT and CD148 knockout mice within the PL-chip (1000 $s^{-1}$). Panel A, flow pressure curves observed in WT (black) and CD148 knock out mice (green). Panel B, distribution of the T-TAS (AUC) measurements on blood from WT (n=5) and CD148 knock out mice (n=4) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and CD148 KO (***p<0.001).

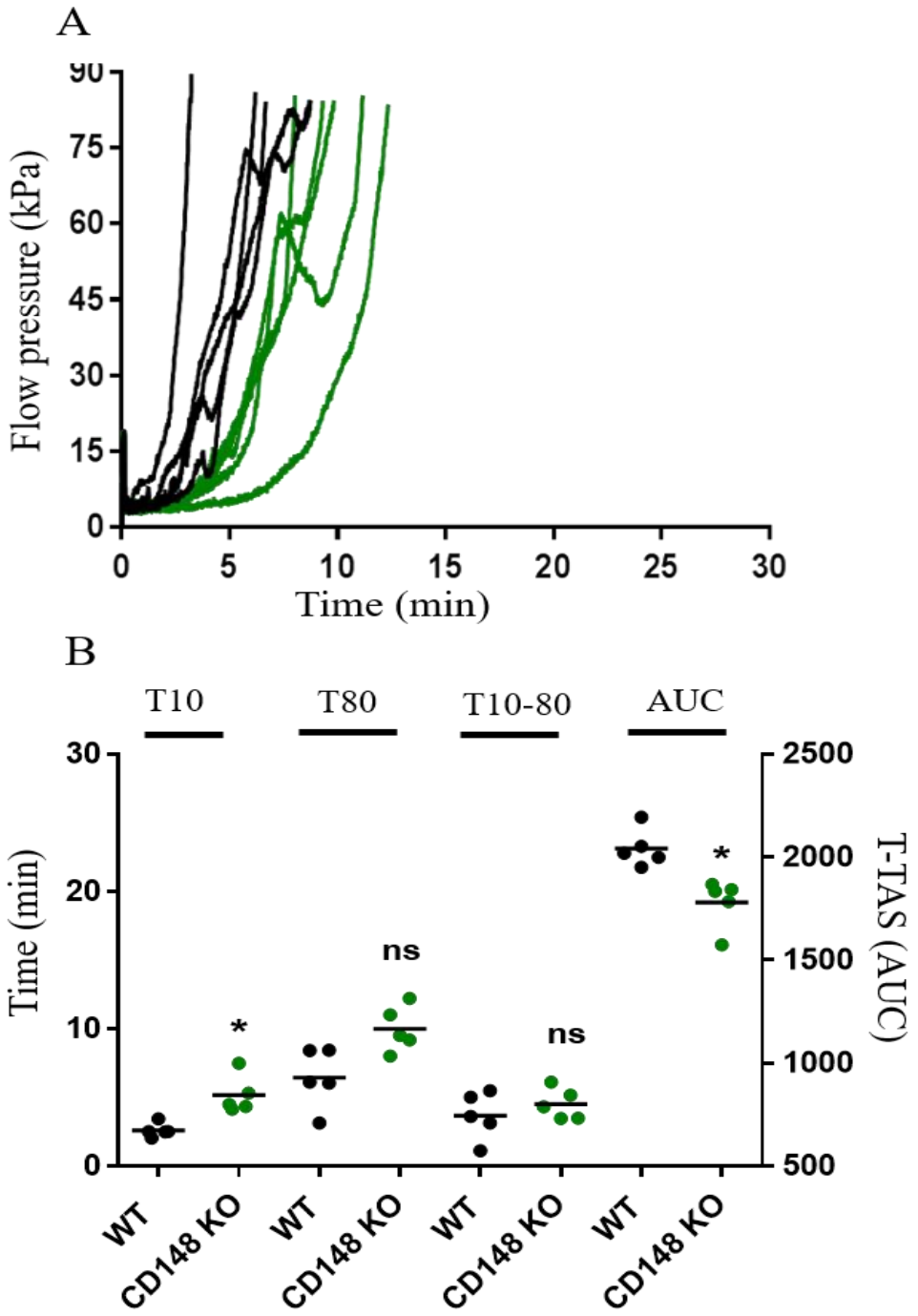



**Figure 6.20** Measurements of thrombus formation in blood from WT and CD148 knockout mice within the AR-chip (240 $s^{-1}$) Panel A, flow pressure curves observed in WT (black) and CD148 knock out mice (green). Panel B, distribution of the T-TAS measurements on blood from WT (n=5) and CD148 knock out mice (n=5) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and CD148 KO model each parameter (*p<0.05).

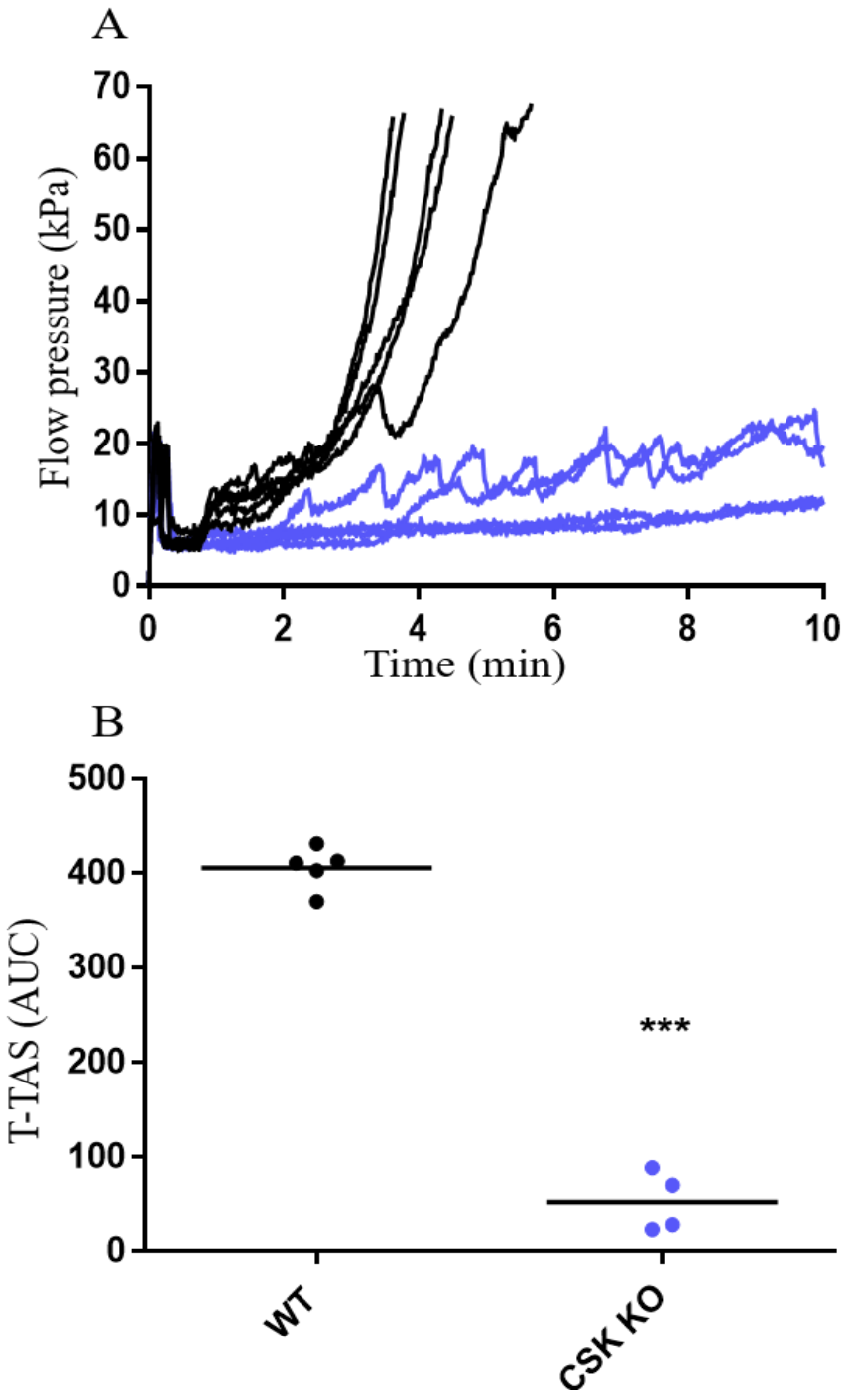



**Figure 6.21** Measurements of thrombus formation in blood samples from WT and CSK KO mice within the PL-chip (1000 $s^{-1}$). Panel A, flow pressure curves observed in WT (black) and CSK KO mice (blue). Panel B, distribution of the T-TAS (AUC) measurements in blood from WT mice (n=5) and CSK KO mice (n=4) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and CSK KO (***$p<0.001$).

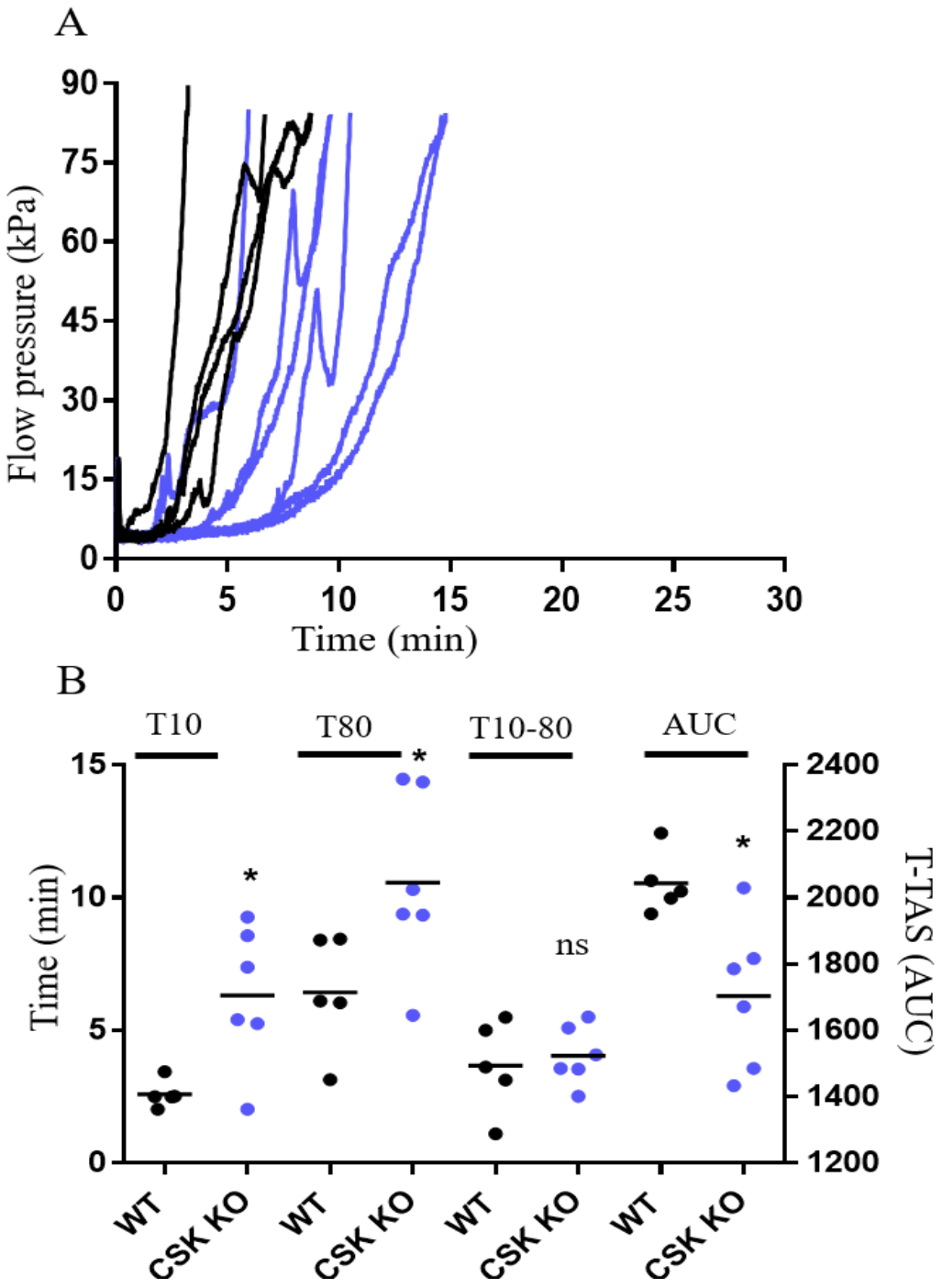



**Figure 6.22** Measurements of thrombus formation with blood from WT and CSK knockout mice within the AR-chip (240 $s^{-1}$ ) Panel A, flow pressure curves observed in WT (black) and CSK KO mice (blue). For panel B, distribution of the T-TAS measurements on blood from WT (n=5) and CSK KO mice (n=6) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and CSK KO in each parameter (*p<0.05).

