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Study of patients with suspected platelet-based bleeding disorders: a search for patients with a defect in the $P2Y_{12}$ ADP receptor

by

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Abstract

Mild platelet-based bleeding disorders are among the most complex bleeding disorders to understand, due to the absence of a 'gold standard' test for diagnosis and the significant overlap with the bleeding phenotype observed in healthy individuals. The work in this thesis is focussed on patients with a clinically diagnosed mild platelet disorder for which no acquired cause was identified by the referring expert clinician. ADP and thromboxane A_2 (TxA_2) are key secondary mediators of platelet aggregation and function in synergy to facilitate robust platelet activation in the event of vascular damage. Less than ten function-disrupting inherited gene defects in the ADP $P2Y_{12}$ receptor and only one in the platelet TxA_2 receptor have been reported, with none in the UK. Over a period of nearly 4 years, patients with a diagnosis of platelet dysfunction made at UK Comprehensive Haemophilia Care Centres were investigated using platelet aggregation and secretion assays alongside controls and reference curves to nine platelet agonists to exhibit an abnormal bleeding phenotype in response to different platelet agonists, focusing in searching on $P2Y_{12}$ receptor defects. In addition, the $P2Y_{12}$ ADP receptor from 148 subjects diagnosed with mild type 1 von Willebrand Disease (VWD) from the EU MCMDM-1VWD study was sequenced in view of the similarity in bleeding phenotype of patients with type 1 VWD and mild platelet disorders and the fact that both conditions show incomplete penetrance consistent with a multifactorial basis for each disorder. The sequencing was performed by Dr Martina Daly in Sheffield. The work in this thesis has led to the identification / characterisation of a patient who is homozygous for an early nonsense mutation in the $P2Y_{12}$ ADP receptor and two patients with heterozygous point mutations in the $P2Y_{12}$ ADP receptor (who also has type 1VWD) and in the TxA_2 receptor. In addition, I studied

platelet aggregation and secretion, along with a number of more specialised assays, in nearly 80 other patients during the course of the thesis and have subdivided these on the basis of the observed defect. Interestingly, in nearly one third of the patients, a platelet defect was not found.

PUBLICATIONS ARISING FROM THE WORK INCLUDED IN THIS THESIS

Andrew D Mumford, **Ban B Dawood**, Martina E Daly, Sherina L Murden, Mike D Williams, Majd B Protty, Jennifer C Spalton, Mark Wheatley, Stuart J Mundell, and Steve P Watson. A novel thromboxane A_2 receptor D304N variant which abrogate ligand binding in a patient with a bleeding diathesis. Blood 2009 in press.

Daly ME, **Dawood BB,** Lester WA, Peake IR, Rodeghiero F, Goodeve AC, Makris M, Wilde JT, Mumford AD, Watson SP, Mundell SJ. Identification and characterization of a novel $P2Y_{12}$ variant in a patient diagnosed with type 1 von Willebrand disease in the European MCMDM-1VWD study. Blood. 2009 Apr 23; 113 (17):4110-3.

Dawood BB, Wilde J, Watson SP. Reference curves for aggregation and ATP secretion to aid diagnose of platelet-based bleeding disorders: effect of inhibition of ADP and thromboxane A(2) pathways. Platelets. 2007 Aug; 18 (5):329-45.

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Abbreviations

AA- Arachidonic acid

AC- Adenylyl cyclase

ADP- Adenosine diphosphate

ATP- Adenosine triphosphate

cAMP- Adenosine 3’,5’-cyclic monophosphate

CRP- Collagen related peptide

COX- Cyclooxygenase

DAG- Diacylglycerol

DMSO- Dimethylsulfoxide

FcR- Fc receptor

GDP- Guanosine diphosphate

GEF- Guanine nucleotide exchange factor

GP- Glycoprotein

GPCR- G-protein coupled receptor

GTP- Guanosine-5'-triphosphate

IP_3- Inositol-1,4,5-trisphosphate

ITAM- Immunoreceptor tyrosine based activation motif

LAT- Linker for activation of T cells

NSAID- Non-steroidal anti-inflammatory drug

PAR- Protease-activated receptor

PBS- Phosphate-buffered saline

PG- Prostaglandin

PGI_2- Prostacyclin

PKC- Protein kinase C

PI-3 K- Phosphatidyl inositol-3 kinase

PIP_2- Phosphatidylinositol-4,5-bisphosphate

PLA_2- Phospholipase A_2

PLC- Phospholipase C

PPP- Platelet-poor plasma

PRP- Platelet-rich plasma

RT- Room temperature

SH2- Src homology 2

SH3- Src homology 3

SPD- Storage pool disease

TP- Thromboxane receptor

TRAPs- Thrombin receptor activating peptides

TxA_2- Thromboxane A_2

TXAS- Thromboxane A synthase

TxB_2- Thromboxane B_2

VWF- Von Willebrand factor

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 Platelet physiology

1.1.1 Platelet genesis, structure and function

Platelets are small discoid cells of 1–3 μm in diameter and with a mean volume of 7–11 fl. They circulate freely in the circulation and under normal circumstances do not form strong interactions with each other or to the vessel wall. They are generated in the bone marrow from proplatelet arms of their precursor, the megakaryocyte (Hartwig and Italiano, 2003), and since they are not created by cell division, they lack a nucleus. In humans, platelets have a life span of 8–10 days (Fritz et al., 1986) and this can be shorter in other species, such as mouse where the life span is in the order 5–6 days (Baker et al., 1997). It is estimated that a single human megakaryocyte can generate between 2000–3000 platelets. Platelets are able to synthesise only a very limited amount of protein from the low level of cytoplasmic mRNA that is carried over from the megakaryocyte. However, it is controversial as to whether this is of physiological significance, with the overall consensus being that this is unlikely to play a critical role. A constant cycle of megakaryopoiesis is required to maintain the normal plasma concentration of platelets of between 1.5–4x10^8/ml. A failure to maintain a sufficient platelet count, either because of reduced platelet production or increased platelet clearance, results in thrombocytopenia which in severe forms is associated with prolonged and even spontaneous bleeding.

The platelet has a highly specialised plasma membrane which is rich in a network of invaginations known as the open canicular system (OCS) through which exchange between the platelet cytoplasm and the surrounding plasma occur. Continuous with the OCS lies the dense tubular system which acts as a Ca^{2+} store and is the site for

enzymes that generate endoperoxides and thromboxanes from arachidonic acid. Below the plasma membrane is a microtubule ring which gives rise to the discoid shape of the non-stimulated resting platelet. The inner leaflet of the platelet plasma membrane contains a high level of phosphatidylserine (PS), a negatively charged phospholipid which is exposed on the platelet surface upon activation and promotes binding of coagulation proteins, a process known as procoagulant activity. The platelet plasma membrane is enriched in a wide range of glycoprotein receptors and G protein-coupled receptors that enable it to respond rapidly to stimulation and thereby mediate adhesive and aggregation events that support the formation of a vascular plug.

The platelet cytoplasm contains three types of secretory granules: dense granules, α-granules and lysosomes. α- Granules contain a range of proteins, including proteins that support thrombus formation e.g. fibrinogen and von Willebrand factor (VWF), chemotaxis of leukocytes and haematopoietic stem cells e.g. SDF-1, and which facilitate wound repair and support angiogenesis e.g. the growth factors VEGF and PDGF. Dense granules contain small molecules and divalent cations, including the powerful feedback platelet agonist ADP and the potent vasoconstrictor, serotonin (also known as 5-HT). Lysosomes contain a mixture of oxidases and other lytic enzymes, which may play a role in the innate defence system although further research is required to confirm this (White, 2007).

Vascular damage results in exposure of a network of proteins in the subendothelial matrix, including collagen and laminin, which mediate adhesion and powerful activation of platelets. In addition, platelets are activated by thrombin which is

generated through the coagulation cascade following exposure of tissue factor which is expressed on the surface of exposed and damaged cells. These pathways work together in the fight against excessive blood loss into the surrounding tissues following injury, a process known as haemostasis. Platelets are therefore critical elements in the maintenance of normal haemostasis working in concert with the coagulation cascade (Gibbins, 2004). Thrombosis, on the other hand, is an inappropriate haemostatic response that leads to a dramatic impediment of blood flow or even occlusion of blood vessels. By necessity, the haemostatic process is tightly controlled. Soluble inhibitors of coagulation and platelet inhibitors include those released from endothelium, namely PGI_2 and NO, in addition to those expressed on the endothelial cells like CD39 (the ecto-ADPase) (Kaczmarek et al., 1996) which removes ADP (a major platelet feedback mediator) and thrombomodulin which converts the procoagulant action of thrombin to an anticoagulant action. These limit thrombus growth and serve to prevent activation within healthy, intact vessels.

In addition to playing a critical role in haemostasis, platelets play an important role in inflammatory responses through release of a number of vasoreactive agents, including chemokines and CD40 ligand which facilitate adhesion of leukocytes to endothelial cells (Gear and Camerini, 2003). Platelets are also implicated in a number of other physiological responses, including liver repair and certain types of angiogenesis.

1.1.2 Platelet activation and release of major secondary mediators

Ordinarily, platelets are unable to interact with each other or with the endothelial cells that surround the vessel walls. However, following injury to the vasculature, platelets undergo 'explosive' activation in order to support the formation of a primary haemostatic plug and thereby prevent excessive bleeding. To achieve this, the subendothelial matrix proteins, most notably collagen, form a highly reactive surface for platelet binding and activation leading to shape change, adhesion, spreading, secretion of the major secondary mediators, ADP and TxA_2, aggregation and enhanced procoagulant activity, which together lead to the formation of a stable platelet-platelet aggregate. These events can be broken down to several defined steps (Watson and Harrison, 2005) (Figure 1.1):

1- **Tethering**: The participating proteins in this step vary according to the shear force in the vessels. Under the intermediate and high shear conditions that are found in arteries and arterioles, platelets are recruited to the site of injury through tethering to VWF which is immobilised on exposed collagen fibres. The platelet receptor for VWF that mediates tethering is the GPIb-IX-V complex, which is a unique receptor in the genome that is expressed only in platelets. The critical feature of the interaction between VWF and GPIb-IX-V is the very fast on-rate of association that enables the tethering to take place. However, this is not a stable interaction due to the fast off-rate of association. In contrast, under the low shear conditions that are found in venous system, platelets are able to bind directly to collagen and other matrix proteins such as laminin and fibronectin via platelets integrins namely $\alpha_2\beta_1$, $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$.

2- **Platelet activation and stable adhesion**: The tethering or capture of platelets enables the low affinity immunoglobulin receptor for collagen, GPVI, to mediate powerful activation of platelets thereby leading to activation of platelet integrins, including $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ which promote stable adhesion through binding to collagen and VWF, respectively. Platelet activation is also mediated through the GPIb-IX-V complex, although this generates a much weaker signal than that of GPVI, thereby questioning the physiological significance of this in the context of thrombus formation.

3- **Spreading:** In order to provide a solid base for the thrombus, platelets undergo a dramatic reorganisation of their cytoskeletons leading to formation of filpodia, lamellipodia and stress fibres that serve to increase their surface area and strengthen their adhesion to the exposed subendothelial matrix and to each other.

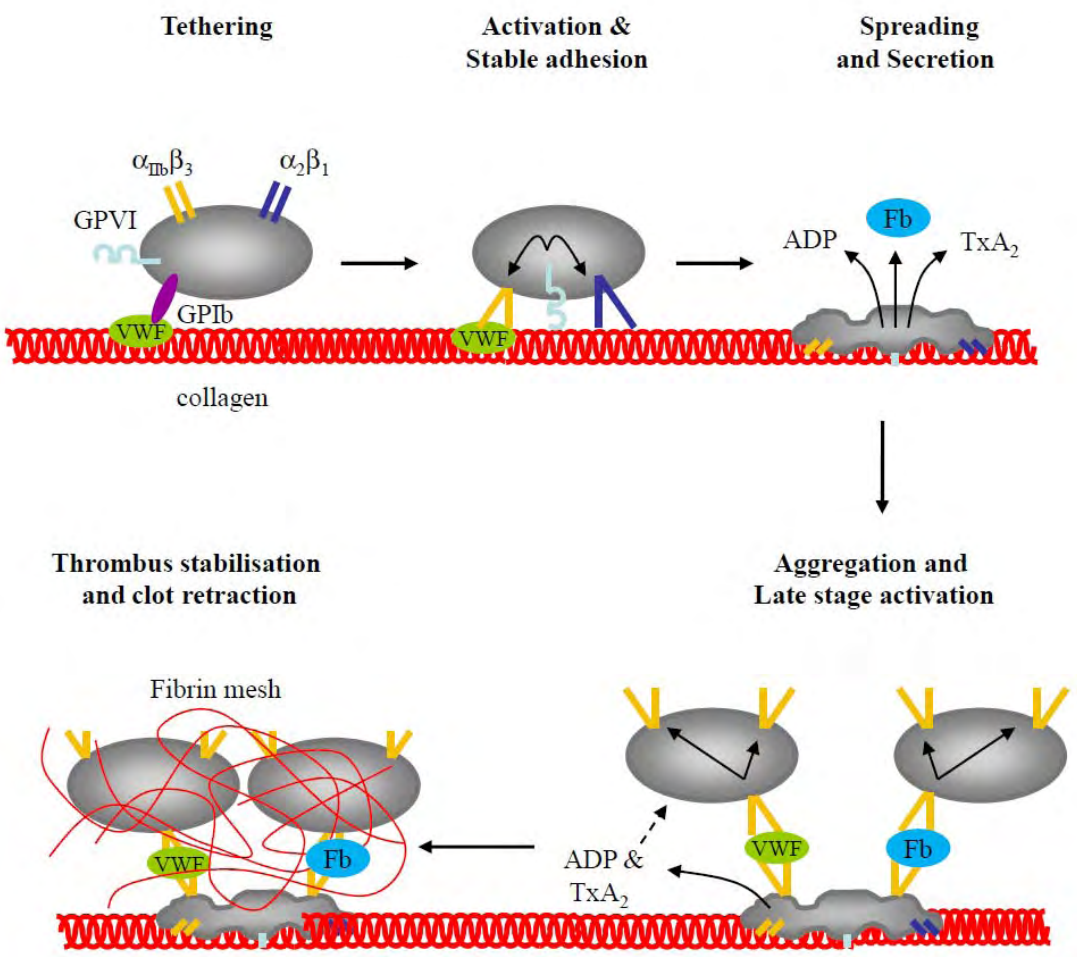
4- **Secretion and aggregation:** Activated platelets undergo rapid, powerful secretion of the contents of the dense and α-granules, and also synthesise thromboxanes from released arachidonic acid. ADP & thromboxane A_2 (TxA_2) are the two major feedback mediators that reinforce platelet activation and thereby help to recruit further platelets into the growing aggregate. Secretion of the α-granule proteins, fibrinogen and VWF, as well as supply of these proteins through the plasma, serves to support the growing platelet aggregate by mediating platelet-platelet interactions (aggregation) via the major platelet integrin, $\alpha_{IIb}\beta_3$, as well as recruitment of further platelets through a continued cycle of tethering (via VWF-GPIb-IX-V) and activation (via ADP and TxA_2).

5- **Thrombus stabilization and clot retraction**: The concomitant generation of thrombin through clotting serves to further activate platelets, in combination with

ADP and TxA_2, and also to convert fibrinogen to the insoluble product fibrin thereby stabilising the growing thrombus. Stabilisation is further reinforced by the process of clot retraction whereby the actin-myosin stress fibres connect to the fibrin mesh through integrin $\alpha_{IIb}\beta_3$.

6- **Procoagulant activity:** The platelets also provide a surface for the generation of coagulation factors through exposure of the procoagulant lipid, phosphatidlyserine. This therefore generates thrombin at the appropriate site to reinforce platelet activation and generate fibrin.

Figure 1.1
Platelet activation



Based on a platelet activation figure from Gemma Fuller's thesis/
University of Birmingham/2006

1.2 Platelet receptors and their agonists

The platelet surface is rich in a variety of receptors for a wide range of agonists, including G protein-coupled receptors (GPCR), immunoglobulins (Ig), integrins, ion channels, cytokine and leucine-rich receptors (Table 1.1). Each of these families of receptors is discussed below:

1.2.1 G protein-coupled receptors

G protein-coupled receptors (GPCRs) are heptahelical transmembrane proteins that mediate their effects through activation of heterotrimeric G proteins, consisting of α, β and γ subunits. In order to achieve their differing signalling effects, GPCRs use four distinct classes of G protein α-subunits, known as $G\alpha_s$, $G\alpha_i$, $G\alpha_q$ and $G\alpha_{12/13}$. Receptor activation promotes dissociation of GDP from the α-subunit thereby enabling binding of GTP. In turn, this causes the α-subunit to dissociate from the βγ-subunits such that it is able to bind to effector proteins, including adenylyl cyclase and phospholipase C β isoforms (see below). The βγ-subunits also target a number of effector proteins.

1.2.2 Gi family of G protein-coupled receptors

Gi proteins are so-named because they were originally shown to mediate inhibition of adenylyl cyclase, thereby opposing the activation of Gs proteins which stimulates formation of cAMP. However, they are now recognised to have a much broader range of effector targets, including PI 3-kinase. Platelets express several members of the Gi family of G proteins, with $G\alpha_{i2}$ and $G\alpha_z$ being the most abundant of this group. Platelets also express a low level of $G\alpha_{i1}$, although interestingly, a patient has been described with a mild bleeding defect attributed to a reduced level of this protein (Patel

et al., 2003). The two major Gi-coupled receptors on platelets are the $P2Y_{12}$ ADP receptor and the α_2-adrenoceptor, as discussed in further detail below. The unique aspect of these two receptors is that neither mediates activation of washed platelets and both mediate only partial activation of platelets in platelet rich plasma in the absence of feedback mediators. On the other hand, both receptors are able to synergise with Ca^{2+}-mobilising receptors to mediate powerful platelet activation. It is this synergy that makes ADP such a critical feedback agonist in regulating platelet activation under normal physiological circumstances.

1.2.2.1 The $P2Y_{12}$ ADP receptor

The $P2Y_{12}$ ADP receptor is the pharmacological target for clopidogrel, which alongside low dose aspirin, is one of the two major antiplatelet drugs used in the long term treatment of individuals who are considered to be at risk of thrombosis. However, several new $P2Y_{12}$ receptor antagonists are at an advanced stage of clinical development and are therefore likely to replace the use of clopidogrel in the near future, which suffers from variability in its effectiveness due to differential metabolism between individuals. Remarkably, clopidogrel was identified before the molecular cloning of the $P2Y_{12}$ receptor, which was achieved independently by two groups for human and rat isoforms in 2001 (Hollopeter et al., 2001, Takasaki et al., 2001). The human form has 342 amino acid residues and has a classical seven transmembrane spanning topography. It has a restricted expression in platelets and subregions of the brain (Hollopeter et al., 2001). The gene is located to chromosome 3 and consists of 3 exons and the coding sequence (1,028 Kb) located entirely within exon 3 (Figure 1.2)

The $P2Y_{12}$ receptor has been shown to be preferentially coupled to $G\alpha_{i2}$ but not to $G\alpha_z$ in platelets. This conclusion is based on studies in mutant mice that show $G\alpha_{i2}$ is a critical component of the signaling pathway for integrin activation by ADP (Jantzen et al., 2001). On the other hand, mice deficient in $G\alpha_z$ show impaired platelet activation by adrenaline. This has been shown to cause the mice to be more resistant to fatal thromboembolism following the injection of collagen together with adrenaline, despite the presence of other $G\alpha_i$ family members (Yang et al., 2000).

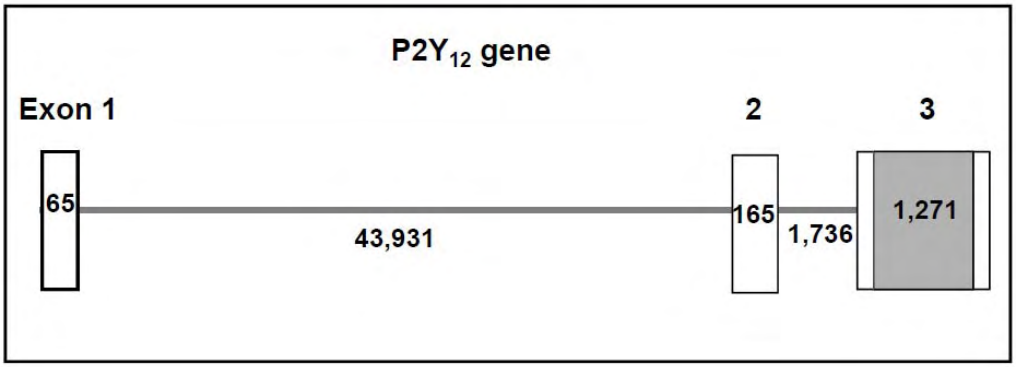
The mechanism underlying the ability of the $P2Y_{12}$ receptor to mediate platelet activation both on its own and in synergy with other receptors has been extensively investigated. Studies from the Haslam group in the late 1970s (Haslam et al., 1978), and more recent studies, have provided strong evidence that inhibition of adenylyl cyclase in otherwise non-stimulated platelets, is insufficient to mediate activation, thereby focussing attention on other signalling events (Daniel et al., 1999, Lova et al., 2003, Lova et al., 2002). A consensus has now emerged that the $P2Y_{12}$ ADP receptor mediates activation through regulation of PI3-kinase and Rap1b (Woulfe et al., 2002, Chrzanowska-Wodnicka et al., 2005) although the full sequence of events that underlie activation are not yet established (Figure 1.3).

Mice deficient in $P2Y_{12}$ show a marked increase in bleeding times (Andre et al., 2003). Further, the importance of $P2Y_{12}$ receptor in mediating platelet activation in haemostasis is emphasised by the identification of patients with mild platelet-based bleeding disorders that are associated with mutations in the receptor, as discussed in further detail in Section 1.4.2.1.

Table 1.1
Some platelets agonists and corresponding receptors

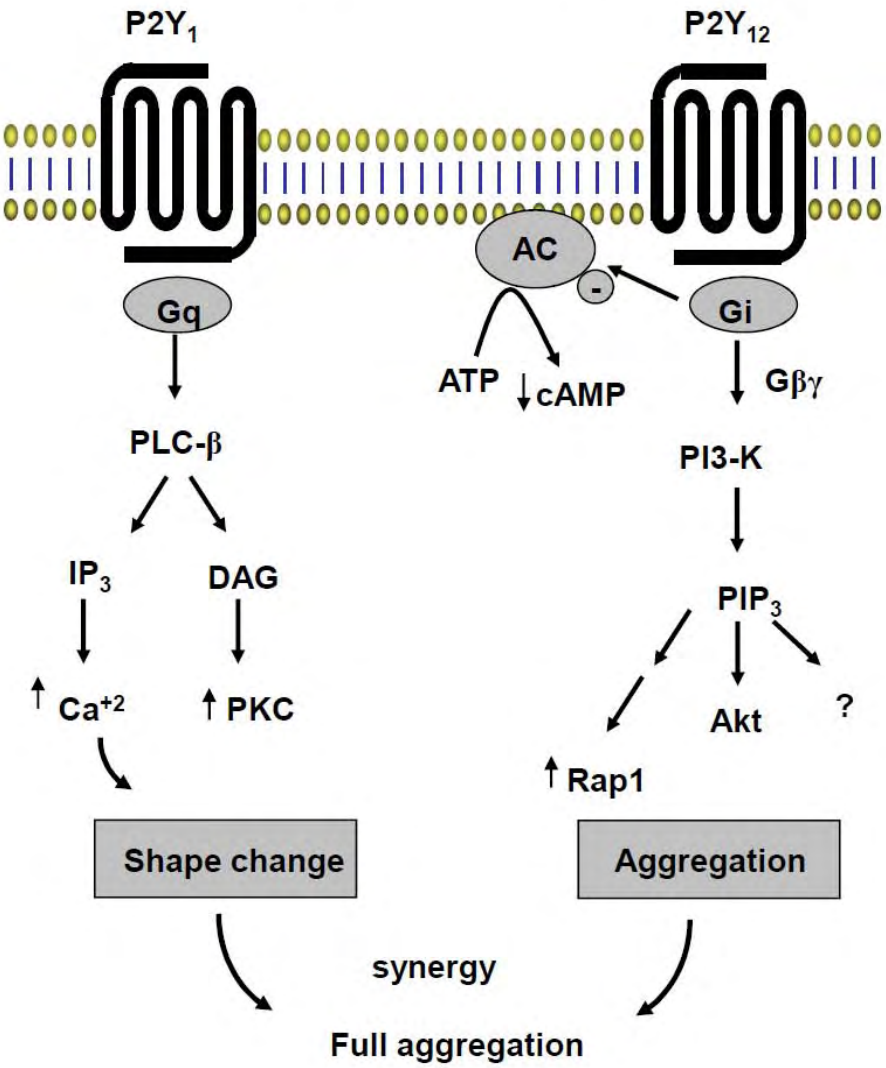
Platelet agonist	Platelet receptor	Receptor type	Signalling pathway
ADP	$P2Y_1$ $P2Y_{12}$	GPCR GPCR	G_q G_{i2}
Adrenaline	α_2-adrenergic	GPCR	G_z
TxA_2 mimetic	TP	GPCR	G_q, G_{13}
PAR1 peptide PAR4 peptide	PAR-1 PAR-4	GPCR GPCR	G_q, G_{13} G_q, G_{13}
Collagen	GPVI $\alpha_2\beta_1$	Ig Integrin	Src and Syk kinases (powerful) Src and Syk kinases (weak)
Collagen related peptide (CRP)	GPVI	Ig	Src and Syk kinases
Ristocetin	GPIb-IX-V GPIIb/IIIa ($\alpha_{IIb}\beta_3$)	Leucine-rich Integrin	Src and Syk kinases (weak)
Fibrinogen	GPIIb/IIIa ($\alpha_{IIb}\beta_3$)	Integrin	

Figure 1.2
The $P2Y_{12}$ gene



- Located on chromosome 3
- 47Kb
- Coding sequence 1,028 Kb located entirely within exon 3
- Transcript = 1502 bases
- Translation product = 342 amino acids

Figure 1.3
The ADP signalling cascade in platelets



$P2Y_1$ and $P2Y_{12}$: ADP receptors
AC: Adenylyl cyclase
PLC: Phospholipase C
PKC: Protein kinase C
Gi and Gq: G proteins
DAG: Diacylglycerol
IP3: Inositol-1,4,5-trisphosphate
PI3-K: Phosphatidyl inositol-3 kinase

1.2.2.2 The α_2-adrenoceptor

The α_2-adrenoceptor is believed to be the major receptor underlying the activation of platelets by adrenaline (Grant and Scrutton, 1979). Historically, the α_2-adrenoceptor had been proposed to be coupled in platelets to $G\alpha_{i2}$ based on studies of adenylyl cyclase inhibition in human platelets (Juska and Farndale, 1999, Simonds et al., 1989) , however since the generation of $G\alpha_z$ (Yang et al., 2000) and $G\alpha_{i2}$ knock out mice (Jantzen et al., 2001) it is now recognized that the α_2-adrenoceptor is preferentially coupled to $G\alpha_{iz}$ at least in mouse platelets. Adrenaline inhibits cAMP accumulation in platelets treated with PGI_2 or other activators of adenylyl cyclase, and does not induce a detectable calcium response in the absence of additional agonist (Woulfe, 2005). In addition, adrenaline stimulates various PI3-K dependent pathways, among them activation of Akt and Rap1 (Kim et al., 2004, Woulfe et al., 2002).

The physiological role of the α_2-adrenoceptor in the activation of human platelets is unclear. There is evidence that a low level of adrenaline is present in platelet dense granules and that it is released upon activation thereby raising the possibility that adrenaline could be an important feedback agonist. However the general consensus is that the $P2Y_{12}$ ADP receptor is the major feedback receptor activating the Gi family of G proteins in platelets, based on studies using $P2Y_{12}$ receptor-specific antagonists. On the other hand, studies in 'knockout' mice deficient in the α_2-adrenoceptor have provided evidence that the adrenoceptor contributes to normal haemostasis, as demonstrated by an increase in tail bleeding (Pozgajova et al., 2006).

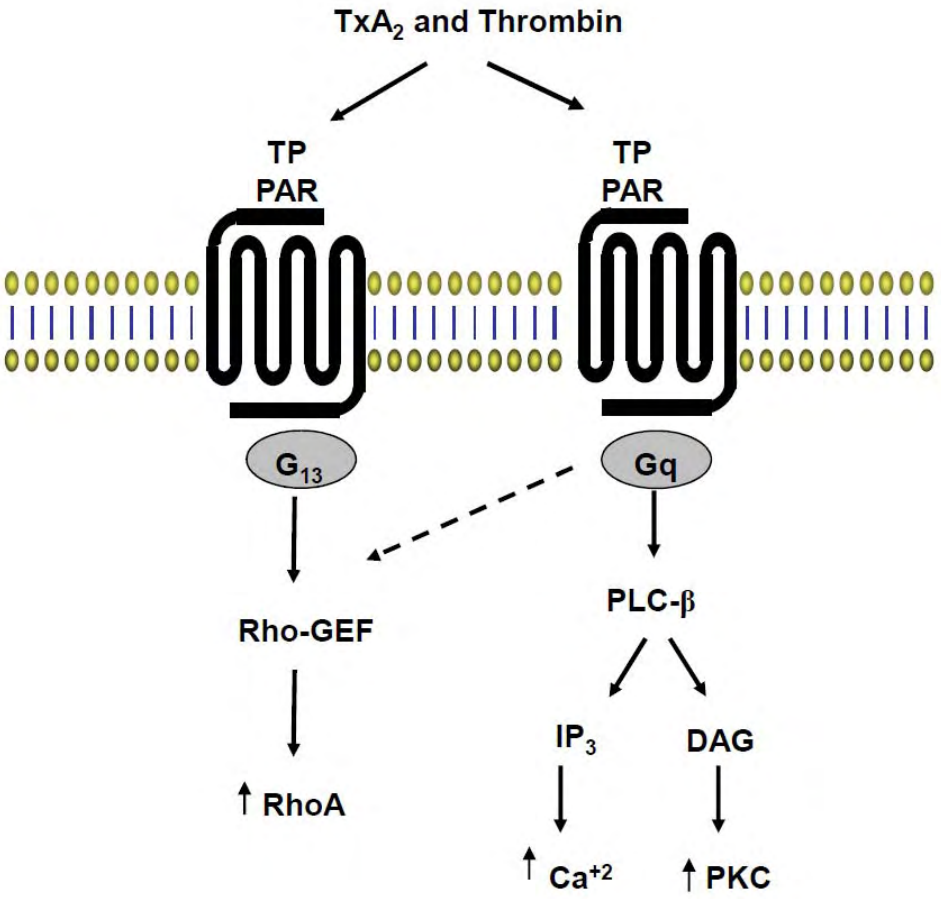
Further work is needed to fully characterise the molecular basis and the physiological role of this receptor in human platelet activation.

1.2.3 G_q protein-coupled receptors

The G_q family of G proteins has two members, G_q and G_{11}, but only the former is expressed in platelets (Offermanns et al., 1997). G_q-coupled receptors mediate platelet activation through regulation of PLCβ isoforms, thereby generating the second messengers inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG), which mobilise Ca^{2+} from intracellular stores and activate protein kinase C (PKC), respectively. PLCβ2 is the major isoform that is expressed in human platelets, although PLCβ3 is also expressed (Lee et al., 1996). The major G_q-coupled receptors in human platelets that play a physiological role in supporting platelet activation are the $P2Y_1$ ADP receptor, the TxA_2 receptor (TP) and the two thrombin receptors, PAR1 and PAR4 (Figures 1.3 and 1.4). Mouse platelets, in contrast, do not express PAR1, but express PAR3 which facilitates activation of PAR4 by thrombin but does not signal on its own.

Many receptors which couple to G_q also couple to the $G_{12/13}$ family of heterotrimeric G proteins, which are also implicated in mediating platelet activation through the p115 GTP exchange factor, which mediates activation of the small G protein, RhoA. Human and mouse platelets are believed to express only the G_{13} member of this family. Studies from the groups of Nieswandt, Offermans and Kunapuli (Nieswandt et al., 2002, Moers et al., 2003, Moers et al., 2004, Dorsam et al., 2002) using G_{13}-deficient mouse platelets and other approaches have demonstrated that this G-protein is able to mediate platelet activation downstream of the PAR4 thrombin receptor, although it is controversial as to whether it contributes to signalling by the other G_q-coupled receptors.

Figure 1.4
The TxA_2 and Thrombin signalling cascade in platelets



TP: TxA_2 receptor
PAR: Protease-activated receptor
PKC: Protein kinase C
Rho: GTPase

G_{13} and Gq: G proteins
DAG: Diacylglycerol
IP3: Inositol-1,4,5-trisphosphate
GEF: Guanine nucleotide exchange factor

1.2.3.1 The $P2Y_1$ ADP receptor

The $P2Y_1$ Gq-coupled receptor plays a critical role in synergising with the $P2Y_{12}$ ADP receptor in mediating full aggregation of platelets to ADP (Hechler et al., 1998, Savi et al., 1998, Jin and Kunapuli, 1998, Gachet, 2006), as is illustrated in the first Results Chapter in this thesis (Chapter 3). Remarkably, this synergy happens despite the relatively low level of expression of the $P2Y_1$ receptor, which is in the order of 150 copies per platelets, thereby emphasising the powerful ability of the $P2Y_{12}$ receptor to synergise with Ca^{2+}-mobilising receptors. Thus, platelet activation by ADP is mediated through the combined action of the $P2Y_1$ and the $P2Y_{12}$ receptors, although of course *in vivo* the $P2Y_{12}$ receptor is able to synergise with any other Ca^{2+}-mobilising receptor. Thus, because of this redundancy, the physiological role of the $P2Y_1$ receptor in mediating platelet activation is unclear, and indeed at the present time no patients have described with a bleeding defect associated with a defect in this receptor. On the other hand, the ability of the $P2Y_1$ receptor to synergise with the $P2Y_{12}$ receptor could reflect a role in the events that *initiate* platelet activation following exposure to ADP. Moreover, studies in mouse platelets have suggested a potential role of the $P2Y_1$ receptor in mediating thrombus formation (Leon et al., 1999, Lenain et al., 2003) giving rise to the proposal that $P2Y_1$ receptor antagonists may represent a novel class of antithrombotics that do not cause bleeding.

1.2.3.2 The thromboxane A_2 receptor

Human platelets express a single thromboxane receptor which has been reported to be alternatively spliced in the C-terminus to two isoforms, α and β, although the significance of this is unclear, and indeed there is evidence that only the TPα form is expressed on the platelet surface (Habib et al., 1999). Both isoforms are encoded by the same gene on chromosome 19, which has four exons and gives rise to classical seven transmembrane spanning confirmation of 343 amino acids. The coding region for the receptor is located in exon 2 and 3 (Nusing et al., 1993) (Figure 1.5).

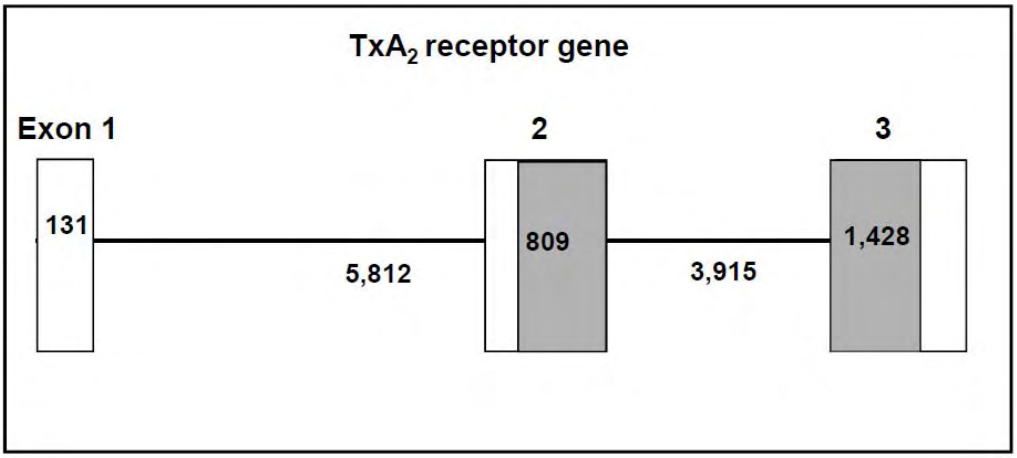
The thromboxane receptor is activated by TxA_2 which is formed from the arachidonic acid that is liberated by the action of cytosolic phospholipase A_2 ($cPLA_2$). $cPLA_2$ is activated by elevation of intracellular Ca^{2+} downstream of Gq-coupled and tyrosine kinase-linked surface receptors. The thromboxane receptor mediates powerful platelet activation in synergy with the $P2Y_{12}$ ADP receptor as discussed above, and it is this synergy that enables it to play such a powerful role as a feedback receptor controlling platelet activation. Indeed, the critical role of the thromboxane receptor in mediating platelet activation is illustrated by the powerful antithrombotic action of low dose aspirin, which selectively inhibits platelet cyclooxygenase-1 (COX-1), thereby preventing thromboxane formation (platelets unlike most other cells cannot make new protein, thereby enabling the use of a reduced aspirin dosage). Further, a role for the thromboxane receptor in haemostasis is indicated by the presence of a significant number of patients with mild, platelet-based bleeding defects that are associated with an impairment in thromboxane formation, although in all but one of these patients, a molecular defect has not been identified (see Chapter 5 for further discussion). The

importance of the thromboxane receptor is further illustrated by the prolonged tail bleeding time in 'knockout' mice that fail to express the receptor (Thomas et al., 1998).

1.2.3.3 The PAR1 and PAR4 thrombin receptors

Thrombin is a soluble serine protease which activates two members of the PAR family of receptors on human platelets, namely PAR1 and PAR4 and two members on mouse platelets, namely PAR3 and PAR4. Whilst PAR1 and PAR4 are both able to activate human platelets, with PAR1 believed to play the major role, only PAR4 is able to activate mouse platelets, with the role of PAR3 being to facilitate the interaction of thrombin with PAR-4 (Kahn et al., 1998, Xu et al., 1998, Brass, 2003). Thrombin cleaves the N-terminal of PAR1 and PAR4, exposing a 'tethered' peptide ligand that mediates receptor activation. Significantly, it is possible to mimic the 'tethered' ligand for each receptor using short thrombin receptor activating peptides (TRAPs) that mediates activation of PAR1 or PAR4 without the need for receptor cleavage. Moreover, these peptides can be used to activate the platelet in platelet rich plasma, whereas the use of thrombin under these conditions generates a platelet-rich clot due to cleavage of fibrinogen to fibrin (Coughlin, 2005). Being a very powerful agonist, thrombin plays an essential role in activating platelets, just as it does in the formation of the fibrin clot. Once ligated, thrombin receptors can activate the heterotrimeric G proteins Gq and G_{13}, which regulate PLCβ and Rho kinase, respectively.

Figure 1.5
The TP receptor gene



- Located on chromosome 19
- 12.154 Kb
- Coding sequence 1,032 Kb located within exons 2 and 3
- Transcript = 2428 bases
- Translation product = 343 amino acids

1.2.4 Glycoprotein receptors

The platelet membrane is rich in glycoproteins that play a crucial role in the activation and aggregation of platelets. Among these is the major signalling receptor for collagen, the GPVI-FcR γ-chain complex, the VWF tethering receptor, GPIb-IX-V, and the major platelet integrin, $\alpha_{IIb}\beta_3$, which is a receptor for a number of adhesion proteins including fibrinogen and VWF.

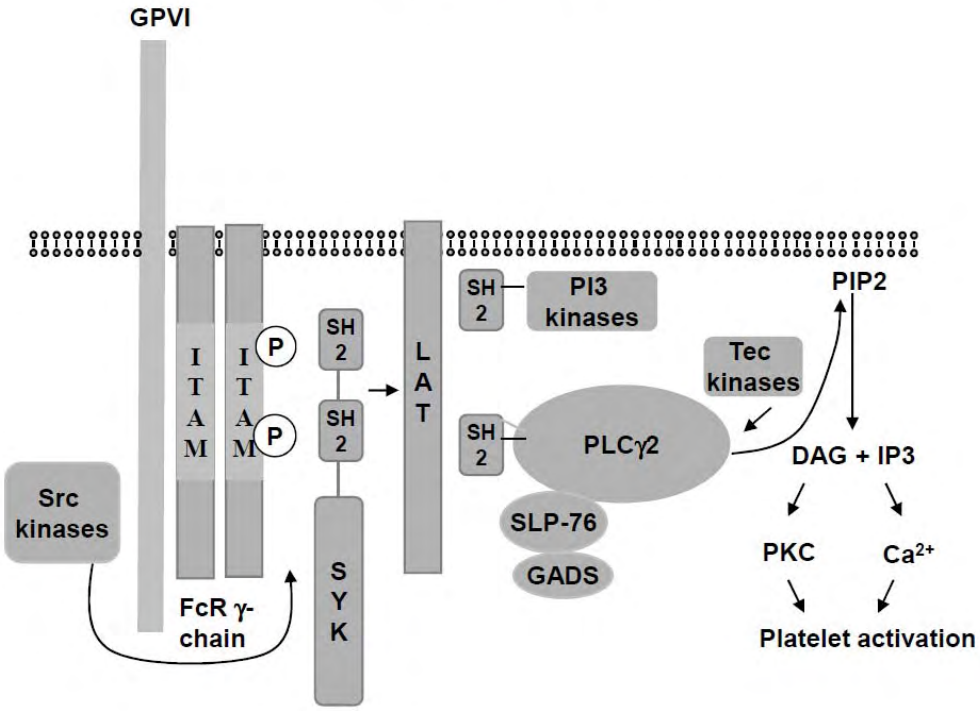
1.2.4.1 Collagen receptors (GPVI and $\alpha_2\beta_1$)

GPVI was first proposed as a major collagen receptor on platelets in 1987 following identification of a patient with a bleeding tendency and an autoimmune thrombocytopenia that was brought about by an autoantibody to GPVI and which resulted in defective platelet aggregation to collagen. However, it was not for another ten years that GPVI became widely recognised as the major signalling receptor for collagen following the demonstration of its association with the FcR γ-chain in the platelet membrane (Gibbins et al., 1996). The latter contains an immunotyrosine-based activation motif (ITAM) that is essential for signalling following clustering of GPVI by collagen (Watson and Gibbins, 1998). GPVI was subsequently cloned by the group of Clemetson and shown to be a member of the immunoglobulin (Ig) family of surface receptors, with two extracellular Ig domains. The majority of, but not all, studies have shown that the level of expression of GPVI is tightly regulated on the platelet surface at around 4,000–6,000 copies per platelet (Best et al., 2003, Chen et al., 2002). Collagen has a relatively low affinity for GPVI, but its interaction with the glycoprotein receptor is facilitated by binding to the other major receptor for collagen on the platelet surface, the integrin $\alpha_2\beta_1$ in a process known as the modified two-site,

two-step model (Nieswandt and Watson, 2003). The significance of GPVI in mediating platelet activation is illustrated by the increase in bleeding time or rebleeding of FcR γ-chain and GPVI deficient mice, respectively, as measured using a tail bleeding assay (Kalia et al., 2008, Kato et al., 2003).

Crosslinking of GPVI mediates platelet activation through sequential activation of Src and Syk family kinases in a pathway that shares many similarities with those used by immune receptors, such as the T- and B-cell antigen receptors. The critical step in this signalling cascade is Src kinase-mediated phosphorylation of the FcR γ-chain ITAM enabling binding of Syk through its tandem SH2 domains and initiation of a downstream pathway that leads to powerful activation of PLCγ2 (Figure 1.6). The integrin $\alpha_2\beta_1$ mediates a much weaker degree of activation of PLCγ2 downstream of Src and Syk kinases, but independent of an ITAM. The synthetic collagen, CRP, which consists of a repeat glycine-proline-hydroxyproline sequence, can be used to activate GPVI independent of $\alpha_2\beta_1$ (Jarvis et al., 2002). From the physiological point of view, Nieswandt et al have shown that mice injected with GPVI antibody are protected from lethal thrombogenic infusions of collagen and adrenaline due to the disappearance of GPVI from their platelet surface (Nieswandt et al., 2001) emphasizing the importance of the Ig receptor in collagen-induced platelet activation.

Figure 1.6
The GPVI signalling cascade



DAG: Diacylglycerol
GADS: Adaptor protein
IP3: Inositol-1,4,5-trisphosphate
ITAM: Immunoreceptor tyrosine-based activation motif
LAT: Linker for activation of T cells
PLC: Phospholipase C
PIP2: Phosphatidylinositol-4,5-bisphosphate
PLC: Phospholipase C
SLP: SH2 domain containing leukocyte protein
Tec: Tyrosine kinase

1.2.4.2 VWF receptor-GPIb-IX-V

The GPIb-IX-V receptor is one of the most highly expressed of the glycoprotein receptors on the platelet surface, with an estimated 25,000 copies per platelet, each made up of two subunits of GPIbα, GPIbβ and GPIX and one subunit of GPV. However, this stoichiometry has recently been challenged as it was demonstrated that Ibα is covalently connected to 2 Ibβ subunits (Luo et al., 2007). As discussed above, GPIb-IX-V plays a critical role in mediating tethering of platelets to immobilised VWF (via collagen) at intermediate and high rates of shear. Qualitative and quantitative defects in the GPIbα, GPIbβ and GPIX components of this receptor give rise to the hereditary disorder, Bernard-Soulier syndrome, which is characterised by large platelets, thrombocytopenia and severe bleeding. The GPIbα subunit also binds to thrombin and, in this way, facilitates its interaction with the PAR1 and PAR4 GPCRs described above (De Marco et al., 1994, Jamieson and Okumura, 1978, Dormann et al., 2000). Binding of VWF to GPIb-IX-V also generates weak intracellular signalling that leads to activation of integrin $\alpha_{IIb}\beta_3$, although the significance of this is unclear (Savage et al., 1996, Yap et al., 2000).

1.2.4.3 The integrin $\alpha_{IIb}\beta_3$

Integrins are heterodimeric proteins consisting of α- and β-subunits. The integrin $\alpha_{IIb}\beta_3$ is the most expressed protein on platelets, where it is present at between 80,000–120,000 copies per platelet, and is restricted to platelets and megakaryocytes. The integrin binds to several adhesion proteins, including VWF and fibrinogen, that mediate cross-linking between platelets to form an aggregate. Activation of the integrin by surface receptors is known as inside-out signalling, whereas clustering of the

integrin by its ligands leads to the generation of outside-in signals that support many aspects of platelet activation including spreading. Patients with mutations or deletions in the genes encoding for either of the two subunits exhibit severe bleeding, a condition known as Glanzmann Thrombasthenia. A similar condition is seen in mice deficient in the αIIb-subunit (Emambokus and Frampton, 2003).

1.2.5 Other receptors

Platelets express many other receptors that have been shown to mediate platelet activation, although for many of these their physiological significance is uncertain or believed to occur at the level of the megakaryocyte. Several examples of these are are shown below, although the list is not exhaustive:

i) the low affinity immune receptor FcγRIIA and the C-type lectin receptor, CLEC-2, whose endogenous ligand is podoplanin, activate platelets through an ITAM or an ITAM-like pathway (Suzuki-Inoue et al., 2007, Fuller et al., 2007).

ii) platelets express a number of further G protein-coupled receptors, including the CXCR4 receptor for SDF-1 which is implicated in the regulation of megakaryocyte migration in the bone marrow (Dhanjal et al., 2007).

iii) the $P2X_1$ receptor is a ligand-gated ion channel for ATP (Mahaut-Smith et al., 2000). Activation of $P2X_1$ mediates rapid entry of Ca^{2+} into the platelet and has been proposed to play a critical role in initiating platelet activation. This receptor undergoes rapid desensitization and this has hampered its investigation using washed platelets.

iv) c-Mpl is a receptor for thrombopoietin, which is recognised as the major cytokine regulating the differentiaton and maturation of megakarocytes in the bone marrow. In addition, TPO potentiates the activation of platelets by a wide variety of G protein-coupled and tyrosine kinase-linked receptors (Rodriguez-Linares and Watson, 1994, Rodriguez-Linares and Watson, 1996). c-Mpl signals through the Janus kinase, JAK2, although the mechanism through which it potentiates platelet activation is unclear. Recently, a V617F mutation in JAK2 has been identified in approximately 50% of patients with essential thrombocythaemia, which is associated with a selective

increase in platelet count (Kralovics et al., 2005, Baxter et al., 2005, Levine et al., 2005).

1.3 Bleeding disorders: acquired and inherited

Bleeding disorders constitute a wide range of disorders in which the patients tend to bleed excessively. Such bleeding may be severe, with bleeding episodes starting in early childhood, or mild, discovered after a haemostatic challenge like surgery, dental procedures, or trauma. The bleeding symptoms may range from nosebleeds, easy bruising or heavy menstrual periods to bleeding into joints, muscles and deep tissues.

In normal healthy individuals, a complex interaction between vessel walls, platelets, coagulation and fibrinolytic proteins occurs in order to maintain the blood within the vascular compartment in a fluid state or to permit thrombus formation at sites of vessel injury. Bleeding disorders occur when any of the above components is abnormal, deficient or dysfunctioning and can be either acquired or inherited. Acquired bleeding disorders are more frequent than the inherited disorders and range from acquired mutations, immune thrombocytopenia, or excessive medication.

1.3.1 Coagulation factor defects

Acquired coagulation factor defects are more frequent than the inherited ones and since many coagulation factors are produced in the liver, several of which are vitamin K dependent, acquired disorders can be caused by liver disease or vitamin K deficiency. Inherited bleeding disorders attributed to ten coagulation factors have been described, and tend to be caused by a deficiency or dysfunction of a single or combined coagulation factor or clotting component. The most common of these are the haemophilias and von Willebrand disease, while others are rare (Laffan and Lee, 2005). Haemophilia, is a group of hereditary genetic disorders that historically existed in three forms:

Haemophilia A (classic haemophilia), is the most common sever inherited bleeding disorder. It has a sex-linked pattern of inheritance, due to the presence of the gene on the X chromosome, with nearly one third resulting from spontaneous mutation. The incidence is 30-100/ 10^6 population. All clinical features of haemophilia A which includes recurrent painful joint bleedings and muscle haematomas are due directly or indirectly to lack of the clotting Factor VIII.

Haemophilia B (Christmas disease), is due to Factor IX deficiency. The inheritance and clinical features are identical to haemophilia A and the two disorders are normally distinguished by specific coagulation factor assays and confirmed by gene sequencing.

Factor XI deficiency (previously called **Haemophilia C**) is an autosomally inherited condition which is particularly common in Ashkenazi Jews caused by mutations of the Factor XI gene on chromosome 4. In comparison with haemophilia A and B, bleeding manifestations in FXI deficiency are much less predictable, even in severe deficiency and is not related to the FXI level (Bolton-Maggs et al., 2004)

.1.3.2 Von Willebrand disease

Von Willebrand disease (or disorder; VWD) is a bleeding disorder of primary haemostasis caused by a complete or partial defect in the concentration, structure, or function of von Willebrand factor (VWF). The estimated prevalence of VWD is 0.8–1.6 per hundred (Rodeghiero et al., 1987, Werner et al., 1993, Miller CH, 1987) making it the most frequent inherited bleeding disorder. The first kindred were identified in 1926. The bleeding events that may suggest VWD includes prolonged epistaxis, cutaneous haemorrhage and bruising, prolonged bleeding from trivial wounds or dental extraction or surgery, in addition to menorrhagia and other mucosal bleedings. The disorder is found to have a greater penetrance in female patients due to increased haemostatic pressures of menstruation and pregnancy.

In 1994, Sadler (Sadler, 1994) published recommendations for the classification of VWD and in 2006 the subcommittee of VWD reevaluated the classification (Sadler et al., 2006), which remains primarily clinical, to facilitate the diagnosis, treatment and counselling of patients. VWD is classified into three primary categories: Type 1 relates to a quantitative partial deficiency, type 2 relates to qualitative defects, and type 3 relates to a virtually complete deficiency of VWF. Type 2 VWD is further divided into four secondary categories. Type 2A includes variants with decreased platelet adhesion caused by selective deficiency of high-molecular-weight VWF multimers. Type 2B includes variants with increased affinity for platelet glycoprotein Ib. Type 2M includes variants with markedly defective platelet adhesion despite a relatively normal size distribution of VWF multimers. Type 2N includes variants with markedly reduced factor VIII binding capacity.

Acquired von Willebrand syndrome is due to an acquired defect in VWF that is associated with a variety of underlying disorders or pharmaceutical agents, and result in clinical symptoms similar to VWD. Federici et al (Federici et al., 2000) by using data from an international registry showed that acquired von Willbrand disease was usually associated with lymphoproliferative and myeloproliferative disorders, neoplasia, immunologic and other miscellaneous conditions. The main mechanisms that account for it are circulating autoantibodies to VWF, adsorption of VWF onto tumoral or activated cells and proteolytic degradation of VWF.

Von Willebrand disorders that have similar symptoms to bleeding disorders of platelets aetiology are described below:

VWD type 1 is defined as an inherited bleeding disorder because of quantitative deficiency of VWF. The molecular mechanism in the large majority of type 1 VWD cases is unknown. A small number of missense mutations have been reported. In classical type 1 VWD , the inheritance is considered to be autosomal dominant and linked to the VWF gene (Laffan et al., 2004).

VWD type 2B is usually inherited as an autosomal dominant trait although some apparently recessive cases have been described. It results from mutations within the anterior region of exon 28, corresponding to the VWF A1 domain, which contains the GPIb binding site hence increasing the affinity of this mutant form of VWF for spontaneous binding to platelets in vivo, followed by clearance of the VWF and platelets. The minimum criteria for diagnosis include a personal or family history of

mucosal bleeding and enhancement of platelet aggregation in the presence of low-dose of ristocetin and by exclusion of platelet-type, or Pseudo, VWD.

Platelet-type, or Pseudo, VWD: is a rare disorder that is transmitted as an autosomal dominant trait and clinically resembles type 2B VWD. It is not due to defects of VWF but is caused by mutations in the platelet GPIbα gene, a gain in the functional phenotype that increases its affinity for VWF, leading to the binding of the biggest VWF multimers which are cleared from the circulation leading to bleeding. Shim K et al (Shim et al., 2008) have also reported that an increase in ADAMTS13 cleavage of platelet-bound VWF under shear may also contribute to the cause of the disease. Platelet size can be increased in platelet-type VWD and may be associated with moderate thrombocytopenia (Nurden and Nurden, 2008). The platelet hyperresponsiveness may be demonstrated with low concentrations of ristocetin. Whereas normal platelets show little or no aggregation at ristocetin concentrations of 0.5 mg/ml or lower, patient platelets show significant binding of VWF, together with strong aggregation (Miller and Castella, 1982).

1.3.3 Blood vessel wall disorders

The vessel wall bleeding disorders are a heterogeneous group characterized by easy bruising and spontaneous bleeding from small vessels which manifest in the skin in the form of petechiae or ecchymoses or both. The underlying abnormality is either in the vessels themselves or in the perivascular connective tissue. These disorders may be inherited or acquired.

Hereditary haemorrhagic telangiectasia is an uncommon disorder, transmitted as an autosomal dominant trait, presents during childhood with dilated microvascular swellings that become more numerous in adult life. The blood moves from the high pressurized arteries into the thinner walled veins, bypassing the capillaries, leading to arteriovenous malformations known as telangiectases. The extra pressure in these vessels, results in compression or irritation of adjacent tissues and frequent episodes of severe bleeding (haemorrhage). Nosebleeds are common, however, more serious haemorrhages may arise from telangiectasia in such vital organs as the lung, brain or the liver.

Acquired vascular defects vary in severity. Some of these defects are due to defective vascular supportive tissues especially collagen, as demonstrated by the bruising seen in senile purpura, steroid purpura, scurvy, and in the case of simple easy bruising which occurs in an otherwise healthy woman of child bearing age. Others are due to immunological defects like Henoch schonlein purpura which is a form of an immune vasculitis affecting venules in the skin, joints, intestinal mucosa and kidney.

Ehlers-Danlos syndrome: a heterogeneous group of inherited connective tissue disorders, the severity of which can vary from mild to life-threatening, is attributed to mutations in connective tissue genes which cause defects in collagen and thus, affects the skin giving hyperelasticity, joints giving hyperextensibility, and blood vessels giving purpura and bruising. The prevalence of each of the six major types that have been recognised vary in the population (Laffan and Lee, 2005).

1.3.4 Platelet disorders

Due to the important role of platelets in haemostasis, acquired or inherited abnormalities in platelet number or function are associated with a risk of bleeding which can range from mild to life threatening. Classically, patients with these disorders have signs and symptoms of mucocutaneous bleedings of variable severity, including nosebleeds, heavy menstrual bleeding and excessive bleeding after trauma or surgery (including dental extractions). However, it is only patients with the most severe of these disorders that are readily identified, with for example the majority of patients with Bernard Soulier syndrome or Glanzmann Thrombasthenia being identified soon after birth because of characteristic petechiae. On the other hand, patients with mild disorders can go undetected for many years until haemostatically challenged e.g. through a tooth extraction.

1.3.4.1 Acquired platelet disorders

In contrast to inherited platelet disorders, which are rare and probably underdiagnosed, acquired disorders of platelet function are relatively common in haematology practice and can arise through a variety of systemic diseases, medications and procedures. Abnormality of platelet function should be considered in patients with mucocutaneous bleeding in the absence of thrombocytopenia, von Willebrand disease. Antiplatelet agents, including aspirin and clopidogrel, are the most common cause of acquired platelet disorders associated with excessive bleeding. In addition, chronic renal failure, liver cirrhosis (leading to a reduction in TPO formation), myeloma, the myeloproliferative disorders polycythemia vera and essential thrombocythemia (which paradoxically is associated with increased thrombosis and increased bleeding) and

cardiopulmonary bypass have long been recognized as clinical situations in which platelet dysfunction may contribute to bleeding (Hassan and Kroll, 2005). This group of disorders are not the intended focus of the work in this thesis and so, as far as possible, we have avoided investigation of patients who fall into this group.

1.3.4.2 Inherited platelet disorders

Inherited platelet-based bleeding disorders are generally classified according to a reduction in platelet count (inherited thrombocytopenias) or function (inherited disorders of platelet function), although some disorders are characterized by both thrombocytopenia and abnormalities of platelet function.

Inherited Thrombocytopenias (excluding Bernard Soulier syndrome):

This is a heterogenous group of uncommon conditions that are likely to have been misdiagnosed in the past. However due to the recent advances in molecular genetics and the widespread use of the electronic cell counters, these conditions are now detected with increased frequency and several new entities have been identified. Various classifications and investigations have been proposed based on variables like platelet size, mode of inheritance and co-existing clinical abnormalities (Balduini et al., 2002, Drachman, 2004). These include MYH-9- related thrombocytopenia syndromes, congenital amegakaryocytic thrombocytopenia, amegakaryocytic thrombocytopenia with radioulnar synostosis, X-linked thrombocytopenia with dyserythropoiesis (GATA1 mutations) and Wiskott-Aldrich syndrome. The recent review on behalf of the UKHCDO (Bolton-Maggs et al., 2006) highlights the diagnosis and current management of each of these specific platelet disorders.

Inherited disorders of platelet function: will be discussed thoroughly in Section 1.4.

1.3.5 Diagnosing bleeding disorders:

History taking is a key part in the assessment of a bleeding disorder and should include the site of bleeding, duration of bleeding**,** precipitating cause and the effects of haemostatic challenges such as surgery or childbirth. History of systemic illnesses, such as liver or renal failure, and a full drug history should be taken as some widely used medications like aspirin can predispose the individual to bleeding and may even unmask an underlying lifelong mild bleeding disorder. A positive family history of bleeding disorder can be helpful to the diagnosis, although the absence of a family history does not rule out a heritable condition, especially as bleeding is often polygenic.

Clinical Examination: A full general examination should be carried out, carefully focusing on the skin, mucous membranes and joints. A mucocutaneous bleeding pattern is more common in patients with a primary haemostatic disorder (i.e. platelet disorder and von Willebrand disease). On the other hand, deep tissue bleeding, haemarthroses and intramuscular haematomas are more common in coagulation disorders such as haemophilia.

Investigations: The extent of the investigations used to diagnose the patient is highly dependant on the probability of having a bleeding disorder. It is very usual to start with a full blood count, blood film and coagulation screen. These tests may be sufficient if the history is not strongly suggestive of a bleeding disorder. However, if the bleeding history is more convincing then specific assays of coagulation factor and tests of

fibrinolysis should be done and if a specific platelet disorder is suspected then platelet function tests, platelet flow cytometry and other platelet specialized tests may be considered (Greaves M, 2001) which will be explained in details in Section 1.5.

1.4 Inherited disorders of platelet function

The subclassification of inherited disorders in platelet function is challenging since many platelet responses are intimately related. Clear distinction on the grounds of altered adhesion, aggregation, secretion and procoagulant activity is in many instances problematic (Cattaneo, 2003). Further, these disorders can give rise to bleeding syndromes of varying intensity. Apart from Bernard-Soulier syndrome, all other platelet function defects associated with thrombocytopenia like the MHY-9 related thrombocytopenia or Wiskott-Aldrich syndrome will not be discussed in this section.

1.4.1 Severe platelet disorders

Inherited hemorrhagic manifestations due to platelet function defects were first identified in patients with the most severe diseases:

Glanzmann Thrombasthenia, first diagnosed in 1918 (Glanzmann, 1918), is an autosomal recessive disease associated with bleeding manifestations that are similar to those of patients with Bernard Soulier syndrome, although usually but not necessarily of lower severity. The disorder is caused by mutations or deletions in the genes encoding for one of the two glycoprotein receptor subunits that form the $\alpha_{IIb}\beta_3$ integrin, leading either to a reduced level or absence of expression, or a qualitative defect in function of one of the subunits. The classical diagnostic criterion of the disease is the lack, or severe impairment, of platelet aggregation induced by all agonists, despite a normal platelet count. Platelet clot retraction is also defective and there is an absence of fibrinogen in platelet α-granules. In cases associated with the absence of expression of the integrin, the condition can be readily diagnosed by flow cytometry using a

specific antibody to either subunit. Conditions associated with normal expression of the integrin but defective activation can also be diagnosed in this way using an antibody that recognises the active state of $\alpha_{IIb}\beta_3$, namely PAC-1.

Bernard-Soulier syndrome (BSS) was first diagnosed in 1948 (Bernard and Soulier, 1948) and is associated with quantitative or qualitative defects of the platelet glycoprotein complex GPIb/IX/V as the result of defects in one of the genes encoding GPIbα, GPIbβ or GPIX. As yet, there are no reports on defects in the gene for GPV that have been associated with the disorder. It is generally considered to have autosomal recessive inheritance and is associated with prolonged bleeding, thrombocytopenia, giant platelets and decreased platelet survival. It can be readily diagnosed as BSS platelets do not agglutinate with ristocetin and this defect is not corrected by the addition of normal plasma.

Quebec platelet disorder (QPD) is an autosomal dominant qualitative platelet abnormality, with high and possibly complete penetrance (McKay et al., 2004), characterized by severe post-traumatic bleeding complications that are unresponsive to platelet transfusion. It is associated with abnormal proteolysis of α-granule proteins, severe deficiency of platelet factor V and multimerin, and often but not always a reduction in platelet count (Tracy et al., 1984, Hayward et al., 1996). The genetic cause of QPD has recently been linked to the urokinase plasminogen activator gene (PLAU) (Diamandis et al., 2009) and so far this syndrome has only been described in Canada.

1.4.2 Mild platelet disorders

The concept of mild bleeding disorders has evolved to indicate disorders characterized by the presence of more frequent and/or more prominent bleeding symptoms than in the normal population. These symptoms usually occur after a recognizable challenge and do not usually lead to major discomfort or organ damage, even in the absence of medical intervention. The boundary between severe and mild bleeding disorders may be considered clinically well defined, but the distinction between normal subjects and patients with mild bleeding disorders is often unclear. For example, the presence of 'severe bruising' or 'heavy nose bleeds' is not sufficient to diagnose a mild bleeding disorder. It is usually the accumulation of evidence that gives confidence to the belief that the patient has a disorder such as, for example, the need for a blood transfusion following surgery in association with a history of severe nose bleeds. This can of course then be reinforced by demonstration of a defect in platelet function (see analysis of platelet function, below). There have been a surprisingly small number of mutations in platelet genes that have been associated with mild bleeding, other than in a few specialist cases that are associated with other dysfunctions such as Hermansky-Pudlak syndrome (these patients can be more readily identified through the associated albinism, visual defects and other symptoms as detailed below) and patients with mutations in the RUNX transcription factor (which have an association with acute myelogenous Leukaemia) (table 1.2).

Table 1.2
Main hereditary disorders of platelet function

1- Disorders of platelet receptors and signalling pathways
- a- Defect in membrane receptors
  - GPIb-IX-V (Bernard-Soulier syndrome)
  - GPIIb/IIIa (Glanzmann Thrombasthenia)
- b- Defect in agonists receptors
  - ADP $P2Y_{12}$ receptor defects
  - TxA_2 receptor defect
  - Collagen receptor defect
  - α_2-Adrenoreceptor defect
- c- Defect in signal transduction pathways
  - TxA_2 signaling pathway
  - defect in Ca^{+2} mobilization
  - defect in PLC & PLA_2 proteins
  - defect in Gq protein

2- Disorders of storage granules
- δ granule defect (HPS and CHS)
- α-granule defect
  - Gray platelet syndrome
  - Paris-Trousseau-Jacobsen syndrome
  - ARC syndrome
  - Quebec platelet disorder
- α and δ granule combined defect

3- Disorders of membrane procoagulant expression
- Scott syndrome and related disorders

4- Disorders of transcription factors
- RUNX1
- GATA1

5- Inherited Thrombocytopenias
- MYH-9 related disorders
- Wiscott-Aldrich syndrome
- others

1.4.2.1 ADP receptor defects

So far, no human pathology of the $P2Y_1$ receptor has been reported. On the other hand, seven $P2Y_{12}$ receptor (P2RY12) mutations associated with excessive bleeding have been described worldwide to date, with four resulting in a failure to express the functional receptor. Homozygous deletions of either 1 or 2 bp in the coding sequence caused frameshifts leading to introduction of premature stop codons and a lack of demonstrable $P2Y_{12}$ expression in patients from two families (Cattaneo et al., 1992, Cattaneo et al., 2000, Cattaneo, 2003). A further patient was homozygous for a missense mutation in the translation initiation codon which resulted in a failure to express functional $P2Y_{12}$ (Shiraga et al., 2005). All patients presented with similar lifelong histories of mucosal bleeding, easy bruising, and/or excessive bleeding in response to haemostatic challenges, and prolonged bleeding times. Platelets from these patients displayed severely impaired ADP-induced aggregation, normal shape change, a failure of ADP to inhibit PGE-stimulated adenylyl cyclase and a reduction in the number of binding sites for radiolabelled ADP (Cattaneo et al., 1992, Cattaneo et al., 2000, Shiraga et al., 2005). A heterozygous carrier of one of the null alleles described above, on the other hand, had a mildly prolonged bleeding time, and platelets which showed only moderate reductions in platelet ADP binding sites, and in adenylyl cyclase inhibition by ADP (Cattaneo et al., 2000). Moreover, the platelets showed a normal aggregation response to high concentrations of ADP (20µM), but reduced and reversible aggregation to 4 µM ADP, as well as impaired or borderline low ATP secretion in response to a range of agonists (Cattaneo et al., 2000). Another patient was heterozygous for a mutated allele which had a 2 bp deletion in the coding region causing a frameshift and introduction of a premature stop codon (Hollopeter et al.,

2001). Although the second allele appeared to have a normal coding sequence, the patient's platelets demonstrated a complete absence of $P2Y_{12}$ receptors and platelet RNA analysis revealed the presence of P2RY12 transcripts derived only from the mutated allele. Platelets from the daughter of this patient, who had one wild-type allele, and one frame-shifted allele, displayed a moderate reduction in ADP binding sites, and impaired ADP-dependent aggregation at low concentrations of ADP (Hollopeter et al., 2001).

A naturally occurring mutation that resulted in expression of dysfunctional $P2Y_{12}$ receptors was identified in a patient with a lifelong history of easy bruising and excessive blood loss in response to surgery and trauma who was compound heterozygous for two mutations predicting substitution of arginine residues 256 and 265 by glutamine and tryptophan, respectively (Cattaneo et al., 2003). Platelets from this patient shape changed normally, but demonstrated reduced aggregation in response to a high concentration of 20 µM ADP, although the fact that a response is observed demonstrates that either one or possibly both mutations gives rise to impairment rather than abolition of function. The R256Q and R265W variants supported normal binding to [^{3}H]2MeS-ADP suggesting a failure in receptor function downstream of agonist interaction (Cattaneo et al., 2003).

Recently, a patient with a haemorrhagic diathesis and a heterozygous P2RY12 mutation (P258T) has been described, whose platelets failed to aggregate in response to low concentrations of ADP (≤4 µM), but showed reduced and reversible aggregation at higher concentrations of ADP (20 µM) (Remijn et al., 2007).

1.4.2.2 Thromboxane A_2 pathway

Many clinics have reported patients with defective platelet aggregation to arachidonic acid and in many cases the patients have been suspected to be under the influence of COX inhibitor. Indeed, there has only been one genetic defect identified which was a defect in TP receptor. Hirata et al (Hirata et al., 1994) described a defective platelet aggregation to TxA_2 in several unrelated Japanese patients giving rise to an inherited bleeding disorder. An alteration in the gene product was suggested and linked to an Arg60 to Leu substitution in the first cytoplasmic loop of the TxA_2 receptor. Aggregation responses to several agonists were impaired with the exception of thrombin. Ushikubi et al and Fuse et al studied the binding and GTPase activity of TxA_2 analogues to these platelets and suggested that the defect was in TxA_2 receptor G-protein coupling. (Ushikubi et al., 1987, Ushikubi et al., 1992, Fuse et al., 1993).

Recently, one publication has shown an inherited human cPLA2α deficiency which is associated with impaired eicosanoid biosynthesis, small intestinal ulceration and platelet dysfunction but not to defective platelet aggregation to arachidonic acid (Adler et al., 2008).

No mutations have yet been identified in the gene encoding the cyclooxygenase enzyme, in spite of descriptions of many patients with COX inhibition like defects, which may be partly due to the challenge in sequencing the large genomic COX gene.

1.4.2.3 Platelet secretion defect (storage pool disease)

This is a heterogeneous group of disorders which most likely represent the largest group of platelet function disorders, resulting from a deficiency of granules (α or δ or both) or defective release of granules upon platelet activation.

δ- storage pool deficiency:

Dense granule (δ) storage pool disorders can appear as a singular clinical disease or as part of other hereditary disorders of lysosome-related organelles such as Hermansky-Pudlack and Chediak-Higashi syndrome. Lumiaggregometry is a helpful diagnostic tool in dense granule disorders in addition to electron microscopy.

Hermansky Pudlak syndrome (HPS) first described in 1959 (Hermansky and Pudlak, 1959), is a rare heterogeneous disorder of variable severity inherited in an autosomal recessive manner. It is more common in some areas of the world, most notably Puerto Rico, where it is the most common form of recessive disorder. It is caused by defects in proteins involved in intracellular vesicle trafficking and the biogenesis of lysosome-related organelles, specifically melanosomes and platelet dense granules.

Eight mutations in human genes have been identified in HPS. These are HPS1, AP3B1/HPS2, HPS3, HPS4, HPS5, HPS6, HPS-7/DTNBP1 (Di Pietro and Dell'Angelica, 2005) and HPS8 (Morgan et al., 2006). An additional nine other genes have been identified in mouse models of HPS, but counterparts have not yet been described in humans. The usual manifestations in all forms of HPS are oculocutaneous albinism and prolonged bleeding, however, additional manifestations like pulmonary fibrosis and immunodeficiency are characteristics of mutations in specific HPS genes.

The bleeding diathesis is due to a storage pool deficiency caused by the lack of platelet dense granules leading to impaired platelet aggregation and secretion induced by several platelet agonists.

Chediak-Higashi syndrome (CHS) is an autosomal recessive disorder affecting haemopoetic (platelets and neutrophils) and non haemopoietic cells, and which in common with HPS, is associated with oculocutaneous albinism and a platelet dense granule disorder, in addition to recurrent infections due to impaired bacteriocidal activity. Death usually occurs in childhood (Cattaneo, 2003). The CHS gene encodes a large protein, CHS1/LYST, that functions in lipid-related protein trafficking. Several mutations throughout this gene have been described upon which the prediction of the severity of the disease depends.

α- granule defects

The defects result from deficiency of proteins which are synthesized in megakaryocytes or endocytosed from plasma and stored in the α-granules. These include conditions like:

Gray platelet syndrome: in which platelets appears gray on peripheral blood smears treated with Wright–Giemsa stain due to the lack of α-granules and their constituents. Patients may have thrombocytopenia in addition. The molecular basis of this syndrome is still not clear, however more than one gene may be involved as both autosomal recessive and autosomal dominant patterns of inheritance have been described.

Paris-Trousseau-Jacobsen syndrome: is a rare syndrome in which the patient is presented with multiple congenital abnormalities, mental retardation and mild bleeding tendency. Giant α-granules with or without thrombocytopenia are present. This syndrome is inherited in an autosomal dominant manner due to deletions in chromosome 11q that including the FLI1 gene, which encodes a transcription factor that is essential for megakaryopoiesis hence giving the characteristic appearance of two morphologically distinct populations of megakaryocytes in the bone marrow (Raslova et al., 2004).

Quebec platelet disorder: (mentioned under severe platelet disorders in section 1.4.1).

ARC syndrome: (arythrogryposis-renal dysfunction and cholestasis) a multisystemic disorder in which affected babies suffer from impairment to kidney and liver function, bleeding problems and weak muscles. It is inherited as an autosomal recessive disorder due to a mutation in the gene VPS33B, which encodes for a protein that is involved in protein trafficking (Gissen et al., 2004, Lo et al., 2005).

The above four conditions are unique to the α-granule pool while other disorders may be associated with inherited deficiencies of the corresponding plasma proteins e.g. fibrinogen in afibrinogenaemia.

α , δ - storage pool deficiency:

Weiss et al have described a storage pool deficiency where both α and δ granules are deficient (Weiss et al., 1979, Weiss et al., 1993). This deficiency shows the same platelet aggregation abnormalities and clinical picture to δ - storage pool deficiency.

1.4.2.4 Other receptor defects

GPVI: Two patients with a congenital GPVI deficiency have been reported. Both patients are compound heterozygotes (Dumont et al., 2009, Hermans et al., 2009). The first patient is a 10 yr old girl presented with ecchymoses since infancy and a prolonged bleeding time. The patient's platelets failed to respond to collagen up to 4 μg/mL but aggregated normally in response to ADP, TRAP, arachidonic acid and ristocetin. GPVI DNA sequencing revealed a R38C mutation in exon 3 of one allele and an insertion of 5 nucleotides in exon 4 of the other allele giving rise to a base-shift (Dumont et al., 2009). The second patient has a lifelong history of bleeding problems and was found to be a compound heterozygous for a 16 bp deletion and a missense mutation S175N of GPVI (Hermans et al., 2009). Subsequent studies demonstrated that the R38C and S175N mutations give rise to non-functional forms of GPVI.

In addition, several patients with autoimmune thrombocytopenia, who are deficient in GPVI receptors and whose platelets are unresponsive to collagen have been reported (Sugiyama et al., 1987, Moroi and Jung, 1997, Kojima et al., 2006). All of these patients have shown a mild bleeding tendency.

α_2- adrenoceptor: Tamponi et al (Tamponi et al., 1987) have described two patients (mother and son) where a decreased platelet response to adrenaline was found to be associated with a congenital defect in the platelet α_2 adrenergic receptors. Another family in which several members had a history of easy bruising and minimal prolongation of bleeding times has also been described and found to have impaired aggregation and secretion in response to adrenaline in association with a decreased

number of platelet α_2- adrenoceptors (Rao et al., 1988). However, the diagnosis was based on radioligand binding experiments and needs to be verified genetically.

PAR1 & PAR4: No human pathology has so far been described in relation to the two thrombin receptors PAR1 & PAR4.

1.4.2.5 Signal transduction defects

Platelet disorders of signal transduction pathways are a heterogenous, ill-defined group of defects that are usually associated with normal platelet count and morphology and includes receptor, G protein and effector defects. Historically, they were classified in the group of primary secretion defects (Rao and Gabbeta, 2000).

The identification of the causative molecular defect in this group of disorders is challenging. A long list of patients with disorders of platelets due to abnormalities in signalling pathways have been reported. These patients showed an inhibition to one or usually more than one platelet agonist due to defects in aggregation, secretion and/or cytoskeletal responses. Beside the clinical manifestations of primary haemostatic defects (mild bleeding), most of these patients are likely to present with additional manifestations due to the contribution of the proteins of these signalling pathways in other cells in the body. Among those already described in this group are patients with impaired Ca^{+2} mobilization, defective inositol-1,4,5-trisphosphate production, reduced phosphorylation of pleckstrin by protein kinase C, a deficiency in phospholipase C-γ2 isoform and a specific decrease in Gαq (Rao, 2003).

1.5 Testing for platelet disorders

Multiple aetiologies exist for platelet-based bleeding disorders. Thorough evaluation of the patient's medical history, concentrating on personal and familial bleeding disorders and current medications should be the initial step in determining whether a bleeding disorder is present. The laboratory evaluation of these disorders is challenging and can range from simple to complex. Moreover, since the last guidelines for the British Committee for Standards in Haematology (BCSH) on platelet function testing were written in the late 1980s (BCSH, 1988), many new tests have become available. Recent advances in genetic testing of platelet defects could also be used to rapidly diagnose defects and receptor polymorphisms in both health and disease, but this is seldom done.

Platelet function tests are primarily utilized to aid in the diagnosis and management of patients presenting with bleeding problems rather than thrombosis. However, nowadays there is also renewed interest in monitoring the efficacy of anti-platelet therapy and measuring platelet hyper-function (Harrison, 2005).

The **bleeding time,** the first physiological test of platelet function, measures the ability of platelets to arrest bleeding from standard sized cuts that are made within the skin of the forearm and, therefore, is a measure of both platelet number and function. In addition, this test also measures the integrity of the connective tissue. While the methodology for performing bleeding times has improved over the years, with the availability of devices to standardize the size and depth of the incisions, the bleeding time remains a test with considerable lack of precision and questionable correlation

with clinically significant patient conditions and has fallen from favor in recent years (Bolton-Maggs et al., 2006).

The **PFA-100** (Platelet Function Analyzer) (Mammen et al., 1998) has been introduced to the market to help monitor antithrombotic drug dosages or thrombotic risk. Recently, it has been widely utilized to diagnose platelet disorders as it simulates high shear platelet adhesion to foreign surfaces (e.g. collagen), mimicking many of the important physiological processes of platelet adhesion, activation and aggregation that occur at higher shear rates in vivo. It provides a potential replacement of the bleeding time as a screening test, and is abnormal in several other conditions most notably von Willebrand disease, which is the most common cause of bleeding. In addition, this procedure is sensitive to platelet count and hematocrit and full clinical use has yet to be established. So is there a single bed-side test that is definitive for a platelet-bleeding defect and indeed is such a test a reality?

Almost certainly, there will never be a universal test on which to make a definite diagnosis in the case of a bleeding defect due to platelet dysfunction. Indeed, in this light, it is possible that the platelet **aggregation test** which was introduced in the early 1960s, will remain as the most commonly used platelet function test used in academic centers and large hospitals. Aggregometers monitor the changes in light transmission that occur in a suspension of platelets that are stimulated with different concentrations of agonists (e.g. ADP, collagen, adrenaline, ristocetin etc). In addition, the **lumiaggregometer** has the ability to simultaneously measure ATP secretion from dense granules using luciferin-luciferase reagent. The pattern of responses obtained

enables the experienced operator to diagnose whether there are defects in platelet receptors, platelet secretion and downstream signaling or metabolic pathways, although it is inevitable that further tests will be needed to determine the cause of the disorder. Although this test is labor intensive and relatively slow and marked thrombocytopenia can make the aggregation responses difficult to interpret, it remain the most widely used and understood test to study platelets function defects.

Platelet nucleotide tests are done to rule out storage pool defects unless the platelet aggregation test and simultaneous measure of the release of ATP from dense granules by using the lumiaggregometer has demonstrated no defect.

Further investigations for the assessment of platelet function:

These are specialist tests and perhaps require a specialist centre.

Platelet flow cytometry is a sensitive and powerful tool for studying platelets with very small quantities of blood even in thrombocytopenia**.** However it requires a trained and experienced operator, and in general is an expensive item of equipment. The major diagnostic use of this technique is to determine the copy density of platelet membrane glycoproteins and receptors confirming the absence of various glycoproteins or receptors in diseases such as Glanzmann Thrombasthenia. Platelet function testing can also be performed and the ability of platelets to degranulate, express activation markers (e.g. P-selectin for α-granules and CD63 for dense granules) and negatively charged phospholipid in response to agonists can all be studied. In addition, dense granule contents measurement using mepacrine uptake and release, microparticle formation

and the recent VASP (Vasodilator Stimulated Phosphoprotein) test for monitoring aspirin and $P2Y_{12}$ drugs have all been applied to platelet function testing with the help of the flow cytometry (Schmitz et al., 1998, Michelson et al., 2007).

Electron microscopy is another helpful tool used in diagnosing platelet defects such as the lack of platelet dense granules in Hermansky Pudlak patients but is only available in few specialized centres.

More specialized tests for platelet enzymes and signal transduction pathways:

Once a defect in a platelet receptor or signaling pathway is suspected then other tests can be done in order to reach a definitive diagnosis, however these tests are not routinely undertaken but may be considered within a research context.

An update on the current clinically available platelet function tests with the advantages and disadvantages of each test has been published recently (Harrison, 2009).

1.6 Aims of the thesis

The overall aim of this thesis is to identify patients with mild bleeding disorders of platelet aetiology and to determine the underlying molecular cause. This will be achieved as follows:

- Generation of standard aggregation and dense granule secretion curves to major platelet agonists and evaluation of the effect of inhibition of the two feedback agonists, ADP and TxA_2, using a Born-lumiaggregometer.
- Testing patients with suspected platelet receptor defects from the Haemophilia Centre in Birmingham and in other Hospitals, notably Bristol and Sheffield, particularly those that appear to have a defect in the $P2Y_{12}$ ADP receptor given the link to mild bleeding, and in comparison to standard curves.
- Testing of patients with type I VWD from the European cohort for possible defects in the $P2Y_{12}$ ADP receptor, in view of the increasing evidence that this is a polygenic disorder, especially in patients with only a partial reduction in VWF. The application will investigate the hypothesis that the similarity in the pattern of bleeding of patients with type I VWD and mild-platelet based disorders, and the incomplete penetrance of bleeding in these two disorders, is because these patients have defects in both pathways. The genetic sequencing for these studies has been performed in Dr Martina Daly's laboratory in the University of Sheffield, and my role has been linked to the functional characterization of the patients.
- More focused tests to identify the nature of the defects discovered for the above patients e.g. cAMP and Ca2+ and then genetic analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Trisodium citrate, ADP, adrenaline, U46619, arachidonic acid, indomethacin, PGI_2 and MRS2179 were purchased from Sigma-Aldrich (Poole, UK). Horm collagen was purchased from Nycomed Austria (Linz, Austria). The PAR1 peptide SFLLRN was purchased from Alta Bioscience Laboratory (University of Birmingham, UK). The PAR4 peptide AYPGKF and CRP was purchased from Dr Richard Farndale (Cambridge University, UK). AR-C67085 was a gift from Astrazeneca (Loughborough, UK). Luciferin/luciferase reagent (Chrono-lume) and ATP standard were purchased from Chrono-log Corporation (Havertown, PA, USA). Platelet aggregation and secretion was performed on a Dual Channel Lumi-Aggregometer (model 460VS, Chronolog). cAMP Biotrak enzyme immunoassay kit and ELISA TxB_2 assay kit were purchased from GE Healthcare UK Ltd. Anti COX-1 mAb and anti TXAS mAb raised in mouse were purchased from Cayman chemical (IDS.LTD, Boldon, UK) Anti GPVI mAb raised in mouse was a gift from Dr. M Moroi-Japan (Moroi et al., 2003). Anti-actin mAb AC-40 raised in mouse was from Sigma Aldrich. The rest of the materials used in this thesis, unless stated, were purchased from Sigma Aldrich (Poole, UK).

ADP (10 mM), adrenaline (100 mM), arachidonic acid (15mM) and PAR1-peptide (10 mM), PAR4-peptide (500 mM), MRS2179 (10mM) and AR-C67085 (10mM) were dissolved in phosphate-buffered saline (PBS) and stored as frozen aliquots at the concentrations shown and diluted in PBS on the day of the experiment. CRP (1.4 mg/ml stock) was cross linked accorded to the recommended protocol as described in Appendix1 and stored at 4°C then diluted in 0.01M acetic acid in PBS containing 0.1% fatty acid-free bovine serum albumin on the day of the experiment. Collagen was stored as a concentrated stock at 1mg/ml as supplied by the manufacturer at 4°C.

Indomethacin (10mM) was stored and diluted in dimethyl sulphoxide (DMSO). U46619 (1mM) was stored in DMSO and diluted in PBS on the day of the experiments. Agents were added in dilutions of 1: 100, with the exception of indomethacin which was added at a dilution of 1: 1000 to keep the DMSO concentration to 0.1%.

2.2 Platelet preparation

2.2.1 Preparation of platelet rich plasma

Blood samples were taken from healthy donors who gave informed consent and answered negatively to routine deferral questions such as ingestion of aspirin and other agents that may influence platelet function. Blood was also taken from patients with a suspected or known platelet defect. Whole blood was anticoagulated with 4% trisodium citrate (trisodium citrate/whole blood ratio, 1:9), by collecting 50 ml blood using a 21G needle into a plastic polypropylene syringe containing anticoagulant. The blood was transferred to 5ml polypropylene tubes, 75 x 12mm, for centrifugation. Platelet rich plasma (PRP) was prepared by centrifugation of anticoagulated blood at 170 g for 10 minutes at room temperature (20 – 22 $^{\circ}$C). The PRP (upper layer) was gently pipetted into a 50ml polypropylene tube using a plastic pipette. The blood was recentrifuged at 190 g for 10 minutes at room temperature and further PRP collected in the same way and pooled. The use of two centrifugation steps increases the yield of PRP. The platelet number was measured using a Coulter Z_2 analyser (Beckman Coulter (UK) Ltd), but was not adjusted prior to experimentation.