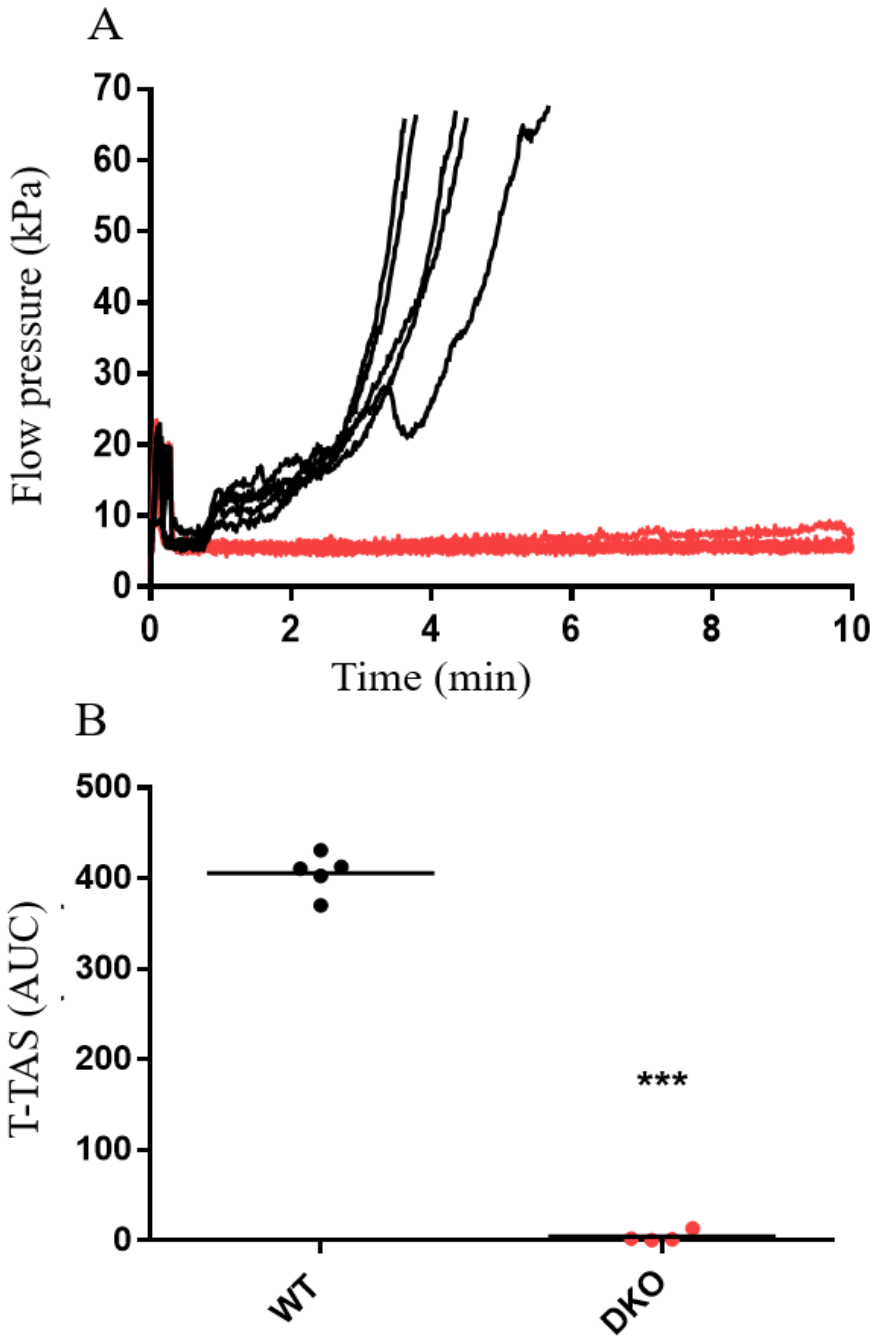



**Figure 6.23** Measurements of thrombus formation in blood from WT and double KO (CD148 and CSK) mice within the PL-chip (1000 $s^{-1}$). Panel A, flow pressure curves observed in WT (black) and double KO mice (red). Panel B, distribution of the T-TAS (AUC) measurements on blood from WT (n=5) and double KO mice (n=7) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and DKO (***p<0.001).

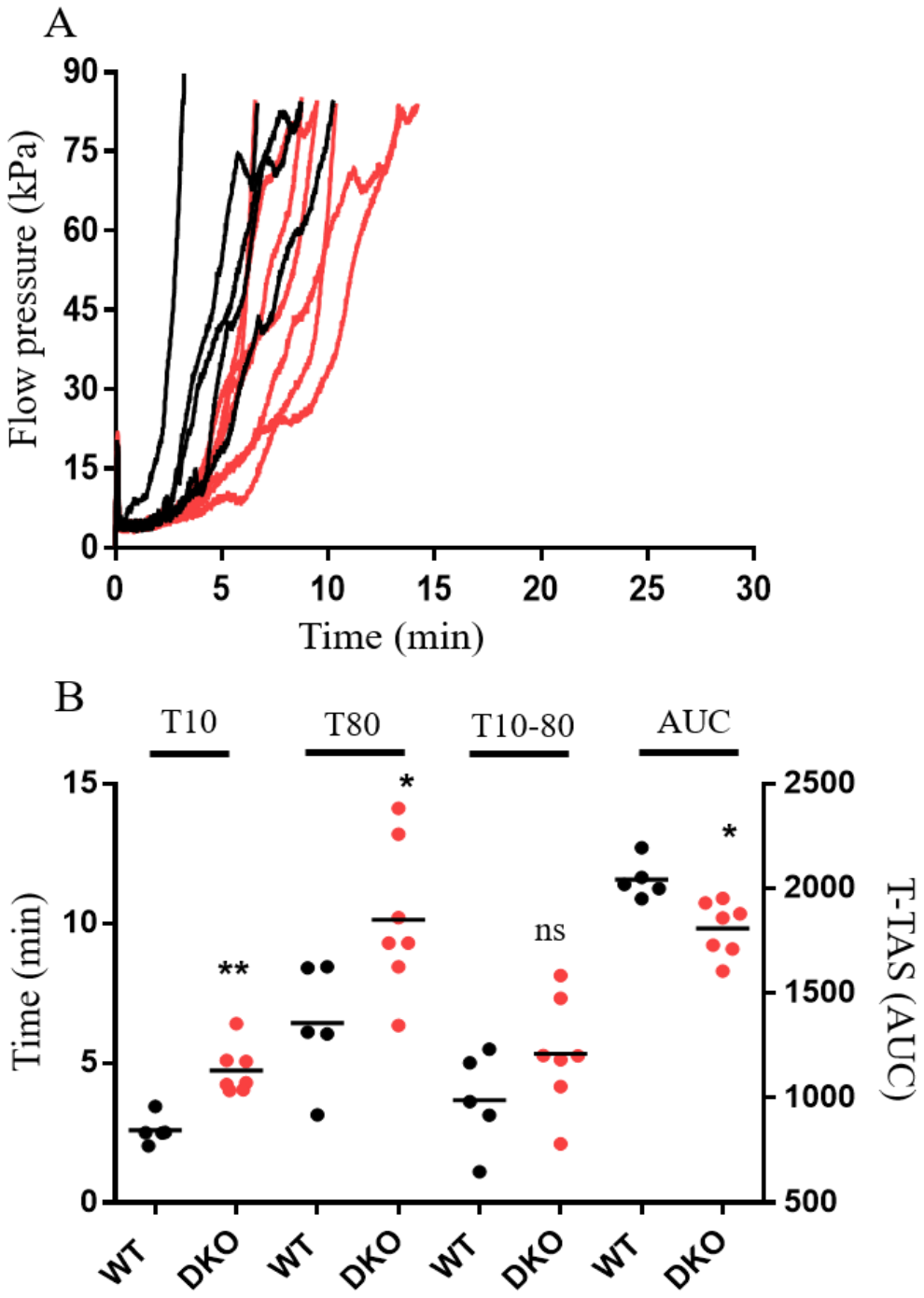



**Figure 6.24** Measurements of thrombus formation in blood samples from WT and double (CD148 + CSK) KO mice within the AR-chip (240 $s^{-1}$) Panel A, flow pressure curves observed in WT (black) and DKO mice (red). Panel B, distribution of the T-TAS measurements within blood from WT mice (n=5) and double knock out mice (n=7) (bars represent mean values). Statistical analysis performed by the Mann Whitney U test. Significance as compared between WT and DKO model each parameter (**p<0.01 and *p<0.05).

## 6.4 Discussion

The T-TAS is a flow chamber system that evaluates thrombus formation using whole blood samples that flow on thrombogenic surfaces under different shear rates. This approach offers an advantage for rapidly assessing thrombus formation in more physiological conditions involving both whole blood constituents and flow. Furthermore, the use of whole blood only requires small blood volumes making it practical for clinical application (Yamaguchi et al., 2013). We therefore sought to evaluate the potential utility of the T-TAS for assessing thrombus formation from GAPP study patients and various mouse platelet receptor knockout models.

In this study T-TAS was performed using 2 disposable chips. 1) The PL chip where thrombus formation is mainly mediated by platelets in the absence of coagulation and fibrinolytic pathways. 2) The AR chip where thrombus formation involves both platelet function and coagulation pathways. Testing in the PL chip was performed with hirudin-anticoagulated blood to maintain physiological calcium levels as recommended by the manufacturer (Hosokawa et al., 2011). In contrast, testing in the AR chip was performed with citrate anticoagulated blood but recalcified with a mixture of $CaCl_2$ in the presence of corn trypsin inhibitor (to prevent the activation of the intrinsic coagulation pathway).

In healthy controls, both the start and end points of thrombus formation varied among individuals within both chips suggesting that measurements obtained using the T-TAS may reflect individual variability of thrombus formation. Within the PL chip, the rate of thrombus formation was shorter at higher shear rate which was reflected in all parameters measured (figure 6.2B). This supports the evidence that high-shear rates favour the GPIb-IX-V/VWF interaction to induce more efficient thrombus formation (Ikeda et al., 1991). In contrast the effect of shear rate within the AR chip was variable, in particular the onset ($T_{10}$) (figure 6.3B) where the value at the high shear was greater than at the low shear. With a reason could not be

explained this finding contradicts with previously publication (Hosokawa et al., 2011, Yamaguchi et al., 2013). However, a decreased rate of thrombus growth ($T_{10-80}$) in accordance with an increase in shear rate was observed on AR chip similar to that on PL chip, indicating that the shear rates accelerated the growth rate of the thrombus. This Shear rate enhance on T-TAS parameters were also reported by Hosokawa et al, and Yamaguchi et al, (Hosokawa et al., 2011, Yamaguchi et al., 2013). In our study, the T-TAS exhibited high intra-assay coefficients of variation (CV) in both chips (table 6.3 and table 6.4). Typical CV's obtained were much higher than those described by Yamaguchi et al although their sample number (n=5) was smaller (Yamaguchi et al., 2013).

We also evaluated the utility of T-TAS in detecting the effect of antithrombotic therapy on haemostasis using two types of drugs. 1) The antiplatelet drug ticagrelor ($P2Y_{12}$ antagonist) and 2) The anticoagulant rivaroxaban (factor Xa inhibitor). Ticagrelor (10µM) completely suppressed thrombus formation within the PL chip. These findings agree with Hokosawa et al. who also previously demonstrated that thrombus formation within the PL chip was inhibited by $P2Y_{12}$ antagonists (Hosokawa et al., 2012, Hosokawa et al., 2013). In contrast, the inhibitory effect of ticagrelor on thrombus formation was not observed within the AR chip.

As thrombus formation within the AR at low shear rate is more dependent upon fibrin formation (Hanson and Sakariassen, 1998, Hosokawa et al., 2011), it is possible that $P2Y_{12}$ inhibitors are therefore expected to have no effect in this chip as platelets play little or no role in thrombus formation at low shear rates (Leonardi and Becker, 2012, Hosokawa et al., 2014a). Secondary, platelets within the AR chip could also be activated via the PAR-1 receptor by thrombin which is generated from activation of the coagulation pathways (Leonardi and Becker, 2012). This was demonstrated by Hokosawa et al. by demonstrating that PAR-1 antagonism reduced thrombus formation within the AR chip (Hosokawa et al., 2014a). In contrast, rivaroxaban (1

μM) incubation with whole blood within the AR chip displayed considerable variability on affecting thrombus formation with a delayed onset despite complete formation of thrombi (figure 6.8). This variability might be a result of an individuals' low responsiveness to the drug. Hokosawa et al indeed demonstrated that rivaroxaban (1 μM) only moderately suppressed thrombus formation on AR chip (Hosokawa et al., 2014b).