2.2.2 Preparation of platelet poor plasma

Platelet-poor plasma (PPP) was prepared by centrifuging the blood remained from the preparation of PRP (discussed above) at 1000 g for 10 minutes at room temperature.

2.2.3 Preparation of washed platelets

Washed platelets were obtained from PRP that was collected after centrifugation of the blood for 20 minutes at 200g as described by Watson et al (Watson et al., 1995). PRP, after the addition of 1 µg/ml prostacyclin (PGI_2), was centrifuged at 1000 g for 10 minutes. The platelet pellet was suspended in 3 ml ACD with 25 ml (pH 7.3) HEPES-buffered Tyrode's buffer (134 mM NaCl, 2.9 mM KCl, 1 mM $MgCl_2$, 20 mM HEPES, 12mM $NaHCO_3$, 5 mM glucose). Following addition of 1 µg/ml PGI_2, a second centrifugation was performed at 1000 g for 10 minutes. Platelets were finally resuspended with HEPES-buffered Tyrode's to a chosen concentration.

2.2.4 Platelet fixation for [^{3}H]2MeSADP binding

Fixed washed platelets were prepared based on the method described by Mundell et al (Mundell et al., 2006). PGI_2 (1µg/ml) was added to PRP, which was then centrifuged at 1000g for 10 minutes before resuspension at 4 x 10^8 platelets/ml in modified Tyrode's-HEPES buffer (145 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, and 5 mM glucose, pH 7.3). Apyrase (0.02 U/ml) and formaldehyde (4 %) were added to the platelet suspension, which left to rotate for 25 minutes at room temperature. The suspension was then centrifuged at 1000g for 10 minutes, and the pellet resuspended in binding buffer (20 mM HEPES and 1mM MgCl2, pH 7.3) to a density of 4x10^8 platelets/ml.

2.3 Platelet functional assays

2.3.1 Platelet aggregation

Platelet aggregation was measured by a turbidimetric method which moniters light transmission (Born, 1962, O'Brien, 1962), using a Dual Channel Lumi-Aggregometer (460VS, Chronolog). This aggregometer incorporates a luminometer that permits simultaneous monitoring of platelet aggregation and release of ATP in siliconized glass cuvettes (Labmedics, Manchester, UK). The PRP and PPP or washed platelets and buffer samples, were used to set the scale prior to the onset of the aggregation recording. The difference between the light transmission of the PRP and PPP samples is taken as 100%, with the initial starting trace representing 0%. Samples (396 µl) were prewarmed at 37 $^{\circ}$C for 120 seconds and stirred at 1200 rpm for 60 seconds before agonist addition. Agonist dilutions are typically 1:100 i.e. 4 µl with the exception of these dissolved in DMSO. Antagonists and inhibitors were given 120 seconds before experimentation. A summary of platelet agonists, antagonists and inhibitors is given in Table 2.1. Platelet aggregation was determined by measurement of the change in optical density (i.e. light transmission) after agonist addition. Each sample was compared relative to the PPP control. Platelet aggregation was monitored for more than 5 minutes. The maximum extent of aggregation was calculated and expressed as a percentage of full-scale deflection as measured by PPP.

Table 2.1 Platelet agonists

Platelet agonist	Low, intermediate and high concentrations	Source
ADP	1, 3, 10, 30 and 100μM	Sigma-Aldrich
Adrenaline	1, 3, 10, 30 and 100μM	Sigma-Aldrich
Arachidonic acid Arachidonic acid (washed platelets)	0.3, 0.5, 1 and 1.5mM 1μM	Sigma-Aldrich
U46619	0.3, 1 and 3μM	Sigma-Aldrich
PAR1 peptide	10, 30 and 100μM	Alta Biosciences, University of Birmingham
PAR4 peptide	10, 30 and 100μM	Dr Richard Farndale Cambridge university
Collagen	0.3, 1 and 3μg/ml	Nycomed Austria-Austria
CRP- XL	1, 3 and 10μg/ml	Dr Richard Farndale Cambridge University
Ristocetin	1, 1.25, 1.5 and 2mg/ml	Sigma-Aldrich
Rhodocytin	10,30,100 and 300nM	Dr Johannes Eble, Universität Münster, Germany
PGH_2 (washed platelets)	1μM	Sigma-Aldrich
PGE1(washed platelets)	1μM	Sigma-Aldrich
Indomethacin	1μM	Sigma-Aldrich
$P2Y_1$ antagonist (MRS 2179)	100μM	Sigma-Aldrich
$P2Y_{12}$ antagonist (AR-C67085)	1μM	A gift from Astrazeneca

Agonists, antagonists, inhibitor, concentrations for PRP (unless stated) and source

2.3.2 Platelet ATP secretion

This is a technique based on firefly bioluminescence. In this assay light is produced in proportion to the concentration of ATP released from platelets. 400 µl PRP was activated by one of the agonists listed in Table 1.2, in the presence of 40 µl of Chrono-lume luciferin/luciferase reagent (Labmedics, Manchester, UK), which was diluted according to the manusfacturer's instructions and given 120 s before experimentation. The ATP secreted was measured with a Dual Channel Lumi-Aggregometer. Changes in luminescence were quantified through calibration with 4 nmol ATP standard for each sample. Data was normalized to the level of secretion per 1x 10^8 platelets.

2.4 Adenylyl cyclase activity (cAMP assay)

Adenylyl cyclase activity in platelets was assessed by measurement of cAMP. In order to quantify cAMP in platelet samples, Amersham cAMP Biotrak enzyme immunoassay kit was used, which is based on competition between unlabelled cAMP and a fixed quantity of peroxidase-labelled cAMP, for a limited number of binding sites on a cAMP specific antibody.

Blood samples from both controls and patients with a suspected ADP receptor/signaling pathway defect were collected. 160 µl of washed platelets, at a concentration of $6x10^7$/ ml in Tyrode's buffer were incubated with 1 µM PGE1 for 15 minutes, after which, platelets were stimulated with either ADP or adrenaline for another 15 minutes followed by the addition of the lysis reagent (2.5% solution of Dodecyltrimethylammonium Bromide in assay buffer) through successive pipetting to facilitate cell lysis. After incubation for 10 minutes at room temperature, the samples were stored at -20° C. Basal and stimulated samples were thawed and cAMP was

measured using protocols stated in the manufacturer's guidelines. The 96-well ELISA plate was analysed on a $VERSA_{max}$ tunable microplate reader (Molecular devices, UK.) Data analysis was subsequently conducted using Graph Pad prism 4 software, using a standard curve constructed from a serial dilution of working standard samples to calculate cAMP production.

2.5 Measurement of platelet nucleotides

In this procedure ATP and ADP levels in platelet are measured using a luminometric assay (Jarvis et al., 1996). The light produced by the firely luciferin/luciferase reagent in the microplate luminometer (Berthold technologies-centro LB960) is proportional to the amount of ATP available in the platelet sample. The ADP level was detected following convertion of ADP to ATP via the phosphoenol pyruvate/pyruvate kinase reaction. PRP for this assay was prepared by centrifugation of citrated blood at 170g for 10 minutes, with no second centrifugation step. This PRP has to be processed within 3 hours of collection. 25µl EDTA (0.1M) , 10% Triton X-100 (vortex, mix well) and 250µl ethanol were added to 250µl PRP and mixed for 30 minutes before storage at -20°C until required. An ATP standard curve was prepared from serial 1/10 dilutions of an ATP standard (10 µM stock) in assay buffer and this was used to calculate the ratio of ADP:ATP and absolute amount of ATP and total ADP+ATP. The final results are expressed as nmoles nucleotide/10^8 platelets. The calculation is done for both total and ATP only. The ADP content is then calculated by subtracting the ATP reading from the total.

2.6 Thromboxane B_2 assay

In order to quantify thromboxane B_2 (TxB_2), the TxA_2 stable metabolite, we used an ELISA TxB_2 assay (GE Healthcare UK Ltd.) which is based on the competition between unlabelled TxB_2 and a fixed quantity of peroxidase-labelled TxB_2 for a TxB_2 antibody. Washed platelets samples (4×10^8 / ml) from controls and patients with a suspected platelet-based TxA_2 pathway defect were stimulated for 5 minutes with 1 µM arachidonic acid. The reaction was then stopped by addition of 50µM indomethacin and 2 mM EDTA. Samples were centrifuged at 14000g for 2 minutes and the supernatant stored at -20° C. All samples (basal and stimulated) were thawed and diluted 1:1000 using Tyrode's buffer prior to commencing the assay. The assay was conducted using protocols stated in the manufacturer's guidelines. The 96-well ELISA plate was analysed on a $VERSA_{max}$ tunable microplate reader (Molecular devices, UK.) Data analysis was subsequently conducted using Graph Pad prism 4 program, using a standard curve constructed from a serial dilution of working standard samples to calculate TxB_2 production in patient samples.

2.7 Protein expression analysis

2.7.1 Western- blotting

Washed platelet samples were prepared (as described in Section 2.2.3). Proteins were extracted from platelet preparations by addition of an equal volume of 2x Laemmli sample buffer (2x Laemmli buffer is: 4% SDS, 10% 2-mercaptoethanol, 20% Glycerol, 10% stacking gel buffer; where stacking gel buffer is: 0.5M tris HCl at PH 6.8). Samples were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) using 10% gradient gels. The gels containing the

separated proteins were transfered into polyvinylidene difluoride (PVDF) membranes using a semi-dry method. Blocking of the membranes were done immediately with 5 % (w/v) non-fat dry milk in TBS-T (0.5 M Tris, 1.5 M NaCl, 0.1 % (v/v) Tween 20, PH 7.4) for an hour then subsequent incubation with the primary antibody at the appropriate dilution in 3 % (w/v) bovine serum albumin with 0.1% sodium azide in TBS-T for 90 minutes. Washings with high salt TBS-T (5 M NaCl) were performed 3 times with a 15 minutes interval between each wash. Blots were incubated with secondary antibody in 5% (w/v) non-fat dry milk in TBS-T for 90 minutes then washing 3 times as before and finally developing using the enhanced chemi-luminescence (ECL) detection system (Pierce Bioscience). This is based on incubation of the western blot with a substrate that will luminesce when exposed to the reporter on the secondary antibody and the light is then detected by photographic film. Quantitation of band volumes was done using the Syngene GeneGnome HR bio imaging system (Synoptics, Cambridge, UK) using the GeneSnap software provided along side the system. To re-probe a membrane, blots were incubated in stripping buffer (2% SDS in TBS-T) plus 2% 2-mercaptoethanol at 80 $^{\circ}$C for 20 minutes, then in stripping buffer for a further 20 minutes at 80 $^{\circ}$C. Blots were washed thouroughly in TBS-T and re-blocked with 5 % (w/v) non-fat dry milk in TBS-T before re-probing.

2.7.2 Measurement of GPVI by flow cytometry

PRP, after being diluted to 1 x 10^7 /ml in PBS with the presence of 1mM EGTA, was stimulated for 5 minutes with platelet agonists or its vehicle at 37 $^{\circ}$C. Platelets (45 µl) were incubated with 5 µl of GPVI antibody (in PBS), bringing it to a final antibody concentration of 10µg/ml, for 20 minutes. The samples were then centrifuged for 5

minutes at 1000g, and a secondary FITC-conjugated anti-mouse antibody added to the pellet, mixed and incubated for 20 minutes. The sample was then diluted to 500 µl before subsequent flow cytometry in the FACS Calibur flowcytometer (Becton Dickinson UK Ltd). Cell Quest programme was used for obtaining and analyzing the data.

2.8 Dense granules evaluation by Transmission Electron Microscopy

A modification of the whole mount procedure for sample preparation explained in "Methods in molecular biology: Platelet and Megakaryocytes" (White, 2004) has been done as follows:

1- Smal1 drops of PRP (prepared as discussed in Section 2.2.1) were placed on six Formvar coated grids (supplied by the electron microscopy centre-University of Birmingham), placed on a sheet of parafilm, rinsed in PBS after 5-7 minutes then fixed with 3-4 drops of 2.5% glutaraldehyde in PBS for 5 minutes. A second rinse for three times in dH_2O was done and then the grids were dried from the edge with pieces of filter paper.

2- The grids were inserted into the Transmission electron microscope (TEM) at the electron microscopy centre-University of Birmingham (Jeol 1200EX TEM -JEOL (U.K.) LTD, Herts, England) and the dense granules were counted in at least 50 platelets spread on the grids.

2.9 Data analysis

2.9.1 Aggregation

The degree of aggregation at 90 s or 180 s was measured and reported as a percentage of the difference in light transmission between the PRP and PPP samples. It should be noted that in cases of transient aggregation, the response at 90 s can be larger than that at 180 s. Results are shown as mean ± S.D. from 3–20 experiments. Concentration-response curves were fitted to a three variable logistic equation, with EC_{50}, Hill slope and maximal response, as variables using Graphpad Prism software. Statistical indications were made using Student's t-test, with $P < 0.05$ taken as the level of significance.

2.9.2 ATP secretion

ATP secretion was calculated by addition of a known concentration of ATP (4 nmol) and normalize the platelet count to $1x\ 10^8$/ platelets.

CHAPTER 3

REFERENCE CURVES FOR AGGREGATION AND SECRETION: THE EFFECT OF INHIBITION OF SECONDARY MEDIATORS

3.1 Aim

The aim of the work in this chapter is to establish the reproducibility of aggregation and dense granule secretion over a variety of parameters such as time, platelet count and between donors, and to establish reference curves to low, intermediate and high concentrations of platelet agonists in the absence and presence of ADP receptor antagonists and cyclooxygenase inhibition. The latter inhibitors were used because they block the action of the two major feedback agonists, ADP and TxA_2, which reinforce platelet activation to all agonists. Importantly, several of the agonists chosen for investigation are not routinely used in clinical testing, but they have been included to help in the diagnosis of what are anticipated to be the more common causes of mild platelet-based disorders. These include the thromboxane analogue, U46619, PAR1 and PAR4 thrombin receptor-specific peptides, and the GPVI collagen receptor agonist, collagen-related peptide (CRP). The results provide a valuable resource of data to aid patient testing and suggest that, if used within certain boundaries, the simultaneous investigation of platelet aggregation and ATP secretion is a powerful way to aid diagnosis of patients with platelet-based bleeding disorders.

3.2 Introduction

Platelet function testing is routinely used in the clinic to diagnose patients with suspected platelet-based bleeding disorders. One of the most widely used tests for assessing platelet reactivity is platelet aggregation in platelet-rich plasma (PRP). However, there is no accepted way to perform or interpret these studies (Zhou and

Schmaier, 2005), with practices varying between laboratories as documented in a review of 46 North American Clinical Laboratories (Moffat et al., 2005). Variables include factors such as sample collection, platelet preparation, platelet count, agonist selection and agonist concentrations. Furthermore, the full value of aggregometry is seldom achieved because of the use of a limited number of agonists and concentrations. Indeed, aggregation methodology has remained largely unchanged since the late 1980s despite important advances in our understanding of platelet activation. For example, the guidelines in the UK for investigation of patients with suspected platelet-based bleeding disorders, including information on platelet aggregation, were drawn up by the British Society for Haematology Task Force in 1988 (BCSH, 1988) before the majority of the platelet surface receptor were first cloned. A more recent report on behalf of the Rare Haemostatic Disorders Working Party of the UK Haemophilia Centre Doctors Organisation (UKHCDO) summarizes heritable platelet disorders and gives guidelines on their analysis and clinical management (Bolton-Maggs et al., 2006). This report emphasizes the importance of aggregation testing in clinical diagnosis but does not give experimental details on how to perform these studies. Further, development of expertise in platelet function testing is hampered by the small number of clinical patients who require testing and by the even smaller number of patients with defined, platelet-based disorders. The interpretation of aggregation concentration response curves in patients is highly specialized because of the complexity of interacting mechanisms that coordinate the aggregation response. A defect in response to more than one platelet agonist could be due to a generalized defect in platelet activation, to impairment in release of the major feedback mediators, ADP and TxA_2, or to a defect in the $P2Y_{12}$ receptor for ADP or in the thromboxane receptor. A defect

in the $P2Y_{12}$ ADP receptor, for example, would result in abnormal responses to low concentrations of nearly all platelet agonists, including arachidonic acid, which is frequently used to investigate defects in the cyclooxygenase pathway. On the other hand, a defect in the $P2Y_1$ ADP receptor would have a more selective effect because it generates a relatively weak signal and its activation is masked by receptors which signal through the same pathway, such as the thromboxane receptor and the two thrombin receptors, PAR1 and PAR4 (Woulfe, 2005). The interpretation of aggregation traces is also influenced by the natural variation in responses that exist within the population, thereby making it essential for each laboratory to have standard reference curves for comparison to patients' responses. For example, a study on aggregation responses in 359 healthy individuals identified a subset of donors with hyper-responsive platelets which was maintained over independent experiments (Yee et al., 2005). Several laboratories have published their own methods for monitoring platelet aggregation (Zhou and Schmaier, 2005, Hayward et al., 2008), but none has used as many platelet agonists as has been used in our investigation or evaluated responses in the presence of inhibitors of the two major feedback inhibitors, ADP and TxA_2. Further, few laboratories have extended their analysis to the simultaneous monitoring of ATP secretion from dense granules, despite the availability of lumi-aggregometers for more than 25 years and recognition of the value of monitoring ATP secretion in diagnosis (Hayward et al., 2006b).

3.3 Results

The results in this chapter are divided into two sections. The first describes the characterisation of the conditions used for monitoring aggregation and secretion, and the concentration response curves to the platelets agonists chosen for investigation. The second describes the effect of inhibition of the two platelet ADP receptors, $P2Y_1$ and $P2Y_{12}$, and cyclooxygenase on aggregation and secretion to low, intermediate and high concentrations of eight platelet agonists.

Section A: Reference curves for platelet aggregation and secretion

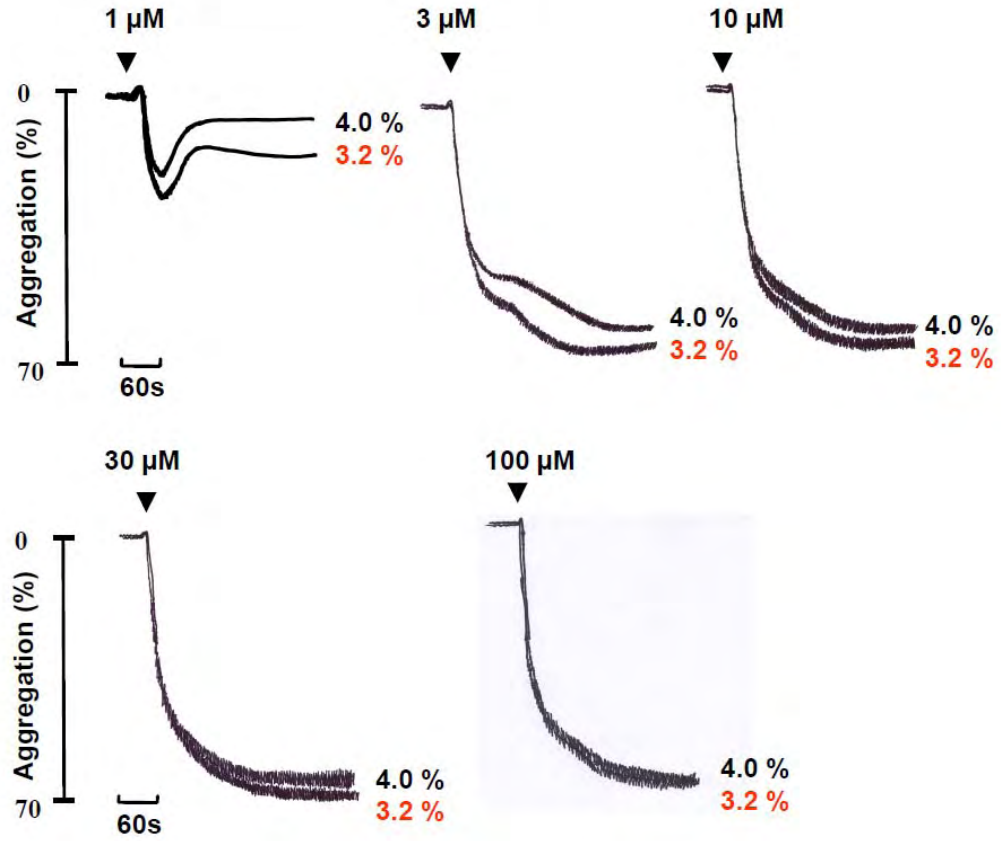
3.3.1 Characterization of aggregation

An initial series of experiments was designed to establish the extent of variation in platelet aggregation to a range of receptor agonists within a population of healthy volunteers who had denied having recently taken medication that is known to alter platelet function. These initial studies were performed with a limited number of platelet agonists: ADP, adrenaline, collagen, PAR1 peptide, arachidonic acid and U46619. Shape change, percentage of maximal aggregation, the rate of aggregation (where the steepest part of a slope versus time was taken) and the pattern of aggregation response were monitored in each experiment.

Sodium citrate was chosen as the anticoagulant as this is routinely used in clinical laboratories for platelet testing at a concentration of either 3.2 or 3.8%. As shown in the aggregation traces to ADP in Figure 3.1 and the dose response curves to representative agonists in Figure 3.2, we observed no difference in response in the

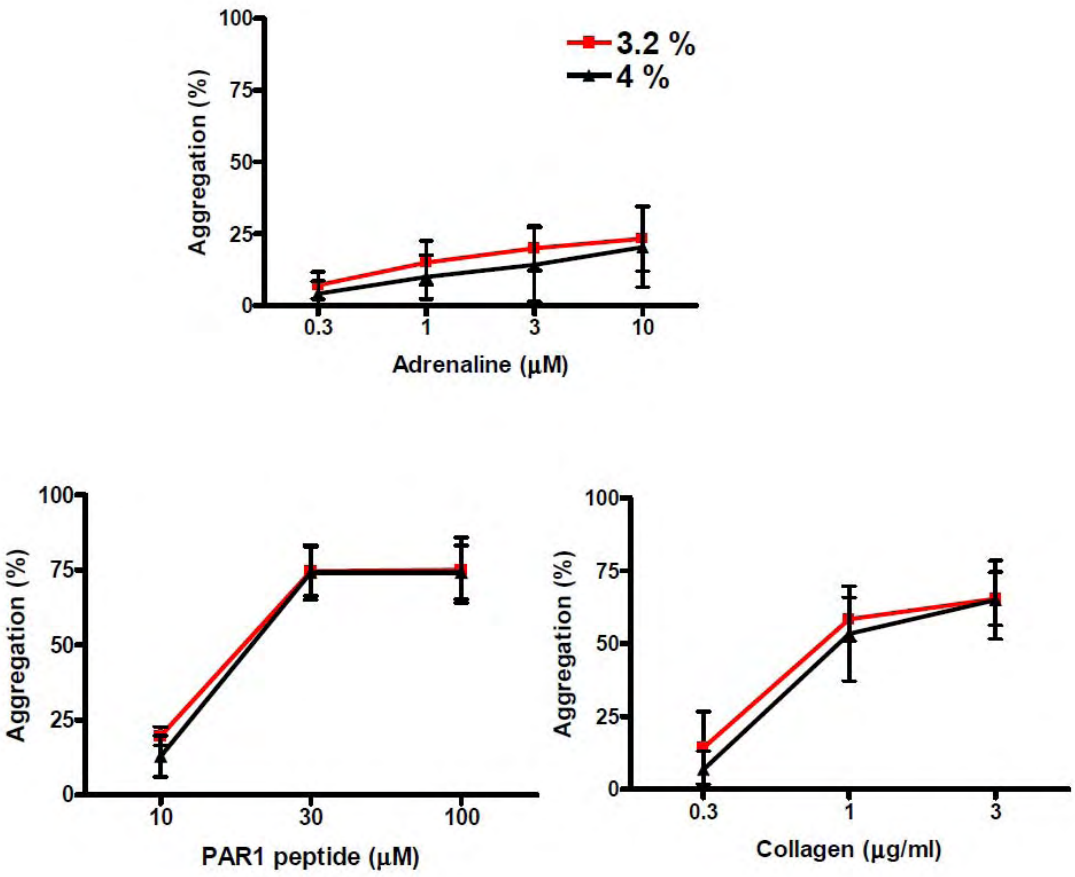
presence of either 3.2 or 4.0% sodium citrate to any of the agonists. Please note in these initial experiments, aggregation was monitored at 90 sec in order to focus on the initial component of the response. For this reason, the maximal level of aggregation to adrenaline is less than that to other agonists, as the second wave of the response peaks after this time (see later). Based on these results, a decision was made to use 4% sodium citrate in subsequent studies, as we have routinely used this in our previous work over the course of many years. This concentration was then used in studies designed to investigate the dependency of aggregation on sex, a limited age range (reflecting those found in our pool of donors), platelet count and time after donation, along with the reproducibility of aggregation within individual donors over time.

Figure 3.1
Platelet aggregation to ADP using two concentrations of sodium citrate as anticoagulant



Aggregation traces obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma using different concentrations (4% or 3.2%) of sodium citrate as anticoagulant. Results are representative of 3 experiments.

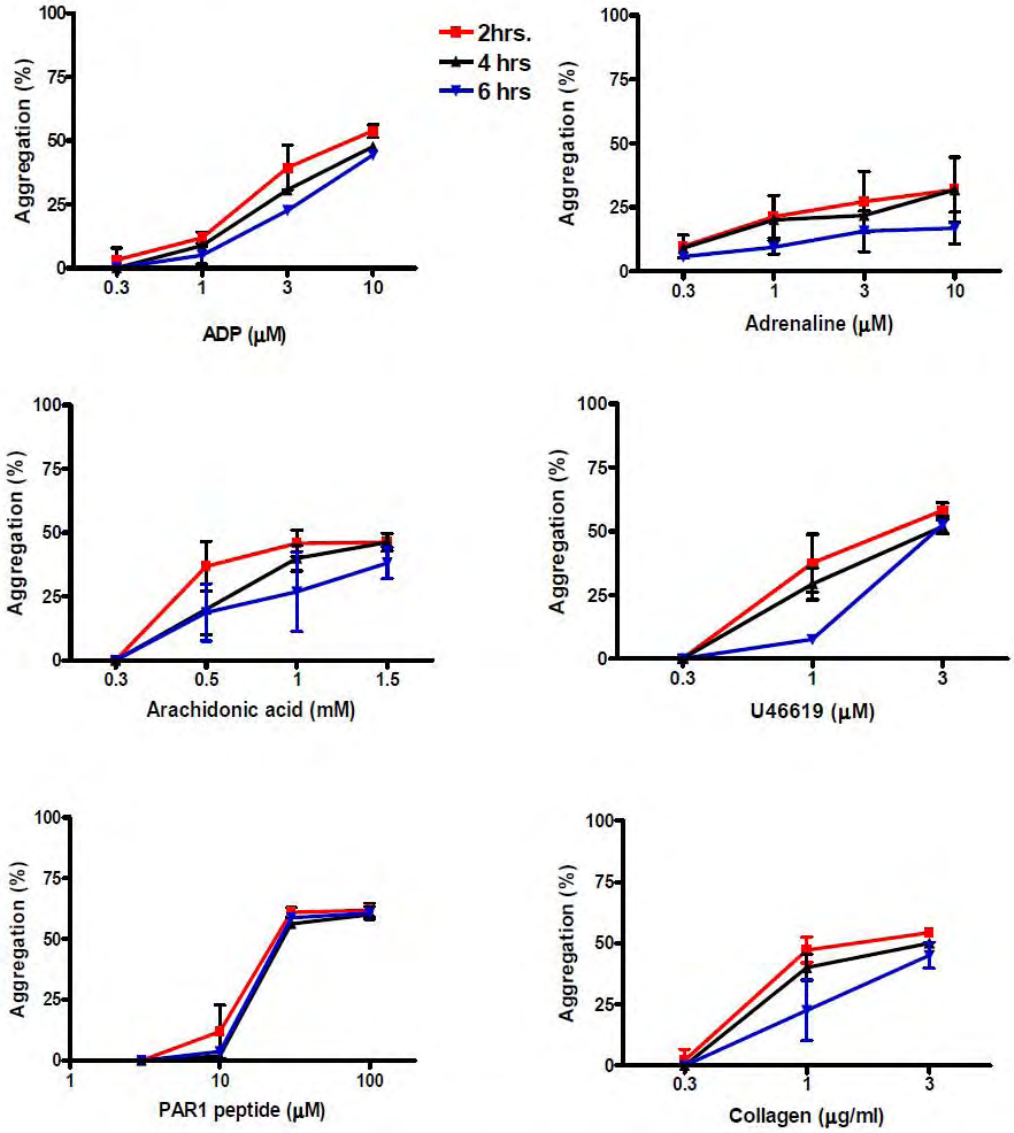
Figure 3.2
Platelet aggregation using two concentrations of sodium citrate as anticoagulant



Platelets aggregation obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Aggregation results, shown as % increase in light transmission, was measured at 90 sec after agonist addition. Results are shown as mean ± s.d. from 3 donors.

I initially investigated the extent to which aggregation deteriorates with time following both donation and platelet preparation. These are two crucial questions as the examination of the aggregation and secretion to low, intermediate and high concentrations of a range of platelet agonists can take several hours. Further, to increase access to patients, it was necessary to establish whether we could transport anti-coagulated blood and subsequently prepare platelets in Birmingham without loss of response. In this regard, I have demonstrated that storage of blood in the presence of sodium citrate (4%) at room temperature for up to 4 hours prior to platelet preparation had a minimal effect on responses to all agonists, although a small reduction in response was observed when blood was left for up to six hours (Figure 3.3). Importantly, when samples were kept in PRP at room temperature for up to 6 hours, platelet responses to all agonists, including ADP, were maintained (not shown). Subsequent studies were therefore usually performed on PRP samples that were prepared within 60 minutes of blood donation and analysed within 6 hours. In later studies using patient samples, this was not always possible due to the need to transport the blood to Birmingham and so a control was always taken alongside for comparison. In all cases, the time of transport to platelet preparation never exceeded four hours.

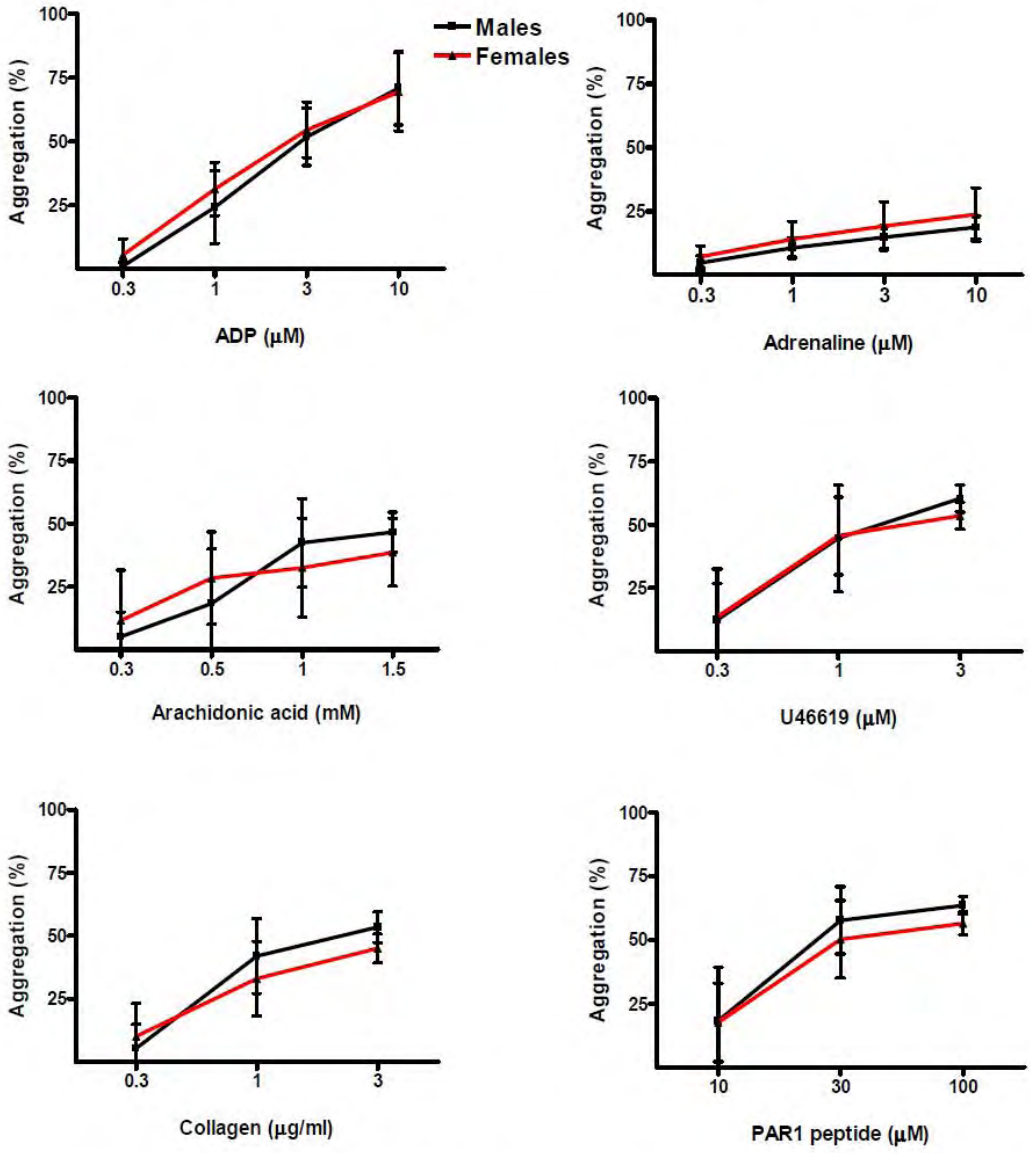
Figure 3.3
Platelet aggregation in relation to time after donation



Platelets aggregation obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Aggregation results, shown as % increase in light transmission, was measured at 90 sec after agonist addition. Results are shown as mean ± s.d. from 3 donors.

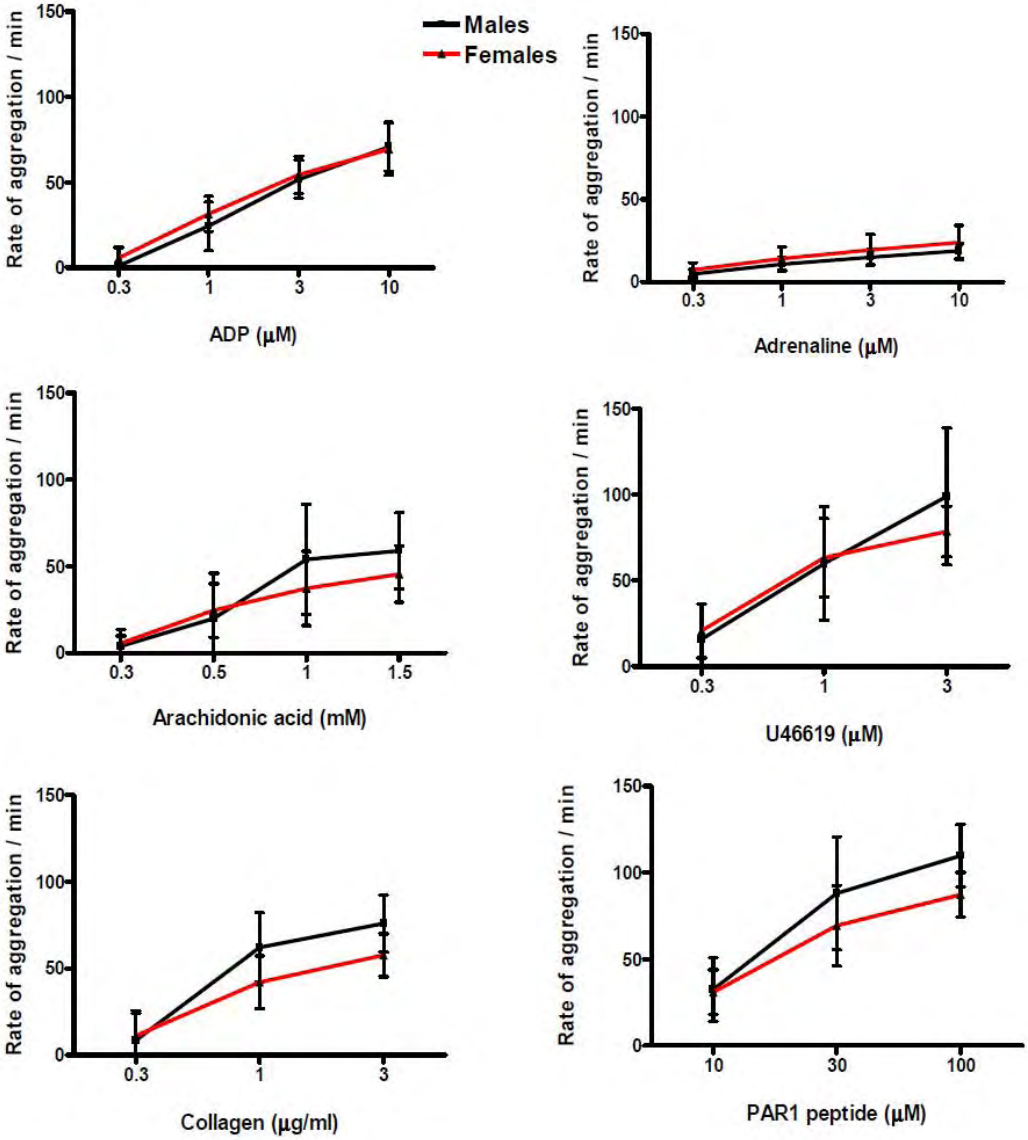
I then evaluated concentration response curves for platelet aggregation to ADP, adrenaline, collagen, PAR1 peptide, arachidonic acid and U46619 in 10 male and 10 female donors aged between 21 and 46 years, reflecting the range of ages of available donors. Similar results were obtained for percentage or rate of aggregation in the two populations (Figures 3.4 and 3.5) demonstrating no significant sex variation in response. Further, because the concentration response curves for the degree and rate of aggregation were similar, a decision was made to only analyse the degree of aggregation in subsequent studies. This work was also extended to investigate the effect of age on the concentration response curves to the above agonists, taking into account the range of ages that are found in our cohort of donors. There was no significant difference in response between donors of below 25 and above 35 yrs of age (Figure 3.6).

Figure 3.4
Comparison of aggregation between males and females



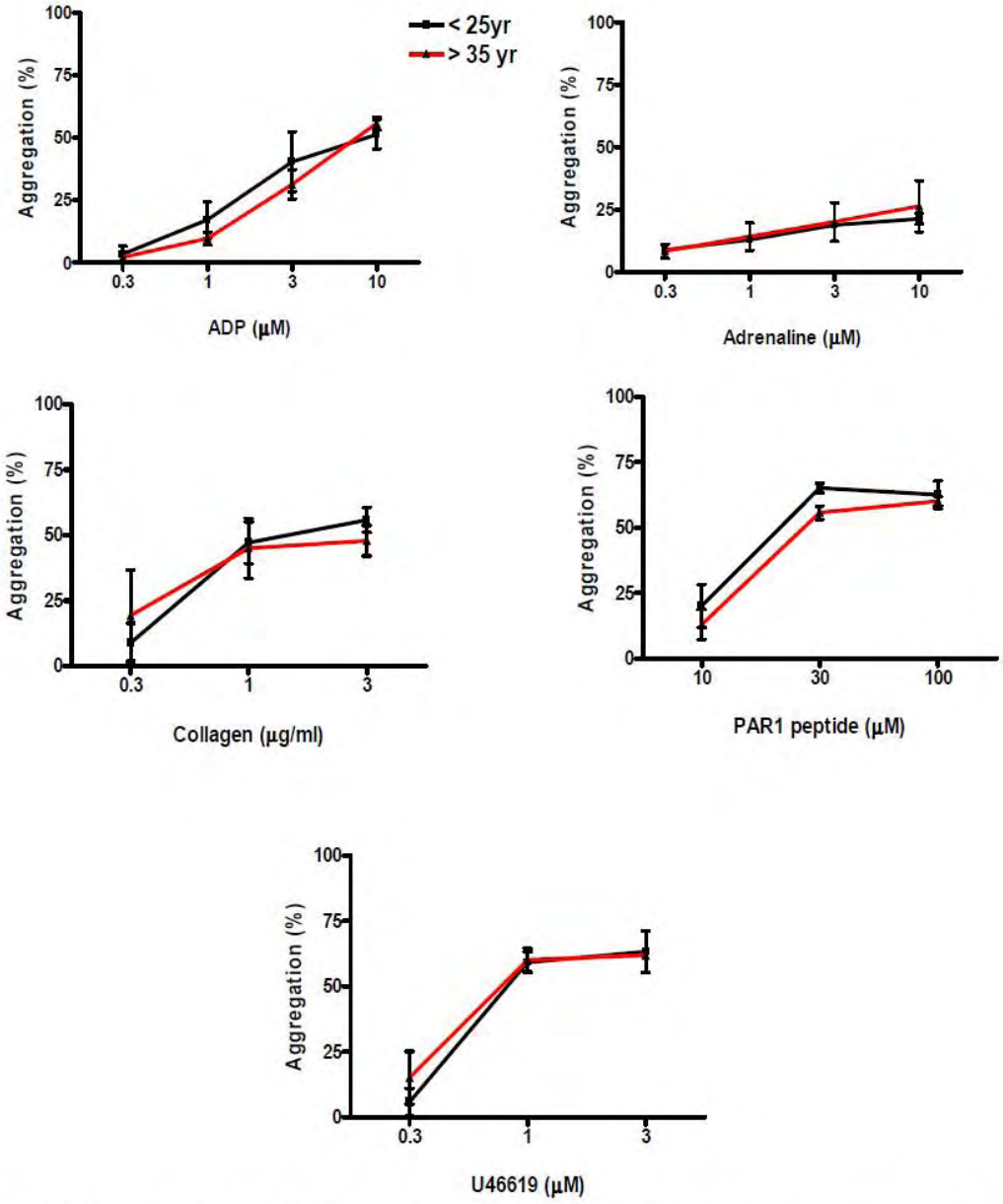
Platelets aggregation obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Aggregation results, shown as % increase in light transmission, was measured at 90 sec after agonist addition. Results are shown as mean ± s.d. from 10 males vesus females.

Figure 3.5
Comparison between males and females in the rate of aggregation



Platelets aggregation obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Rate of aggregation was calculated. Results, shown as mean ± s.d. from 10 males versus females.

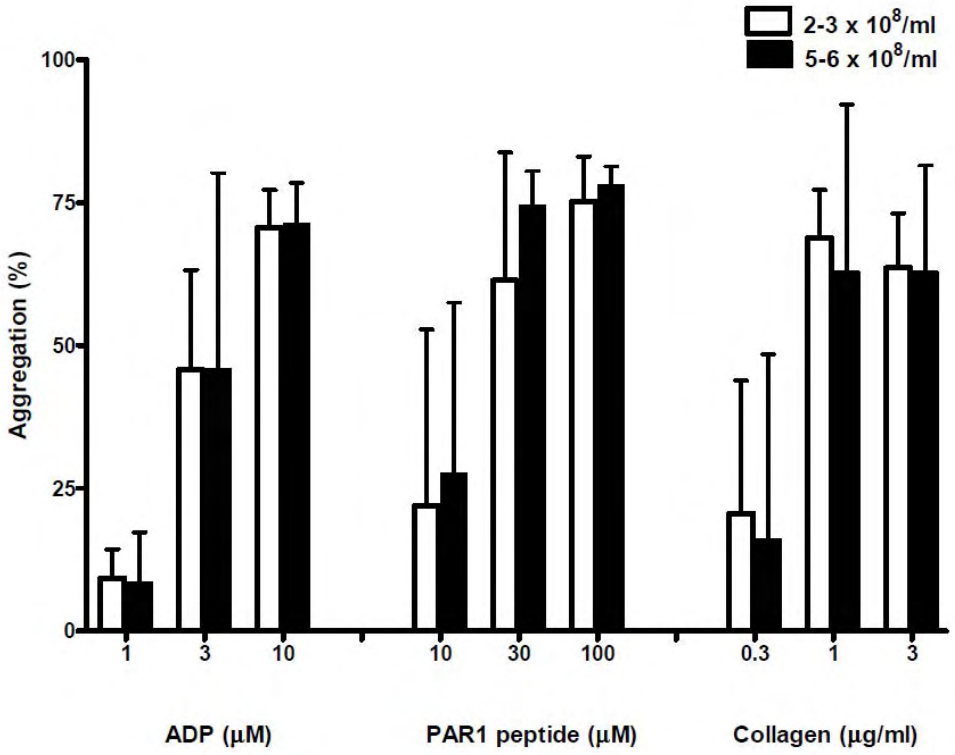
Figure 3.6
Comparison of aggregation in donors aged < 25 yr and > 35 yr



Platelets aggregation obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Aggregation results, shown as % increase in light transmission, was measured at 90 sec after agonist addition. Results are shown as mean ± s.d. from 3 donors.

A potentially important variable in these studies is platelet count, as many laboratories routinely normalise the count between controls and patients through dilution using platelet poor plasma (PPP). Further, it was anticipated that a significant number of patients with suspected mild platelet disorders would be thrombocytopenic. The practice of diluting platelet rich plasma (PRP) with PPP has however been shown to reduce platelet activation in several independent studies, thereby bringing this into question (Lecchi, 2005, Cattaneo et al., 2007, Mani et al., 2005). For this reason, the platelet concentration was not adjusted and aggregation curves were compared in relation to platelet count. To justify this I divided controls into those with a platelet count in PRP of between 2–3 x 10^8/ml or 5–6 x 10^8/ml and compared the concentration response curves to ADP, PAR1 peptide and collagen. As shown in Figure 3.7, there was no significant difference between the two sets of curves for low, intermediate and high concentrations of the three agonists. In further studies (not shown), performed on washed platelets, I observed a shift to the right in the dose response curve to collagen when the platelet count was reduced to less than 1.5 x 10^8/ml, thereby restricting aggregation studies (without alteration of platelet count) to between 1.5–6 x 10^8/ml in PRP.

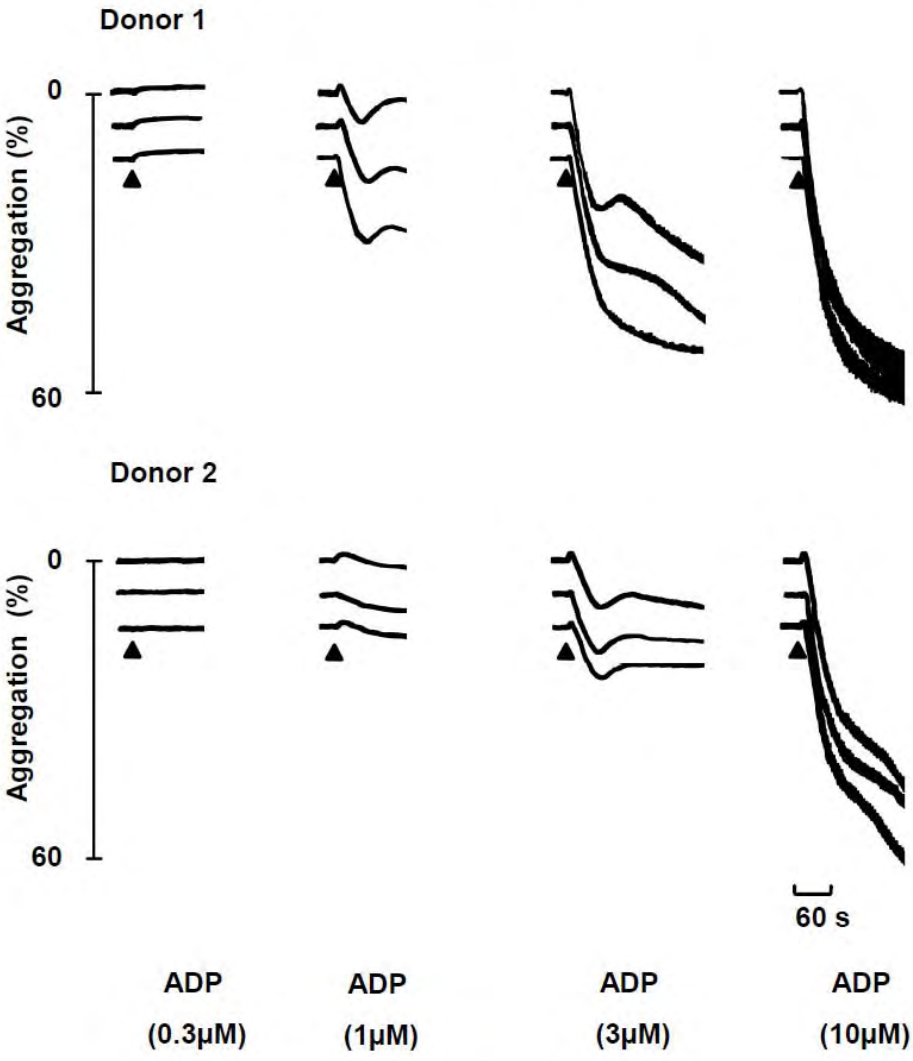
Figure 3.7
Effect of platelet number on aggregation



Platelets aggregation obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Aggregation results, shown as % increase in light transmission, was measured at 180 sec after agonist addition. Results are shown as mean ± s.d. from 5 donors each.

Despite the high level of reproducibility, small differences between donors in the concentration response curves for specific platelet agonists were observed, with the most marked difference being seen with ADP, although all of the response curves fell within a 2–3 fold concentration range. The explanation as to why the greatest variation in response is seen with ADP may reflect that it mediates activation through the synergistic interaction of two receptors, one of which, the $P2Y_1$, is expressed at very low level (approximately 150 copies per platelet). Importantly, differences in the agonist response curves were maintained between donors, as illustrated by the response to ADP in two example donors in Figure 3.8, performed within 10–12 weeks of each other. In this example, donor 1 has a greater sensitivity to ADP than donor 2. This is particularly apparent at 1 or 3 μM ADP which generate reversible or biphasic irreversible aggregation respectively, in donor 1, compared with shape change or reversible aggregation respectively, in donor 2. Further, at the higher concentration of 10 μM ADP, the response in donor 2 can clearly be seen to be biphasic whereas only a single, rapid aggregation response is observed in donor 1. Indeed, small differences in response to multiple agonists between individuals who have donated blood on multiple occasions have been maintained over a course of several years in our laboratory (not shown). This variation in response between donors emphasizes the importance of generating reference curves within a population of healthy volunteers.

Figure 3.8
Reproducibility of concentration response curves for platelet aggregation to ADP in two donors

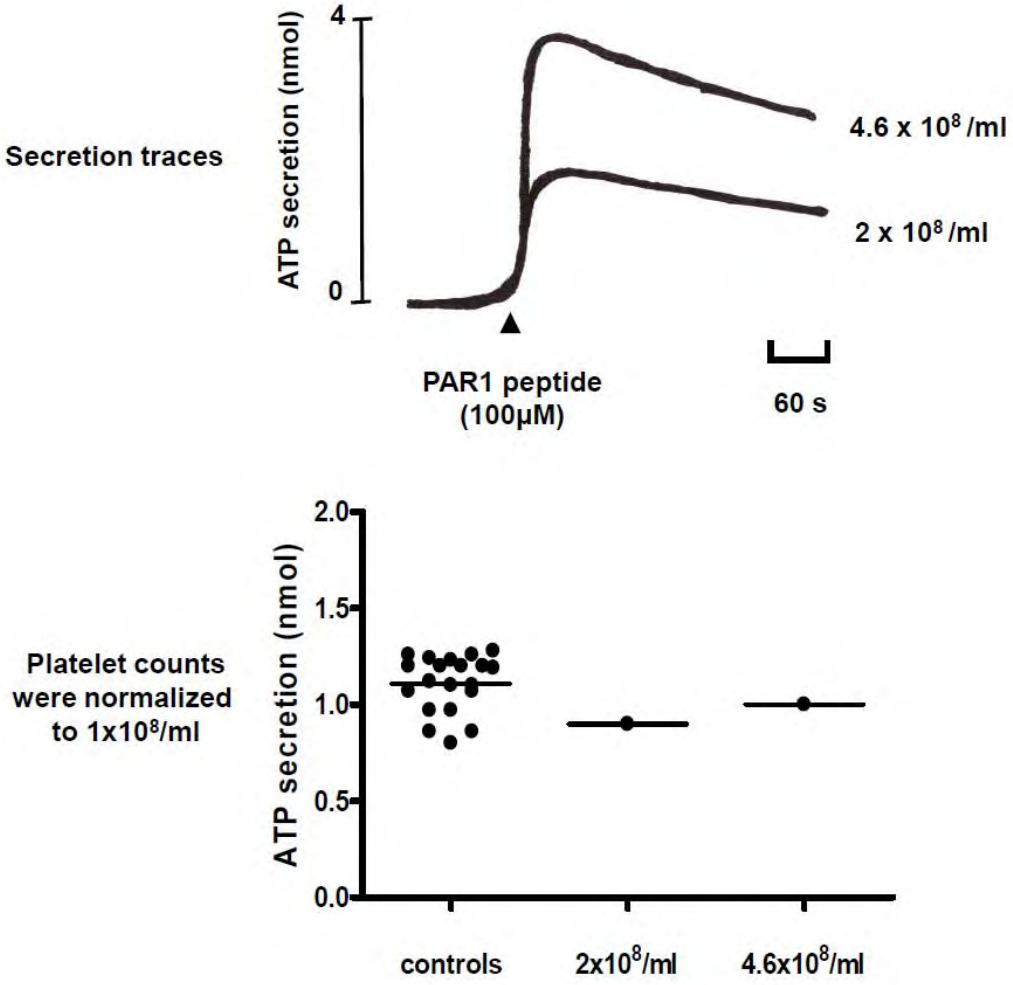


Traces of platelet aggregation in response to ADP, shown in two healthy individuals on three occasions at intervals of 10–12 weeks

3.3.2 Initial characterization of secretion responses

Following the above studies, measurement of ATP release as a monitor of dense granule secretion alongside aggregation was undertaken in healthy volunteers using luciferin-luciferase. ATP is secreted from dense granules in a constant ratio to ADP of 2:3 (BCSH, 1988, Akkerman et al., 1983). Data was normalized to the level of secretion of ATP per 10^8 platelets. Normalization of the data in this way was found to be essential because of the variation in platelet count, as illustrated for platelets stimulated by (100 μM) PAR1 (Figure 3.9).

Figure 3.9
ATP secretion in two donors with different platelet counts in response to 100μM PAR1 peptide

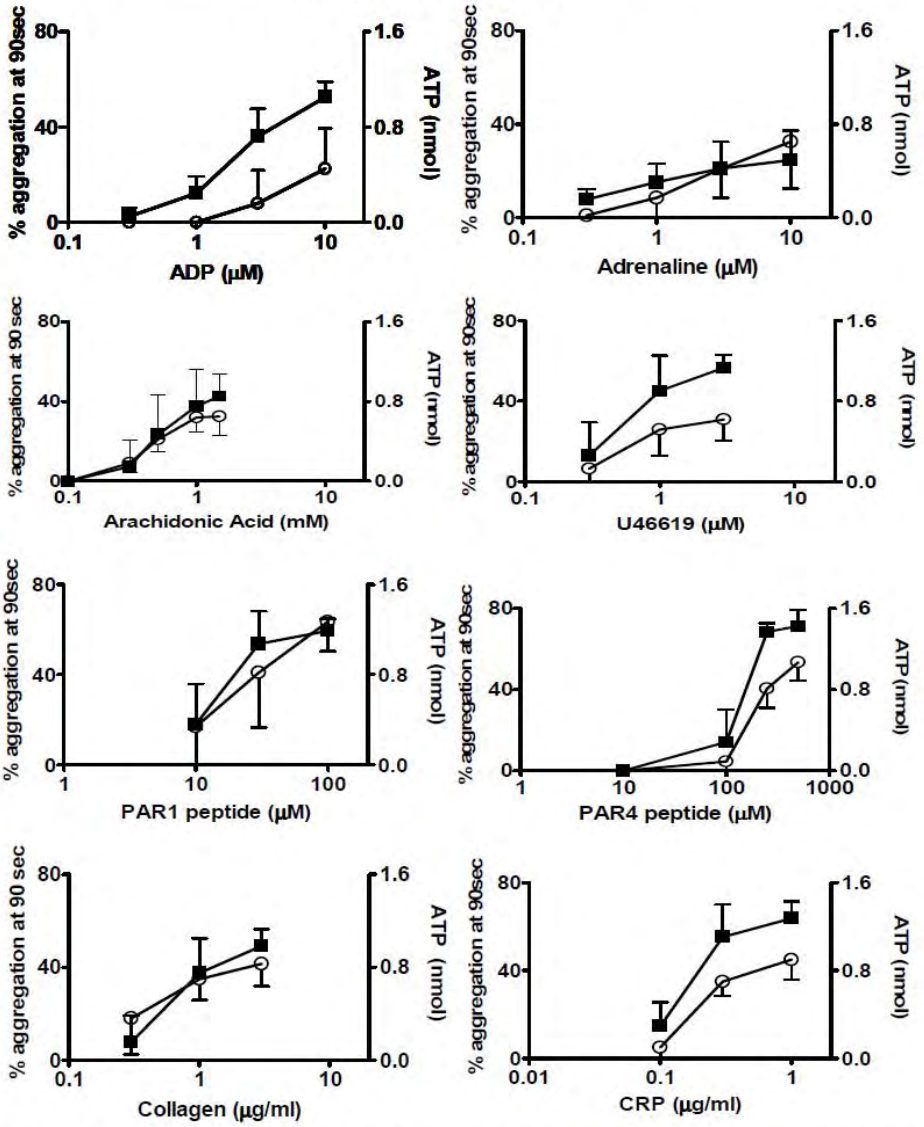


ATP secretion in platelets obtained from two healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. ATP secretion was measured 180 sec after agonist addition using luciferin-luciferase reagents and normalised to 1x 10^8 platelets.

3.3.3 Reference concentration response curves

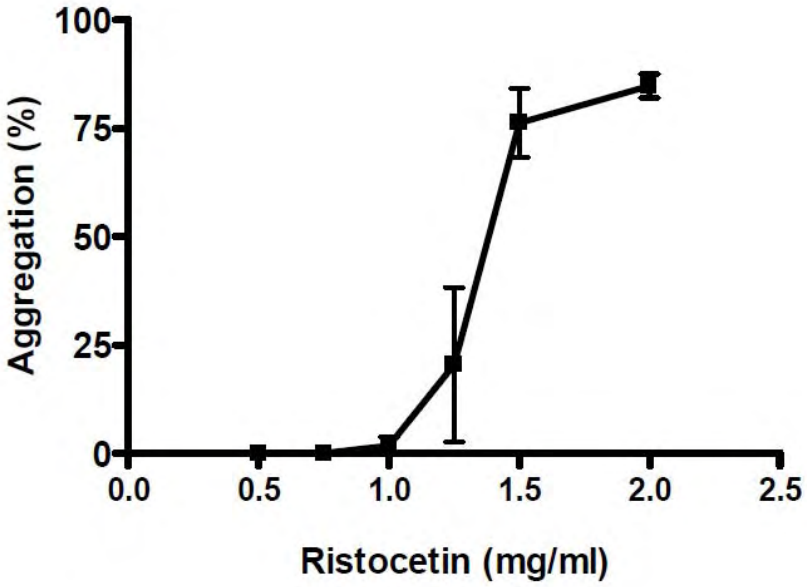
The previously described studies were performed with ADP, adrenaline, collagen, PAR1 peptide, arachidonic acid and U46619. To this list, I added the synthetic collagen CRP, PAR4 petpide and ristocetin. CRP was included because it has been shown in mouse platelets to more readily detect a reduction in the level of the collagen receptor GPVI relative to collagen, which also binds to integrin $\alpha_2\beta_1$. The PAR4 peptide selectively activates the PAR4 receptor, which is generally considered to be the more minor of the two thrombin receptors. Ristocetin mediates platelet agglutination through via the GPIb-IX-V complex. The concentration response curves for aggregation and secretion, measured at 90 and 180 sec, respectively, were determined to the above agonists in 8–20 controls. There was no adjustment for platelet count (2–6x10^8/ml), age (20–55 years) and sex, and all experimentation was performed within 6 hours of platelet preparation. The reference curves are shown in Figure 3.10 and, in the case of ristocetin, Figure 3.11. It was not possible to record the ATP secretion to ristocetin due to interference of ristocetin with the luciferin luciferase reagent. There was a high level of reproducibility in the response curves in all cases, with nearly all standard deviation values falling within 20% of the mean. The pattern of aggregation response to each of the agonists used will be discussed in detail in Section B.

Figure 3.10
Concentration response curves for aggregation and ATP secretion



Aggregation and ATP secretion in platelets obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Aggregation (■) results are shown as % increase in light transmission. ATP secretion (○) was measured using luciferin-luciferase reagents and normalised to 1x 10^8 platelets. Aggregation and secretion were measured 90 sec and 180 sec after agonist addition, respectively. Results are shown as mean+s.d. from between 8 – 20 donors.

Figure 3.11
Concentration aggregation curve for Ristocetin



Platelets aggregation obtained from healthy volunteers were measured in a Born-lumiaggregometer in platelet rich plasma as described in the methods. Aggregation results are shown as % increase in light transmission and measured at 90 sec after agonist addition. Results are shown as mean ± s.d. from 10 donors.

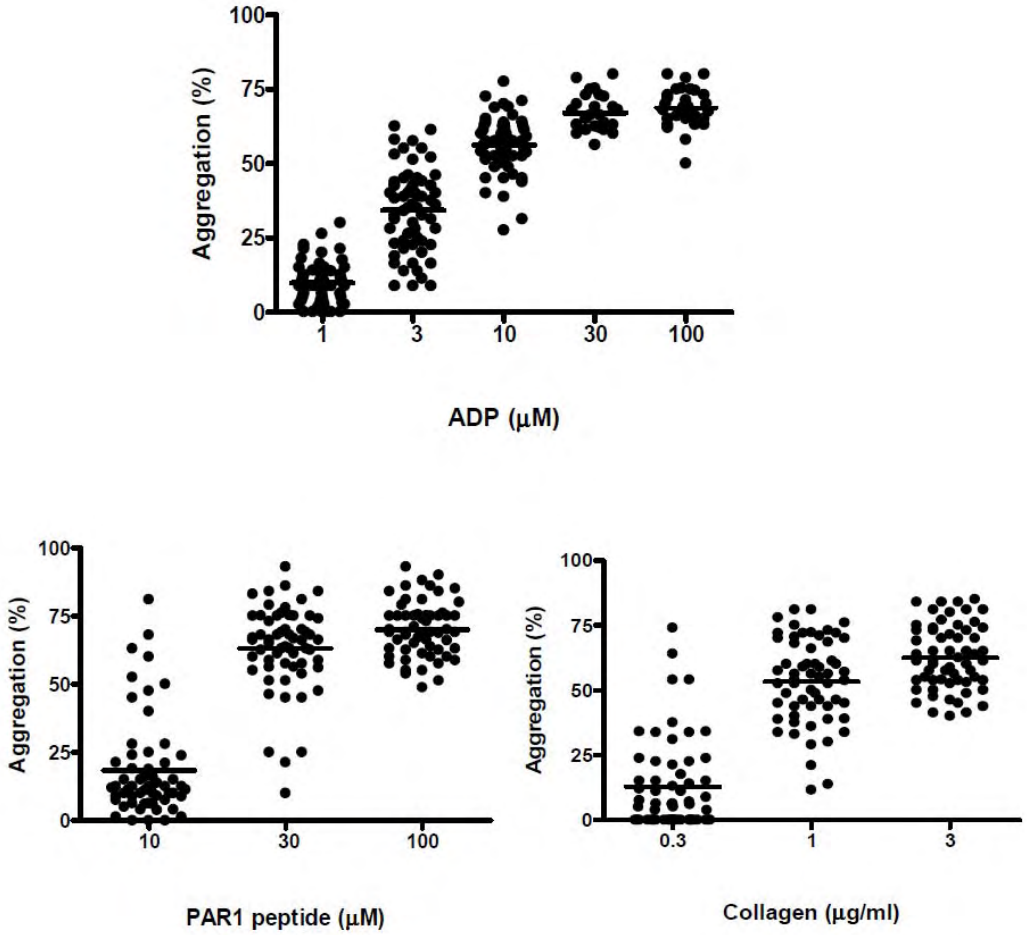
The location of the concentration response curves for ATP secretion corresponded to those for aggregation for all agonists with the exception of ADP, where the concentration response curve for ATP secretion was located approximately three-fold to the right of that for aggregation. This indicates that secretion of ADP reinforces aggregation to all agonists with the exception of ADP itself. The former statement is confirmed by the studies in Section B which investigate the effect of inhibition of $P2Y_1$ and $P2Y_{12}$ receptors on aggregation. PAR1 peptide stimulated the largest level of secretion of ATP, although maximal responses to all agonists fell within 50% of this response (Figure 3.10). Thus, these results indicate a high level of reproducibility in ATP secretion, thereby supporting previous studies that have indicated ATP secretion as a valuable marker of platelet activation.

3.3.4 Aggregation and secretion data in 60 controls

The studies described above were performed at the outset of the project, and before studies on patients had begun. Over the following period of more than 3 years, platelet aggregation and secretion responses to the above agonists were continually monitored in controls performed alongside patients. This has now increased the number of controls that have been analysed to over 60 and has enabled us to further assess the reproducibility in response between individuals. This data is summarised in Figure 3.12 with the response of each individual represented by a single dot for each concentration of agonist. Since several individuals have been analysed on more than one occasion, I have only included the results from the first experiment on that individual. I have chosen to illustrate the data as the response of each individual as outliers can be masked through meaning of data and not each concentration of agonist

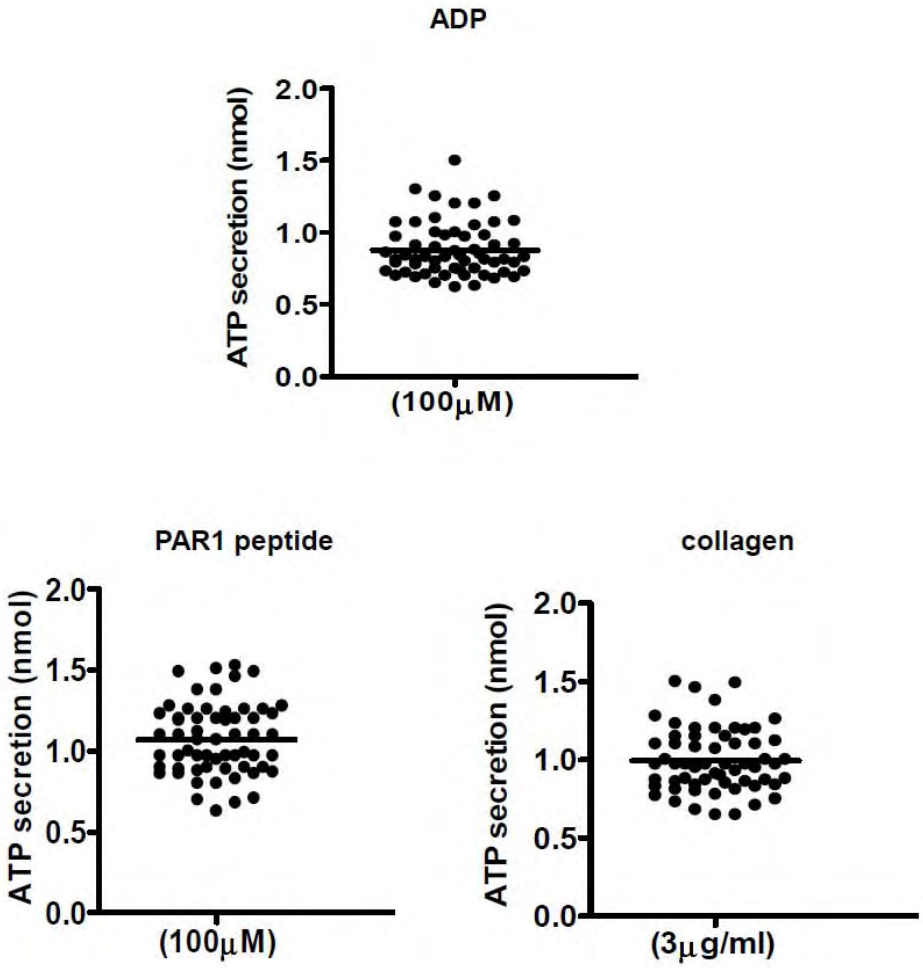
was used on each control, as the study was guided by the results in the patient. Thus, for example, if the response to a submaximal concentration of an agonist such as PAR1 peptide was normal, I may not have gone on to monitor the response to a lower / higher concentration, taking into consideration also volume of blood, time and the response to other agonists. Data has been presented for three agonists, ADP, PAR1 and collagen, as these signal through a distinct set of receptors. Two higher concentrations of ADP (30 and 100 μM) have been included. ATP secretion from dense granules was also measured in this group of controls in response to the highest concentration of each agonist and is shown in Figure 3.13.

Figure 3.12
Aggregation to ADP, PAR1 peptide and collagen in 60 controls



The concentration response relationships for platelet aggregation were measured in response to ADP, PAR1 peptide and collagen. Responses were measured 180 seconds after agonist addition.

Figure 3.13: ATP secretion in 60 controls in response to ADP, PAR1 and collagen



ATP secretion in platelets from 60 healthy volunteers (controls) induced by maximal concentrations of ADP, PAR1 peptide and collagen as indicated. ATP secretion was measured 180 sec after agonist addition using luciferin-luciferase reagent and normalised to $1x10^8$ platelets

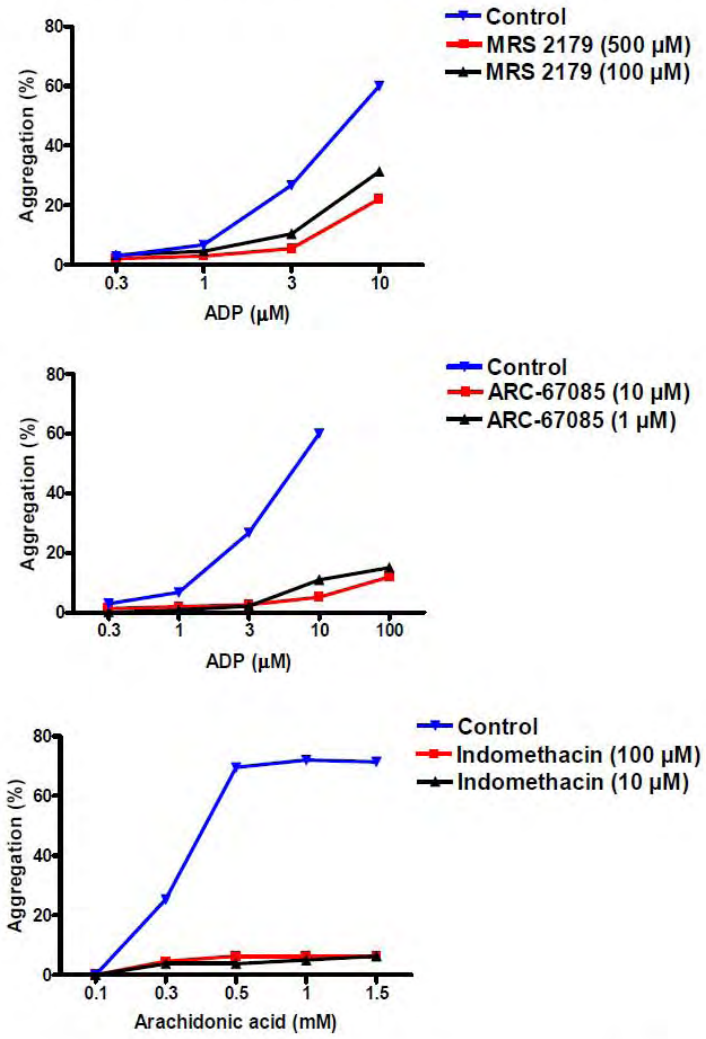
The striking observation from this data is the level of reproducibility which confirms the conclusion from the smaller studies described above. The most variable response was seen with ADP (3 μM), which ranged from < 10% to over 50% aggregation. Importantly, however, at a three fold lower concentration, the response to ADP was always less than 30%, while at a threefold higher concentration, it was equal to or greater than 30%. A small number of individuals did show a hyper response to the lowest concentration of PAR1 peptide that was used (10 μM), whereas in contrast, 4 individuals showed a reduced response to 30 μM peptide. Overall though the level of reproducibility is impressive and we can say with confidence, for example, that a patient with a reduced aggregation to single concentrations of ADP (30 μM), PAR1 (100 μM) and collagen (3 μg/ml) must have a platelet defect.

Section B

3.3.5 Analysis of aggregation and dense granule secretion to platelet agonists in the presence of ADP receptor antagonists and cyclooxygenase inhibition

ADP and TxA_2 play a critical feedback role in mediating platelet activation. A defect in their release or in the function of their receptors has a widespread inhibitory effect on platelet activation. However, the bleeding phenotype of the defect differs according to whether secretion is disrupted or receptor function is impaired. To characterize this in full, we have investigated aggregation and dense granule secretion induced by low, intermediate and high concentrations of platelet agonists in the absence and presence of antagonists of the two ADP receptors, $P2Y_1$ and $P2Y_{12}$, namely MRS 2179 (100 μM) and ARC-67085 (1μM), respectively, and the cyclooxygenase inhibitor, indomethacin (10 μM), which blocks the liberation of TxA_2. All of the inhibitors were used at maximally-effective concentrations, as shown by the observation that a 5-10 fold higher concentration of each inhibitor did not have an additional effect on platelet activation to ADP or arachidonic acid, even though both agonists caused a small response in the presence of the two receptor antagonists or cyclooxygenase, respectively (Figure 3.14). The residual response to ADP that is seen in the presence of either of the receptor antagonists can be explained by the presence of the other ADP receptor as discussed below. The weak, residual response to arachidonic acid, which was manifest as shape change and very weak aggregation (< 10% of maximal aggregation) indicates a cyclooxygenase-independent actions, as also described by others (Frelinger et al., 2006).

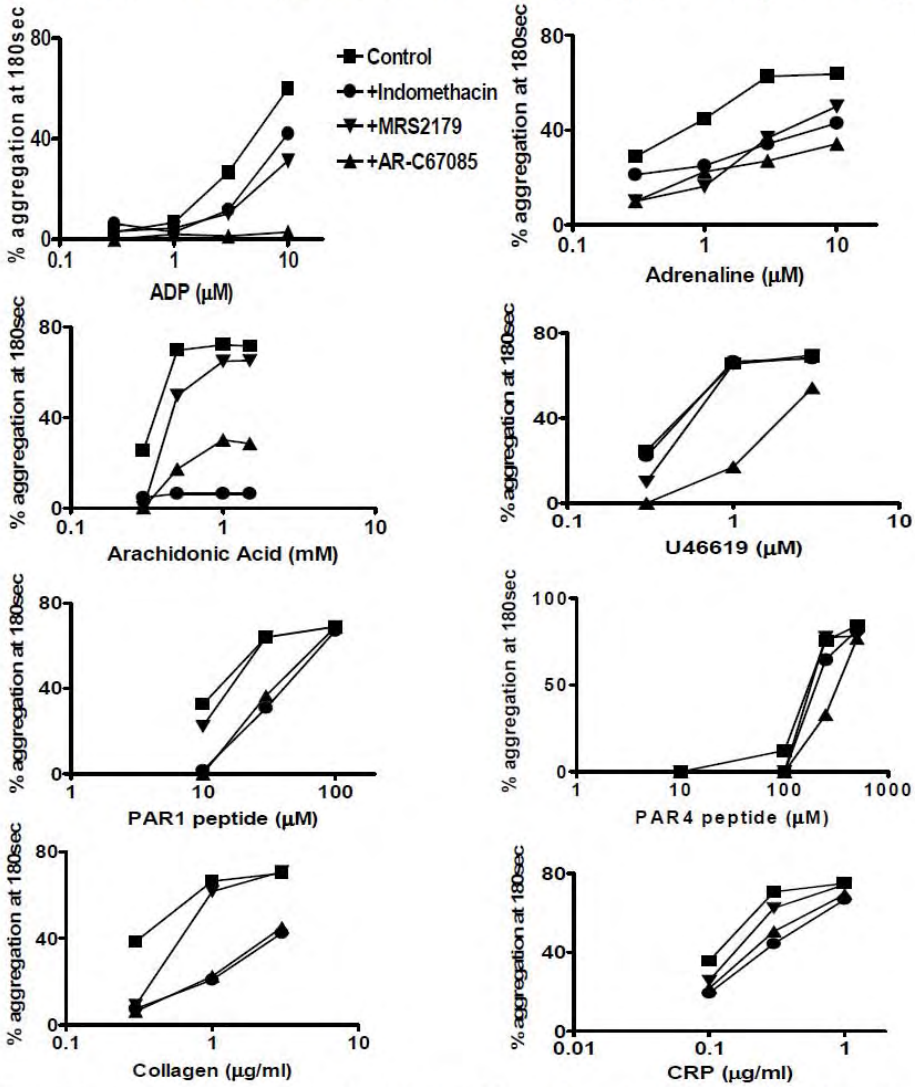
Figure 3.14
The effect of higher concentrations of ADP receptor antagonists and COX-inhibitor on aggregation responses



The concentration response relationships for platelet aggregation were measured in response to ADP in the absence and presence of the $P2Y_1$ receptor antagonist MRS2179 (100 and 500µM) and the $P2Y_{12}$ antagonist AR-C67085 (1 and 10µM) and in respose for arachidonic acid in the presence of the cyclooxygenase inhibitor indomethacin (10 and 100µM), Inhibitors were given 3 min prior to the agonists. Responses were measured 180 seconds after agonist addition. Results represent 2 experiments. (n=2).

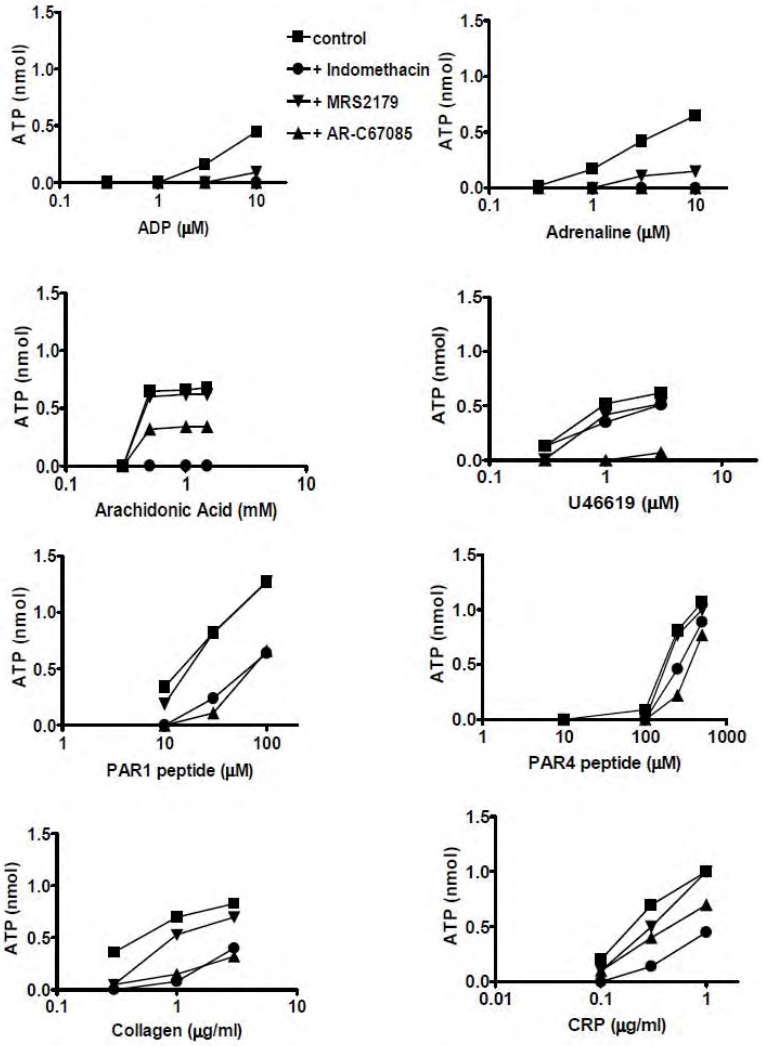
The effect of the two ADP receptor antagonists or cyclooxygenase inhibition on aggregation and dense granule secretion to ADP, adrenaline, PAR1 and PAR4 peptides, collagen, CRP, U46619 and arachidonic acid was investigated in a minimum of three donors for each agonist (Figure 3.15 and 3.16). All responses were monitored at 180 sec as secretion has peaked by this time and biphasic aggregation can be readily seen in the majority of controls. Each aggregation response curve was fitted to a three variable logistic equation using Graphpad Prism software to calculate the EC_{50} and 95% confidence levels as shown in Table 3.1. A similar analysis was not performed for ATP secretion because of uncertainty in the maximal response. The effect of the inhibitors on the above agonists and the pattern of response to low, intermediate and high concentrations of each agonist are discussed below.

Figure 3.15
The effect of inhibition of ADP receptors and cyclooxygenase activity on the concentration response curves for aggregation to platelet agonists



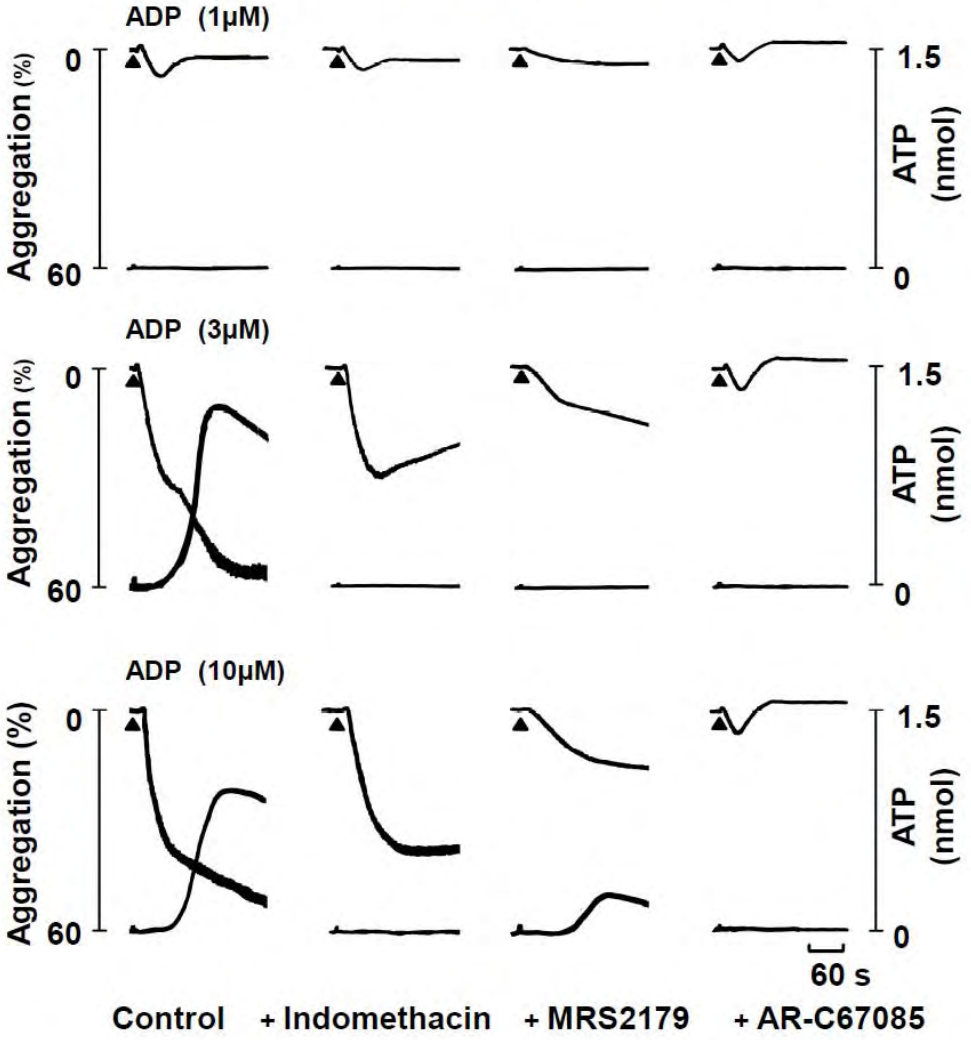
The concentration response relationships for platelet aggregation were measured in response to eight different platelet agonists in the absence and presence of the cyclooxygenase inhibitor indomethacin (10µM), the $P2Y_1$ receptor antagonist MRS2179 (100µM) and the $P2Y_{12}$ antagonist AR-C67085 (1µM). Inhibitors were given 3 min prior to the agonists. Responses were measured 180 seconds after agonist addition. Results represent the mean+s.d. of a minimum of three experiments. (n=3).

Figure 3.16
The effect of inhibition of ADP receptors and cyclooxygenase activity on the concentration response curves for ATP secretion to platelet agonists



The concentration response relationships for ATP secretion were measured in response to eight different platelet agonists in the absence and presence of the cyclooxygenase inhibitor indomethacin (10µM), the $P2Y_1$ receptor antagonist MRS2179 (100µM) and the $P2Y_{12}$ antagonist AR-C67085 (1µM). Inhibitors were given 3 min prior to the agonists. Responses were measured 180 seconds after agonist addition. Results represent the mean+s.d. of a minimum of three experiments. (n=3).

Figure 3.17
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to ADP.



Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of ADP in the absence or presence of cyclooxygenase inhibitor indomethacin (10µM), the $P2Y_1$ receptor antagonist MRS2179 (100µM) and the $P2Y_{12}$ antagonist AR-C67085 (1µM). Traces are representative of a minimum of 3 experiments.