We also performed a comparative evaluation of the T-TAS using PL chip with GAPP patients with suspected platelet function defects and compared the results with the gold standard of lumi-LTA. Comparison of the overall results in 30 patients with normal platelet counts (Table 6.5) demonstrated a good agreement between T-TAS (PL chip) and lumi-LTA with concordance in 87% of samples tested. As 73% of samples were defined as normal by lumi-LTA, T-TAS therefore gave a negative predictive value of 86% suggesting that the test may be a potentially novel, reliable and useful tool for the screening of platelet function disorders. Interestingly, T-TAS also detected all patients with COX-like defects and secretion defects (figure 6.13). This finding is consistent with earlier reports showed that T-TAS was able to detect all patients diagnosed with SPD (Minami et al., 2015). T-TAS also showed high consistency in detecting abnormalities in patients with multiple defects. This findings also support the earlier report suggest that T-TAS is reliable in detecting more severe forms of platelet defects such BSS (Minami et al., 2015). In contrast T-TAS failed to detect thrombus formation in all patients with thrombocytopenia including three patients that gave normal responses by lumi-LTA (figure 6.15). This might suggest that T-TAS is not reliable for testing samples with low platelet counts and that thrombus formation is dependent on normal platelet number. An earlier study on healthy subjects demonstrated a good correlation between normal platelet counts and T-TAS parameters (Yamaguchi et al., 2013). Further studies to evaluate a

correlation between platelet count and T-TAS on samples with low platelet count would be of clinical value.

T-TAS only requires a small blood volume make it practical in studying thrombus formation in small animal models. To evaluate the utility of T-TAS for potentially testing mouse blood we then analysed blood from both WT and three knock out mouse models (CD148 KO, CSK KO and Double KO mice). As preliminary data (*in vivo* and *in vitro*) within these mouse models also demonstrated impaired platelet interaction with collagen this suggests they are a suitable model to test within the T-TAS (manuscript in preparation). Indeed testing of normal WT blood demonstrated the same influence of shear rates of thrombus formation within the PL-chip as with human blood (figure 6.16). Total thrombus formation however was much more rapid within mice blood compared to human. Similar results were reported on studies of blood from micro minipigs (Miura et al., 2013), suggesting that these animals might exhibit a high thrombotic tendency than humans, probable due to high platelet count (table 6.7). As expected, all KO models displayed poor thrombus formation within the PL chip concurring with previous in vivo and in vitro findings (manuscript in preparation). In contrast, full thrombus formation was observed within the AR chip with all knockouts indicating that the coagulation pathways are normal and that platelets probably have no so significant contribution to thrombus formation at a shear rate of 240 $s^{-1}$.

There is no single parameter that has been described to be more useful in defining haemostatic abnormalities within the T-TAS. Almost all studies evaluating bleeding disorders in the T-TAS have used $AUC_{10/30}$ and to a lesser extent the $T_{10}$ (Minami et al., 2015, Ito et al., 2016, Nogami et al., 2016, Ogiwara et al., 2015). AUC is particularly useful in quantifying a decrease in thrombus formation when occlusion time is not achieved during the time period of assay. In our study almost all samples (7/8) with abnormalities detected by T-TAS exhibited low values of

$AUC_{10}$. One patient however, gave normal values of $AUC_{10}$ but with delayed $T_{10}$ parameters. This might therefore suggests that a combination of $T_{10}$ and AUC parameters are sufficient to identify any haemostatic abnormalities on T-TAS.

Our present study has several limitations. First, the study was performed with only a small group of patients suspected of platelet function disorders. Therefore for future studies it would be necessary to enrol a larger number of patients with known platelet function defects as well as other haemostatic abnormalities such as haemophilia and VWD. Second, the study of antithrombotic drugs was performed using a single concentration of either ticagrelor or rivaroxaban. It would be of interest in future studies to evaluate the T-TAS with a full range of antiplatelet drugs with different molecular targets and at different concentrations. Finally, the mouse models were only studied within the AR chip at a low shear rate only. It would be of interest to find out if high shear rates would also have detected platelet defects in these mice.

In summary, T-TAS has demonstrated a good agreement with LTA suggesting that the technique could be applied in screening patients with platelet function defects. Moreover, the device might be a useful tool in monitoring antithrombotic therapy. Finally, we demonstrated that the T-TAS could provide valuable assistance in rapidly studying samples from animal models.

# CHAPTER SEVEN: PROSPECTIVE STUDY TO EVALUATE THE REMOTE PLATELET FUNCTION TEST (RPFT)

## 7.1 Introduction

A range of techniques are now available to test platelet function which are simple, less time consuming and require relatively small blood volumes. However, one of the major limitations is that the testing needs to be performed with a fresh blood sample (within 4-6 hours) following venipuncture (Harrison et al., 2011, Cattaneo et al., 2013). This still limits platelet function testing to specialized laboratories where appropriate technique(s) and experienced staff are available. These limitations therefore remain significant obstacles for rapid and easy diagnosis of platelet function defects and may also contribute to under-estimation of their true prevalence.

A recently developed Remote Platelet function Test (RPFT, Platelet Solutions) by Professor Stan Heptinstall at the University of Nottingham could overcome these limitations and may offer a completely new approach to platelet function testing. The kit essentially uses a series of vials with lyophilized platelet agonists and fixative solution (PAMFix) that sequentially activate and fix platelets in whole blood with proven stability for up to 9 days. Furthermore, the RPFT kit is also a closed point of care (POC) system without requiring pipetting or specialized laboratory equipment. Fixed samples from remote locations can also then be shipped at ambient temperature to a central laboratory for flow cytometric analysis (Dovlatova, 2015).

For platelet stimulation, anticoagulated whole blood is simply added into 4 vials containing (i) saline only as a negative baseline control, (ii) Arachidonic acid/epinephrine, (iii) ADP/U46619 and (iv) PAR-1 peptide the thrombin receptor agonist. Samples are then mixed and activated for 5 mins at $37^0$C within a heat pouch provided in the kit. The samples are then fixed and sent in sealed containers via post to the central laboratory. Upon receipt of the samples the degree

of platelet activation is assessed by measuring the expression of p-selectin (CD62p) and CD63 as markers of alpha granular and dense granular secretion respectively by using flow cytometry.

Several studies have assessed the utility of RPFT for monitoring several types of antiplatelet therapy and for prediction of the risk of recurrent thrombotic events in patients with acute coronary syndromes (Fox et al., 2009, Thomas et al., 2014, Keeler et al., 2015). The RPFT has also been evaluated for the diagnosis of mild bleeding disorders and demonstrated a good agreement with lumi-LTA (Dovlatova et al., 2014). However, in this study the test was used with GAPP samples received at the University of Birmingham and not as a true POC test within the clinic.

### 7.2 Aim

To perform a prospective study to evaluate the POC utility of RPFT in the diagnosis of platelet function defect in patients with mild bleeding disorders by comparison with the gold standard of Light transmission lumi-aggregometry (LTA). Initially, a training session was performed in each participating centre to demonstrate how the RPFT test is optimally performed and processed.

## 7.3 Results

### 7.3.1 Sample recruitment and determination of cut off values

A total of 122 RPFT kits were distributed to 6 haemophilia centres across the UK. Only 63 (52%) of kits with samples were returned for analysis. 10 of these samples were also rejected because of low platelet counts, haemolysis or because of excessive dilution of blood samples. The list of kits distributed to each haemophilia centre and number of samples returned for analysis is summarised in table 7.1. The highest number of samples were recruited from Lincoln where 76% of the kits were returned for analysis, followed by Nottingham 12(50%), Canterbury 10(100%), Leicester (40%) and Manchester 5(23%). Unfortunately no samples were recruited from St Thomas's Hospital in London. 27 healthy volunteers were also recruited and measured by RPFT as controls to determine a normal range and to define cut off values for classification of normal or abnormal results. As shown within table 7.2, the cut off MFI values for P-selectin were 1507 (AA/EPI), 2536 (ADP/U46619) and 2434 (PAR-1), and for CD63 were 211(AA/EPI), 343 (ADP/U46619) and 440 (PAR-1). When the above cut off values were applied to the group of healthy controls only 3/27 participants presented with reduced platelet response by the RPFT (figure 7.1).

| Haemophilia centre | No. of kits supplied | No. of samples received | No. of samples rejected | No. of samples analysed | No. of normal results | No. of abnormal results |
|---|---|---|---|---|---|---|
| Lincoln | 42 | 32 | 2 | 30 | 10 | 20 |
| Nottingham | 24 | 12 | 2 | 10 | 5 | 5 |
| Canterbury | 10 | 10 | 2 | 8 | 4 | 4 |
| Leicester | 10 | 4 | 1 | 3 | 2 | 1 |
| Manchester | 22 | 5 | 3 | 2 | 2 | 0 |
| St Thomas (London) | 14 | 0 | 0 | 0 | 0 | 0 |
| Total | 122 | 63 | 10 | 53 | 23 | 30 |

**Table 7.1** List of RPFT kits distributed to 6 haemophilia centres showing the number of kits supplied and the number of samples returned for analysis.

| | P-selectin (MFI) | | | CD63 (MFI) | | |
|---|---|---|---|---|---|---|
| | AA/EPI | ADP/U46619 | PAR-1 | AA/EPI | ADP/U46619 | PAR-1 |
| RPFT (mean ± SD) | 3279 ± 886 | 3457 ± 460 | 3437 ± 501 | 638 ± 213 | 775 ± 216 | 961 ± 260 |
| RPFT cut off values | 1507 | 2536 | 2434 | 211 | 343 | 440 |

**Table 7.2** Summary of p-selectin and CD63 expression (MFI) measured with RPFT in samples from healthy controls (n=27) after stimulation with various agonists, the data are presented as mean and SD. The cut-off values were calculated as the $5^{th}$ percentile of the corresponding measurements in healthy controls.

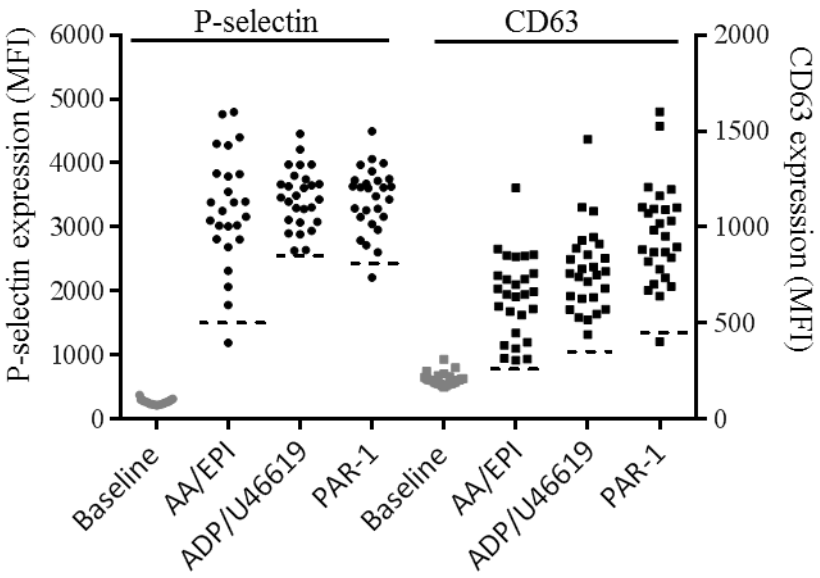



**Figure 7.1** P-selectin and CD63 (MFI) measured with RPFT in healthy controls (n=27) with baseline controls and after stimulation with various platelet agonists. Horizontal dotted lines represent the 95$^{th}$ percentile (cut off value) used to determine abnormal results.

### 7.3.2 Overall patient results

53 patients (15 male and 38 female) with a median age of 37 ± 17 (median ± SD) ranging from 2-80 years were analysed by the RPFT and results were compared with lumi-LTA. Whole blood platelet count and MPV values (mean ± SD) were normal in all participants (250 ± 61x$10^9$/L and 11.0 ± 0.9fl, respectively). Overall, 30(57%) patients gave an abnormal RPFT with at least one parameter falling below the cut off values obtained from healthy controls as shown in table 7.2. Of these 30, all exhibited low levels of P-selectin expression mainly in response to ADP/U46619 and PAR-1 peptide (figure 7.2). In contrast, only 16/30 patients gave low levels of CD63 expression (figure 7.3). According to lumi-LTA, 17 out of 53 patients were considered to have a platelet defect as defined using previous criteria (Dawood et al., 2012). The overall results of all 53 patients tested are shown in figure 7.4.