3.3.6 Aggregation and dense granule secretion in the presence of ADP receptor antagonists and cyclooxygenase inhibition

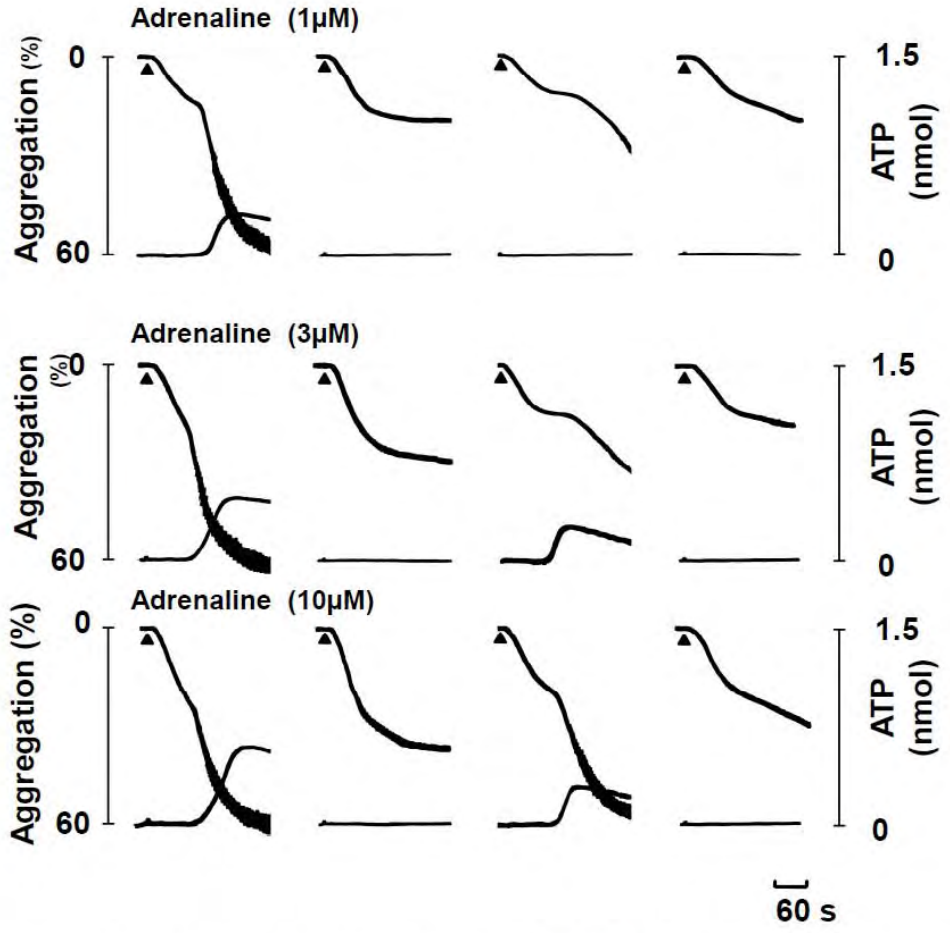
Example traces showing aggregation and dense granule secretion in response to low, intermediate and high concentrations of platelets agonists, in the presence and absence of inhibitors, are shown in Figures 3.17 –3.25 and are discussed below.

ADP

ADP stimulates platelet aggregation through a synergy between $P2Y_1$ and $P2Y_{12}$ receptors which are coupled to the G_q and G_{13} families, and the G_i family of heterotrimeric G proteins, respectively. The $P2Y_{12}$ receptor is recognized to undergo marked synergy with receptors that signal through G_q and G_{13} to mediate powerful aggregation and secretion. In the representative trace, an intermediate concentration of ADP (3 μM) induces biphasic aggregation as shown by an initial rapid response followed by a delayed second phase (Figure 3.17). This pattern of response is seen in most but not all donors as illustrated in Figure 3.8. The second phase is absent at a lower concentration of ADP (1 μM) and the two phases are less distinct at a higher concentration of ADP (10 μM). Sustained aggregation at intermediate and high concentrations of ADP (3 and 10 μM respectively) is dependent on release of thromboxane, as illustrated by deaggregation in the presence of indomethacin, although this is notably much slower at the higher concentration of ADP (Figure 3.17). Indomethacin also causes a complete inhibition of dense granule secretion to ADP (Figure 3.17). The loss of the second wave of aggregation is most likely due to the absence of synergy between TxA_2 and ADP rather than loss of secretion, as the latter is not thought to contribute to ADP-induced aggregation (see Section 3.3.3 and Figure

3.10), a result that was confirmed using platelets from two patients with Hermansky-Pudlak syndrome-8 (HPS-8) (see Chapter 4-Figure 4.3). HPS-8 is a recently identified form of the dense granule disorder that is caused by a mutation in BLOC1S3 and so they are unable to form platelet dense granules. In contrast, blockade of the two P2Y ADP receptors causes a qualitatively distinct pattern of inhibition to that seen with indomethacin. The $P2Y_1$ receptor antagonist, MRS2179, completely inhibits shape change (i.e. the initial transient increase in optical density) to all concentrations of ADP and also markedly reduces the extent of aggregation and dense granule secretion (3.17). Significantly, however, a low level of aggregation to ADP in the presence of MRS2179 is sustained and dense granule secretion is induced by a high concentration of ADP (Figure 3.17). On the other hand, the $P2Y_{12}$ receptor antagonist, AR-C67085, has no effect on shape change but converts aggregation to a transient response that returns to the base-line within 2 minutes at all three concentratons of ADP that were investigated (Figure 3.17). In addition, dense granule secretion is abolished to all concentrations of ADP in the presence of AR-C67085 (Figure 3.17). The results demonstrate a critical role for the $P2Y_1$ receptor in mediating shape change and in synergising with the $P2Y_{12}$ ADP receptor to mediate aggregation and secretion. The inhibitory effect of AR-C67085 demonstrates the critical role of the $P2Y_{12}$ ADP receptor in initiating and sustaining aggregation, as well as in mediating secretion. Thus, these results demonstrate that blockade of the two ADP receptors or cyclooxygenase has distinct effects on aggregation and secretion to ADP.

Figure 3.18
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to adrenaline.



Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of adrenaline in the absence or presence of cyclooxygenase inhibitor indomethacin (10µM), the $P2Y_1$ receptor antagonist MRS2179 (100µM) and the $P2Y_{12}$ antagonist AR-C67085 (1µM). Traces are representative of a minimum of 3 experiments.

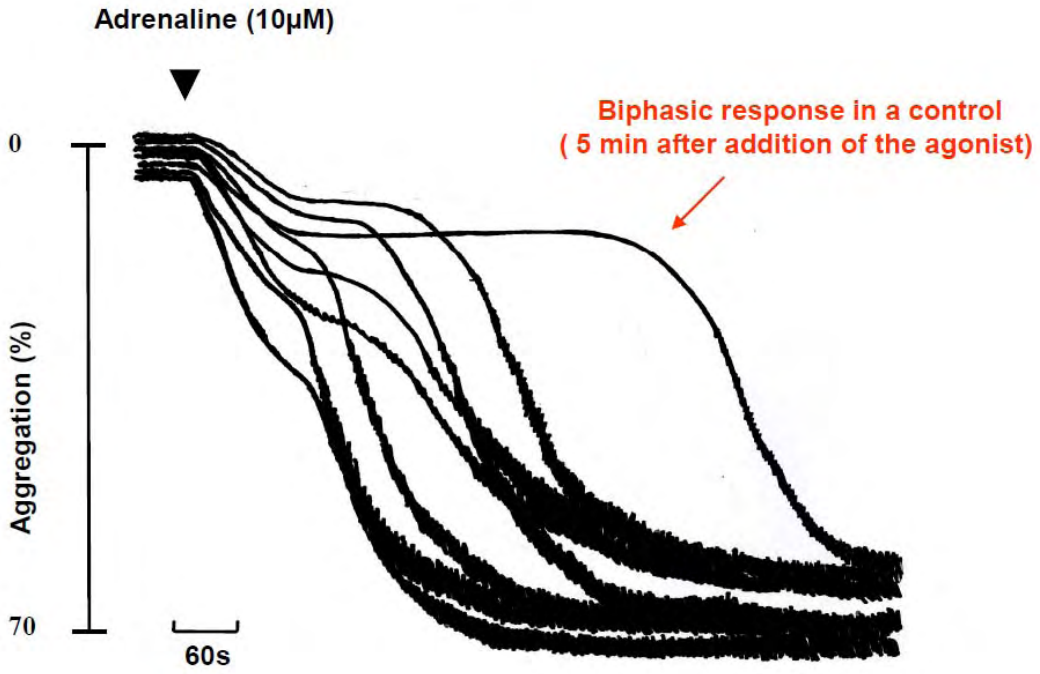
Adrenaline

Adrenaline stimulates aggregation through the α_{2A}-adrenoreceptor, which is coupled to the G_i family of heterotrimeric G proteins. Since this is the same family of G proteins that is regulated by the $P2Y_{12}$ ADP receptor, it is important to compare responses to adrenaline and ADP in patients that have a defect in one or both of these agonists, although it should be borne in mind that studies in mice have demonstrated major roles for distinct G_i isoforms in signalling by the two receptors (Jantzen et al., 2001, Yang et al., 2000). Specifically, $P2Y_{12}$ is coupled to $Gi_{\alpha 2}$ and the α_{2A}-adrenoceptor is coupled to Gz. The response to adrenaline is characterized by the absence of shape change and a biphasic aggregation in which the second phase occurs concomitantly with release of dense granules as indicated by release of ATP (Figure 3.18). Importantly, this phase of aggregation and secretion are completely blocked in the presence of indomethacin (Figure 3.18). In contrast, blockade of the $P2Y_1$ ADP receptor has a relatively mild effect on aggregation and secretion to adrenaline that is manifest as a small shift to the right in the concentration response curves for the two responses (Figure 3.18). Blockade of the $P2Y_{12}$ ADP receptor by AR-C67085 in this control also inhibits the second phase of aggregation and the secretion of ATP. However, following investigation of other controls, I have shown that blockade of the $P2Y_{12}$ ADP receptor can have either a partial or no effect on the second phase of adrenaline-induced aggregation, but nevertheless there is always a dramatic reduction in secretion (see Chapter 4-Figure 4.2).

It is important to emphasize that biphasic aggregation to adrenaline was observed in all controls that were investigated in this study in view of reports in the literature that a proportion of the population exhibit only primary wave aggregation. For example,

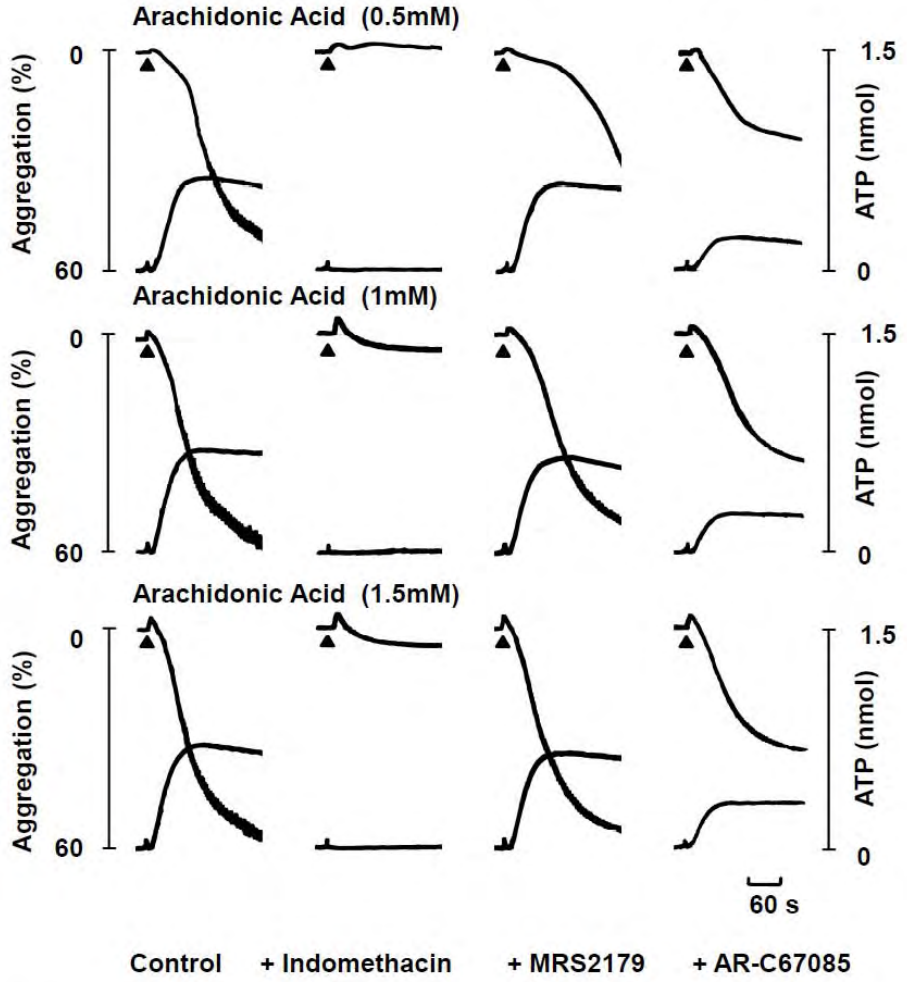
Weiss and Lages observed secondary aggregation to adrenaline in 117 out 130 donors i.e. approximately 90% of donors (Weiss and Lages, 1988). The explanation as to why all of the donors in the present study were found to undergo secondary aggregation to adrenaline may be because we allowed aggregation to proceed for several minutes, as 2 of the 60 controls exhibited a delay in the secondary phase, which took more than 5 minutes to be begin as illustrated in Figure 3.19. Indeed, I have investigated one of these two controls on five separate occasions and each time have observed a prolonged delay in the onset of the second wave.

Figure 3.19
Aggregation responses to (10 μM) adrenaline in several healthy donors



Examles of biphasic platelet aggregation responses to adrenaline (10μM) measured in healthy controls. The traces proceed for more than 5 minutes.

Figure 3.20
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to arachidonic acid



Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of arachidonic acid in the absence or presence of cyclooxygenase inhibitor indomethacin (10μM), the $P2Y_1$ receptor antagonist MRS2179 (100μM) and the $P2Y_{12}$ antagonist AR-C67085 (1μM). Traces are representative of a minimum of 3 experiments.

Arachidonic acid and the thromboxane mimetic U46619

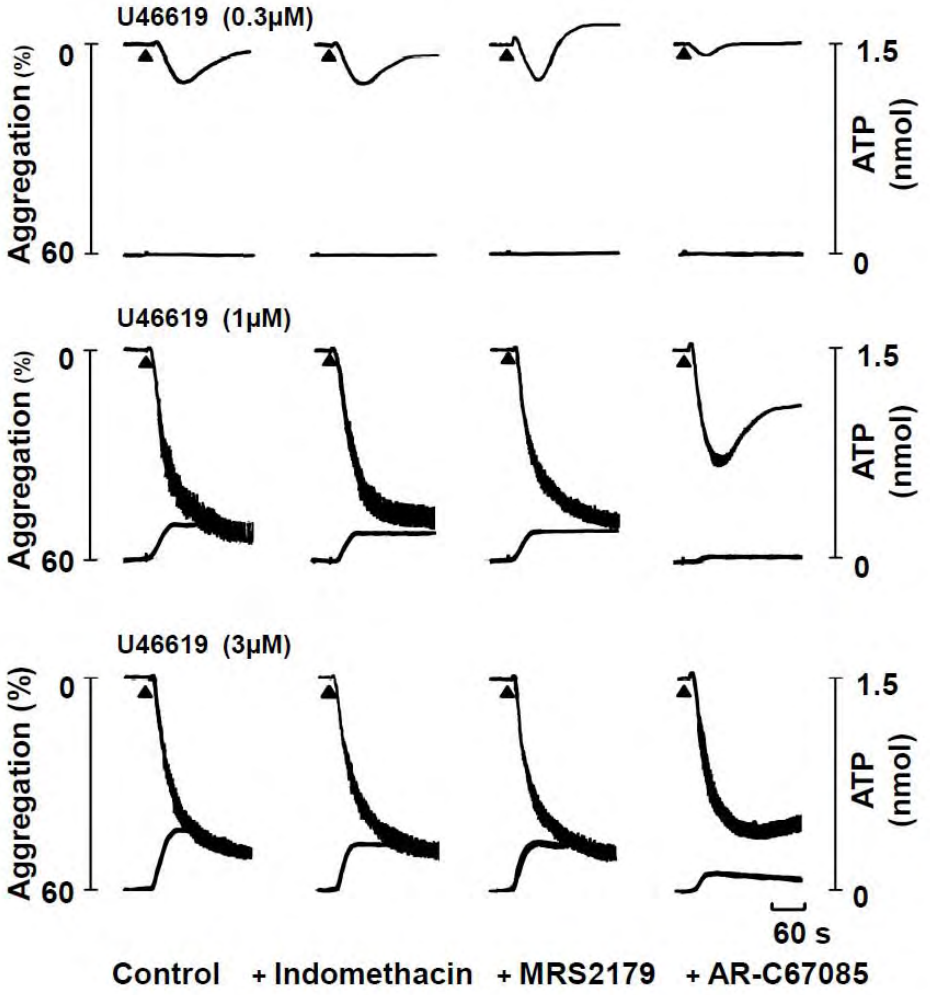
Arachidonic acid is converted to TxA_2 by platelet cyclooxygenase, which induces platelet activation through the G_q and G_{13}-coupled thromboxane receptor. TxA_2 has a very short half-life and so the stable analogue, U46619, was used to induce platelet activation through the thromboxane receptor under *in vitro* conditions.

Arachidonic acid was observed to induce biphasic aggregation at low concentrations and monophasic aggregation at higher concentrations, along with robust secretion (Figure 3.20). Powerful, sustained aggregation and secretion were abolished in the presence of indomethacin, although there was a residual shape change response and weak aggregation as discussed above in Section 3.3.5 and consistent with the results of Frelinger et al (Frelinger et al., 2006). In comparison, the $P2Y_1$ ADP antagonist, MRS2179, has a minor inhibitory effect on aggregation and ATP secretion to low but not high concentrations of arachidonic acid, while the $P2Y_{12}$ ADP antagonist, AR-C67085, reduced the second component of aggregation, although the extent of this reduction varied between controls. The $P2Y_{12}$ ADP antagonist, AR-C67085, also partially inhibited ATP secretion (Figure 3.20). Thus, arachidonic acid mediates full aggregation and secretion through a synergy between the thromboxane receptor and the $P2Y_{12}$ ADP receptor, but at high concentrations it can partially overcome the effect of $P2Y_{12}$ ADP receptor blockade. In addition, arachidonic acid stimulates shape change independent of thromboxane formation.

Strikingly, the effect of the above inhibitors on the response to U46619, a stable analogue of TxA_2, was distinct from that observed against arachidonic acid (Figure

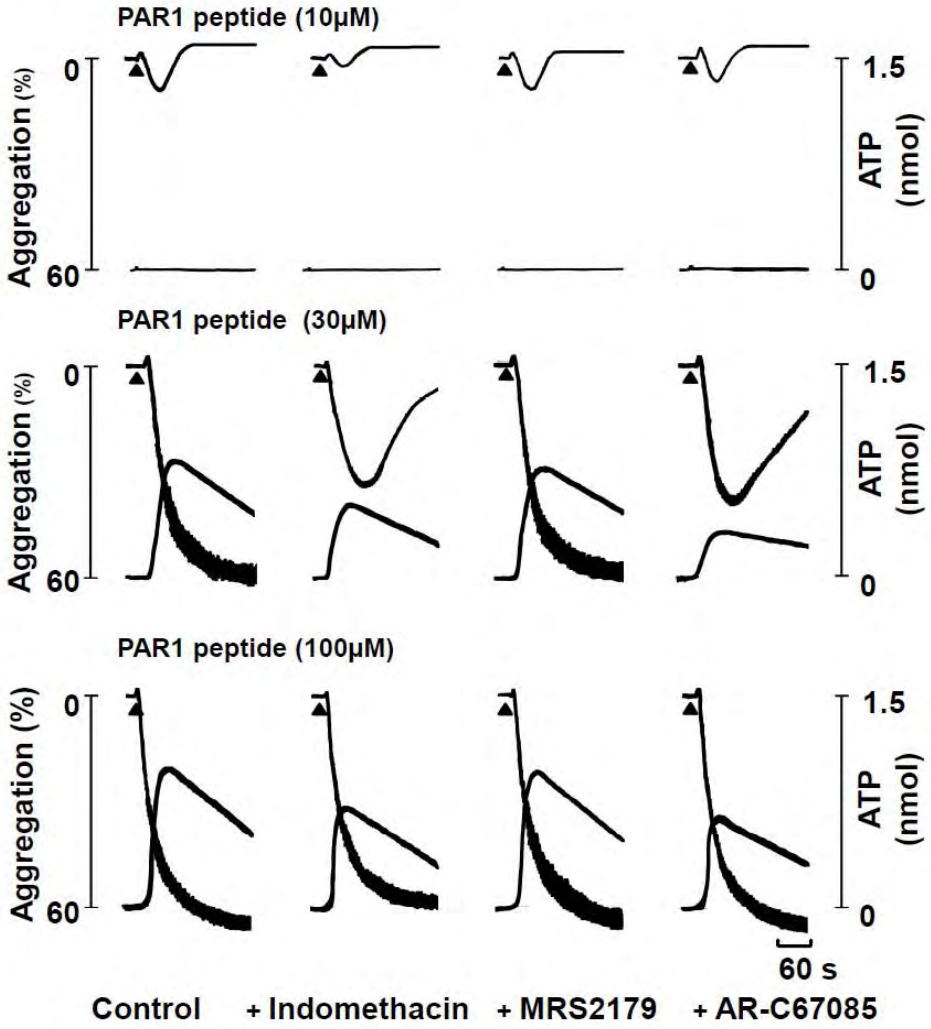
3.21). In particular, aggregation and secretion induced by U46619 was not altered, or was only marginally reduced, in the presence of indomethacin (Figure 3.21). On the other hand, the second phase of the aggregation response and the stimulation of secretion by U46619 were partially inhibited in the presence of the $P2Y_{12}$ receptor antagonist, AR-C67085, as was the case for arachidonic acid. The $P2Y_1$ receptor antagonist, MRS2179, has a very minor effect on the response to low but not higher concentrations of U46619 (Figure 3.21), as was the case with arachidonic acid. This minor effect is presumably because of redundancy in that the TxA_2 and $P2Y_1$ receptor signal through the same pathway and the former is expressed at approximately one order of magnitude higher level. Thus, comparison of responses to arachidonic acid and U46619 allows differentiation between a defect at the level of platelet cyclooxygenase or the platelet thromboxane receptor. Specifically, platelet activation by arachidonic acid is inhibited in the presence of the cyclooxygenase inhibitor indomethacin, whereas the response to U46619 is not. On the other hand, the response to both stimuli would be inhibited by a defect in the thromboxane receptor.

Figure 3.21
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to U46619



Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of U46619 in the absence or presence of cyclooxygenase inhibitor indomethacin (10μM), the $P2Y_1$ receptor antagonist MRS2179 (100μM) and the $P2Y_{12}$ antagonist AR-C67085 (1μM). Traces are representative of a minimum of 3 experiments.

Figure 3.22
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to PAR1 peptide

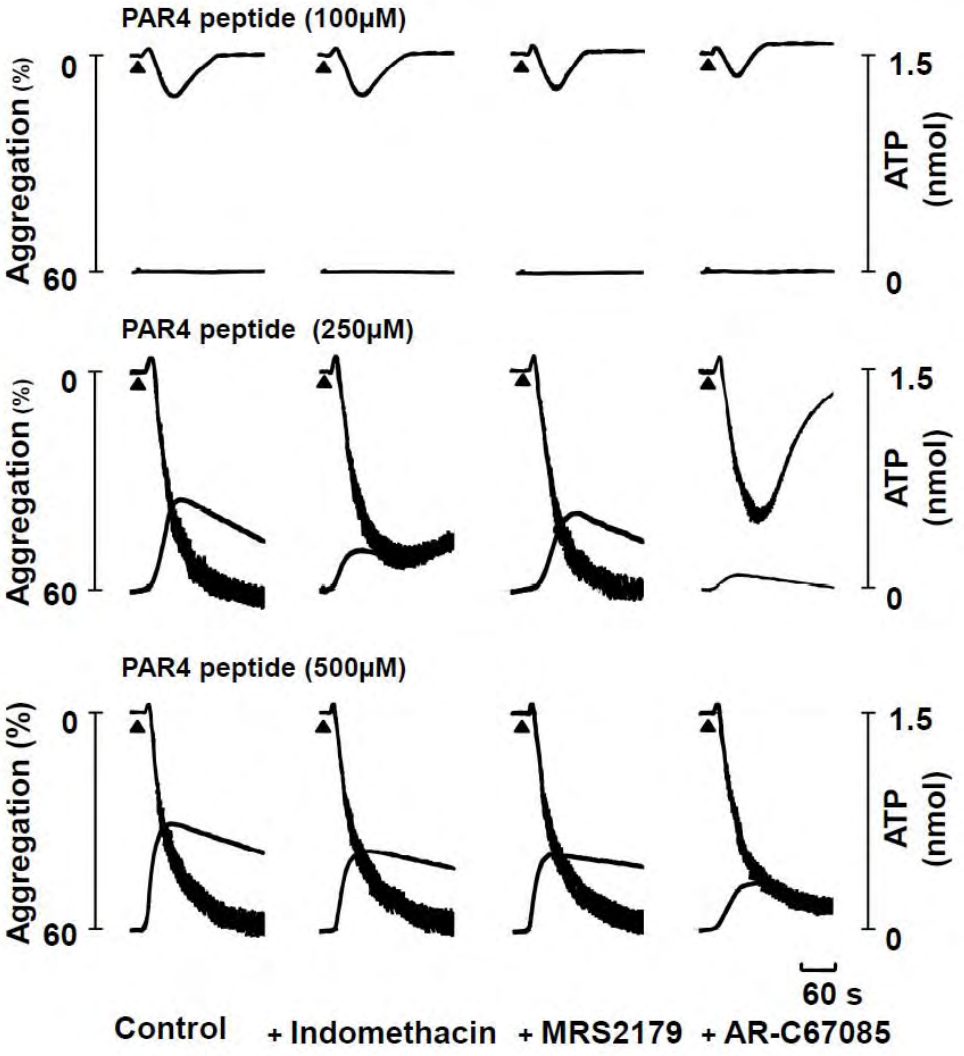


Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of PAR1 peptide in the absence or presence of cyclooxygenase inhibitor indomethacin (10μM), the $P2Y_1$ receptor antagonist MRS2179 (100μM) and the $P2Y_{12}$ antagonist AR-C67085 (1μM). Traces are representative of a minimum of 3 experiments.

PAR1 and PAR4 peptides

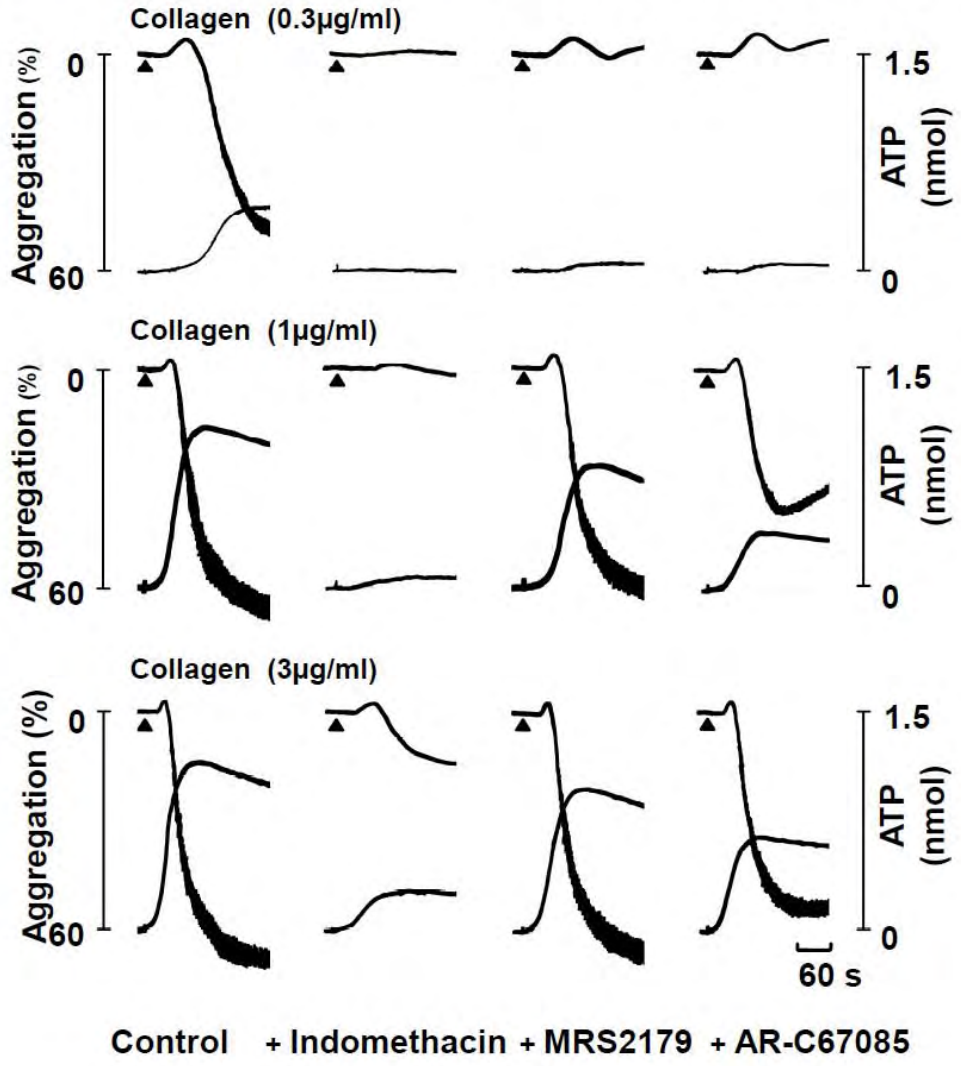
The ability of the thrombin receptors, PAR1 and PAR4, to mediate platelet activation can be monitored using thrombin receptor activating peptides (TRAPs) that are specific for each receptor (Coughlin, 2005). In the present study, we have used SFLLRN and AYPGKF to activate PAR1 and PAR4, respectively. Both thrombin receptors are coupled to G_q and G_{13} heterotrimeric G proteins. The two PAR1 and PAR4-specific peptides induce similar patterns of aggregation and secretion and their responses are altered in a similar way in the presence of indomethacin and the $P2Y_{12}$ receptor antagonist. For both peptides, the $P2Y_1$ receptor does not appear to contribute to aggregation or secretion, presumably because of redundancy in their signalling pathways as is the case for the thromboxane receptor (Figures 3.22 and 3.23). On the other hand, the sustained aggregation induced by intermediate concentrations of the PAR1 and PAR4-specific peptides is converted to a transient response in the presence of indomethacin and the $P2Y_{12}$ receptor antagonist, AR-C67085, with a corresponding decrease in dense granule secretion (Figures 3.22 and 3.23). Higher concentrations of the two peptides induce sustained, maximal aggregation in the presence of indomethacin and AR-C67085, although dense granule secretion is reduced. Thus, these results demonstrate that platelet activation by the PAR1 and PAR4-specific peptides is reinforced by release of TxA_2 and secretion of ADP, and that this is most readily seen at intermediate concentrations of the two peptides.

Figure 3.23
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to PAR4 peptide



Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of PAR4 peptide in the absence or presence of cyclooxygenase inhibitor indomethacin (10µM), the $P2Y_1$ receptor antagonist MRS2179 (100µM) and the $P2Y_{12}$ antagonist AR-C67085 (1µM). Traces are representative of a minimum of 3 experiments.

Figure 3.24
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to collagen



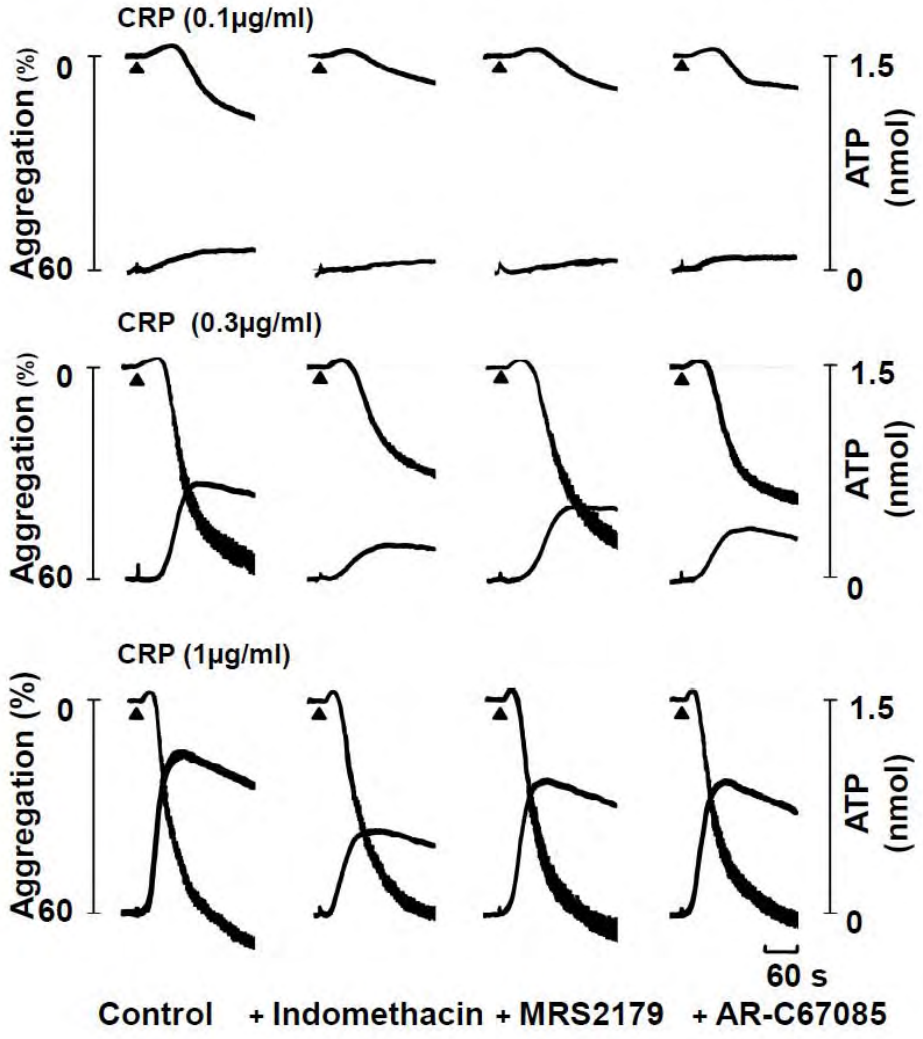
Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of collagen in the absence or presence of cyclooxygenase inhibitor indomethacin (10µM), the $P2Y_1$ receptor antagonist MRS2179 (100µM) and the $P2Y_{12}$ antagonist AR-C67085 (1µM). Traces are representative of a minimum of 3 experiments.

Collagen and CRP

Collagen initiates platelet activation through the two glycoprotein receptors, GPVI and integrin $\alpha_2\beta_1$ (Nieswandt and Watson, 2003). Crosslinking of GPVI generates powerful tyrosine kinase-dependent signals that mediate activation. In addition, the interaction of collagen with GPVI is reinforced by binding to integrin $\alpha_2\beta_1$, thereby part of the function of the integrin is to increase binding to GPVI. Integrin $\alpha_2\beta_1$ also generates weak tyrosine kinase-based intracellular signals, although the significance of these is not known (Inoue et al., 2003). The synthetic collagen, CRP, induces platelet activation independent of integrin $\alpha_2\beta_1$ and can therefore be used to distinguish between defects in GPVI and $\alpha_2\beta_1$. The ability of a low concentration of collagen to induce full aggregation and dense granule secretion is markedly inhibited in the presence of indomethacin and by the two ADP receptor antagonists, MRS2179 and AR-C67085 (Figure 3.24). This demonstrates the critical role of the two ADP receptors and thromboxane formation in mediating platelet activation to low concentrations of collagen. In comparison, the $P2Y_1$ receptor does not contribute to activation by higher concentrations of collagen (Figure 3.24). On the other hand, aggregation and ATP secretion to higher concentrations of collagen is powerfully inhibited in the presence of indomethacin, whereas blockade of the $P2Y_{12}$ receptor has a less marked inhibitory effect (Figure 3.24). Thus, these results demonstrate that platelet activation to collagen is critically dependent on generation of thromboxanes and, to a lesser extent, the $P2Y_{12}$ receptor. This can be explained by the fact that only a small proportion of platelets undergo adhesion to collagen and it is the release of the two secondary mediators that mediate activation of other platelets, especially at lower concentrations of collagen.

In comparison, responses to all concentrations of CRP are independent of the $P2Y_1$ receptor and have only a partial dependency on liberation of thromboxanes and the $P2Y_{12}$ receptor (Figure 3.25). Thus, a high concentration of CRP is able to induce full aggregation in the presence of indomethacin and AR-C67085, although dense granule secretion is partially reduced. Indomethacin and AR-C67085 cause a greater level of inhibition of aggregation and ATP secretion to an intermediate concentration of CRP (Figure 3.25). Thus, responses to CRP and collagen can be distinguished in terms of their dependency on thromboxanes and the $P2Y_{12}$ ADP receptor. The ability of CRP to induce powerful activation that is largely independent of secondary mediators is important in the context of testing whether there is a generalized defect in platelet responses or a defect that is restricted to activation mediated through G protein coupled receptors or dense granule secretion. Further, the use of CRP and collagen is important in distinguishing between patients with defects in the two collagen receptors, GPVI and integrin $\alpha_2\beta_1$. The response to CRP is also more heavily influenced by a reduction in the density of GPVI, as shown in mouse studies, because its ability to crosslink the receptors is the product of its (low) affinity for GPVI and avidity, whereas for collagen, the increased affinity for integrin $\alpha_2\beta_1$ reduces the reliance on receptor density.

Figure 3.25
The effect of inhibition of ADP receptors and cyclooxygenase activity on platelet aggregation and ATP secretion to CRP



Example traces of platelet aggregation and ATP secretion induced by low, intermediate and high concentrations of CRP in the absence or presence of cyclooxygenase inhibitor indomethacin (10µM), the $P2Y_1$ receptor antagonist MRS2179 (100µM) and the $P2Y_{12}$ antagonist AR-C67085 (1µM). Traces are representative of a minimum of 3 experiments.

Table 3.1
The EC50 and the 95% CI of the agonists ± inhibitors

Agonist	Control	+ Indomethacin	+ MRS2179	+ AR-C67085	
ADP (μM)	2.0	3.3	5.9		lower
	3.2	**6.3**	**9.6**	**N/A**	**EC50**
	5.0	12	15		upper
Adrenaline (μM)	0.17	1.0	1.4		lower
	0.38	**2.1**	**2.5**	**N/A**	**EC50**
	0.85	4.1	4.2		upper
Arachidonic Acid (mM)	0.32		0.12		lower
	0.32	**N/A**	**0.47**	**N/A**	**EC50**
	0.33		1.9		upper
U46619 (μM)	0.35	0.30	0.27	1.0	lower
	0.36	**0.37**	**0.46**	**1.7**	**EC50**
	0.38	0.46	0.79	2.7	upper
PAR1 peptide (μM)	9.6	31	12	26	lower
	10	**32**	**13**	**29**	**EC50**
	11	33	13	32	upper
PAR4 peptide (μM)	147	151	18	267	lower
	151	**208**	**190**	**279**	**EC50**
	155	287	2070	293	upper
Collagen (μg/ml)	0.24	1.5	0.38	1.5	lower
	0.27	**2.1**	**0.54**	**1.9**	**EC50**
	0.31	2.9	0.77	2.3	upper
CRP (μg/ml)	0.10	0.16	0.12	0.18	lower
	0.10	**0.22**	**0.14**	**0.18**	**EC50**
	0.11	0.31	0.16	0.18	upper

The effect of inhibition of cyclooxygenase activity and ADP receptors on the EC50 and 95% confidence limits for platelet aggregation to various agonists. The effects of indomethacin, MRS2179 and AR-C67085 on platelet aggregation were calculated from the aggregation traces generated in the experiments as described for figure 3.15. Each aggregation response curve was fitted to a three variable logistic equation using Graphpad Prism software to calculate the EC50 and 95% confidence levels. Results are representative of three experiments. N/A refers to cases where de-aggregation prohibited calculation of maximal aggregation and therefore EC50.

3.4 Discussion

Born-aggregometry is the most widely used test in clinical research for investigating patients with suspected platelet-based bleeding disorders. Despite this, there are no established guidelines on how to perform these studies, with practices varying widely both within and between countries (Zhou and Schmaier, 2005, Moffat et al., 2005, Cattaneo et al., 2009). Further, the interpretation of aggregation traces is complex and hampered by the relatively few patients that are sent for testing. There have also been surprisingly few changes in recent years in the way that aggregation testing is performed in the laboratory, especially in light of our increased understanding of the mechanisms that underlie platelet activation. As a consequence, the majority of clinical laboratories are unclear on many aspects of Born-aggregometry, such as agonist selection, agonist concentration and whether to correct for platelet number etc. This has naturally led many clinical laboratories to consider whether other tests are more appropriate in analysing platelet function and to question the usefulness of aggregation in identifying conditions other than severe defects in platelet function such as those seen with Glanzmann Thrombasthenia or Bernard Soulier Syndrome.

The goal of the work in this chapter was to investigate the reproducibility of aggregation responses to platelet agonists within a population of healthy volunteers and to establish the dependency of response on factors such as platelet count, time after donation and individual donor variation. In parallel, simultaneous measurement of ATP secretion from dense granules using a lumi-aggregometer has demonstrated that measurement of both parameters provides valuable information on the effect of

inhibition of the two major feedback mediators, ADP and TxA_2, on platelet activation. The data generated during this work are an important resource of information to aid in testing of platelet samples. Importantly, the study demonstrates that aggregation and secretion responses are relatively robust within a population of healthy volunteers over the range of platelet concentrations that are found within healthy volunteers (150–600 $x10^9$ platelets/litre) and that there is no major variation in response curves with sex, age or platelet number over defined ranges. The work also demonstrates that platelet activation is maintained over several hours when PRP is stored at room temperature or as whole blood. In comparison, many clinical testing laboratories in the UK dilute the PRP to a constant platelet concentration, usually 200 x 10^9/litre, and attempt to complete the study within 2 hours in line with guidelines of the British Society of Haematology Task Force (BCSH, 1988). Not only does this impose significant constraints on the number of agonists that can be tested, there is now evidence that dilution of samples with PPP can modify aggregation (Lecchi, 2005, Cattaneo et al., 2007, Mani et al., 2005). However, in the case of thrombocytopenic donors, where the platelet count is less than 150 x 10^9 platelets/litre, aggregation is impaired and so there is a need to either dilute the PRP from the control donor to the same count or to use washed platelets in order to increase the platelet concentration.

Although the present standard curves are a powerful resource of information, it should be stressed that each clinical laboratory should generate their own standard concentration response curves from within a population of healthy individuals because of the potential impact of subtle differences in methodology. It is also recommended that studies are performed on a healthy volunteer alongside those on a patient to

control for any untoward experimental difficulties. Inherent in the use of a control volunteer in this way, however, is the possibility that the volunteer's platelets may not appear normal, possibly because of unknown ingestion of a platelet modifying agent or because of donor-specific differences in the concentration-response relationships. For example, the present work has described donor-specific concentration response differences to ADP which are maintained over time. Nevertheless, the availability of reference curves such as those generated in this work provides confidence on the response within a healthy donor for a particular agonist.