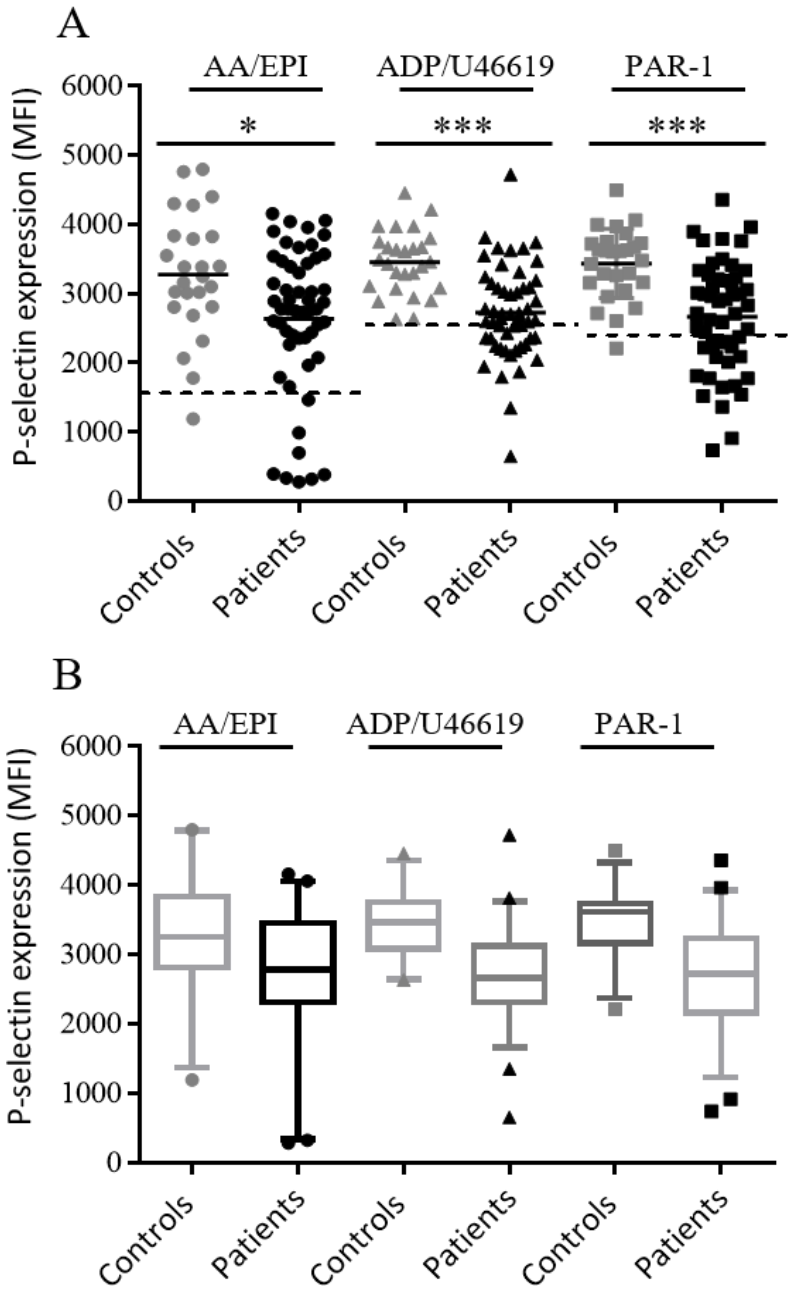



**Figure 7.2** P-selectin (MFI) measured with RPFT in healthy controls (n=27) and patients (n=53) after stimulation with various platelet agonists. For panel A, horizontal bars represent mean values and horizontal dotted lines represent the 95$^{th}$ percentile (cut off value) used to determine abnormal results. Statistical analysis was performed by the Mann Whitney U test. Significance was compared to healthy controls (*$p<0.05$ and ***$p<0.001$). For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

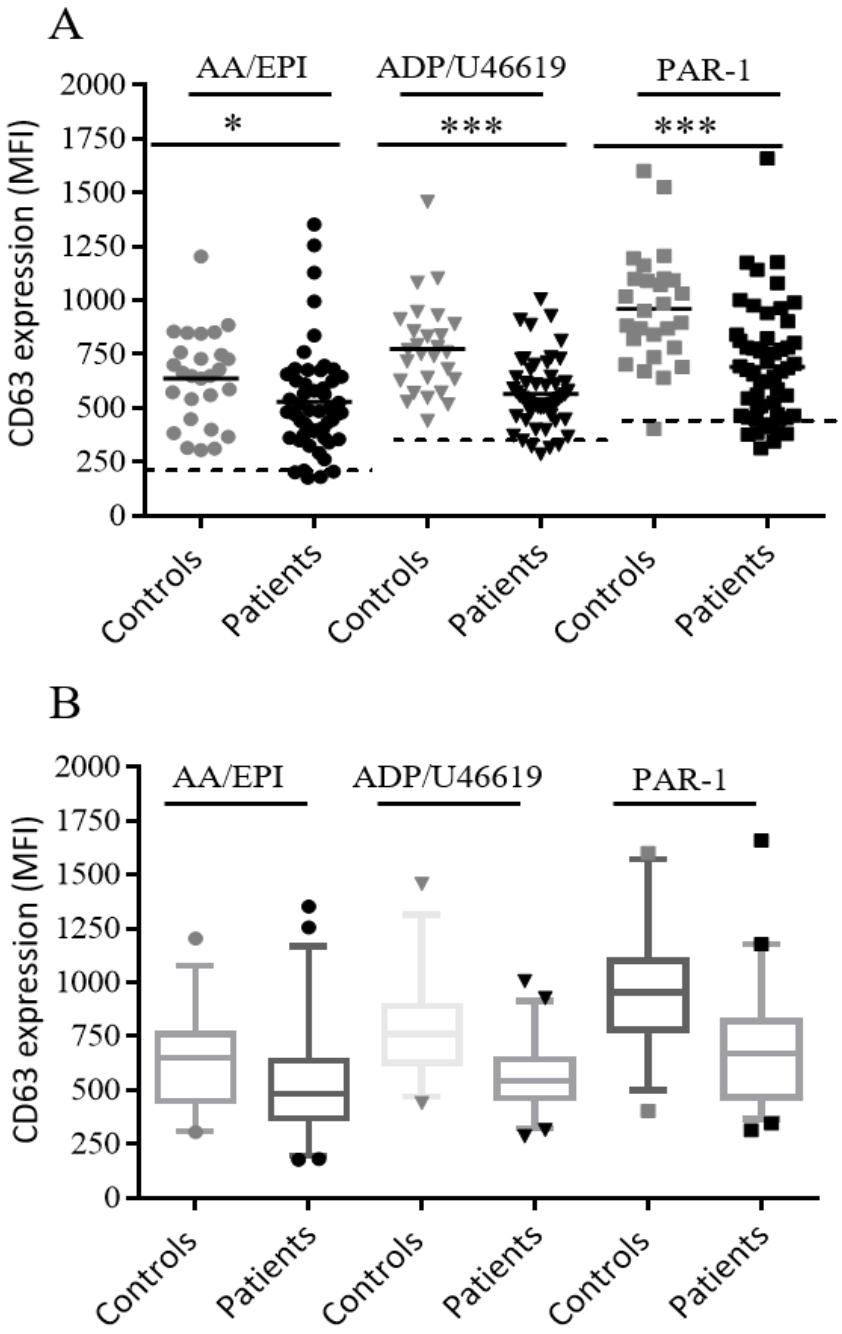



**Figure 7.3** CD63 (MFI) measured with RPFT in healthy controls (n=27) and patients (n=53) after stimulation with various platelet agonists. For panel A, horizontal bars represent mean values and horizontal dotted lines represent the 95$^{th}$ percentile (cut off value) used to determine abnormal results. Statistical analysis performed by the Mann Whitney U test. Significance was compared to healthy controls (*$p<0.05$ and ***$p<0.001$). For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles and outlying points are represented by dots.

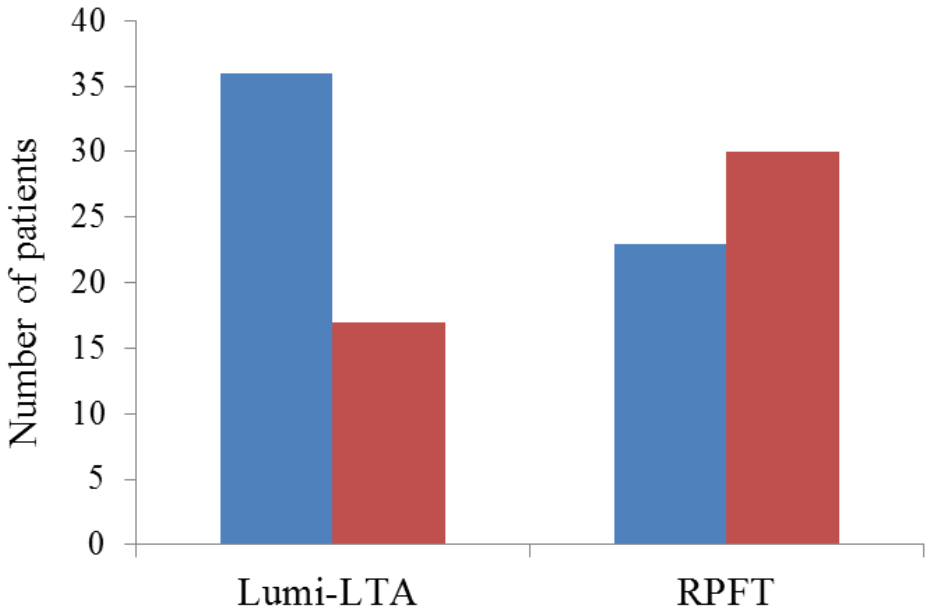



**Figure 7.4** Classification of patients results (n=53) analysed by lumi-LTA and RPFT. Normals and abnormals are shown in blue and red respectively. Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (by RPFT) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy individuals as shown in table 7.2

### 7.3.3 Overall agreement between lumi-LTA and RPFT

The overall agreement between the lumi-LTA and RPFT was poor (table 7.3). Only 30/53 samples gave identical results by both tests. In contrast, there was disagreement in 23/53 samples. RPFT gave abnormal results in 18 patients that were normal by lumi-LTA. However, 5 samples were normal by RPFT but abnormal by lumi-LTA. A similar poor overall agreement between the two techniques was also observed when cut off values obtained from a ROC analysis were also applied. A comparison between lumi-LTA and RPFT was then analysed using each platelet marker separately. Summary of overall results are shown in figure 7.5. By using P-selectin and CD63 individually abnormal platelet function was detected in 30 and 16 patients respectively, compared to 17 patients detected by lumi-LTA. In contrast, P-selectin and CD63 were normal on 23 and 37 samples respectively whereas 36 samples were normal by lumi-LTA. Overall, a poor agreement was also observed when lumi-LTA was compared with p-selectin expression as there was disagreement in 22(42%) samples (table 7.4A). In contrast a better agreement was observed when lumi-LTA was compared with CD63 expression with concordance in 43(81%) samples (table 7.4B). When P-selectin and CD63 where compared to one another there was agreement within 39(74%) samples, In contrast, the two markers disagreed on 14 samples, the majority of which (13) exhibited reduced expression of P-selectin only (table 7.4C).

| | Lumi-LTA (Abnormal) | Lumi-LTA (Normal) | |
|---|---|---|---|
| RPFT (Abnormal) | 12 | 18 | PPV=40% |
| RPFT (Normal) | 5 | 18 | NPV=78% |
| | Sensitivity=71% | Specificity=50% | |

**Table 7.3** Overall classification of results by lumi-LTA and RPFT in patient samples (n=53) displaying sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

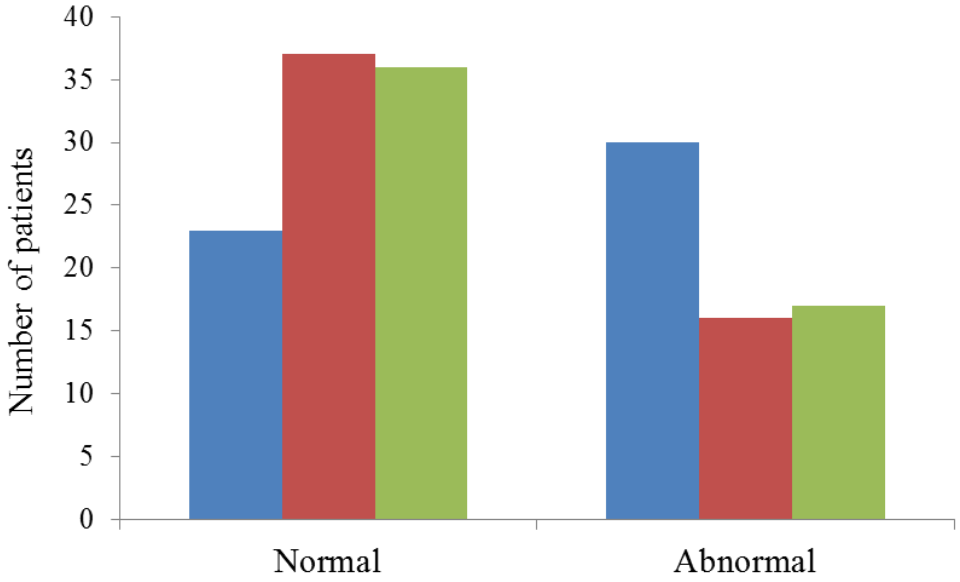



**Figure 7.5** Classification of overall patient results (n=53) showing normal and abnormals in comparison between samples detected by P-selectin (blue), CD63 (red) and lumi-LTA (green). Abnormal results by Lumi-LTA were determined based on our previously published methodology that is based upon the cut-off values that were calculated as the 5$^{th}$ percentile from the healthy volunteers as well as both the magnitude and time course of response (Dawood et al., 2007, Dawood et al., 2012) and (RPFT) when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown in table 7.2