The experiments with the $P2Y_1$ and $P2Y_{12}$ ADP receptor antagonists and the cyclooxygenase inhibitor indomethacin provide important information on the distinct roles of these pathways in mediating platelet aggregation and secretion to different platelet agonists. This is important in helping to diagnose a patient with platelets which exhibit a defect in response to more than one of the conventional platelet agonists, especially as a defect in the generation of either of these feedback agonists is anticipated to be a common cause of mild bleeding (given their critical roles in supporting platelet activation). Significantly, in many cases, the effect of inhibition of $P2Y_1$ and $P2Y_{12}$ ADP receptors or cyclooxygenase is only revealed by analysis of responses to low/intermediate concentrations of agonists, thereby emphasizing the importance of studying multiple agonist concentrations. This is pertinent in the context that testing in some but not all clinical laboratories is restricted to a single concentration of an agonist. Further, it is anticipated that these reference profiles will facilitate distinguishing of patients with defects in the two $P2Y_1$ and $P2Y_{12}$ ADP

receptors from those with a ‘secretion disorder’, as only the former are characterized by a loss of response to ADP.

The present work has investigated the concentration response relationships for a number of platelet agonists, several of which are not routinely used in clinical laboratories, namely the stable thromboxane mimetic, U46619, the synthetic collagen, CRP, and PAR1 and PAR4 specific peptides. Investigation of the effect of CRP provides important information on whether a loss of response to collagen is mediated at the level of GPVI or integrin $\alpha_2\beta_1$. A partial defect in GPVI would only cause a small reduction in response to collagen because of the presence of integrin $\alpha_2\beta_1$, whereas it would have a much more dramatic effect on the response to CRP as shown by studies on mouse platelets that express a reduced level of GPVI (Snell et al., 2002). The use of U46619 provides important information on whether a diminished response to arachidonic acid is mediated at the level of cyclooxygenase or through the thromboxane receptor. The PAR-specific peptides would identify defects in the two thrombin receptors, PAR1 and PAR4. Indeed, the fact that these two PAR-specific peptides are not routinely tested in most clinical laboratories may explain why, as yet, no patients with defects in PAR1 and PAR4 have been described.

3.5 Conclusion

The present observations serve to demonstrate the robustness of platelet aggregation and secretion over a range of parameters and also emphasise the known benefit of simultaneous monitoring of ATP secretion as a marker of dense granule release. The generated response relationships and patterns of aggregation are an important resource for the clinical laboratory in the testing of patients. The results also demonstrate the importance of using additional agonists, such as the two PAR-specific peptides, in the routine testing of patients, especially those with a clinical history suggestive of a platelet disorder but where a defect has not been found. The further development and refinement in the use of aggregation testing and coanalysis of ATP secretion is likely to increase the diagnosis of patients with mild, platelet-based bleeding disorders. This increase in testing will require extra resources, but in the long term will benefit both the patient and the clinic.

CHAPTER 4

SEARCH FOR $P2Y_{12}$ RECEPTOR DEFECTS

4.1 Aim

A proportion of patients with mild bleeding that is believed to be due to platelet dysfunction are expected to have quantitative and/or qualitative defects in the platelet ADP receptor $P2Y_{12}$. This would be consistent with the major feedback role of ADP in potentiating platelet activation through the $P2Y_{12}$ receptor and the recent identification of several patients with defects in the receptor. The overall aim of the work in this chapter was to search for patients with defects in the $P2Y_{12}$ ADP receptor using two distinct approaches:

(i) monitoring of aggregation and secretion in patients with platelet dysfunction.

(ii) sequencing of the $P2Y_{12}$ receptor in the 152 index cases with type1 VWD from the EU MCMDM-1VWD study coordinated in the University of Sheffield.

4.2 Introduction

Patients with a severe bleeding problem caused by a platelet dysfunction such as Glanzmann Thrombasthenia, Bernard Soulier syndrome or marked thrombocytopenia are usually identified early in life. However, patients with a mild bleeding disorder that is due to platelet dysfunction often remain undiagnosed until early adulthood or later because the individual may not have been exposed to an appropriate haemostatic challenge such as a tooth removal or minor surgery. The majority of these patients will have a history of excessive bruising and/or nose bleeds, but similar symptoms are found in healthy individuals and so on their own are not diagnostic.

Surprisingly few patients have been identified with inherited, genetically-mapped mutations in the collagen receptor GPVI, the major ADP receptor, $P2Y_{12}$, and the TxA_2 receptor as summarized in Table 4.1. Moreover, none of these patients are from the UK. Perhaps even more striking is the observation that no patient has been described with a bleeding disorder that is attributed to a defect in either of G protein-coupled receptors for thrombin, PAR1 and PAR4, or the ADP $P2Y_1$ receptor, despite evidence from mouse studies demonstrating that PAR4 and $P2Y_1$ contribute to platelet activation *in vivo* (mice platelets do not express PAR1). The difficulty in identifying patients with defects in these receptors is likely to be the consequence of the mild nature of the bleeding disorders and current limitations in platelet testing in the clinic, although it must also be borne in mind that defects in some of these receptors may not cause excessive bleeding in human due to redundancy.

Table 4.1
Gene-mapped mutations in platelet receptors in patients with mild bleeding disorders

Receptor	Patient No.	Description	Reference
Collagen receptor (GPVI)	1	Compound heterozygous mutations (R38C and insertion of 5 nucleotides	Jandrot-Perrus et al Blood 2009
	2	Compound heterozygous mutations (a 16 bp deletion with a missense S175N	Frenson et al JTH-2009
ADP receptor ($P2Y_{12}$)	1	Homozygous 2 bp deletion in codon 98	Conley et al., Blood, 2001, abstract
	2	2 bp deletion in codon 240 uncharacterised defect on the second allele	Hollopeter et al., 2001, Nature
	3 & 4	1 bp deletion in codon 126 (hemizygote)	Cattaneo et al., ATVB, 2000 Fontana et al., Haematologica, 2009
	5	Compound heterozygous for 2 missense mutations; R256Q and R265W	Cattaneo et al., PNAS, 2003
	6	M1R (homozygous translation start site defect)	Shiraga et al., JTH 2005
	7	P258T (heterozygous patient)	Remijn et al., 2007 Clin Chem Lab Med
TxA_2 receptor (TP)	1	Arg^{60} to Leu (homozygous)	Hirata, Kakizuka et al. JCI, 1994

In the present chapter, I used the reference concentration response curves for aggregation and ATP secretion described in Chapter 3 to search for patients with defects in the $P2Y_{12}$ ADP receptor. This work was undertaken as 'proof-of-principle' that more extensive use of platelet lumiaggregometry would facilitate diagnosis of platelet disorders. The $P2Y_{12}$ ADP receptor was chosen as the focus on this work because several patients with mild bleeding linked to mutations in this receptor have been identified. As a result of these studies, several patient samples were sent for genomic DNA sequencing of $P2Y_{12}$ to the laboratory of Dr. M. Daly in the University of Sheffield (see Appendix 2).

Alongside this approach, the $P2Y_{12}$ receptor gene was sequenced by Dr. Daly in 148 of the 152 index cases enrolled in the MCMDM-1VWD study (Goodeve et al., 2007). Given the role of VWF in primary haemostasis, and the similarities in clinical phenotypes of patients with type 1 VWD and platelet-based bleeding disorders, it is possible that the bleeding tendency in patients diagnosed with type 1 VWD is influenced by defects in one or more platelet receptors. The $P2Y_{12}$ ADP receptor was again chosen as 'proof-of-principle' for this hypothesis. This approach identified two heterozygous mutations in $P2Y_{12}$ and I was fortunate in that one of the index cases, along with several family members, were located in greater Birmingham and were available for further testing.

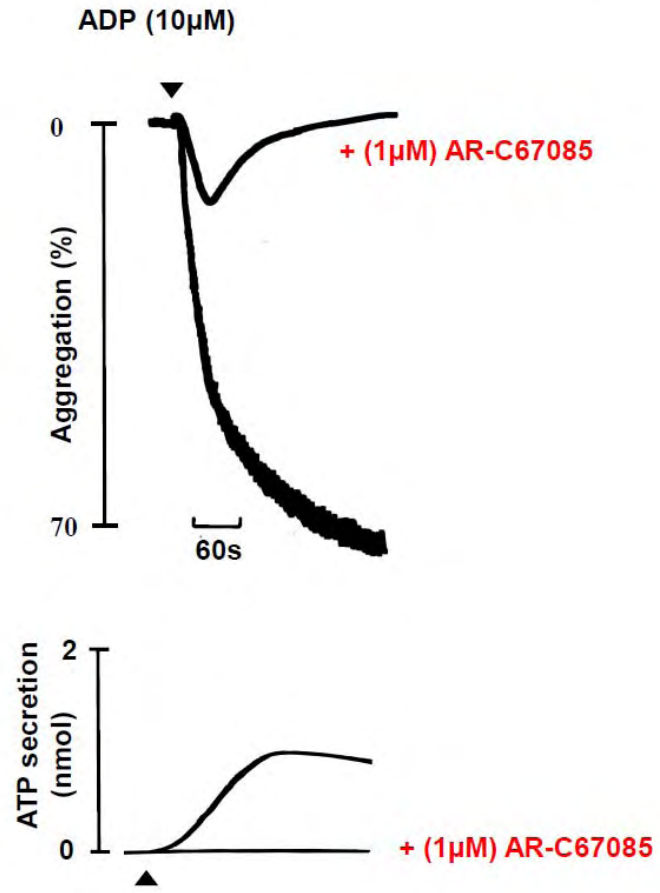
4.3 Results

4.3.1 The effect of the $P2Y_{12}$ receptor antagonist, AR-C67085, on platelet activation by ADP and adrenaline

The $P2Y_{12}$ ADP receptor and α_A-adrenoceptor signal via the Gi family of G proteins, albeit through distinct Gi α-subunits, namely $G\alpha_{i2}$ and G_z, respectively, at least as shown using mouse platelets (Jantzen et al., 2001, Yang et al., 2002). Thus, signalling defects that lie downstream of Gi should result in reduced responses to both agonists. Further, there is also evidence from the studies through the use of $P2Y_1$ and $P2Y_{12}$ receptor antagonists that ADP plays an important feedback role in platelet activation to adrenaline through both receptors, as reported in Chapter 3 and described in more detail below.

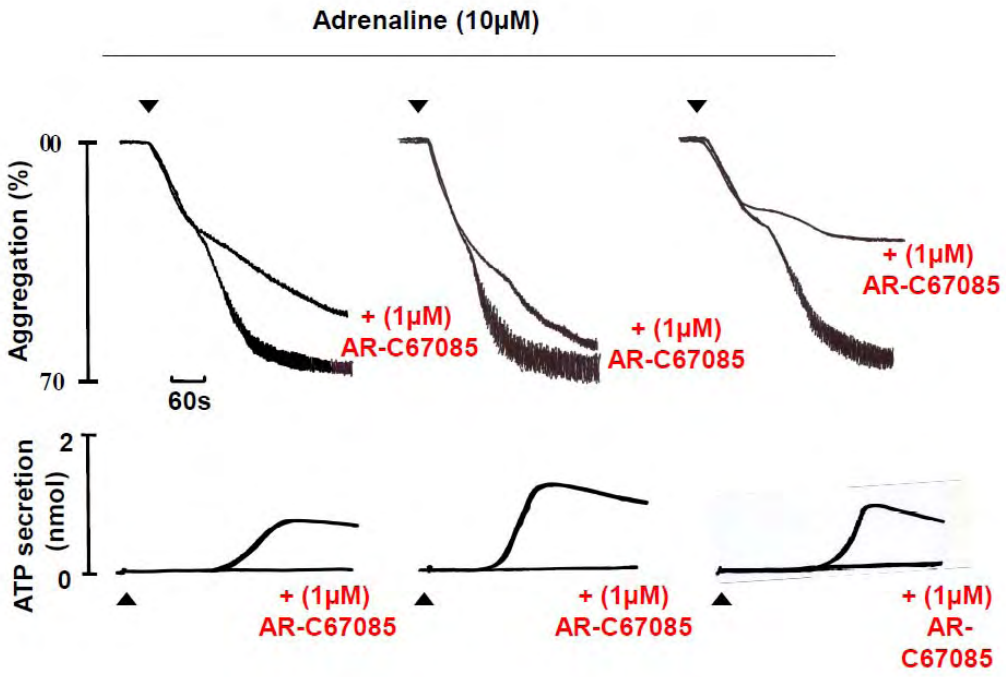
The effect of the $P2Y_{12}$ receptor antagonist, AR-C67085, on the response to a concentration of ADP (10 μM) that induces sustained aggregation in over 60 controls was evaluated. AR-C67085 has no effect on shape change, which is mediated through the $P2Y_1$ receptor, but induces a transient aggregation response that returns to the base-line within 2 minutes. The trace in Figure 4.1 is representative of the response in over 10 controls and demonstrates the critical role of the $P2Y_{12}$ ADP receptor in mediating sustained aggregation. In contrast, AR-C67085 had not effect on the primary wave of aggregation to adrenaline but caused a slight but significant reduction in the second phase of the response which was associated with a dramatic reduction in secretion (Figure 4.2).

Figure 4.1
ADP-induced platelet aggregation and secretion ± (AR-C67085)



Aggregation of platelets in PRP from a healthy volunteer in response to ADP (10μM) in the absence and presence of the $P2Y_{12}$ antagonist AR-C67085 (1μM) which was given 180s earlier.

Figure 4.2
Adrenaline-induced platelet aggregation and secretion ± (AR-C67085) in 3 different controls

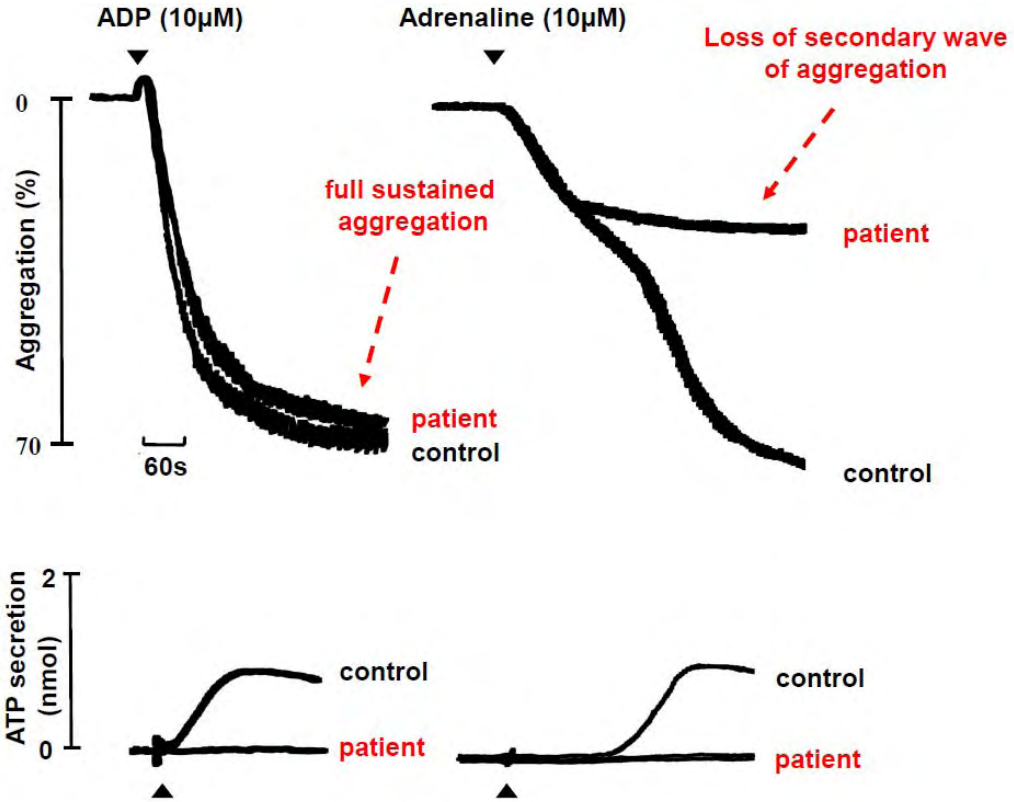


Aggregation of platelets in PRP from a healthy volunteer in response to adrenaline (10µM) in the absence and presence of the $P2Y_{12}$ antagonist AR-C67085 (1µM) which was given 180s earlier.

4.3.2 ADP and adrenaline aggregation in a patient with Hermansky-Pudlak syndrome

The role of dense granule secretion in the aggregation response to ADP and adrenaline was further investigated in two related patients with a newly identified form of Hermansky-Pudlak syndrome (HPS-8) caused by a base pair deletion in a gene *BLOC1S3* that encodes for a dense granule protein (Morgan et al., 2006). Full sustained aggregation was noticed in response to ADP (10 μM) whereas only a primary wave of aggregation was observed in response to adrenaline (10 μM) in both patients (Figure 4.3).

Figure 4.3
Aggregation response to ADP and adrenaline in a patient with Hermansky-Pudlak syndrome (HPS)



Platelet aggregation and secretion in response to ADP and adrenaline (10µM each) in citrated PRP from a healthy volunteer (control) and from the patient with HPS

4.3.3 Patients with Gi-like defect

Over the course of four years, we investigated patients who had been clinically diagnosed with a platelet defect by the consulting consultant. The majority (>60%) of these patients were referred by Dr Jonathan Wilde from the Queen Elizabeth Hospital, but we also received over ten patients each from the clinics of Drs Makris and Mumford in Sheffield and Bristol respectively, as well as one or two patients from other consultants, including Dr Mike Williams in the Birmingham Children's Hospital and Dr David Keeling in Oxford. The majority (90%) of the patients had been previously investigated in the clinic. The vast majority of the patients that had previously investigated had not been found to have any evidence of a coagulation defect or type 1 VWD, and most had been shown at least a partial defect in platelet aggregation to ADP. The patient selection was therefore intentionally biased towards patients with a decrease in aggregation to ADP consistent with the primary goal of searching for defects in the $P2Y_{12}$ ADP receptor. However, patients categorized with other platelet defects such as storage pool disease or a cyclooxygenase defect were also included, in part because of difficulty in diagnosing such disorders.

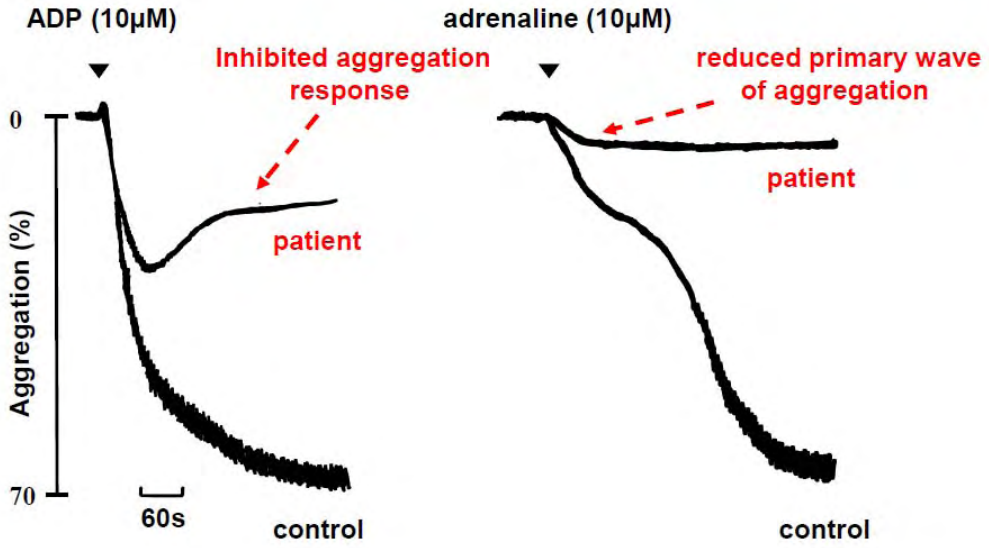
Within the group of 80 index patients that were investigated in this study, 14 unrelated patients had a combined defect in aggregation to both ADP and to adrenaline, while a further patient (Birm-JW-100708) showed a selective defect in response to ADP as described in further detail in section 4.3.4 below. Importantly ATP secretion in response to a high concentration of collagen related peptide (CRP) in these patients was within the normal range thereby ruling out a dense granule defect. All of these patients also had reduced aggregation to low concentrations of other agonists, but these

were attributed to the reduced response to ADP and so are not described in further detail.

Given that ADP, through the $P2Y_{12}$ receptor, and adrenaline signal via the Gi family of heterotrimeric G proteins, this group of patients have therefore been categorized with a 'Gi-like defect'. In this context, it is important to remember that, in mouse platelets, the $P2Y_{12}$ receptor and adrenaline have been shown to signal via distinct Gi family G proteins, namely $Gi_{\alpha 2}$ and G_z, respectively (Yang et al., 2000, Yang et al., 2002), although it is not known if this is the case in humans. There is however a residual component of signalling by both receptors in mice platelets that is independent of $Gi_{\alpha 2}$ and G_z, and also of $Gi_{\alpha 3}$, thereby implicating $Gi_{\alpha 1}$ in signalling by both receptors. There may also be a difference in the coupling of the two receptors to Gi proteins between human and mouse, which may explain why adrenaline is unable to induce aggregation in mouse platelets.

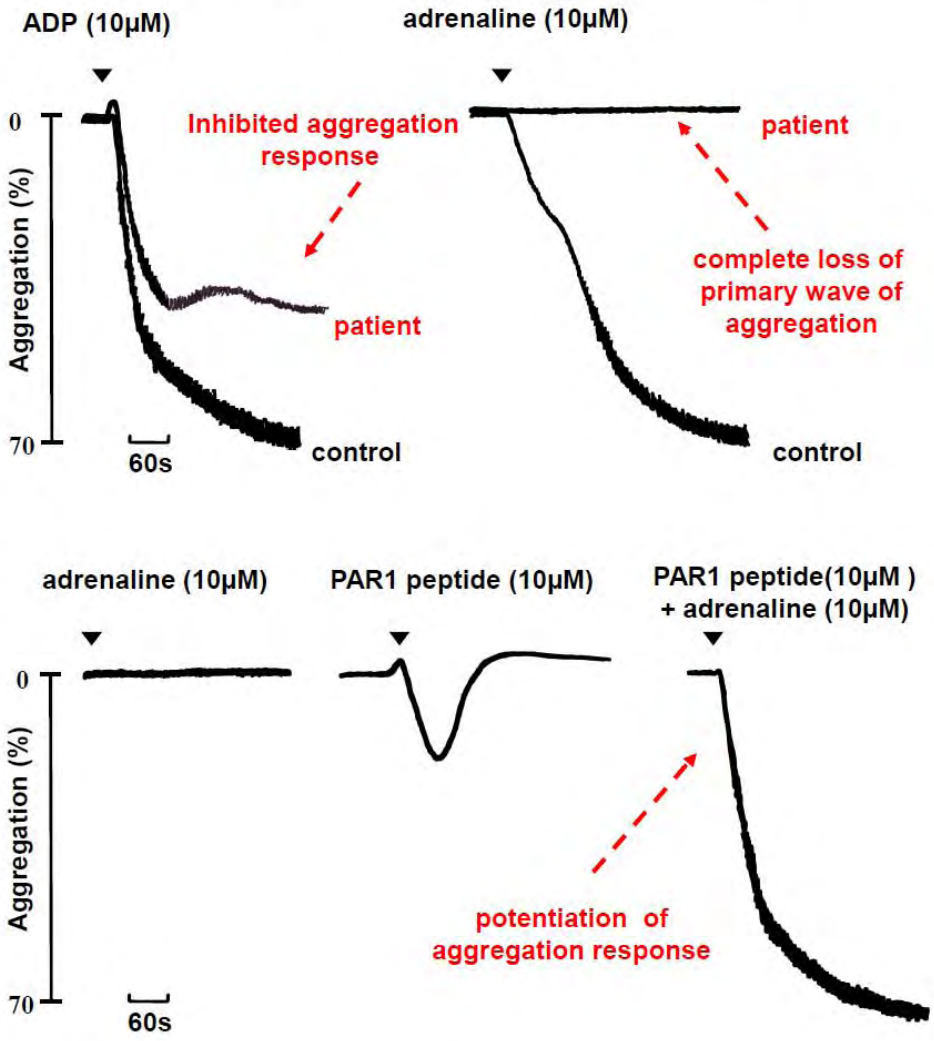
Patients with a 'Gi-like' defect were further subdivided into two groups dependent on the presence of partial or complete absence of primary wave aggregation to adrenaline. Thus Group 1 (9 patients) showed a reduced primary wave and no second wave of aggregation to ADP and to adrenaline as illustrated in Figure 4.4. Group 2 (5 patients) showed a partial/complete loss of the second wave of aggregation to ADP and a complete loss of both the primary and secondary wave to adrenaline as illustrated in Figure 4.5.

Figure 4.4
Aggregation responses to ADP and adrenaline in patients with Gi-like defect (group1)



Platelet aggregation in response to ADP and adrenaline (10μM each) in citrated PRP from a healthy volunteer (control) and a patient with Gi-like defect (group1). Similar profile was seen in other patients within the same group.

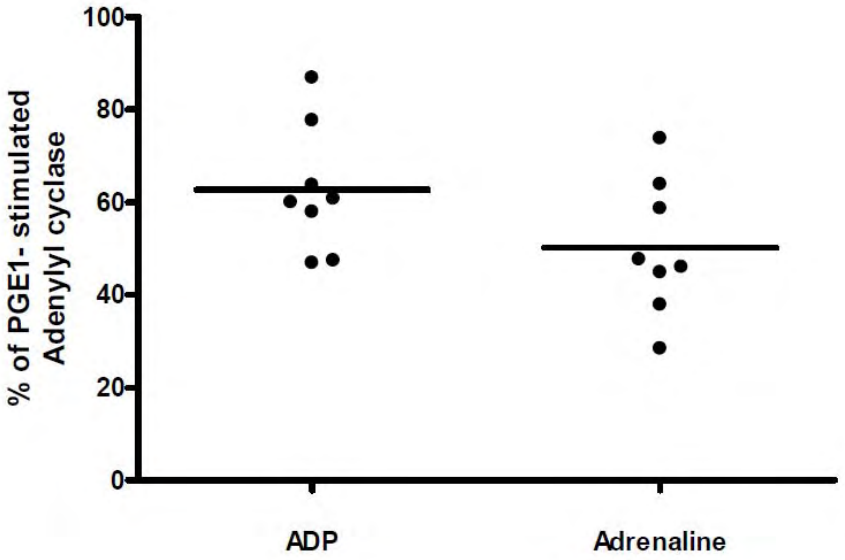
Figure 4.5
Aggregation responses to ADP, adrenaline and PAR1 peptide in patients with Gi-like (group 2)



Platelet aggregation in response to ADP, adrenaline and/or PAR1 peptide (10µM each) separate or added together in citrated PRP from a healthy volunteer (control) and from the patient with Gi-like defect (group 2). Similar profile was seen in other patients within the same group.

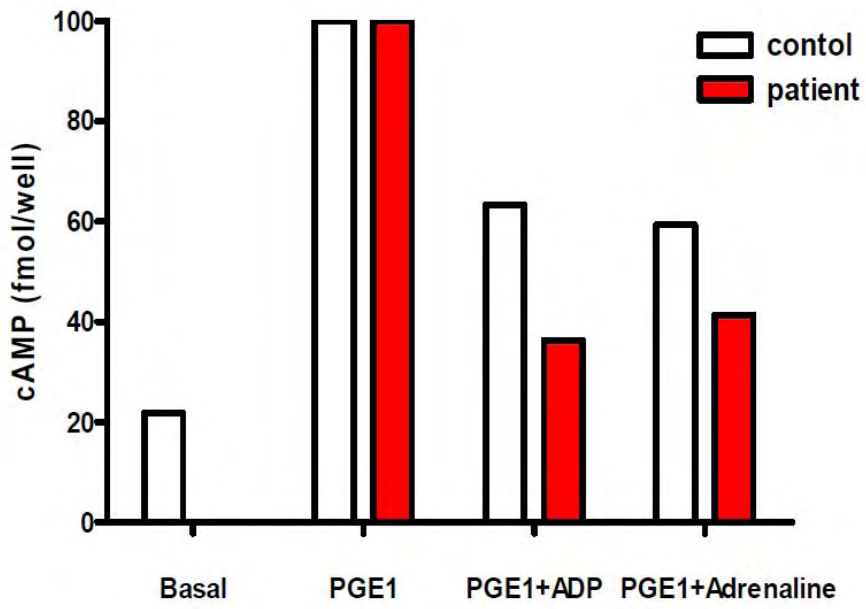
The complete abolition of aggregation to adrenaline in Group 2 could be explained by a mutation in the α_2-adrenoceptor, but this does not account for the associated loss of response to ADP. Studies were therefore undertaken to investigate whether adrenaline was still able to synergise with other platelet receptor agonists and to inhibit the formation of cAMP in the patient's platelets. As shown in Figure 4.5, at a concentration that had no effect on aggregation, adrenaline (10 μM) converted the response to the PAR1 peptide (10 μM) from a transient to a maximal, sustained aggregation, with a similar result being observed in the other 4 patients that were investigated from this group. To investigate the effect on inhibition of cAMP formation, platelets were treated with PGE1, which elevates cAMP formation through the prostacyclin receptor (Moncada et al., 1976, Miller and Gorman, 1976, Gorman et al., 1978), in the absence or presence of ADP / adrenaline. The ability of the two Gi-coupled receptor agonists to inhibit formation of cAMP was initially assessed in 8 healthy controls as shown in Figure 4.6. ADP and adrenaline decreased the level of cAMP by 62.66 ± 13.78 and 50.16 ± 14.62, respectively (Figure 4.6). Both adrenaline and ADP suppressed the elevation of cAMP by PGE1 to a similar extent to that seen in controls in the two available patients who failed to give primary wave aggregation as illustrated in Figure 4.7. These results therefore demonstrate that the α_2-adrenoceptor is still able to signal in this group of patients, despite in its inability to induce primary wave aggregation, consistent with the associated impairment in aggregation to ADP.

Figure 4.6
The effect of ADP and adrenaline on PGE1-stimulated cAMP formation in platelets from healthy individuals



Washed platelets from healthy controls were incubated for 15 min with (1μM PGE1) in the absence or presence of ADP (20μM) or adrenaline (20μM). Initial values were obtained from PGE1-stimulated platelets and results following agonist stimulation are expressed as percentages of the initial values. n=8

Figure 4.7
The effect of ADP and adrenaline on PGE1-stimulated cAMP formation in platelets from a patient with Gi-like defect (group 2)



Washed platelets from healthy volunteers, control and the patient were incubated for 15 min with (1μM PGE1) in the absence or presence of ADP (20μM) or adrenaline (20μM). Initial values were obtained from PGE1-stimulated platelets and results following agonist stimulation are expressed as percentages of the initial values. n=1

Although both of these groups of patients showed defects in response to ADP and to adrenaline, we chose to sequence the $P2Y_{12}$ gene from three patients in Group 1 and three in Group 2 in view of the possibility that the defect was due to a feedback role of ADP in platelet activation by adrenaline. In this context, it is important to emphasize that the effect of $P2Y_{12}$ blockade on adrenaline induced aggregation varies between controls as described in Chapter 3 and discussed further below. Samples were sent to Dr. Martina Daly at Sheffield University for sequencing of $P2Y_{12}$ gene. No genetic mutations in the encoding region of the gene was found in the six samples that were analysed, with three from each of the two groups.

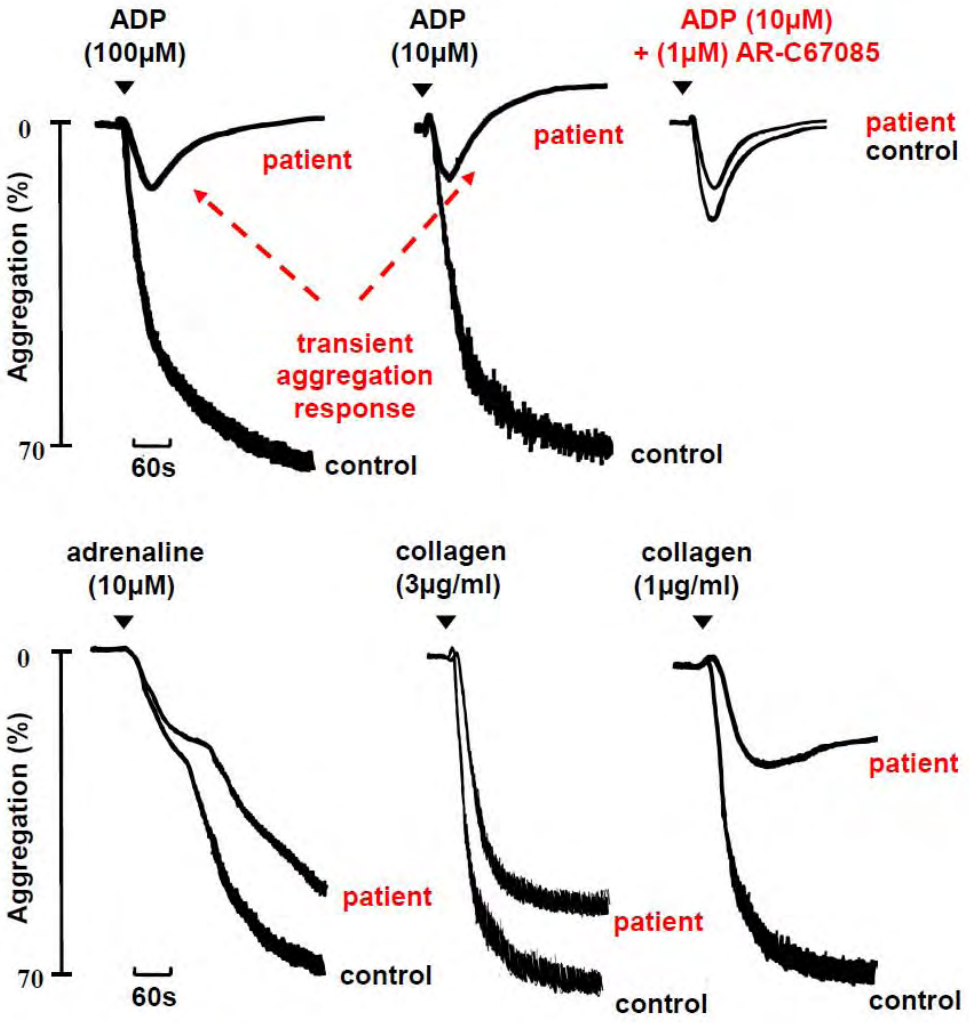
4.3.4 A Patient with homozygous $P2Y_{12}$ receptor mutation

As discussed above, one of the patients that was investigated in this study, coded Birm-JW-100708, exhibited a selective defect to ADP whereas the response to adrenaline was normal. Birm-JW-100708 was a 31 year old Asian female with a life long history of bruising and prolonged bleeding from cuts, and who had exhibited excessive bleeding following a caesarean section. Her parents were first cousins, although neither had a history of bleeding. The brother of Birm-JW-100708 also had a life long history of prolonged bleeding from cuts, but was not available for investigation. The full blood count and coagulation tests in patient Birm-JW-100708 were within the normal range.

The platelets from patient Birm-JW-100708 exhibited a marked defect in response to all concentrations of ADP, which was manifest as a weak, transient aggregation response and abolished ATP secretion to a concentration of 100 μM (Figures 4.8 and 4.9). On the other hand, shape change induced by ADP was retained

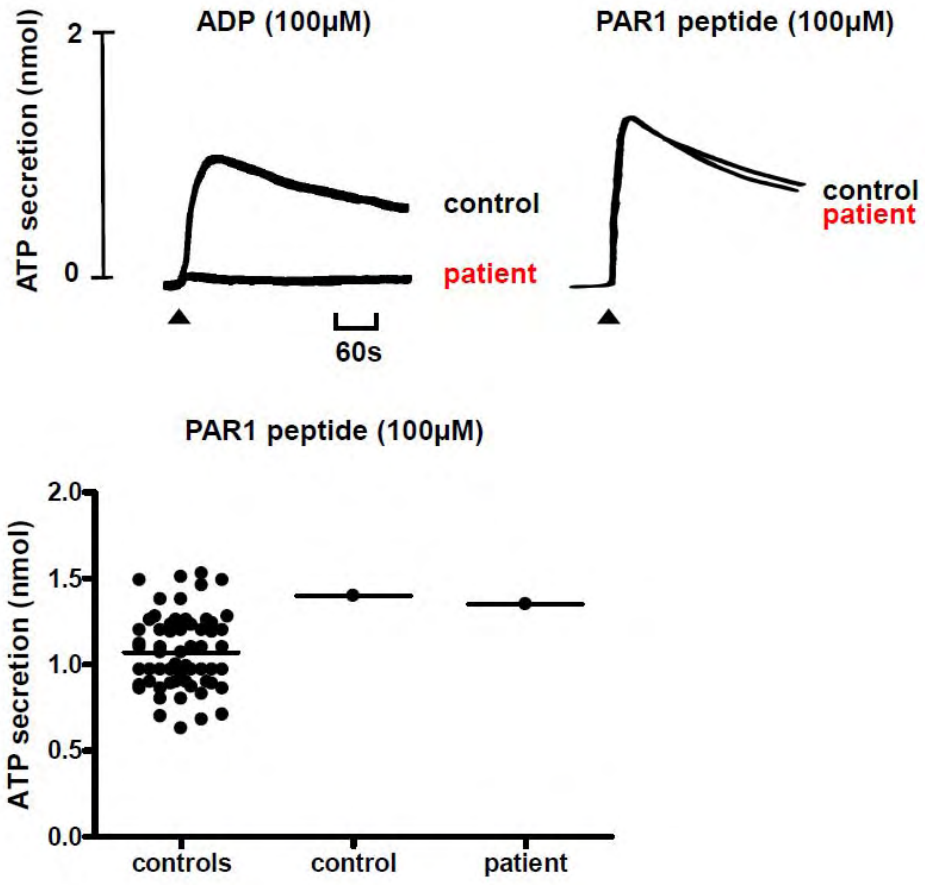
and the platelets exhibited a normal pattern of biphasic aggregation to adrenaline (Figure 4.8). A reduction in magnitude and transient aggregation response of this nature is indicative of a defect in the $P2Y_{12}$ rather than the $P2Y_1$ ADP receptor, which mediates shape change (see chapter 3). Consistent with this, the ADP receptor antagonist, AR-C67085 (1μM), reduced the aggregation response in the control to the same level as that seen in the patient, whereas it had no effect on the response of the patient (Figure 4.8). Furthermore, ADP was unable to inhibit formation of cAMP levels in PGE1-stimulated platelets in the patient, whereas adrenaline induced marked suppression in formation of the second messenger (Figure 4.10). ADP and adrenaline both inhibited formation of cAMP in platelets from a control performed alongside (Figure 4.10).

Figure 4.8
Aggregation response to ADP and adrenaline in a patient Birm-JW-100708 with homozygous $P2Y_{12}$ mutation



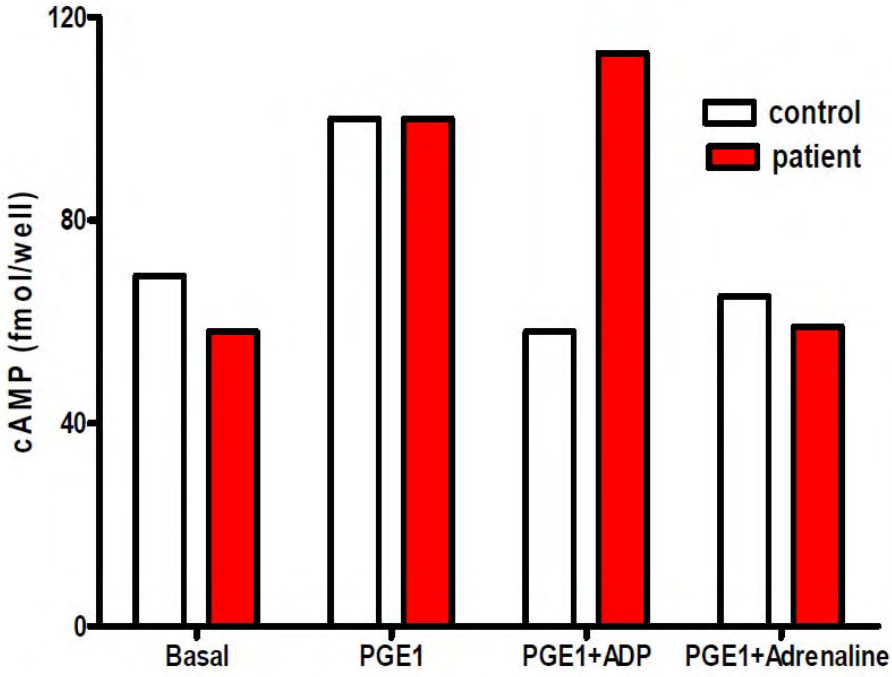
Aggregation of platelets in PRP from a healthy volunteer (control) and from the patient Birm-JW-100708 in response to the indicated concentrations of ADP, adrenaline and collagen. AR-C67085 (1µM) was given 180s earlier to ADP (10µM). n=1

Figure 4.9
Platelet secretion induced by ADP and PAR1 peptide in the patient Birm-JW-100708 with homozygous $P2Y_{12}$ mutation



Maximal levels of ATP secretion in PRP from a healthy volunteer (control) and the patient Birm-JW-100708 induced by (100μM) of ADP and PAR1 peptide shown as indicated, PAR1 peptide results measured at the same day and compared with maximal ATP secretion levels in PRP from 60 control subjects studied on separate occasions.

Figure 4.10
The effect of ADP and adrenaline on PGE1-stimulated cAMP formation in a patient Birm-JW-100708 with homozygous $P2Y_{12}$ mutation



Washed platelets from the control or the patient Birm-JW-100708 were incubated for 15 min with 1μM PGE1 in the absence or presence of ADP (20μM) or adrenaline (20μM). Initial values were obtained from PGE1-stimulated platelets and results following agonist stimulation are expressed as percentages of the initial values. n=1

The patient's platelets exhibited a defect in aggregation to other platelet agonists, which was consistent with defect in the $P2Y_{12}$ receptor. Thus, for example, there was a blunted aggregation response to collagen at low concentrations of the matrix protein (Figure 4.8). On the other hand, the level of ATP secretion from the dense granules induced by maximal concentrations of all agonists other than ADP fell within the normal range indicating that the reduced aggregation was not due to a defect in secretion (Figure 4.9).

The above observations suggest a defect at the level of the $P2Y_{12}$ ADP receptor. DNA from the patient was therefore sent for sequencing, which was performed by Dr Martina Daly in Sheffield. Genomic DNA sequencing revealed that the patient was homozygous for a single base deletion at nucleotide position 36 in the coding sequence of $P2Y_{12}$. This mutation is predicted to cause a frame shift and introduction of a premature stop codon (c.36delG, p.Gly12fs). This mutation therefore predicts a complete failure of $P2Y_{12}$ receptor expression.

4.3.5 Patient with a heterozygous $P2Y_{12}$ receptor mutation

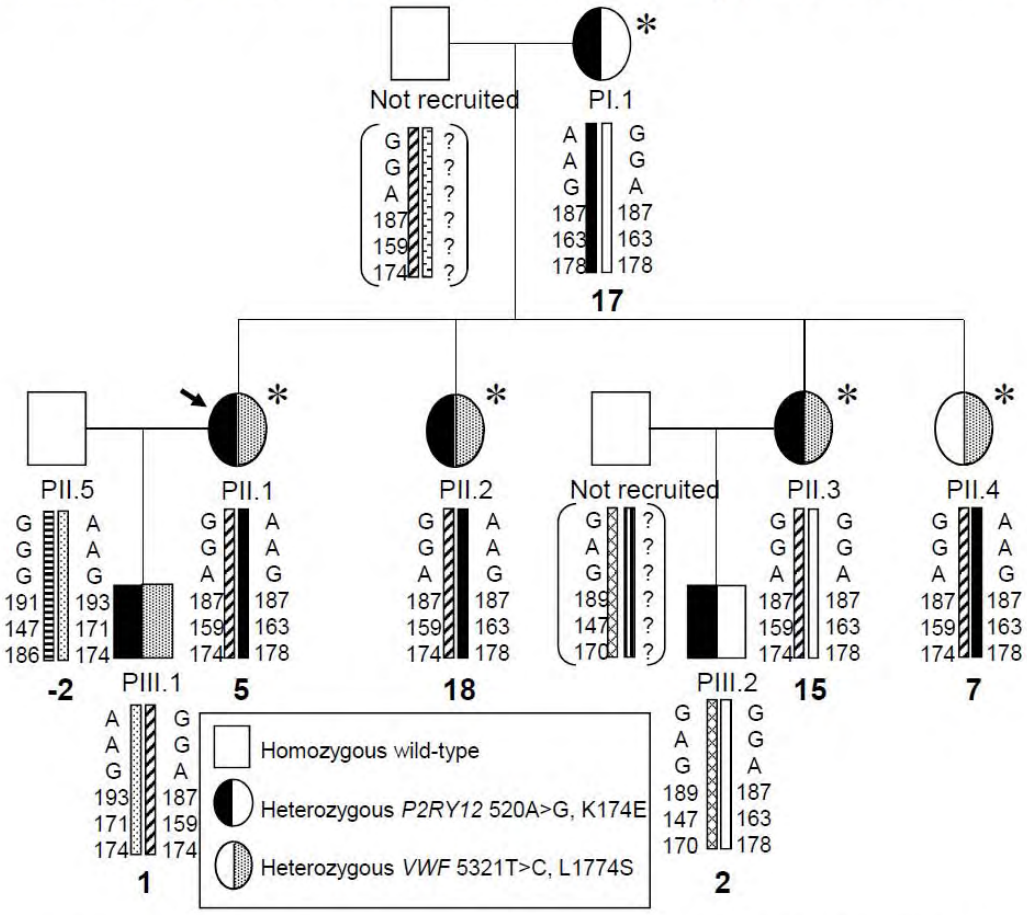
A second approach that used to search for patients with a defect in the $P2Y_{12}$ gene was to sequence the gene (consent was obtained) in the 152 index cases enrolled in the EU MCMDM-1VWD study that was led by Drs Ian Peake and Anne Goodeve in Sheffield (Goodeve et al., 2007). The sequencing was coordinated by Dr Martina Daly. This approach identified two index cases with heterozygous mutations that predicted a lysine to glutamate mutation at amino acid position 174 (K174E) in the second extracellular loop, and a proline to alanine at position 341 (P341A) which lines in a putative PDZ binding domain at the C-terminus of the receptor. The index case with

the K174E mutation, and several family members, live in the Birmingham area and was available for further experimentation. The second index case and his relatives were from Milan and so were not available to me for experimentation.

The index case (PII.1) with the K174E mutation was heterozygous for a A>G transition at nucleotide 520 of the $P2Y_{12}$ cDNA (where +1 is the A of the initiator ATG codon). This mutation predicted substitution of lysine by glutamate at amino acid position 174 (520A>G; K174E) which is located in the second extracellular domain adjacent to the cysteine which is covalently modified by clopidogrel. The 520A>G alteration did not occur in 80 unrelated healthy control subjects (160 alleles) recruited in the same centre as PII.1, supporting the likelihood that this mutation was functionally relevant rather than a novel polymorphism.

The family tree shown in Figure 4.11 reveals the distribution of $P2Y_{12}$ receptor mutation alongside the mutation in the VWF gene that was also found in the index case (Figure 4.11). The mother of the index case, PII.1, was heterozygous for the mutation in the $P2Y_{12}$ receptor but not the VWF gene, even though she had one of the highest bleeding scores in the family. The bleeding score (a quantitative measure of bleeding) of each of the members of the family is also shown and is based on the questionnaire used in the EU study (which can be found in the Appendix). The bleeding symptoms were recorded retrospectively for all subjects using the questionnaire to derive the bleeding score and for each individual, a summative bleeding score was computed as the sum of each symptom-specific grading, and could theoretically range from - 3 (no spontaneous bleeding symptom, no bleeding after surgeries, teeth extractions and deliveries) to +45 (major bleeding for all symptoms), a value greater than 3 being considered abnormal (Tosetto et al., 2006).

Figure 4.11
Inheritance of *P2RY12* and *VWF* defects in family of index case PII.1.

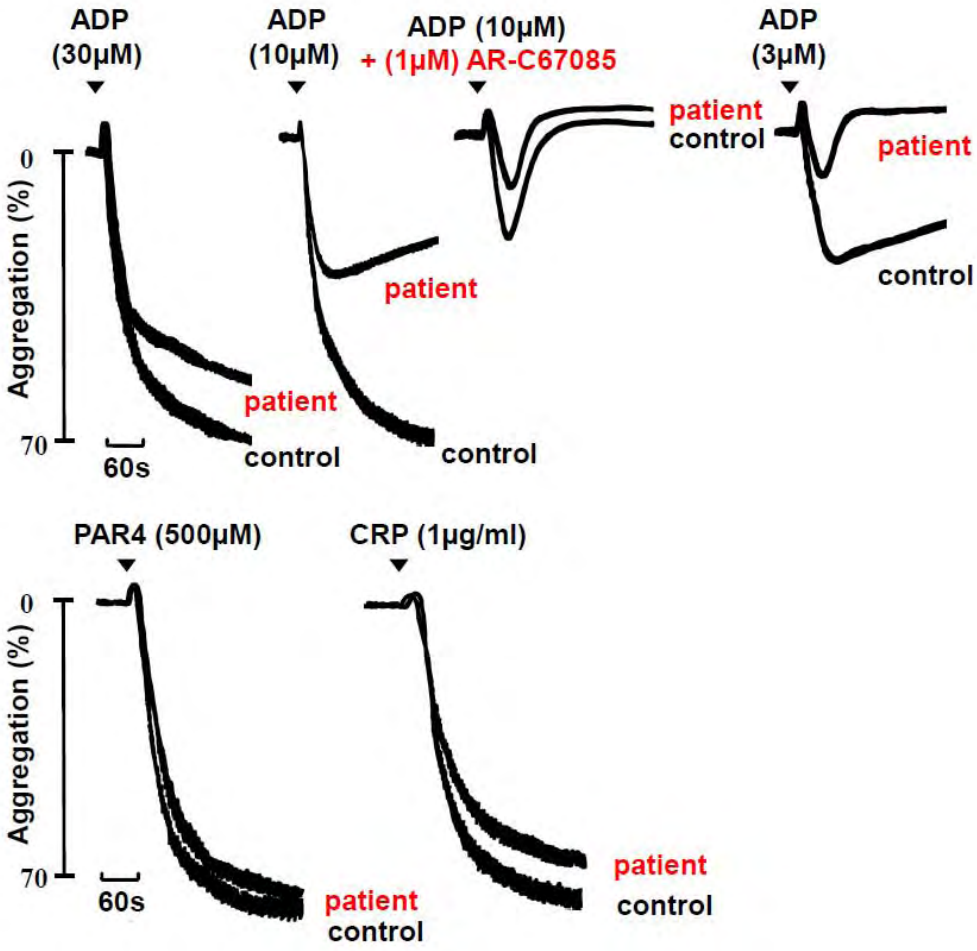


Males are represented by squares and females by circles. The index case is arrowed and individuals with a historic diagnosis of type 1 VWD are indicated by asterisks. Heterozygosity for the *P2RY12* 520A>G (K174E) mutation is indicated by black filling on the left side of the symbols, while shading on the right side indicates heterozygosity for the *VWF* 5321 T>C (L1774S) mutation. Genotypes for three *VWF* SNPs (rs1800378, rs1063856, rs216311) and allele sizes in bp for three short tandem repeat markers (VWP, VNTR3 and VNTR2) are shown below each symbol and identical *VWF* haplotypes are indicated by bars. Deduced haplotypes are shown in parentheses. The bleeding scores for individuals enrolled in the MCMDM-1VWD study are shown below the bars.

Diagram courtesy of Dr. M. Daly

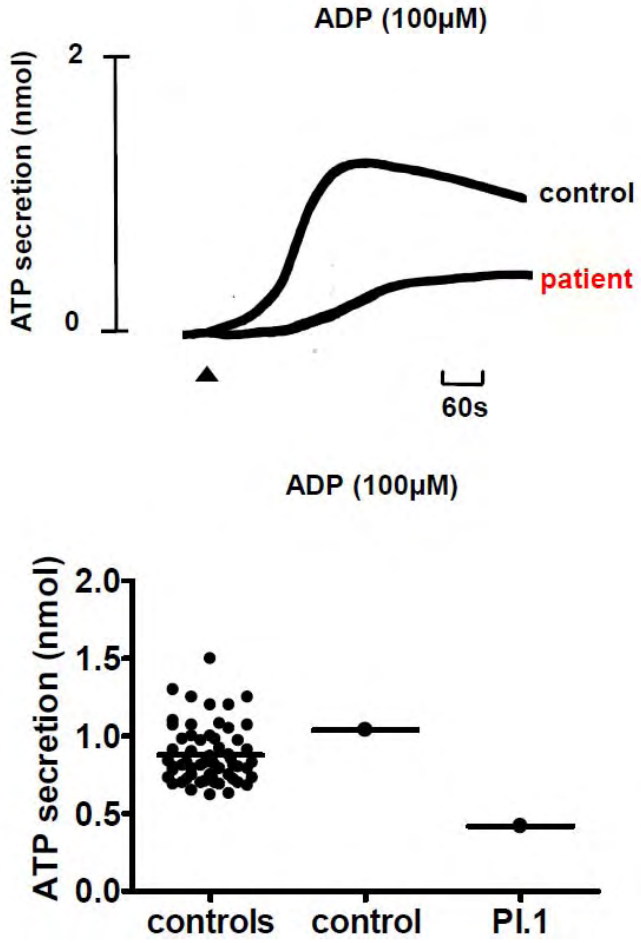
The index case, PII.1, and two relatives who also carried the K174E mutation, namely her mother (PI.1) and sister (PII.2), were available for assessment of platelet function. Platelets from all three subjects underwent shape change in response to stimulation by ADP over the concentration range of 3 – 30 μM (Figure 4.12), which is mediated through activation of the $P2Y_1$ ADP receptor. In contrast, platelets from the three patients exhibited reduced and transient aggregation to a concentration of ADP (10 μM) that induced maximal, sustained aggregation in samples from over 60 control subjects that were analysed over the period of the last four years, including the control who was investigated on the day of the experiment (Figure 4.12 and Chapter 3 Figure 3 .12). There was also a reduction in response to a lower concentration of ADP (3 μM) relative to controls, whereas a higher concentration of ADP (30 μM) induced maximal, sustained aggregation in all three patients with $P2Y_{12}$ receptor gene mutation (Figure 4.12). These findings therefore demonstrate a partial defect in the $P2Y_{12}$ receptor. Consistent with this, the $P2Y_{12}$ receptor antagonist, AR-C67085, caused a further reduction in the response to ADP (10 μM) such that, in the presence of the $P2Y_{12}$ receptor antagonist, the platelets from the patients and control showed a similar response (Figure 4.12). The partial nature of the defect in the response to ADP was further illustrated by the reduction in the level of dense granule secretion induced by ADP, as monitored by measurement of ATP release using luciferin-luciferase in one of the three patients in which this study was performed (Figure 4.13). Thus, the presence of the heterozygous K174E mutation induces a partial loss in aggregation and secretion to ADP.

Figure 4.12
ADP-induced platelet aggregation in a heterozygous carrier of the $P2Y_{12}$ K174E mutation



Aggregation of platelets in citrated PRP from a healthy volunteer (control) and from the patient who is heterozygous for K174E mutation (PII.2) in response to the indicated concentrations of ADP, PAR4 peptide, CRP and to 10µM ADP in the absence and presence of AR-C67085 (1µM) which was given 180s earlier. A similar profile was seen in the other 2 patients enrolled in the study

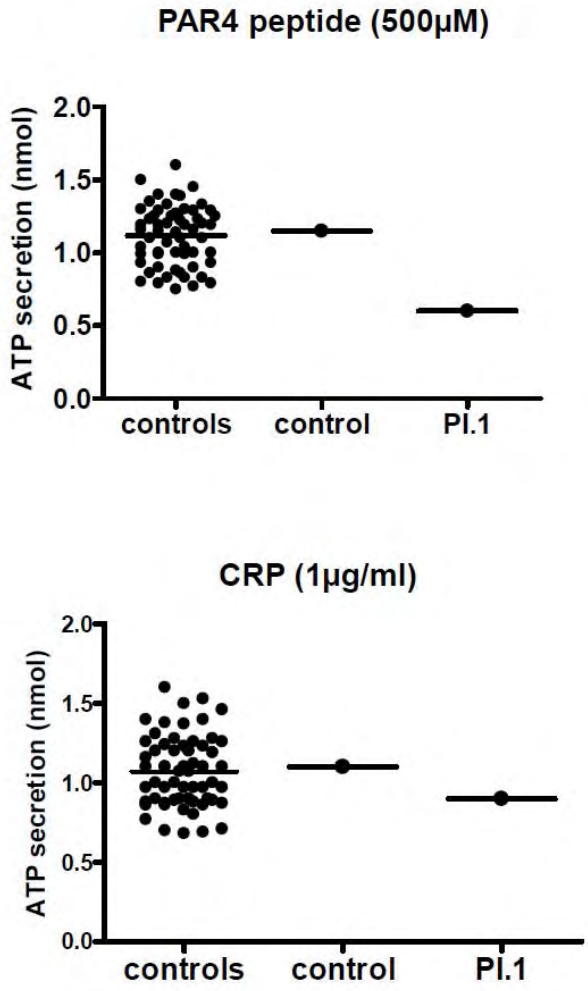
Figure 4.13
ADP-induced platelet secretion in a heterozygous carrier of the $P2Y_{12}$ K174E mutation



ATP secretion in PRP from a control subject and the patient who is heterozygous for K174E mutation (PI.1) after stimulation with ADP (100 μM). The studies were performed alongside each other and shown as traces (above) and as maximal level of secretion in each sample in comparision with the maximal levels of ATP secretion in PRP from 60 control subjects on separate previous occasions (below).

The aggregation of platelets from the index case and PI.1 to other agonists was also examined. Sustained aggregation was observed in platelets from both the index case and PI.1 in response to high concentrations of CRP (1 μg/ml), collagen (3 μg/ml), PAR-1 peptide (100 μM), PAR-4 peptide (500 μM) and U46619 (3 μM) as shown by the example traces in Figure 4.12. In contrast, reduced aggregation responses to lower concentrations of the above agonists were observed in the patient, consistent with a positive feedback role for ADP. An approximate 50 % reduction in ATP secretion was observed in response to high concentrations of the above agonists in the two patients with the exception of CRP as illustrated by the example traces in Figure 4.14.

Figure 4.14
Platelet secretion induced by GPVI and PAR-4 specific agonists in a heterozygous carrier of the $P2Y_{12}$ K174E mutation (PI.1)

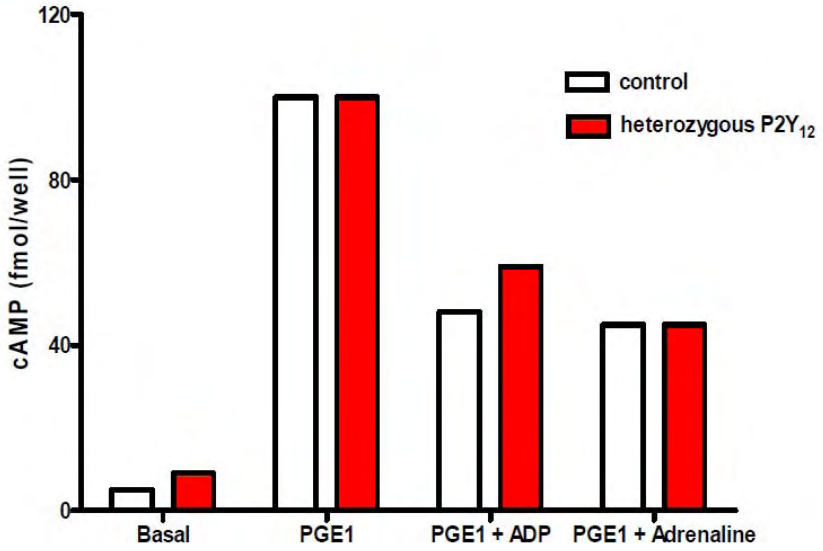


Maximal levels of ATP secretion in PRP from a healthy volunteer (control) and the patient who is heterozygous for K174E mutation (PI.1) induced by a PAR4 peptide (500µM) and the GPVI receptor agonist, CRP (1µg/ml) measured on the same day as indicated and compared with maximal ATP secretion levels in PRP from control subjects studied on separate occasions. A similar profile was seen in the other 2 patients enrolled in the study.

A series of further studies were performed to further characterize the defect. The ability of ADP to inhibit cAMP levels in the PGE1-stimulated platelets was within the normal range in these heterozygous patients (Figure 4.15). This shows that cAMP assay is not sufficiently sensitive to detect heterozygous function disrupting mutations in $P2Y_{12}$ receptor. The binding of [^{3}H]2MeS-ADP to the $P2Y_{12}$ receptor in platelets from two affected individuals was reduced by 50% relative to controls that were analysed at the same time, while binding to the K174E mutant in a transfected cell line was abolished despite a similar level of surface expression to that of the wild type receptor (Figures 4.16 and 4.17). The binding and cell line studies were performed by Dr Stuart Mundell in Bristol.

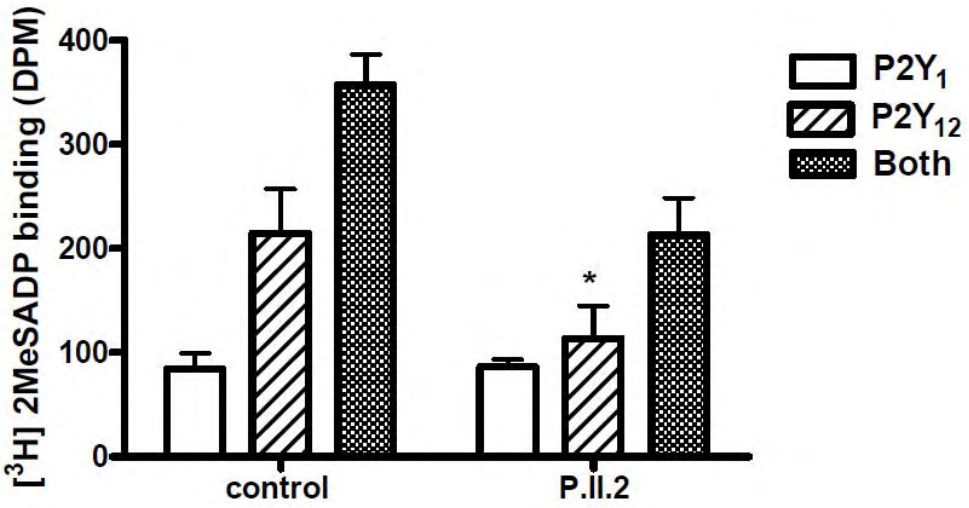
Together these results demonstrate that the K174E mutation causes a marked inhibition in the binding of ADP to the $P2Y_{12}$ ADP receptor.

Figure 4.15
The effect of ADP and adrenaline on PGE1-stimulated cAMP formation in a heterozygous carrier of the $P2Y_{12}$ K174E mutation



Washed platelets from control or patient who is heterozygous for K174E mutation were incubated for 15 min with 1 μM PGE1 in the absence or presence of ADP (20μM) or adrenaline (20μM). Initial values were obtained from PGE1-stimulated platelets and results following agonist stimulation are expressed as percentages of the initial values, n=1.

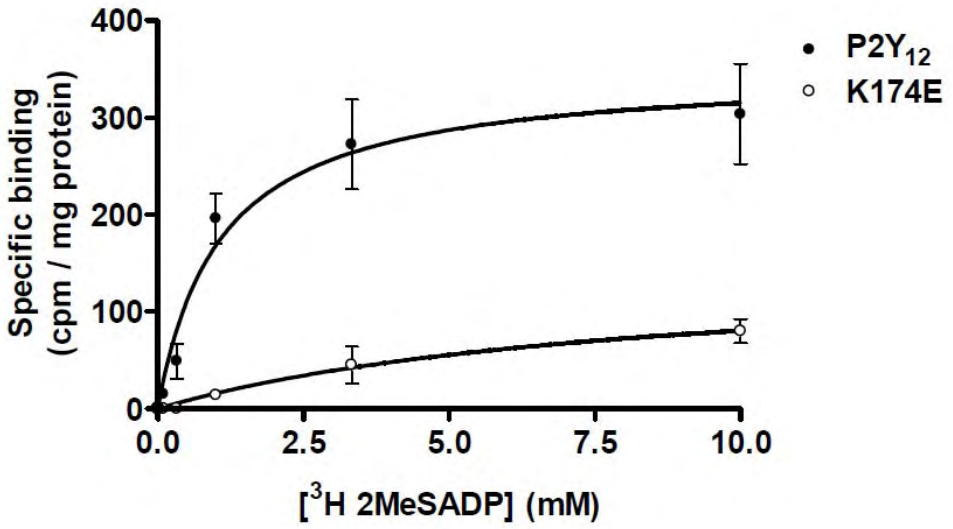
Figure 4.16
Reduced binding of the non-selective P2Y receptor ligand 2MeS-ADP to the $P2Y_{12}$ receptor in K174E patient



$P2Y_1$ and $P2Y_{12}$ surface receptor levels were measured in fixed platelets using [3H]2MeS-ADP (100nM) in the presence of either the $P2Y_1$ receptor antagonist A3P5P (1mM) or the $P2Y_{12}$ receptor antagonist AR-C69931MX (1µM). Data are expressed as [^{3}H]2MeS-ADP binding (DPM) and represent means ± S.E.M. of three independent experiments. *Statistically significant reduction in $P2Y_{12}$ binding levels at $p<0.05$ for data compared with respective control data (Mann-Whitney U-Test).

Work done by Dr. Stuart Mundell / University of Bristol

Figure 4.17
Markedly impaired binding to $P2Y_{12}$ K174E in a stable cell line



Receptor levels were measured in CHO cells stably expressing wild-type or K174E receptor using [3H]2MeS-ADP (0.1-10μM) in the presence of the $P2Y_{12}$ receptor antagonist AR-C69931MX (1μM) to determine specific binding. Data are expressed as specific binding of [^{3}H]2MeS-ADP (cpm) per mg protein and represent means ± S.E.M. of three independent experiments.

Work done by Dr. Stuart Mundell / University of Bristol

4.4 Discussion

In this Chapter, two approaches were used to search for mutations in the $P2Y_{12}$ ADP receptor in patients with a clinically diagnosed bleeding disorder. The first involved the functional characterization of platelets from patients registered at Haemophilia Care Centres throughout the UK, with the majority from the Birmingham Queen Elizabeth Hospital, combined with targeted gene sequencing of selected individuals. Eighty patients were studied over the course of this thesis in this way. The initial functional test was that of lumi-aggregometry, but where appropriate, other tests were performed, including measurement of cAMP levels in PGE1-stimulated platelets. This test is diagnostic for defects in Gi-coupled receptors. This approach identified a single patient who was homozygous for a base pair deletion that gives to a premature stop codon very early on in the coding sequence. 14 other patients were shown to have a defect in platelet activation by both ADP and adrenaline thereby pointing towards one or more mutations in the Gi-signalling axis. Sequencing of the $P2Y_{12}$ ADP receptor in six of these individuals failed to identify any mutations in the receptor consistent with a defect later in the signalling cascade. The second approach that was used to search for defects in the $P2Y_{12}$ ADP receptor was that of sequencing of 148 out of 152 index cases from the EU MCMDM-1VWD study. This identified two candidate mutations that had not been previously identified as polymorphisms and which were absent in approximately 100 controls from the same area as the index case. Only one of these two index cases, along with other family members, was available to me for aggregation and other functional studies as they were from the Birmingham area. This work, performed in collaboration with Dr Stuart Mundell in Bristol, has revealed defective

platelet activation to ADP as a consequence of impaired binding to the G protein-coupled receptor. Studies on the other mutation, which was in the PDZ binding domain of the receptor, has revealed that this causes an impairment in desensitization and resensitisation of the receptor, leading to a net reduction in the surface expression of the $P2Y_{12}$ ADP receptor (Stuart Mundell, unpublished). Together these studies bring the total number of known mutations in the $P2Y_{12}$ ADP to ten, of which interestingly, five are heterozygotes.

There has been much debate on the use of Born lumiaggregometry in diagnosing patients with mild bleeding disorders of platelet aetiology. In the two cases described above, a clear defect in aggregation was observed which was characterized by a transient response to an intermediate concentration of ADP (10 μM) which was shown to cause full and sustained aggregation in platelets from over 60 controls. Interestingly, one of these mutations was heterozygous, thereby emphasizing the potential of this approach, if used correctly, to pick up defects in ADP-induced platelet activation. This is particularly relevant in clinical laboratories where aggregometry is currently the technique most widely used when investigating patients with platelet bleeding disorders. Further, platelets from PI.1 showed a reduction in ATP secretion at high doses of agonists like PAR1 and PAR4 emphasizing the importance of this approach and suggesting the possibility of a secondary secretion defect, which could contribute to the clinical phenotype observed in this individual.

It is generally held that the mild bleeding defect associated with $P2Y_{12}$ deficiency is inherited as an autosomal recessive trait and that the milder abnormalities in platelet aggregation and secretion displayed by heterozygous individuals are benign (Cattaneo et al., 2003). The identification of the K174E defect challenges this

assumption, a result that is further emphasized by the fact that five out of the ten known inherited defects in the $P2Y_{12}$ receptor are heterozygote. Interestingly, the K174E mutation was identified in an index case that had been diagnosed with mild type 1 VWD. While this is consistent with a growing body of evidence that mild type 1 VWD is multifactorial in origin (and thereby helping to explain the variable heritability of the disease), it is important to emphasise that the mother of the index case also carries the K174E mutation and has one of the highest bleeding scores, and yet does not have type 1 VWD. Since not all individuals who are heterozygous for a $P2Y_{12}$ mutation have a clinically diagnosed bleeding tendency, this indicates that the mother has a second, unidentified defect.

The present results have important implications for patients taking antiplatelet agents such as the $P2Y_{12}$-targetted medication clopidogrel or aspirin, as these may unmask previously unrecognised defects in platelet activation in the patients and thereby give rise to serious bleeds. Thus, just as the expression of a thrombotic tendency is determined by the combination of inherited and acquired prothrombotic risk factors, the same may apply in this case such that the bleeding in disorders such as type 1 VWD (particularly in mild cases) or a $P2Y_{12}$ deficiency should be considered multifactorial in origin.

4.5 Conclusion

We identified two novel $P2Y_{12}$ receptor mutations associated with bleeding tendency, a homozygous mutation in an otherwise normal individual and a heterozygous mutation in patients with a historic diagnosis of type 1 VWD. The co-existence of type 1 VWD and a $P2Y_{12}$ receptor has not been previously described. This studies also emphasizes that an ADP receptor defect should be suspected whenever ADP at a concentration of 10 µM or higher induces a reversible aggregation preceded by a normal shape change. Tests such as inhibition of adenylyl cyclase can be used to further specify the condition, but genetic analysis will be essential to confirm the defect. The bleeding diathesis which is associated with this hereditary defects in the $P2Y_{12}$ receptor further emphasize the critical role of ADP and its signaling pathway in the aetiology of mild platelet-based bleeding disorders.

Further functional studies combined with the sequencing of tens of genes is required to identify the defects in the other patients who have been grouped together with a Gi-like defects.

CHAPTER 5

DEFECTS IN THROMBOXANE A_2 SIGNALING PATHWAY: IDENTIFICATION OF A CYCLOXYGENASE ENZYME-LIKE DEFECT AND A NOVEL TxA_2 RECEPTOR DEFECT

5.1 Summary

Thromboxane A_2 (TxA_2) is one of the major secondary mediators that interacts with other agonists to mediate powerful aggregation and secretion to ensure robust platelet activation at sites of damage to the vasculature. Consistent with this critical role, I have observed a defect in platelet activation in response to arachidonic acid in approximately 14% of patients diagnosed with platelet dysfunction. In this chapter, I report the characteristics of the defect in platelet activation in these patients and focus on two, one of which has an unknown defect in arachidonate metabolism, and a second with a novel mutation in the TxA_2 receptor.

5.2 Introduction

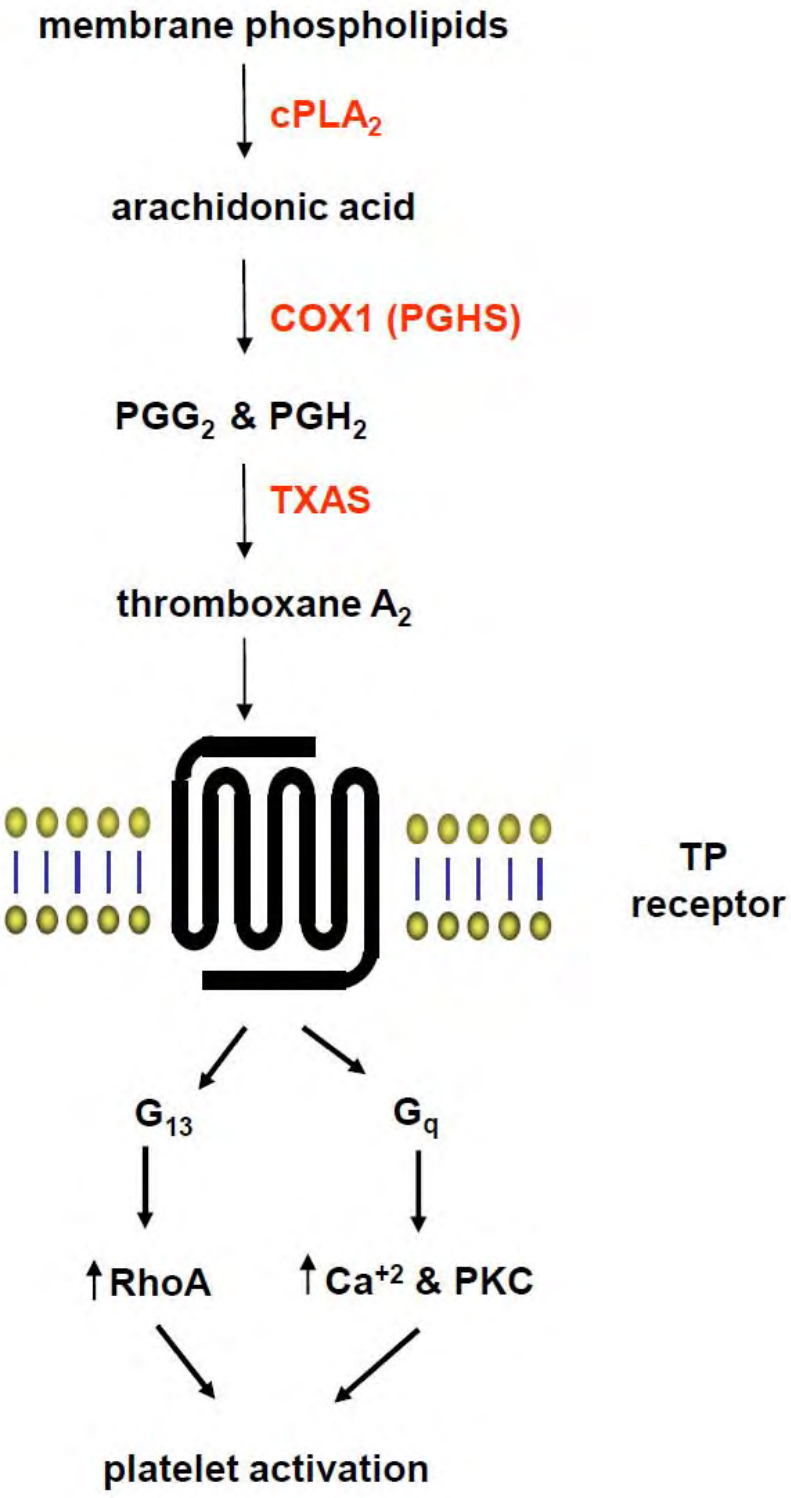
TxA_2 is a key secondary mediator in platelet activation that synergises with other agonists including ADP to induce powerful platelet activation. As a consequence, defects in the formation of TxA_2 or in the function of the TxA_2 receptor cause impairment in platelet activation. However, because of redundancy in the mechanisms of platelet activation and the fact that we are not continuously exposed to events that can give rise to serious bleeds, defects in the thromboxane pathway are predicted to give rise to a mild bleeding disorder or to be symptomless, as is illustrated by the fact that millions of non-steroidal agents, including aspirin, are taken every day by healthy individuals without serious bleeding episodes. Nevertheless, it is accepted that chronic administration of aspirin can give rise to excessive bleeding in a small number of healthy individuals or those at risk of thrombosis, and indeed the latter is an important

risk to benefit consideration in the chronic prescribing of aspirin in this group of patients.

Upon platelet activation, TxA_2 is generated through sequential activation of cytosolic phospholipase A_2 ($cPLA_2$), cyclooxygenase-1, (COX-1; also known as prostaglandin H_2 synthase) and thromboxane A synthase (TXAS) as shown in Figure 5.1. Earlier reports that platelets also contain COX-2 have not been confirmed. Activation of $cPLA_2$ is the rate limiting step in this pathway and therefore plays a crucial role in the regulation of TxA_2 synthesis. Activation of $cPLA_2$ is critically dependent on the mobilisation of intracellular Ca^{2+} from intracellular stores by IP3 and is therefore mediated downstream of any receptor that induces activation of phospholipase C. In platelets, the activity of $cPLA_2$ is also increased through phosphorylation by mitogen activated protein kinases, although this appears to be a relatively minor pathway of regulation (Borsch-Haubold et al., 1995). Activation of $cPLA_2$ leads to hydrolysis of the SN_2 acyl bond in membrane phospholipids to generate arachidonic acid which is then metabolised by COX-1 on intracellular membranes. COX-1 is a dual-functioning enzyme, possessing both oxygenase and peroxidase activities, which converts arachidonic acid to prostaglandin G_2 (PGG_2) and then to prostaglandin H_2 (PGH_2). The latter has been shown to cause weak activation of the platelet thromboxane receptor but is ordinarily rapidly converted to TxA_2 by thromboxane synthase which induces powerful activation of the TP receptor. Activation of the G protein-coupled TxA_2 receptor leads to powerful platelet activation through G_{q-} and G_{13}-regulated signalling pathways (Figure 5.1). There is also evidence that arachidonic acid is able to mediate weak platelet activation independent of its metabolism by COX-1 and conversion to the prostanoids (Frelinger et al., 2006). It is

presently unclear whether this is mediated directly by arachidonic acid or following its metabolism by platelet lipooxygenase to downstream metabolites. However, the weak nature of this effect means that it is generally considered to play a non-significant role in mediating platelet activation.

Figure 5.1
TxA_2 signaling pathway in platelets



Patients with an 'aspirin-like' defect have been diagnosed in clinic testing laboratories ever since the anti-platelet activity of aspirin was first reported just over 40 years ago. In a significant number of these cases, the identified defect in activation by arachidonic acid has been attributed to the ingestion of aspirin or an alternative non-steroidal anti-inflammatory, emphasizing the importance of obtaining an accurate drug history prior to platelet function studies. However a significant number of these patients have been diagnosed with a defect in platelet cyclooxygenase (Malmsten et al., 1975, Horellou et al., 1983, Lagarde et al., 1978, Pareti et al., 1980, Rak and Boda, 1980, Roth and Machuga, 1982, Matijevic-Aleksic et al., 1996, Rolf et al., 2009) or thromboxane synthase deficiency (Defreyn et al., 1981, Mestel et al., 1980). In all these cases, there is impairment in arachidonic acid metabolism leading to reduced TxA_2 synthesis. However, a causative mutation in either of these enzymes has not been found in any patient (Rao, 2003) and it is notable that, with the exception of a recent study (Rolf et al., 2009), these references are more than ten years old and the majority appeared prior to the sequencing of the two enzymes.

The prevalence of genetic defects in COX-1, thromboxane synthase and the TxA_2 receptor was estimated to be approximately one hundred patients *worldwide* with ten of these in the UK (Bolton-Maggs et al., 2006), while a second study states that 'approximately 40 cases of haemostatic defects that have been published since the early 1970s may be regarded as aspirin-like defect' (Rolf et al., 2009). In both cases, however, the basis of these estimates is unclear and the number of patients is low in comparison to the number of patients diagnosed with Glanzmann Thrombasthenia where more than 1,000 have been identified worldwide.

To date, only two mutations in the thromboxane pathway have been reported, a mutation in the TxA_2 receptor which was identified in several unrelated Japanese families (Hirata et al., 1994) and the second in $cPLA_2$ in a white American male of Italian descent (Adler et al., 2008). In the first case, a single amino acid substitution (Arg^{60}→Leu) in the first cytoplasmic loop of the TxA_2 receptor was reported to give rise to an inherited bleeding disorder characterized by a defective platelet response to TxA_2. One of the first cases to be identified is a 53-year-old man who was referred due to excessive bleeding from wounds, gum bleeding and the need for blood transfusion after prolonged postoperative bleedings (gasterectomy at age 42 and a removal of a urinary stone at age 43). His parents were not consanguineous. No signs of petechiae, bruising, ecchymosis, or joint involvement were found on physical examination. The platelet (count, size and morphology) and coagulation factors were all within normal limits. The mutant receptor expressed in CHO cells showed decreased agonist-induced second messenger formation despite normal ligand binding affinity. The original study (Hirata et al., 1994) described the mutation of having a dominant pattern of inheritance suggesting that the mutation produces a dominant negative TxA_2 receptor. This and later studies however demonstrated that while platelet aggregation to the stable thromboxane mimetic, STA2, was abolished in patients who were homozygous for the mutation (shape change was retained; Hirata, Kakizuka et al., 1994), a partial or normal response was observed to STA2 in heterozygotes, depending on the parameter measured (e.g. platelet aggregation showed a partial defect, second messenger formation showed no defect (Fuse et al., 1996, Higuchi et al., 1999). This argues against the idea that the mutant receptor is acting solely as a dominant negative. Further, the bleeding history of many of the heterozygote patients has not been

reported, making it unclear whether bleeding is inherited in a dominant or recessive manner. For the mutant $cPLA_2$, two heterozygous single base pair mutations and a known SNP were found in the coding regions of the cPLA2α gene (p.[Ser111Pro, Arg485His]+[Lys651Arg]) in a 45-year-old white American male of Italian descent with a life-long history of occult gastrointestinal blood loss and frequent bouts of abdominal pain as a child and young adult. Repeated episodes of acute gross gastrointestinal bleeding late in his fourth decade and multiple episodes of small bowel perforation required 5 surgical interventions between 38 and 45 years of age. Surgical exploration of the small intestine and intra operative endoscopy revealed multiple recurrent ulcerations. The use of non steroidal anti-inflammatory and corticosteroid medications was specifically denied. There was no family history of ulcers (Adler et al., 2008).

By reference to the concentration response curves for aggregation and ATP secretion described in Chapter 3, 11 out of 80 index cases have been classified as having a cyclooxygenase like defect, with every reasonable effort having been made to rule out the ingestion of aspirin or any other non-steroidal cyclooxygenase inhibitor as the causative factor. Further, in one of these cases, the defect was mapped to a heterozygote mutation in the thromboxane receptor. The phenotype of the 11 index cases is described below, with special focus on the patient with a TP receptor mutation and a second patient with a suspected COX-1-like defect.

5.3 Results

5.3.1 Identification of patients with a COX-like defect

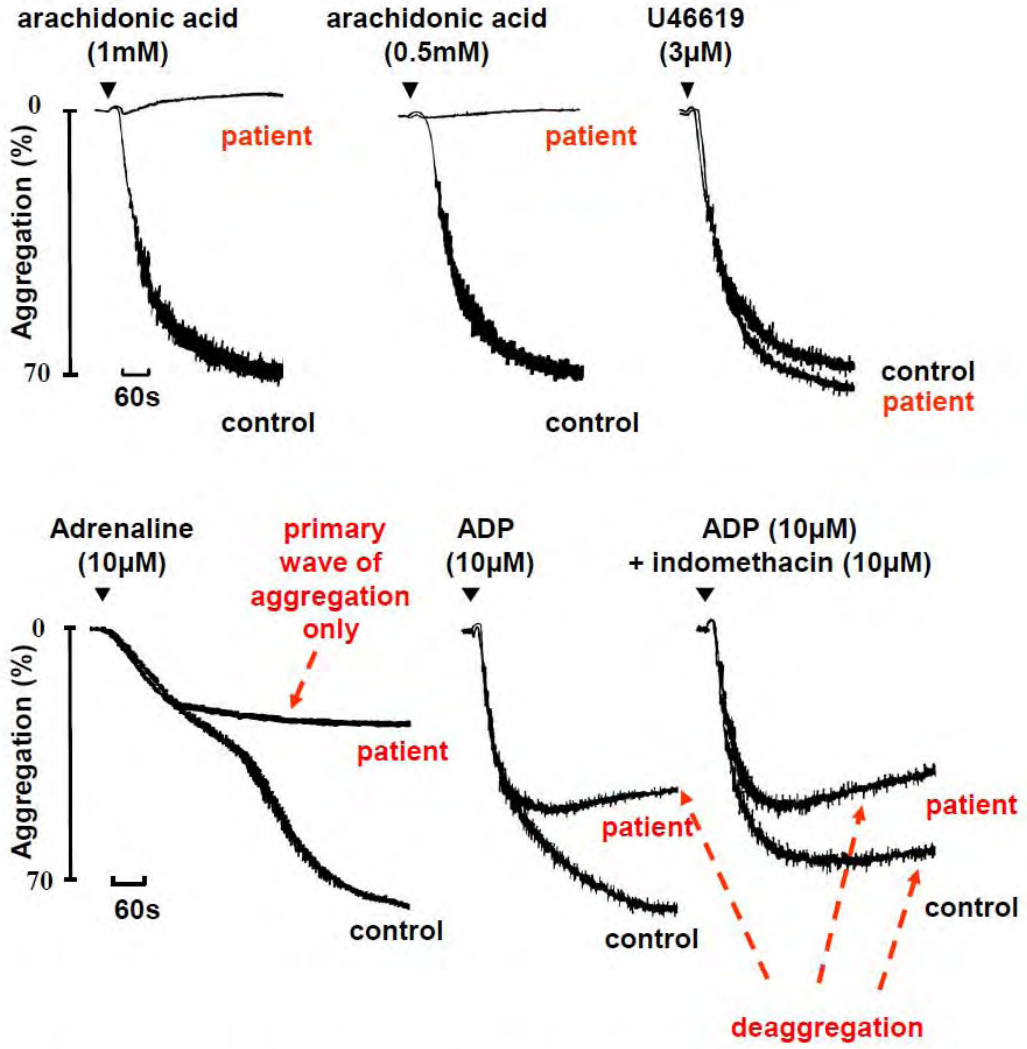
Over the course of the last four years, I have investigated 80 patients diagnosed with a mild bleeding defect by consultants in Birmingham, Bristol, Cambridge and Sheffield. Of these, eleven have been classified as having a defect in the thromboxane pathway as revealed by a defect in aggregation to arachidonic acid (0.5–1.5 mM) relative to other platelet agonists. Additional testing is required to identify patients with a defect in $cPLA_2$ but this was not done as part of the initial testing of each patient. Of these, one patient was found to have a partial defect in the response to the stable thromboxane mimetic, U46619, which was retained in the presence of the cyclooxygenase inhibitor indomethacin thereby pointing towards a defect at the level of the thromboxane receptor. This patient is described in Section 5.3.3 below. The other ten patients had a partial or complete loss of aggregation to arachidonic acid but a normal aggregation response to U46619, in line with data in Chapter 3 that activation of platelets by the stable TxA_2 mimetic is not altered in the presence of indomethacin. These patients also had defective platelet aggregation and secretion to (low) concentrations of several other platelet agonists, consistent with the loss of the feedback role of the thromboxane pathway. The nature of this defect is illustrated by series of studies on one of these patients, Birm-JW-240108, as described below.