A

| | Lumi-LTA (Abnormal) | Lumi-LTA (Normal) | |
|---|---|---|---|
| P-selectin (Abnormal) | 12 | 17 | PPV=41% |
| P-selectin (Normal) | 5 | 19 | NPV=79% |
| | Sensitivity=71% | Specificity=53% | |

B

| | Lumi-LTA (Abnormal) | Lumi-LTA (Normal) | |
|---|---|---|---|
| CD63 (Abnormal) | 12 | 5 | PPV=71% |
| CD63 (Normal) | 5 | 31 | NPV=86% |
| | Sensitivity=71% | Specificity=86% | |

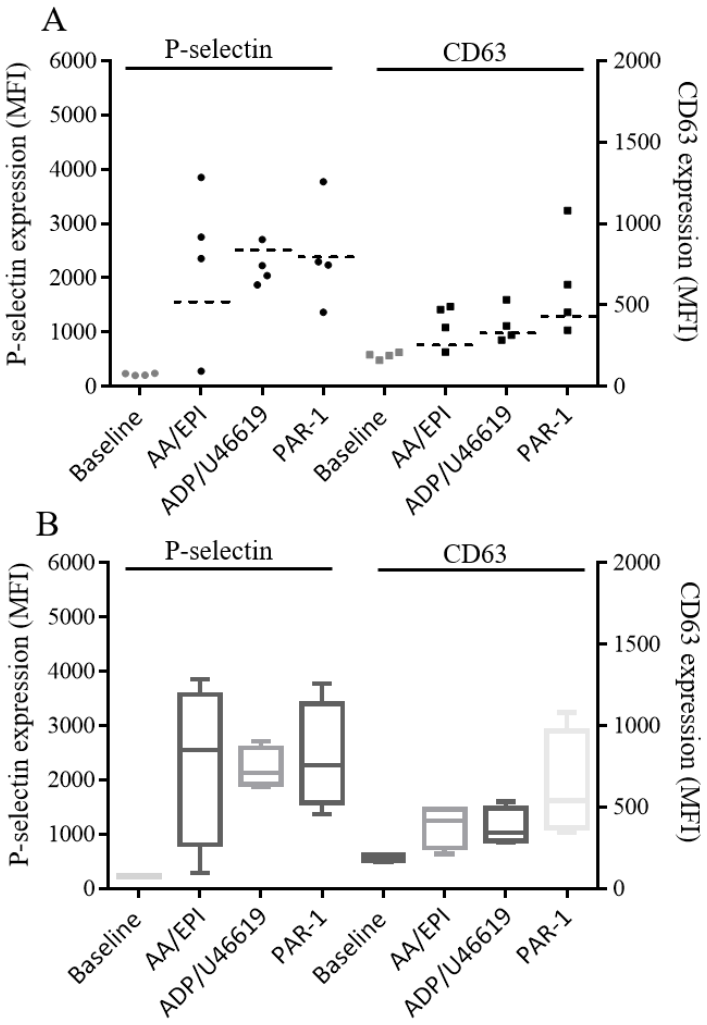
C

| | P-selectin (Abnormal) | P-selectin (Normal) | |
|---|---|---|---|
| CD63 (Abnormal) | 16 | 1 | |
| CD63 (Normal) | 13 | 23 | |
| | | | |

**Table 7.4** Classification of RPFT results in patient samples (n=53) comparing between lumi-LTA and P-selectin (A), lumi-LTA and CD63 (B) and, P-selectin and CD63 (C), displaying sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV).

### 7.3.4 Performance of RPFT in patients with Gi and dense granular secretion defects as classified by lumi-LTA

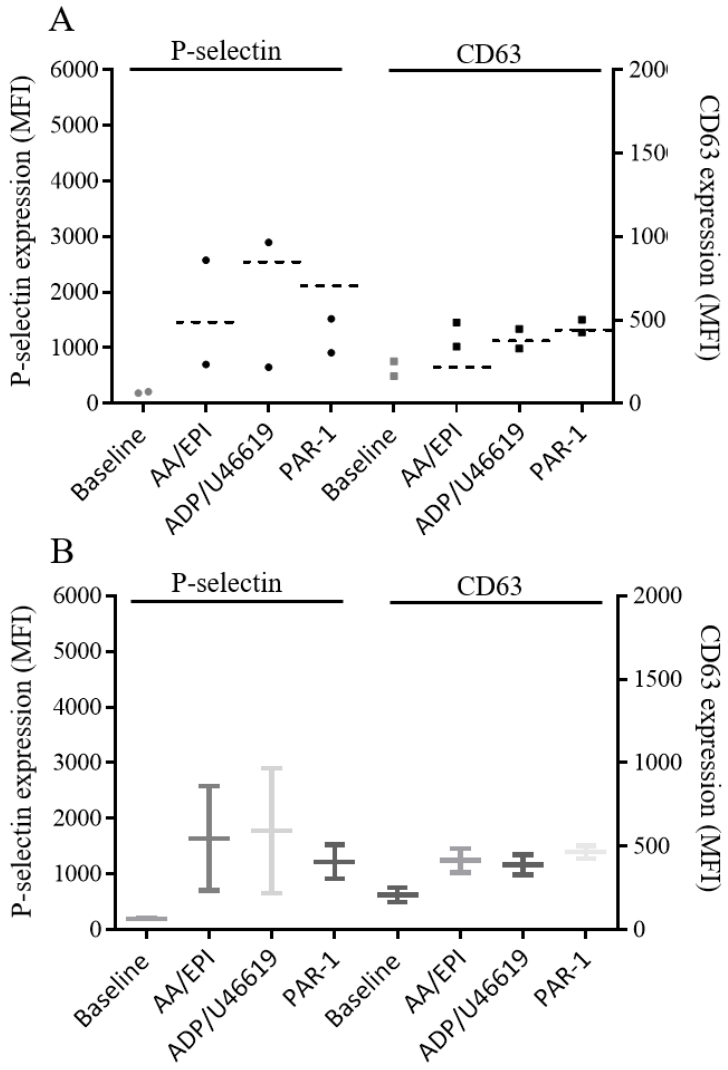
The 17 patients with platelet defects identified by lumi-LTA were further subdivided into four categories based on their patterns of aggregation responses as previously described (Ban et al., 2012): defects in Gi signalling (n=4), defects in dense granular secretion (n=2), defects in cyclooxygenase pathway (n=5) and multiple or complex defects (n=6). All participants who presented with Gi signalling defect (figure 7.6) or dense granular secretion defect (figure 7.7) on lumi-LTA were also found to give abnormal results by the RPFT. Interestingly, all of these patients exhibited a reduced level of both P-selectin and CD63 expression in response to at least one agonist. In the Gi defect group, 3/4 patients gave an abnormal result by RPFT in response to ADP/U46619, whereas one patient gave an abnormal response to PAR-1 peptide. In dense granular secretion defects however, both patients gave abnormal levels of both platelet markers in response to PAR-1 peptide.




C

| | P-selectin expression (MFI) | | | CD63 expression (MFI) | | | | |
|---|---|---|---|---|---|---|---|---|
| Patient code | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | Results obtained from RPFT | Results obtained from Lumi-LTA |
| P01 | 282 | 1874 | 2238 | 210 | 373 | 625 | Abnormal | Gi defect |
| P02 | 2751 | 2225 | 2298 | 472 | 316 | 455 | Abnormal | Gi defect |
| P03 | 2355 | 2707 | 1366 | 362 | 533 | 345 | Abnormal | Gi defect |
| P04 | 3854 | 2040 | 3771 | 491 | 285 | 1080 | Abnormal | Gi defect |

**Figure 7.6** P-selectin and CD63 (MFI) measured by RPFT in patients (n=4) with a defect in Gi pathway as determined by lumi-LTA. For panel A, patients results at baseline and after stimulation with the corresponding agonist, horizontal dotted line represents the 95$^{th}$ percentile (cut off value) used to determine abnormal results. For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles. For table C patient results are presented as an absolute number (MFI) after stimulation with the corresponding agonists with abnormal results shown in red text. The results were considered to be abnormal when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown table 7.1.



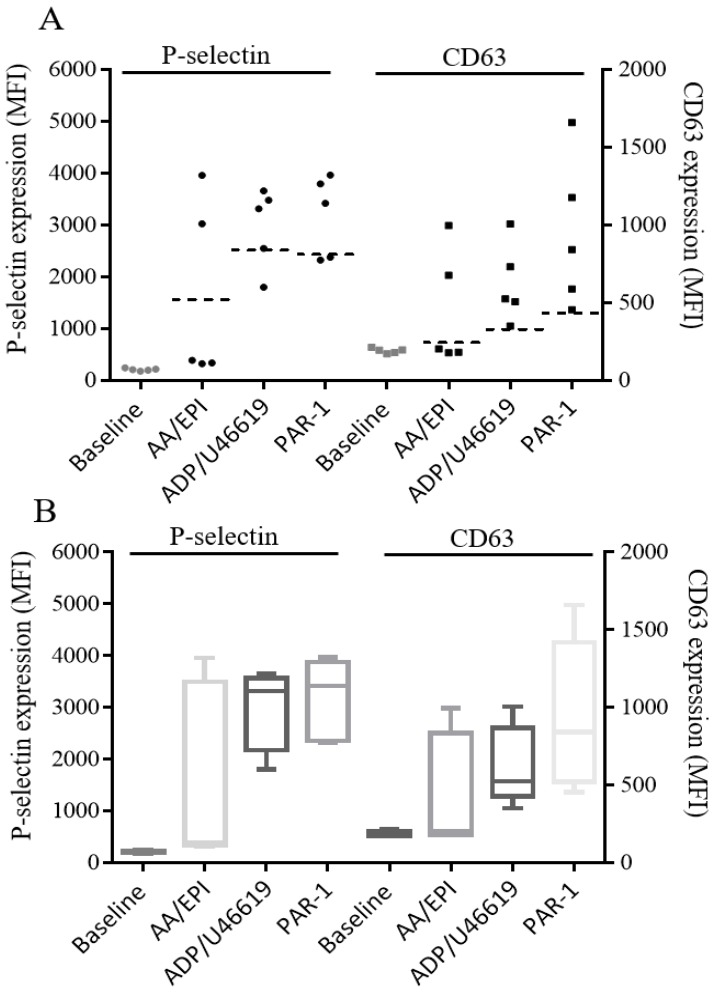

C

| | P-selectin expression (MFI) | | | CD63 expression (MFI) | | | | |
|---|---|---|---|---|---|---|---|---|
| Patient code | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | Results obtained from RPFT | Results obtained from Lumi-LTA |
| P10 | 701 | 654 | 912 | 341 | 329 | 503 | Abnormal | Secretion defect |
| P11 | 2577 | 2899 | 1520 | 486 | 448 | 425 | Abnormal | Secretion defect |

**Figure 7.7** P-selectin and CD63 (MFI measured by RPFT in patients (n=2) with a defect in dense granular secretion as determined by lumi-LTA. For panel A, patients results are shown at baseline and after stimulation with the corresponding agonist, horizontal dotted line represents the 95$^{th}$ percentile (cut off value) used to determine abnormal results. For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles. For table C patient results are presented as an absolute number (MFI) after stimulation with the corresponding agonists with abnormal results shown in red text. The results were considered to be abnormal when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown table 7.1.

### 7.3.5 Performance of RPFT in patients with Cox and multiple platelet defects as classified by lumi-LTA

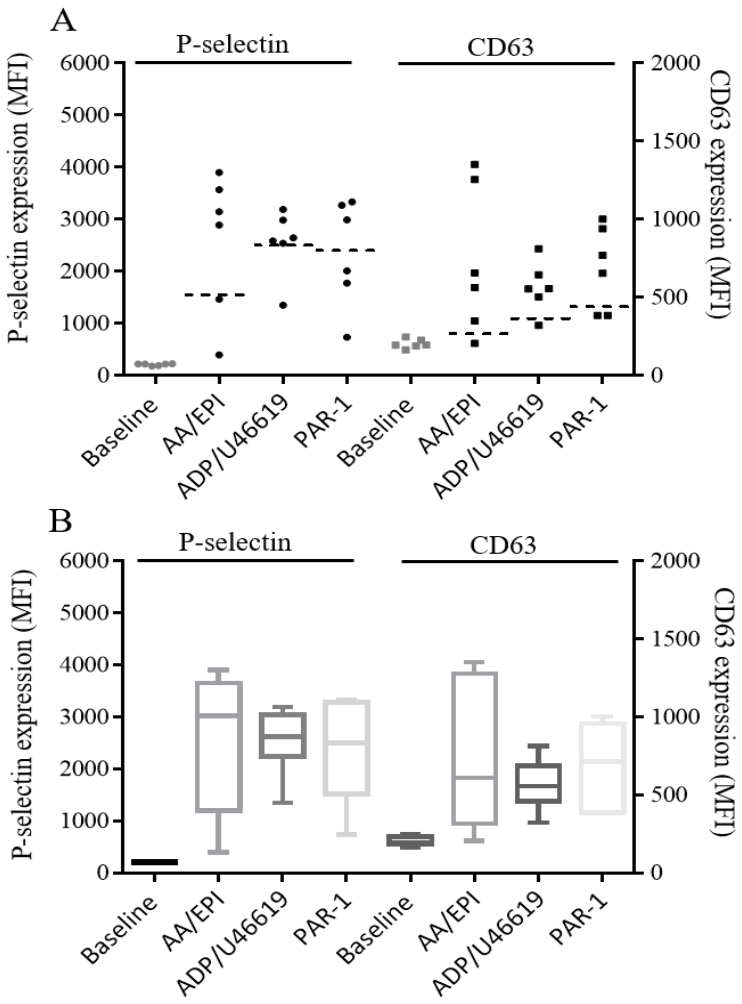
In patients who presented with defects in the cyclooxygenase pathway as detected by lumi-LTA, only 3/5 gave an abnormal response by RPFT (figure 7.8). Interestingly, all of 3 patients gave abnormal responses to Arachidonic acid/epinephrine (more details are shown in figure 7.8C). In contrast, 3/6 patients with multiple and complex defects, gave abnormal responses by RPFT, especially PAR-1 peptide (more details are shown in figure 7.9C).




C

| Patient code | P-selectin expression (MFI) | | | CD63 expression (MFI) | | | Results obtained from RPFT | Results obtained from Lumi-LTA |
|---|---|---|---|---|---|---|---|---|
| | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | | |
| P05 | 338 | 1797 | 2378 | 203 | 349 | 588 | Abnormal | Cox defect |
| P06 | 388 | 2545 | 2320 | 180 | 506 | 454 | Abnormal | Cox defect |
| P07 | 321 | 3314 | 3962 | 178 | 524 | 1175 | Abnormal | Cox defect |
| P08 | 3958 | 3655 | 3793 | 995 | 1006 | 1659 | Normal | Cox defect |
| P09 | 3024 | 3479 | 3419 | 675 | 731 | 841 | Normal | Cox defect |

**Figure 7.8** P-selectin and CD63 (MFI) measured by RPFT in patients (n=5) with defects in the cyclooxygenase pathway as determined by lumi-LTA. For panel A, patients results are shown at baseline and after stimulation with the corresponding agonist, horizontal dotted line represents the 95$^{th}$ percentile (cut off value) used to determine abnormal results. For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles. For table C patient results are presented as an absolute number (MFI) after stimulation with the corresponding agonists with abnormal results shown in red text. The results were considered to be abnormal when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown table 7.1.



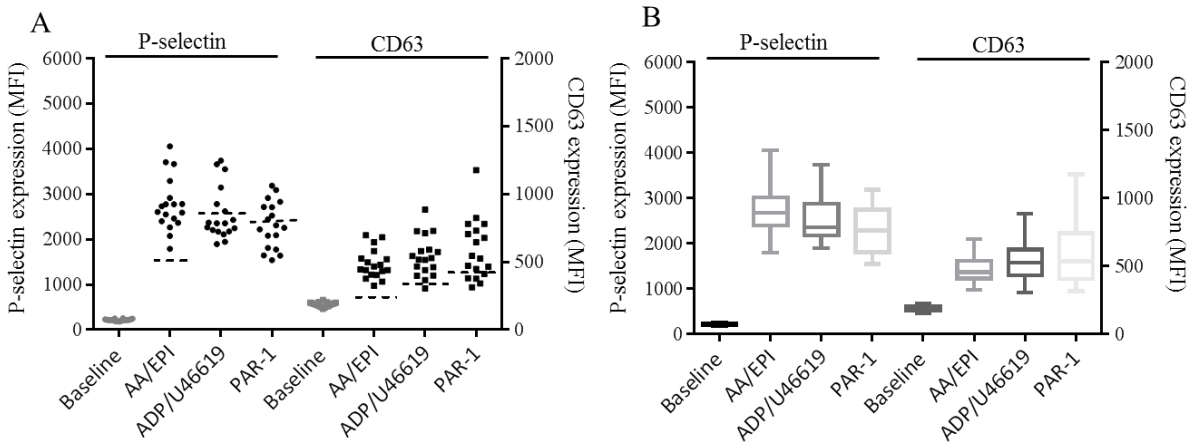

C

| Patient code | P-selectin expression (MFI) | | | CD63 expression (MFI) | | | Results obtained from RPFT | Results obtained from Lumi-LTA |
|---|---|---|---|---|---|---|---|---|
| | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | AA (0.5mM)/ EPI (100µM) | ADP (10µM)/ U46619 (1µM) | PAR-1 (20µM) | | |
| P12 | 399 | 3191 | 1779 | 205 | 557 | 385 | Abnormal | Cox + Sectretion defect |
| P13 | 3902 | 2541 | 736 | 1255 | 813 | 386 | Abnormal | Gi + Sectretion defect |
| P14 | 1468 | 1353 | 2010 | 350 | 323 | 656 | Abnormal | Gq defect |
| P15 | 3145 | 2585 | 3339 | 1353 | 504 | 940 | Normal | Gi + Cox defect |
| P16 | 3573 | 2989 | 2992 | 657 | 555 | 770 | Normal | Gi + Cox defect |
| P17 | 2891 | 2649 | 3272 | 564 | 645 | 1002 | Normal | Un-classifeid |

**Figure 7.9** P-selectin and CD63 (MFI) measured by RPFT in patients (n=6) with multiple defects as determined by lumi-LTA. For panel A, patients results are shown at baseline and after stimulation with the corresponding agonists, horizontal dotted line represents the 95$^{th}$ percentile (cut off value) used to determine abnormal results. For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles. For table C patient results are presented as an absolute number (MFI) after stimulation with the corresponding agonists with abnormal results shown in red text. The results were considered to be abnormal when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown table 7.1.

### 7.3.6 False positive samples identified by RPFT when compared to lumi-LTA.

18 patients presented with abnormal responses by RPFT, but with normal responses to all agonists by lumi-LTA (figure 7.10). Almost all patients (17/18) had reduced level of P-selectin expression in response to ADP/U46619 (n=12) and to PAR-1 peptide (n=11) agonists. In contrast only 5(28%) patients gave low expression of CD63, all of which gave an abnormal response to PAR-1 peptide with one giving an abnormal response to ADP/U46619 (figure 7.10C). Interestingly, all participants had normal responses to Arachidonic acid/epinephrine. 13 out of 18 patients gave abnormal expression of P-selectin but CD63 was normal. Conversely, only 1/18 patient gave abnormal expression of CD63 with normal levels of P-selectin.




C

| Patient code | P-selectin expression (MFI) | | | CD63 expression (MFI) | | | Results obtained from RPFT | Results obtained from Lumi-LTA |
|---|---|---|---|---|---|---|---|---|
| | AA (0.5mM)/ EPI (100µM) | ADP(10µM)/ U46619 (1µM) | PAR-1 (20µM) | AA (0.5mM)/ EPI (100µM) | ADP(10µM)/ U46619 (1µM) | PAR-1 (20µM) | | |
| **P1** | 2368 | 2371 | 1649 | 467 | 441 | 314 | **Abnormal** | **No defect** |
| **P2** | 2733 | 2357 | 2083 | 408 | 574 | 465 | **Abnormal** | **No defect** |
| **P3** | 3669 | 3660 | 2094 | 498 | 581 | 525 | **Abnormal** | **No defect** |
| **P4** | 2777 | 2355 | 2916 | 581 | 399 | 730 | **Abnormal** | **No defect** |
| **P5** | 2783 | 2432 | 2220 | 479 | 529 | 472 | **Abnormal** | **No defect** |
| **P6** | 2403 | 2176 | 3093 | 441 | 401 | 546 | **Abnormal** | **No defect** |
| **P7** | 3708 | 3744 | 1544 | 434 | 713 | 380 | **Abnormal** | **No defect** |
| **P8** | 1792 | 1949 | 3185 | 520 | 729 | 1178 | **Abnormal** | **No defect** |
| **P9** | 2918 | 3148 | 2447 | 697 | 590 | 378 | **Abnormal** | **No defect** |
| **P10** | 4057 | 3554 | 1775 | 646 | 462 | 449 | **Abnormal** | **No defect** |
| **P11** | 3294 | 1896 | 1643 | 440 | 305 | 342 | **Abnormal** | **No defect** |
| **P12** | 2079 | 2265 | 2833 | 355 | 516 | 780 | **Abnormal** | **No defect** |
| **P13** | 2607 | 2781 | 2313 | 683 | 886 | 780 | **Abnormal** | **No defect** |
| **P14** | 2266 | 2113 | 2711 | 378 | 518 | 824 | **Abnormal** | **No defect** |
| **P15** | 2779 | 2623 | 1811 | 526 | 728 | 646 | **Abnormal** | **No defect** |
| **P16** | 2464 | 2173 | 2719 | 325 | 367 | 412 | **Abnormal** | **No defect** |
| **P17** | 2553 | 2248 | 2529 | 446 | 544 | 705 | **Abnormal** | **No defect** |
| **P18** | 2590 | 2206 | 2259 | 404 | 467 | 678 | **Abnormal** | **No defect** |

**Figure 7.10** P-selectin and CD63 (MFI) expression measured by RPFT in patients (n=18) with false positive results when compared to lumi-LTA. For panel A, patients results are shown at baseline and after stimulation with the corresponding agonists, horizontal dotted line represents the 95$^{th}$ percentile (cut off value) used to determine abnormal results. For panel B, the boxes represent median and interquartile ranges, whiskers represent 5$^{th}$ and 95$^{th}$ percentiles. For table C patient results are presented as an absolute number (MFI) after stimulation with the corresponding agonists with abnormal results shown in red text. The results were considered to be abnormal when they fall below the cut-off values (5$^{th}$ percentile) calculated from healthy controls as shown table 7.1

## 7.4 Discussion

This chapter presents the results from a prospective study that was performed to assess the potential of the RPFT in comparison to lumi-LTA for the potential diagnosis of PFDs. The main advantage of RPFT is the use of small volumes of unseparated whole blood that minimises the time of sample processing. Furthermore, the use of a fixative allows testing to be performed remotely as a POC test avoiding the need of immediately processing of the blood sample before sending to a central laboratory for flow cytometry. This approach could therefore improve the diagnosis of platelet function which is currently restricted to specialized laboratories.

Abnormal RPFT results were defined based on the cut-off values that were calculated from the healthy volunteers. When the same cut off values were applied in the group of healthy controls a small number of participants presented with reduced platelet response on RPFT. This small degree of overlap in platelet responsiveness among healthy population has also been reported in other tests of platelet function and haemostasis (Quiroga et al., 2007).

RPFT directly measures both alpha and dense granular secretion. When both markers were analysed together a poor agreement between lumi-LTA and RPFT was observed with an overall concordance of 57% in 30 samples. However, when each marker was analysed independently, a better agreement was observed between lumi-LTA and RPFT using CD63 expression with overall concordance of 81% of 43 samples. The latter result is very similar to a previous study where the overall agreement between lumi-LTA and RPFT using both markers (P-selectin and CD63) was shown to be concordant in 84% of cases (Dovlatova et al., 2014).

In this study, the RPFT correctly identified a platelet function defect in all of the patients classified with either Gi signalling or dense granular secretion defects by lumi-LTA. Previously, the RPFT was reported to correctly identify all patients with secretion defects

identified by lumi-LTA. This suggests that the RPFT is very sensitive for identifying abnormalities related to platelet secretion defects (Dovlatova et al., 2014). This is not unexpected given that the test is a direct measurement of granular secretion and also provides further validation that lumi-LTA is sensitive to secretion defects. However, of the two markers, CD63 expression has shown to be more reliable for identifying platelet defects with a specificity of 86% and PPV of 71% (table 7.4B). Similarly, the most sensitive agonist used within the RPFT is Arachidonic acid/epinephrine, where abnormal results were obtained in 3 patients with defects in the cyclooxygenase pathway with a NPV of 100% (figure 7.10).

RPFT however identified false positives within 18(34%) samples that gave normal responses by lumi-LTA, the majority (94%) showing reduced levels of P-selectin expression. Previously, Dovlatova et al, also reported a false positive rate of 10% within patient samples. However, only one of these patients presented with a reduced level of P-selectin expression alone (Dovlatova et al., 2014). A low level of P-selectin expression in patients with normal aggregation and normal dense granules secretion may indicate impaired α-granular secretion (Blair and Flaumenhaft, 2009). However, deseases associated with α-granule secretion defects are rare and so this is an unlikely cause of the defects observed in this study.

A number of possibilities may have contributed to the higher false positive rates reported here, particularly as this study was performed as a true POC test in each Haemophilia Centre. As the test was used within busy clinical environments away from a well-controlled laboratory it is possible that the conditions of platelet activation were more poorly controlled in this study e.g. temperature and/or time of incubation resulting in the observed reduced platelet activity. It is possible given the length of the study that the long term stability of the agonists may have also been compromised even though the tests were performed within recommended expiry dates.

However, the even distribution of false positive results throughout the duration of the study would suggest that reagent stability was not the issue.

One of the challenges faced in this study was sample recruitment. Out of 122 RPFT kits distributed to 6 haemophilia centres only 53(43%) samples were analysed over a 1.5 year period. Furthermore, 57% of these samples were recruited from a single centre. This has not only undermined the overall objective and power of this study but also made the drawing of final conclusions more difficult. Future studies should therefore be performed with higher sample numbers and include multiple centres. Although adequate training of staff performing the RPFT test in each centre was performed it is possible that inter-individual variation between different centres and staff may have contributed to some of the discrepancies in this study. Furthermore, a more accurate comparison would be conducted by measuring samples that had been tested by using the RPFT both as a POC test and within the central laboratory at Birmingham simultaneously. This would validate whether the recommended POC conditions are being adhered to in each centre. This however, would not only be more costly and require fresh samples to be sent for RPFT analysis in the central laboratory but also introduce the variable of sample ageing. At the end of the study a questionnaire form was sent to all participated centres and were asked to provide a feedback and suggestions about their experience in using the RPFT kits. Overall the feedback was positive, however, addition training and availability of a training video to each centre was highly suggested. This might then improve sample handling and processing. (The full questionnaire form and the feedback responses are in appendix 4)

# CHAPTER EIGHT: GENERAL DISCUSSION

## 8.1 General discussion

Platelet function disorders (PFDs) are a heterogeneous group of disorders with a variety of causes and functional consequences. Individuals with PFDs often experience mild day-to-day bleeding symptoms which sometimes can have impact upon the quality of life and activities of daily living. Furthermore the bleeding can occasionally be life-threatening in response to an appropriate challenge such as surgery, injury, menstruation, and childbirth. As such diagnosis of underlying PFDs has important clinical implications as it not only facilitates correct treatment but appropriate management of patients over their life time.

This thesis has built on the previous GAPP project by further examining patients with suspected PFDs (Watson et al., 2013). The GAPP study uses a genotyping and platelet phenotyping approach with the ultimate aim of detecting the underlying causes of patients bleeding symptoms. An additional goal was to not only identify mutations in either known or novel platelet/MK genes to explain the cause of inherited platelet defects but to further understand basic platelet biology which may improve both diagnosis and treatment.

For the success of this study, multi-centre collaboration was crucial to obtain sufficient numbers of patients with good geographical coverage across the UK. Over the past 8 years the GAPP project have recruited nearly 1000 patients and healthy volunteers in 28 haemophilia referral centres, of these, 318 participants (206 patients and 112 healthy volunteers) were investigated during the period of this thesis. Similar to previous studies, patients were recruited based on the inclusion criteria as detailed in the methods section (chapter 2) including negative screening for coagulation defects and VWD. Exclusion of other haemostatic defects is also important to ensure that the bleeding symptoms presented are more likely to be platelet related. The bleeding

symptoms of recruited cases were also evaluated using the ISTH BAT score. Assessment of the ISTH BAT score in this study and in other two studies previously published including one from within the GAPP project demonstrated that the ISTH BAT score value although unable to predict the likelihood of diagnosing platelet function defect has proved to be a powerful tool in documenting bleeding symptoms among patients with excessive haemorrhage (Tosetto et al., 2011, Lowe et al., 2013).

A qualitative defect in platelet function was observed in 44% of patients (90/206) that were tested by lumi-LTA. This is slightly lower than a previously reported higher frequency of 58% platelet defects within the early stages of the GAPP study (Dawood et al., 2012). The high frequency of patients with undetectable platelet function disorder observed in this study was surprising. This however, may highlight the importance of the patients' clinical evaluation to ensure that other defects that are non-related to platelets are excluded, This may also suggest that there has been a selection bias in centres possibly sending their best cases during the early phases of GAPP but also shows the importance of developing more accurate screening techniques that give high negative predictive value to exclude patients with non-platelet related disorders.

The platelet categories of defects observed in this study (dense granular defects, Gi and Gq signalling defects, and cyclooxygenase pathway defects) were similar to those found in national guidelines for the laboratory investigation of platelet function disorders and those previously published within the GAPP project (Harrison et al., 2011, Dawood et al., 2012). There were however, a slight high number of cases with dense granular defects than previously reported (Watson et al., 2013). This observed increase of secretion defects in this study may have been

caused by inclusion of 39(19%) patients with thrombocytopenia. This group of patients have previously reported to have high numbers of defects in dense granules (Johnson et al., 2016).

Analysis of WES in this cohort has identified variants in known platelet genes that could suggest an underlying mutation in 59% of the cases studied. This is slightly lower than previous finding of 68% of the cases reported within the GAPP study (Johnson et al., 2016). However, as the latter study analysed patients with thrombocytopenia this might suggests that genetic mutations that underlie thrombopoiesis are more common than those related to platelet function. The use of platelet phenotyping in this study was also crucial to guide the genetic investigations by providing important supportive information to any candidate variants that were identified in genes previously known to cause platelet function defects. For example some candidate genes identified in this study were able to be linked either to Gi signalling or platelet secretion defects that were consistent with the patient phenotype. Moreover, 5 variants previously reported to be associated with platelet function in individuals with aspirin resistance were also identified in patients with defects in the cyclooxygenase pathway. However, the link between the defect in these candidate genes and the aspirin-like defect observed in these patients is still unclear. In general, the fact that genetic defects identified do match with patient's phenotype cannot be considered evidence for pathogenicity. Especially in this cohort where almost all of the candidate genes identified were of uncertain significance. However, due to the multifactorial nature of platelet function defects, a possible additional mutation might be present along with current identified variants. In combination, these might give rise to excessive bleeding and the platelet phenotype observed. Further investigation will still be required to confirm the contribution of these variants to the bleeding symptoms presented in these patients. Further investigation will still be required to confirm the contribution of these variants to the bleeding symptoms presented in these patients.

In contrast, platelet function testing results have also enabled the exclusion of number of variants that were not consistent with patients phenotype. For example, *MYH13*, *NBEAL2* and *LYST* were identified in patients but with no evidence of a functional secretion defect. Furthermore *ANKRD26* was identified in patients with normal platelet count, and *ITGA2B* was identified in two patients with no features of GT. This finding indicates that these candidates are unlikely to be the probable cause of defects in these patients.

The unavailability of family members in the majority of cases in this study has also significantly hampered the final identification of possible causative variants. As a result, the number of candidate genes identified per individual was high and in many cases multiple variants were consistent with patient phenotype. This made it difficult to select the most appropriate candidate gene without comparison with extended family members. This is exemplified by studying family two where the inclusion of four affected members guided selection of candidate genes to only one possible variant that was consistent with their phenotype. However, if additional unaffected family members had been included, it would have provided additional confirmation of this variant. This has further emphasised the importance of recruiting family members with and without bleeding symptoms.

Identification of the *VWF* gene in four patients was consistent with previous studies where genetic defects in platelet function have been identified in patients with VWD including defects in the $P2Y_{12}$ ADP receptor (Daly et al., 2009, Stockley et al., 2015). As such, this study has provided additional evidence of possible linkage between VWD and platelet function defects, and further supports the growing body of evidence that both VWD and PFD can sometimes be multifactorial in nature. This is consistence with the fact that a number of genetic mutations (e.g. TxA2 mutation;Asp304Asn and $P2Y_{12}$ mutation;Lys174Glu) previously identified in patients with bleeding diatheses are in heterozygous state. In the first example, the heterozygous

mutation (Asp304Asn) is unlikely on its own to be the cause of the increase bleeding as an extended family member with the same genetic mutation is exist without apparent history of bleeding (Mumford et al., 2010). In the second example, the heterozygous mutation (Lys174Glu) was identified in co-existence with type 1 VWD (Daly et al., 2009). Suggesting that, in both cases a possible combination of mutation might gives rise to excessive bleeding observed in these patients. Furthermore, one of the VWF mutation identified in this cohort (Tyr1584Cys) has recently been reported in an Italian family with coinheritance of mild haemophilia A and type 1 von Willebrand disease. Interestingly, only family members with both mutations are exhibiting excessive bleeding (Daidone et al., 2017). This finding has important clinical implications in the investigation of patients with either VWD or PFD. We anticipate that in the future genetic investigation of patients with VWD and PFD may allow assessment of their dual roles in the contribution to bleeding tendency in individuals with haemorrhagic disorders.

The lack of variant identification in the remaining 41% of cases could have two possible explanations, one is that genetic variant(s) causative of disease occur in genomic regions beyond the coding exome which was not analysed in this cohort. The second is that the variant(s) occur in novel genes not yet known to affect platelet function. However, the analysis of these genes were beyond the scope of this study but is being continued by the GAPP team.

The limitations of current methods for diagnosing inherited platelet function disorders and the urgent need for alternative new methods also encouraged us to assess a number of new techniques which are relatively easier to use, less time consuming and require smaller blood volumes compared to lumi-LTA. One of these is MEA which has the advantage of rapidly assessing platelet function in more physiological conditions using smaller quantities of blood. MEA has previously been reported to be useful for detecting severe PFDs (Awidi et al., 2009,

Albanyan et al., 2015) and it is widely applied for measuring antiplatelet therapy (Paniccia et al., 2015, Velik-Salchner et al., 2008). However, in this study MEA demonstrated a lack of sensitivity in identifying patients with mild bleeding symptoms associated with abnormal platelet function and platelet secretion defects (Al Ghaithi et al., 2017). As MEA is largely an end point technique, it is possible that lack of detailed information provided by lumi-LTA such as the kinetics, biphasic responses, reversible aggregation and lack of secretion measurement may have contributed to the lack of sensitivity observed in this study. It is also possible that the MEA may be more sensitive to pre-analytical variables associated with time delays in platelet function testing as it is a whole blood technique (Seyfert et al., 2007, Kaiser et al., 2012).

The T-TAS has the advantage of assessing both platelet function and coagulation pathways under different shear rates using a very small blood volume under more physiologically relevant conditions. A number of studies has demonstrated the utility of the T-TAS in monitoring antithrombotic drugs (Hosokawa et al., 2011, Hosokawa et al., 2013) and detecting defects in platelet function and coagulation abnormalities within humans and animal models (Ogawa et al., 2012, Minami et al., 2015). In this study T-TAS has demonstrated a good agreement with lumi-LTA in detecting platelet function defects in patients with mild bleeding disorders. With a very high NPV, the technique could possibly play a role as a screening test so that intensive platelet function investigations could be avoided. With an ability to detect platelet inhibition by various pathways, the T-TAS could also be a useful tool in monitoring antithrombotic therapy. Finally, the study showed the ability of T-TAS to detect thrombus formation in blood from mice. This could provide valuable assistance in rapidly characterising the phenotype of a variety of gene defects in mouse models of human disease.

The simplicity in the use of the RPFT and the use of a fixative with a small blood volume gives a clear advantage of this method as a potential POC test. This approach could therefore

significantly improve the diagnosis of platelet function testing which is currently restricted to specialized laboratories. An initial evaluation of the RPFT demonstrated a good agreement with lumi-LTA but was performed in the laboratory on GAPP samples and not as a POC test (Dovlatova et al., 2014). In this study however, RPFT showed a poor agreement with lumi-LTA with higher false positive rates measuring the expression of P-selectin. However, in contrast as CD63 expression yielded very similar results with the study performed by Dovlatova *et al,* (Dovlatova et al., 2014) the test still has potential as a POC test. The false positive sensitivity of P-selectin alone might indicate that the conditions of platelet activation such as temperature and/or time of incubation were more poorly controlled in the POC setting or that the receptor is more vulnerable to being shed under the conditions of the assay and subsequent transport to Birmingham. Also as higher sample numbers were analysed in multiple centres this could have contributed to further variability and increased false positive results

## Study limitations and Future work

The majority of candidates recruited in this study were individual patients. As such, recruitment of more family members with and without bleeding symptoms would significantly help define candidate gene identification and therefore provide more accurate confirmation of any plausible candidate variants identified.

Genetic analysis in this study was only focused on genes previously known to be associated with platelet defects. It is advised in the future to analyse variants in novel genes outside of the coding exomes especially on those individuals whom causative variants were not identified.

Evaluation of the T-TAS was carried out to determine defects only on patients with platelet function defects. It would be of interest in the future to analyse samples from patients with VWD and coagulation defects to demonstrate the utility of this technique in identifying

abnormalities in other haemostasis pathways as this makes the test a potential valuable screening tool for all bleeding disorders.

RPFT was performed as a true POC test in each Haemophilia Centre by non-laboratory professionals. Despite training this could suggest that conditions of platelet activation e.g. temperature and/or time of incubation might have been more poorly controlled. Furthermore, the recruitment of samples was very limited, and the majority were referred from a single centre which made it impossible to compare results between different centres. Future studies should therefore be performed with higher sample numbers and include multiple centres. Adequate training should also be provide to each centre including training videos that may help to improve sample handling and processing. It is also suggestive that more accurate comparison should be conducted by measuring samples that had been tested by using the RPFT both as a POC test and within the central laboratory simultaneously as an internal control to ensure that sample activation conditions are adequate.

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**Table 1. Bleeding score**

| **SYMPTOMS (up to the time of diagnosis)** | SCORE | | | | |
|---|---|---|---|---|---|
| | 0§ | 1§ | 2 | 3 | 4 |
| ***EPISTAXIS*** | No/trivial | - > 5/year<br>or<br>- more than 10 minutes | ***CONSULTATION ONLY**** | Packing or cauterization or antifibrinolytic | Blood transfusion or replacement therapy (use of hemostatic blood components and rFVIIa) or desmopressin |
| Cutaneous | No/trivial | For bruises 5 or more (> 1cm) in exposed areas | Consultation only* | Extensive | Spontaneous hematoma requiring blood transfusion |
| Bleeding from minor wounds | No/trivial | - > 5/year<br>or<br>- more than 10 minutes | ***CONSULTATION ONLY**** | Surgical hemostasis | Blood transfusion, replacement therapy, or desmopressin |
| Oral cavity | No/trivial | Present | ***CONSULTATION ONLY**** | Surgical hemostasis or antifibrinolytic | Blood transfusion, replacement therapy or desmopressin |

| | | | | | |
|---|---|---|---|---|---|
| GI bleeding | No/trivial | Present (not associated with ulcer, portal hypertension, hemorrhoids, angiodysplasia) | Consultation only* | Surgical hemostasis, antifibrinolytic | Blood transfusion, replacement therapy or desmopressin |
| Hematuria | No/trivial | Present (macroscopic) | ***CONSULTATION ONLY**** | Surgical hemostasis, iron therapy | Blood transfusion, replacement therapy or desmopressin |
| Tooth extraction | No/trivial or none done | Reported in ≤25% of all procedures, no intervention** | ***REPORTED IN >25% OF ALL PROCEDURES, NO INTERVENTION ****** | Resuturing or packing | Blood transfusion, replacement therapy or desmopressin |
| Surgery | No/trivial or none done | Reported in ≤25% of all procedures, no intervention** | ***REPORTED IN >25% OF ALL PROCEDURES, NO INTERVENTION ****** | Surgical hemostasis or antifibrinolytic | Blood transfusion, replacement therapy or desmopressin |
| Menorrhagia | No/trivial | Consultation only* or<br>- Changing pads more frequently than every 2 hours or | ***- TIME OFF WORK/SCHOOL > 2/YEAR OR<br>- REQUIRING*** | - Requiring combined treatment with antifibrinolytics and hormonal therapy<br>or | - Acute menorrhagia requiring hospital admission and emergency treatment<br>or |

| | | | | | |
|---|---|---|---|---|---|
| | | - Clot and flooding or<br>- PBAC score>100[#] | ***ANTIFIBRINOLYTICS OR HORMONAL OR IRON THERAPY*** | - Present since menarche and > 12 months | - Requiring blood transfusion, Replacement therapy, Desmopressin, or<br>- Requiring dilatation & curretage or endometrial ablation or hysterectomy) |
| Post-partum hemorrhage | No/trivial or no deliveries | Consultation only*<br>or<br>- Use of syntocin<br>or<br>- Lochia > 6 weeks | - Iron therapy<br>or<br>- Antifibrinolytics | - Requiring blood transfusion, replacement therapy, desmopressin<br>or<br>- Requiring examination under anaesthesia and/or the use of uterin balloon/package to tamponade the uterus | - Any procedure requiring critical care or surgical intervention (e.g. hysterectomy, internal iliac artery legation, uterine artery embolization, uterine brace sutures) |
| Muscle hematomas | Never | Post trauma, no therapy | ***SPONTANEOUS, NO THERAPY*** | Spontaneous or traumatic, requiring desmopressin or replacement therapy | Spontaneous or traumatic, requiring surgical intervention or blood transfusion |
| Hemarthrosis | Never | Post trauma, no therapy | ***SPONTANEOUS, NO THERAPY*** | Spontaneous or traumatic, requiring desmopressin or replacement therapy | Spontaneous or traumatic, requiring surgical intervention or blood transfusion |
| CNS bleeding | Never | - | **-** | Subdural, any intervention | Intracerebral, any intervention |

| | | | | | |
|---|---|---|---|---|---|
| Other bleedings^ | No/trivial | Present | ***CONSULTATION ONLY**** | Surgical hemostasis, antifibrinolytics | Blood transfusion or replacement therapy or desmopressin |

In addition to the guidance offered by the table, it is mandatory to refer to the text for more detailed instructions.

§ Distinction between 0 and 1 is of critical importance. Score 1 means that the symptom is judged as present in the patient's history by the interviewer but does not qualify for a score 2 or more

* Consultation only: the patient sought medical evaluation and was either referred to a specialist or offered detailed laboratory investigation

** Example: 1 extraction/surgery resulting in bleeding (100%): the score to be assigned is 2; 2 extractions/surgeries, 1 resulting in bleeding (50%): the score to be assigned is 2; 3 extractions/surgeries, 1 resulting in bleeding (33%): the score to be assigned is 2; 4 extractions/surgeries, 1 resulting in bleeding (25%): the score to be assigned is 1

# If already available at the time of collection

^ Include: umbilical stump bleeding, cephalohematoma, cheek hematoma caused by sucking during breast/bottle feeding, conjunctival hemorrhage or excessive bleeding following circumcision or venipuncture. Their presence in infancy requires detailed investigation independently from the overall score

Appendix 3 – List of known 357 platelet related genes

| Gene code | Gene code | Gene code | Gene code | Gene code |
|---|---|---|---|---|
| ABCA12 | BLOC1S1 | FERMT3 | ITGA2 | MYO18B |
| ABCB4 | BLOC1S2 | FGD3 | ITGA2B | MYO3A |
| ABCC4 | BLOC1S3 | FGR | ITGA5 | MYO5A |
| ABCG5 | BLOC1S4 | FHOD1 | ITGB1 | MYO5B |
| ABCG8 | BLOC1S5 | FLI1 | ITGB3 | NAPA |
| ACSL4 | BLOC1S6 | FLII | ITPR1 | NAPG |
| ACTN1 | BMP4 | FLNA | JAK2 | NBEA |
| ACVRL1 | BTBD9 | FMNL1 | JMJD1C | NBEAL2 |
| ADAMTS13 | BTK | FMNL3 | KIAA1109 | NFE2 |
| ADCY3 | C14orf133 | FYN | KIAA2018 | NIPSNAP3A |
| ADCY6 | C19orf55 | GATA1 | LAIR1 | NOTCH1 |
| ADCY7 | C20orf42 | GDI2 | LAT | NOX1 |
| ADORA2B | C6orf25 | GFI1 | LCP2 | NRG3 |
| ADRA2A | CD226 | GFI1B | LPAR1 | NSF |
| ADRA2B | CD36 | GNA12 | LTBP1 | NXF1 |
| ADRBK1 | CHD3 | GNA13 | LY6G6F | ORAI1 |
| AK3 | CLEC1B | GNAI1 | LYN | P2RX1 |
| AKT1 | CLEC4F | GNAI2 | LYST | P2RY1 |
| AKT2 | CNO | GNAQ | MAP2K2 | P2RY12 |
| ALOX12 | CSK | GNAZ | MAP2K4 | P2RY13 |
| ANKRD12 | CTTN | GNB2 | MAP3K9 | PDE2A |
| ANKRD18A | CYCS | GNB3 | MAPK1 | PDE3A |
| ANKRD18B | DAAM1 | GNG11 | MAPK13 | PDE4D |
| ANKRD26 | DIAPH1 | GNG12 | MAPK14 | PDE5A |
| ANKRD33 | DIAPH2 | GNG13 | MAPK8 | PDPK1 |
| AP3B1 | DIAPH3 | GNG5 | MDS1 | PDZD3 |
| AP3D1 | DNAH11 | GP1BA | MECOM | PDZK1 |
| AP3M1 | DNM1L | GP1BB | MKL1 | PEAR1 |
| AP3S1 | DNM2 | GP5 | MLK1 | PECAM1 |
| APC | DNM3 | GP6 | MLPH | PGM3 |
| ARHGAP1 | DTNBP1 | GP9 | MMP17 | PHOX2A |
| ARHGAP17 | EFNB1 | GRAP2 | MNX1 | PIK3CA |
| ARHGAP32 | EPHA4 | GRB2 | MPL | PIK3CB |
| ARHGAP6 | EPHB1 | GRK5 | MRPS34 | PIK3CD |
| ARHGDIA | ERG | GRK6 | MUC16 | PIK3CG |
| ARHGDIB | ETS1 | GUCY1A3 | MUC2 | PIK3R1 |
| ARHGEF12 | ETV6 | GUCY1B3 | MUTED | PIK3R3 |
| ARHGEF3 | EXOC1 | HBB | MYB | PIK3R5 |
| ARRB1 | F2R | HOOK3 | MYH10 | PLA2G4A |
| ASPN | F2RL3 | HOXA11 | MYH13 | PLA2G4C |
| BAK1 | FARP2 | HPS1 | MYH9 | PLCB2 |
| BCL2L1 | FCER1G | HPS4 | MYL9 | PLCB3 |
| BCOR | FCGR2A | HTR2A | MYLK | PLCG2 |
| BET1L | FERMT1 | INPP5D | MYLK2 | PLDN |

Appendix 3 – List of known 357 platelet related genes

| Gene code | Gene code | Gene code | Gene code |
|---|---|---|---|
| PPP1CA | RAP1GAP2 | STXBP1 | VPS52 |
| PPP1CB | RAP1GDS1 | STXBP2 | VPS8 |
| PPP1CC | RASGRP2 | STXBP3 | VWF |
| PPP1R12A | RBM8A | STXBP4 | WAS |
| PPP1R12C | RGS10 | STXBP5L | WDR66 |
| PPP1R14A | RGS18 | STXBP6 | ZFPMI |
| PPP1R2 | RGS19 | SUZ12 | |
| PRKACA | RGS20 | SYK | |
| PRKACB | RGS9 | SYTL3 | |
| PRKACG | RHOA | SYTL4 | |
| PRKAR1A | RHOC | TAL1 | |
| PRKAR2A | RHOF | TAOK1 | |
| PRKCA | ROCK1 | TBXA2R | |
| PRKCB | ROCK2 | TEC | |
| PRKCD | RUNX1 | TGFBR3 | |
| PRKCQ | SCAMP2 | THPO | |
| PRKD1 | SCAMP5 | TLN1 | |
| PRKG1 | SCFD1 | TLR2 | |
| PRKG2 | SELP | TMCC2 | |
| PTEN | SERPINE2 | TPM1 | |
| PTGIR | SH2B3 | TPM4 | |
| PTGS1 | SIRPA | TRAF4 | |
| PTK2 | SLC35D3 | TREML1 | |
| PTPN1 | SLC9A3R1 | TRPM7 | |
| PTPN11 | SLC9A3R2 | TTC37 | |
| PTPN12 | SLFN14 | TUBA3C | |
| PTPN18 | SMAD1 | TUBB1 | |
| PTPN2 | SMAD6 | UNC13A | |
| PTPN6 | SNAP23 | UNC13B | |
| PTPN7 | SNAP25 | VAMP2 | |
| PTPN9 | SNAP29 | VAMP3 | |
| PTPRA | SNAPIN | VAMP7 | |
| PTPRC | SNX1 | VAMP8 | |
| PTPRJ | SRA1 | VAV1 | |
| RAB27A | SRC | VAV2 | |
| RAB27B | SRF | VAV3 | |
| RAB38 | STIM1 | VPS11 | |
| RAB4A | STOM | VPS16 | |
| RABGGTA | STX11 | VPS18 | |
| RAC1 | STX12 | VPS33A | |
| RAF1 | STX2 | VPS33B | |
| RAI1 | STX4 | VPS39 | |
| RAP1B | STX6 | VPS41 | |
| RAP1GAP | STX7 | VPS4B | |