5.3.2 Diagnosis of a COX-like defect in the patient Birm-JW-240108

The index case was a 50 year old female (Birm-JW-240108) with a clinical history indicative of a mild bleeding disorder from early childhood. This included prolonged bleeding from cuts for more than 20 minutes and the need for a blood transfusion following tooth extraction in early childhood and later in life following tonsillectomy at 34 yr of age. On the other hand, Birm-JW-240108 had given birth to three children by vaginal delivery without the need for a blood transfusion. The platelets from Birm-JW-240108 were investigated for a platelet defect on two separate occasions, separated by an interval of more than one year. On both occasions, Birm-JW-240108's platelets did not respond to 1 mM arachidonic acid whereas the control gave a robust and sustained aggregation (Figure 5.2), as was the case in greater than 95% of the other controls investigated over the course of this thesis (see Chapter 3). Importantly, the response of Birm-JW-240108's platelets to the stable TxA_2 mimetic, U46619, was within the normal range (Figure 5.2), thereby localizing the defect upstream of the TP receptor i.e. at the level of COX-1 or thromboxane synthase. Further, the response of the patient's platelets to other agonists was consistent with a defect in arachidonate metabolism as determined by comparison to the reference curves determined in the presence of indomethacin as described in Chapter 3. For example, ADP (10 μM) induced full aggregation followed by deaggregation in Birm-JW-240108's platelets (Figure 5.2) whereas it induced a sustained aggregation response in greater than 98% of controls. However, the addition of indomethacin (10 μM) had no effect on the response to ADP in Birm-JW-240108's platelets, whereas it converted the response to a transient aggregation in the control that was studied alongside (Figure 5.2). A similar difference between the response of Birm-JW-240108's platelets and both to the control

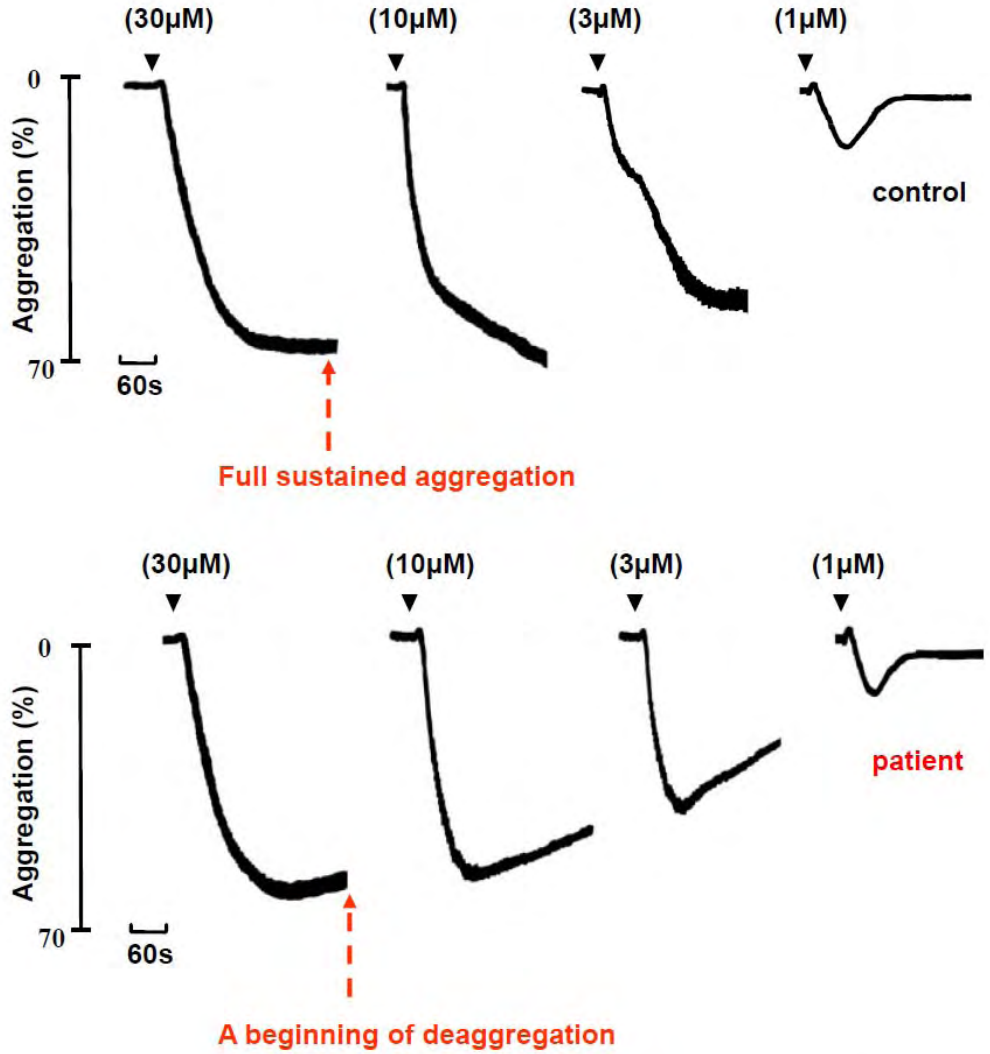
that was analysed on the same day or over the course of the last four years was observed throughout the dose response curve to ADP (Figure 5.3) and to other agonists such as adrenaline, which only induced primary wave aggregation in Birm-JW-240108's platelets (Figure 5.2). Further, sustained aggregation, similar to that observed in controls was observed in response to single high concentrations of collagen (3 μg/ml), PAR1 (100 μM), PAR4 peptides (500 μM) and CRP (1 μg/ml), whereas a reduced aggregation response was observed to lower concentrations of the above agonists, consistent with a positive feedback role for TxA_2 (data not shown). To further localise the defect, I analysed the response to the COX-1 metabolite PGH_2 (3μM) in the patient alongside the control. The response was similar in both cases (Figure 5.4) thereby indicating that the defect in Birm-JW-240108's platelets is in the metabolism of arachidonic acid by COX-1. In line with the above results, ATP secretion from dense granules was abolished in Birm-JW-240108's platelets in response to a maximal concentration of arachidonic acid while ATP secretion in response to U46619 was similar to the control (Figure 5.5). Furthermore, ATP secretion in response to CRP and the PAR1 peptide was also within the normal range (Figure 5.5) thereby ruling out a secretion defect.

Figure 5.2
Aggregation responses to different agonists in Birm-JW-240108 patient with the COX-like defect



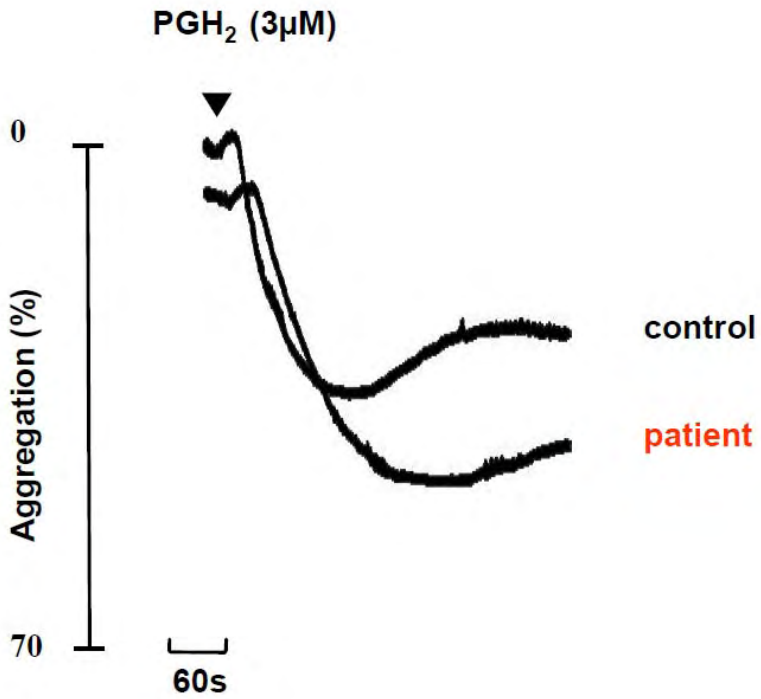
Platelet aggregation in response to the indicated concentrations of arachidonic acid, U46619, adrenaline, ADP and ADP in the presence of (10μM) indomethacin. Responses were measured in citrated PRP from a healthy volunteer (control) and from a patient diagnosed with a COX-like defect (a similar profile was seen in nine other patients diagnosed with the same defect).

Figure 5.3
Aggregation responses to ADP in patients with TxA_2 pathway defect



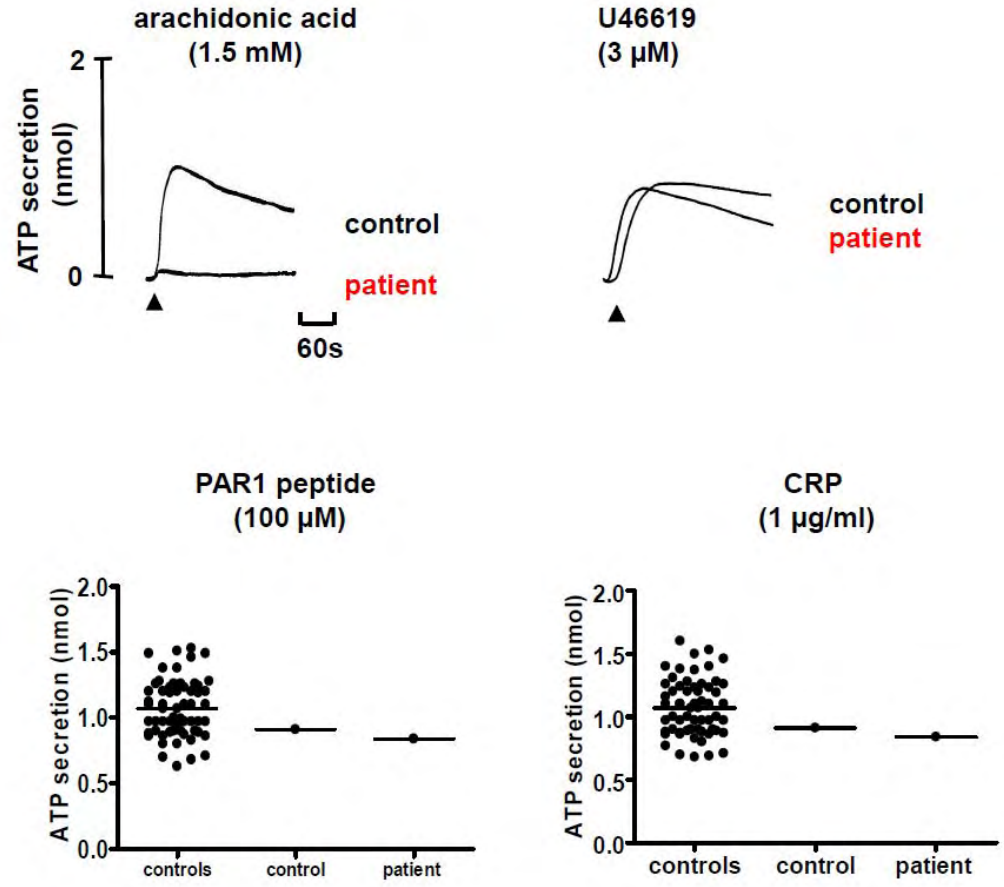
Platelet aggregation in response to the indicated concentrations of ADP in citrated PRP from a healthy volunteer (control) and from a patient with COX like defect. (a similar profile was seen in all eleven patients diagnosed with a defect in the TxA_2 pathway).

Figure 5.4
Aggregation responses to PGH_2 in washed platelets from Birm-JW-240108 patient with the COX-like defect



Platelet aggregation in response to (3µM) PGH_2 in washed platelets from a healthy volunteer (control) and from the patient Birm-JW-240108 with COX-like defect (similar profile was seen in other patients of the same defect).

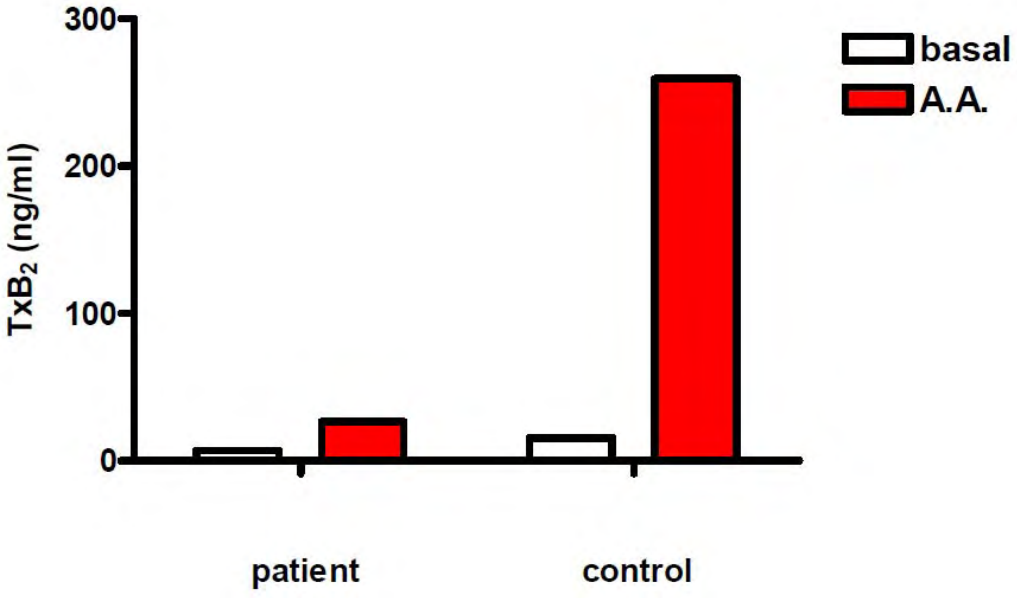
Figure 5.5
ATP secretion in response to different agonists in Birm-JW 240108 patient with the COX-like defect



ATP secretion in PRP from a healthy volunteer (control) and Birm-JW-240108 patient with COX-like defect induced by (1.5 mM) arachidonic acid, (3µM) U46619 (shown as secretion traces) (1µg/ml) CRP and (100µM) PAR1 peptide compared with maximal ATP secretion levels in PRP from control subjects studied on separate occasions (similar profile was seen in the other 9 patients diagnosed with a COX-like defect).

To further investigate the defect in the patient, I measured the conversion of arachidonic acid to TxA_2 synthesis via a TxB_2 ELISA assay, as an indirect measure of TxA_2 production. The formation of TxB_2 was almost abolished in Birm-JW-240108 platelets, consistent with a COX-1 defect, whereas the response to the control was within the normal range (Figure 5.6).

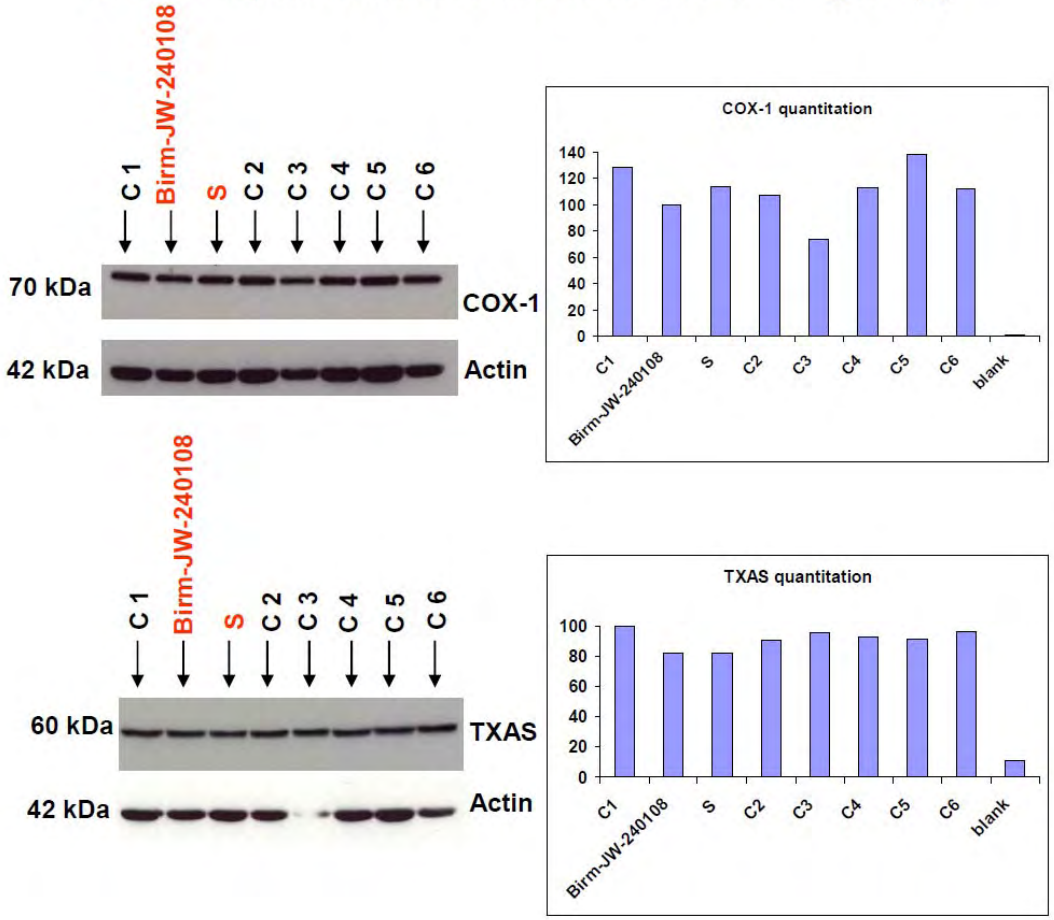
Figure 5.6
Quantification of TxA_2 production via a TxB_2 ELISA assay in Birm-JW-240108 patient with COX-like defect



Data is shown for Birm-JW-240108 patient with COX-like defect alongside a control. The concentration of TxB_2 produced was determined from a standard curve of TxB_2 concentrations constructed using a serial dilution ranging from 0-64ng/ml. Results for unstimulated platelets with the addition of PBS are shown in white and results for platelets stimulated with (1µM) arachidonic acid (A.A.) are shown in red.

The above results point to a defect in COX-1. To investigate this further, the expression level of COX-1 was measured in platelets from Birm-JW-240108 alongside six other controls and her daughter (S), who does not have a bleeding defect, by quantitative western blotting (Figure 5.7). The level of expression of COX-1 in Birm-JW-240108's platelets was similar to that in the controls and in her daughter's platelets (daughter is labeled S in Figure 5.7). Furthermore a similar result was obtained for quantitation of thromboxane synthase by western blotting (Figure 5.7). Thus, the defect in Birm-JW-240108's platelets does not appear to be due to a reduced level of expression of COX-1 or thromboxane synthase.

Figure 5.7
Protein expression analysis for COX-1 and TXAS enzymes in Birm-JW-240108 patient with COX-like defect and her daughter (S)



Whole cell lysates from the patient Birm-JW-240108, her daughter(S) and 6 controls (equalised for protein quantity) were run on 1-D SDS-PAGE gel, transferred to PVDF and blotted for COX1 and TXAS, then reprobed for Actin. Quantitation of band volumes was done using the Syngene GeneGnome HR bio imaging system.

The above results raise the possibility of an underlying point mutation in the coding region of COX-1. To investigate this, the cDNA of COX-1 was generated by reverse transcription of RNA isolated from white cells and sequenced. This work was performed by Dr. Jen Spalton in the laboratory. No defect in the sequence was identified (not shown – Appendix-5).

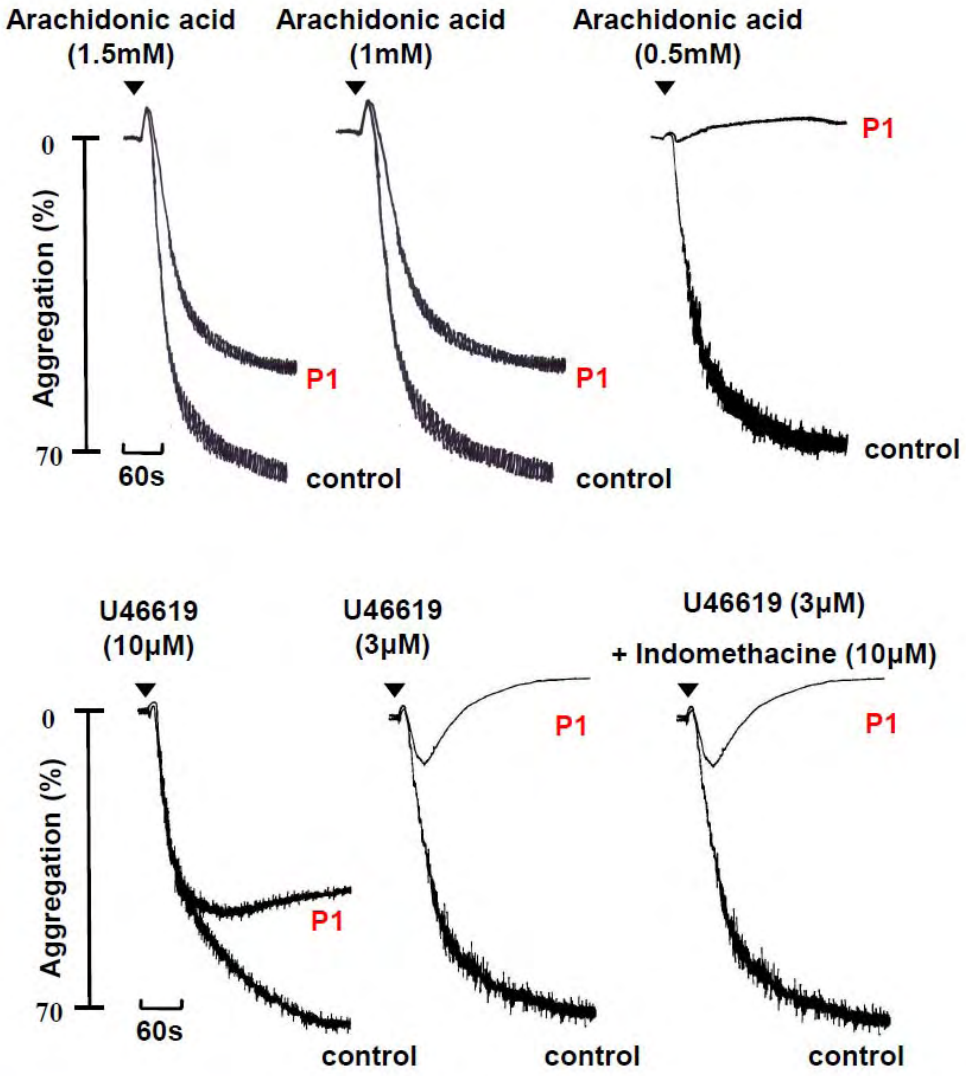
5.3.3 Investigation of a patient with a TxA_2 receptor defect

A 14 year old male, Birm-MW-030408, (labeled as P1) with a significant history of recurrent episodes of epistaxis where local effects in nasal blood vessels had been ruled out, and with no other haemostatic challenges from surgery and trauma, was referred for platelet function testing. The patient's platelets underwent weak shape change to a concentration of arachidonic acid (0.5 mM) that induced full aggregation in a control which was analysed alongside, but underwent full, sustained aggregation to higher concentrations of the lipid agonist (Figure 5.8). Importantly, the concentration response curve for the control was similar to that in over 50 other controls that have been analysed over the course of the last four years as described in Chapter 3. Furthermore, the patient also exhibited a marked defect in aggregation to intermediate and high concentrations of the stable thromboxane mimetic, U46619 (1–10 μM), with reduced aggregation observed at lower concentrations and deaggregation at higher concentrations (Figure 5.8). In contrast, U46619 (1–3 μM) stimulated full sustained aggregation in a control which was analysed alongside, as is the case in over 50 other controls that have been studied throughout the course of this work (see Chapter 3). Furthermore, there was no diminution in the aggregation response to U46619 (3 μM) following incubation with indomethacin (10 μM) (Figure 5.8), which demonstrate that

the defect is independent of COX-1 and thromboxane synthase. This result therefore indicates that the defect is at the level of the thromboxane receptor.

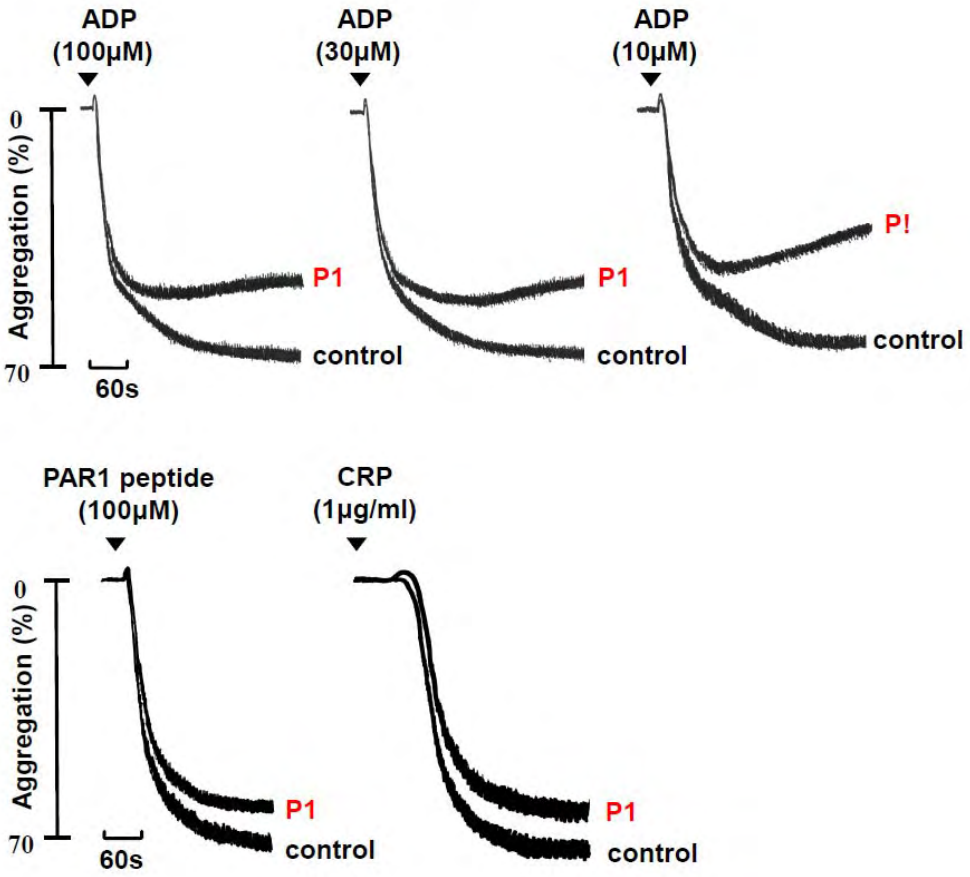
The aggregation results obtained for the other platelet agonists are consistent with a defect in the TxA_2 pathway. For example, although a high concentration of ADP (100 μM) stimulated maximal aggregation, the response was not sustained (Figure 5.9) as is seen in controls treated with indomethacin (10 μM) (see Chapter 3). Loss of the secondary wave of aggregation to adrenaline was also observed (not shown). Sustained full aggregation, similar to that in controls, was observed in response to high concentrations of other agonists including collagen, peptides specific for the thrombin receptors, PAR1 and PAR4, and the GPVI collagen receptor agonist CRP (Figure 5.9), with a corresponding reduction in response to submaximal concentrations due to the feedback role of the thromboxane pathway in platelet activation (not shown).

Figure 5.8
Aggregation responses to arachidonic acid and U46619 In the patient (P1) with the TP receptor defect



Platelet aggregation in response to (1.5, 1 and 0.5 mM) arachidonic acid in citrated PRP from a healthy volunteer (control) and from the patient Bim-MW-030408 labelled (P1). n=2

Figure 5.9
Aggregation responses to some agonists in the patient (P1) with the TP receptor defect

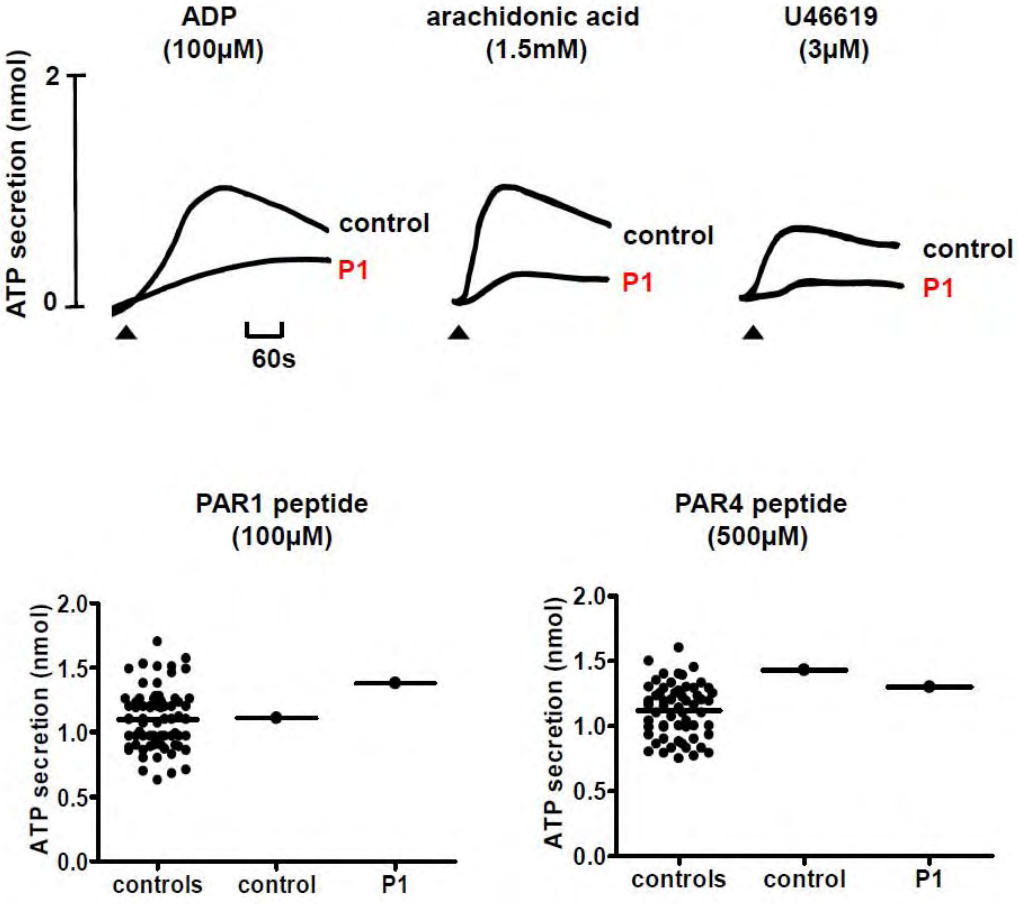


Platelet aggregation in response to different concentrations of ADP, PAR1 peptide and CRP (as indicated) in citrated PRP from a healthy volunteer (control) and from the patient Bim-MW-030408 labelled (P1). n=1

The patient's platelets also exhibited a reduction in the secretion of ATP from dense granules in response to high concentrations of ADP, arachidonic acid and the TxA_2 mimetic, consistent with a defect in the thromboxane receptor (Figure 5.10). On the other hand, high concentrations of the two PAR peptides induced a normal level of secretion of ATP alongside that of the control that was analysed on the same day and by comparison to more than 60 other controls measured on different days (Figure 5.10). This therefore demonstrates that the nucleotides levels and the release mechanisms from the dense granules are within the normal range, thus ruling out a secretion defect in this patient.

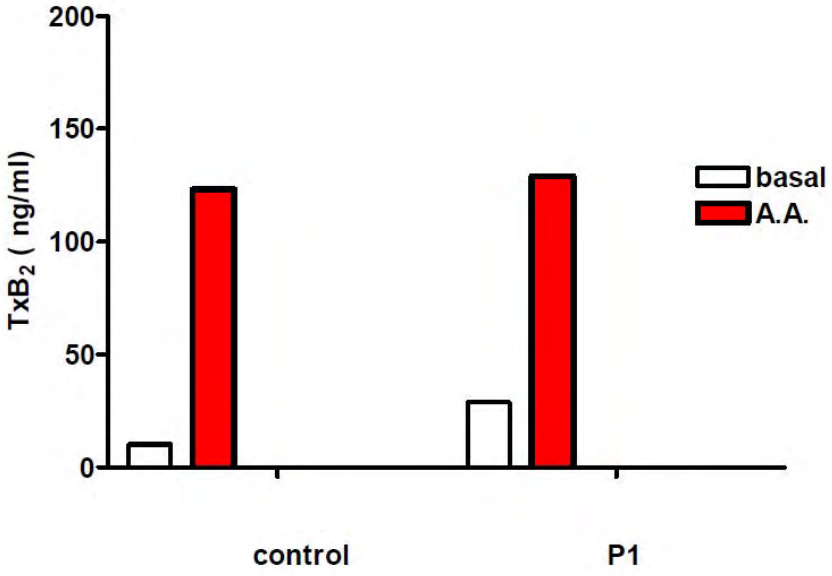
Together, the aggregation and secretion traces in the patient appear point to a partial defect at the level of the thromboxane receptor, therefore compromising the ability of the patient's platelets to respond to both endogenous and exogenous sources of TxA_2. Furthermore, the patient's platelets generated a similar level of TxB_2 to that of the control in the presence of arachidonic acid (1 μM) thereby confirming that the phenotype is not due a defect in metabolism (Figure 5.11).

Figure 5.10
ATP secretion in response to different agonists in the patient (P1) with the TP receptor defect



Maximal levels of ATP secretion in PRP from a healthy volunteer (control) and the patient with the heterozygous D304N mutation induced by (100μM) ADP, (1.5mM) arachidonic acid and (3μM) U46619 (shown as secretion traces) and (100μM) PAR1 and (500μM) PAR4 peptides (shown as graphs and compared with maximal ATP secretion levels in PRP from control subjects studied on separate occasions).

Figure 5.11
Thromboxane B_2 formation in the patient P1 with the TP receptor defect



Data is shown for the patient P1 with the TP receptor defect alongside a control. The concentration of TxB_2 produced was determined from a standard curve of TxB_2 concentrations constructed using a serial dilution ranging from 0-64ng/ml. Results for unstimulated platelets with the addition of PBS are shown in white and results for platelets stimulated with 1μM arachidonic acid (A.A.) are shown in red.

5.3.4 Identification of the D304N mutation and further confirmatory tests

In order to search for a possible mutation in the TP receptor, genomic DNA sequencing was conducted in Bristol by Dr Andrew Mumford. The patient was found to be heterozygote for a candidate mutation of an aspartic acid to an asparagine at position 304 in the 7^{th} transmembrane region of the receptor (Figure 5.12). This candidate mutation is not present in any of the available databases suggesting that it is not a known polymorphism.

The binding of the TxA_2 receptor antagonist [^{3}H]-SQ29548 to fixed platelets was measured by Dr. Stuart Mundell at University of Bristol. The platelets were fixed in Birmingham and transported to Bristol by courier. The number of binding sites on the platelets from the patient was reduced by approximately 50%, whereas the affinity constant was not altered relative to the platelets from the patient's mother (P3) or to an unrelated healthy control (Figure 5.13). These results indicate a defect in either receptor expression or ligand binding. To distinguish between these two possibilities, the binding of [^{3}H]-SQ29548 to CHO cells stably expressing variant D304N TxA2R was investigated. [^{3}H]-SQ29548 exhibited specific binding to the wild-type TxA2R but not to the D304N mutant (Figure 5.14; this work was performed by Drs Stuart Mundell, Jen Spalton and Andrew Mumford) thereby demonstrating a defect at the level of ligand binding.

Figure 5.12
A diagram showing D304N heterozygous mutation on TP receptor

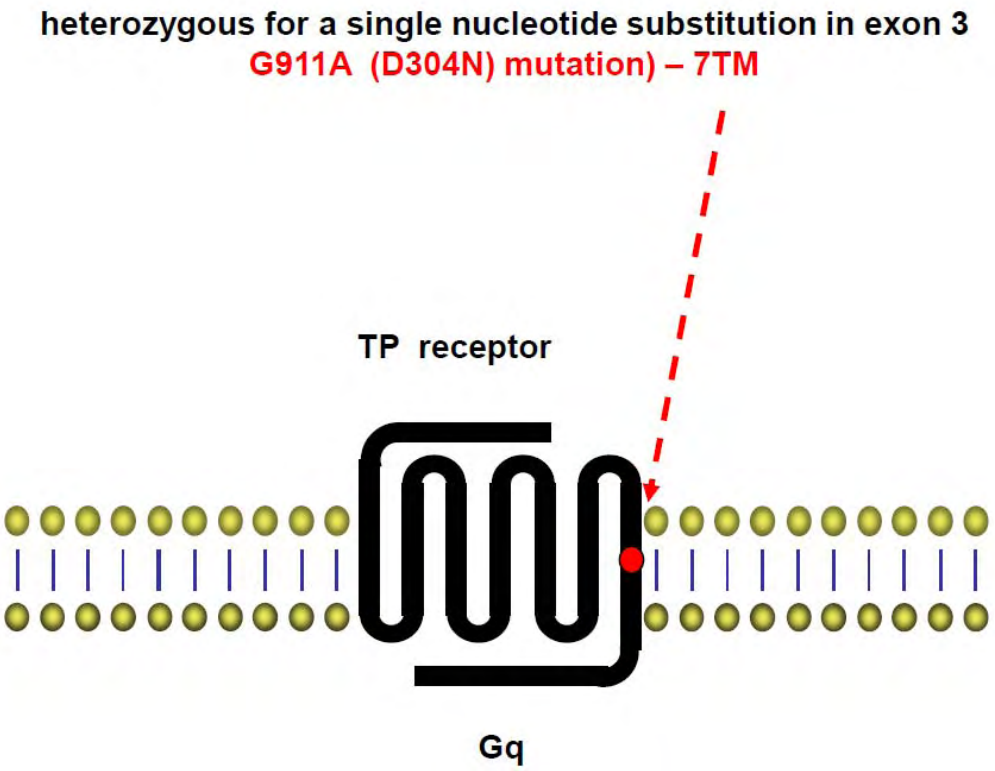
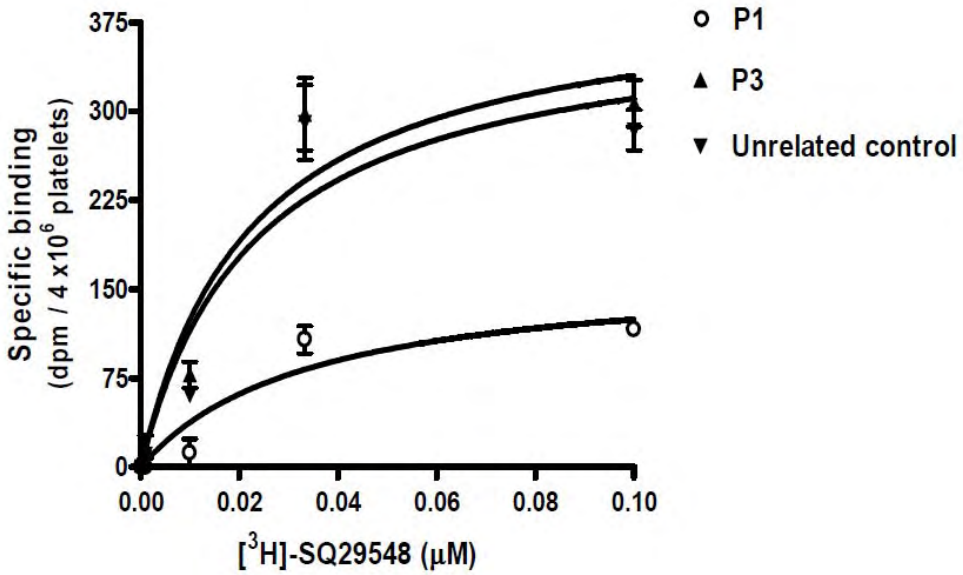


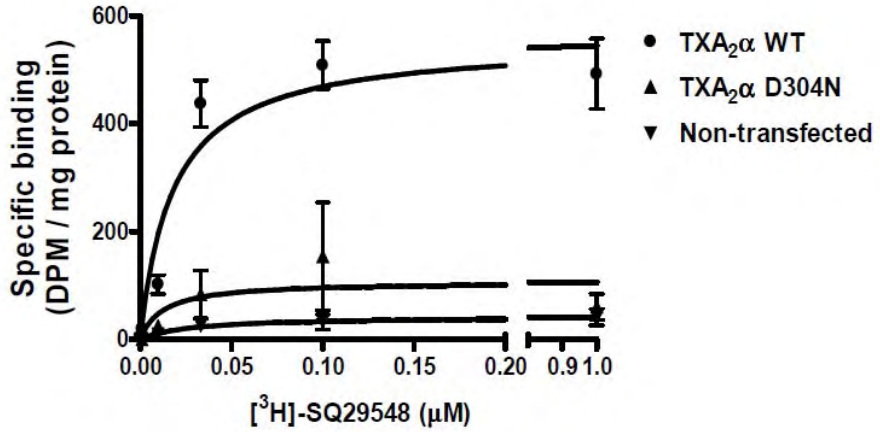
Figure 5.13
[^{3}H]- SQ29548 binding in subjects heterozygous for the TxA_2 D304N substitution. (P1 and P3)



Ligand binding to TXA2R on fixed platelets from P1 was measured using the thromboxane A_2 antagonist [^{3}H]-SQ29548. Comparison was made to platelets from and unrelated control and family member P3 who showed normal platelet aggregation. Data are expressed as the mean (±SEM) of 3 independent experiments.

Work done by Dr. Stuart Mundell / University of Bristol

Figure 5:14
Analysis of D304N variant TxA2Rα in heterologous cells.



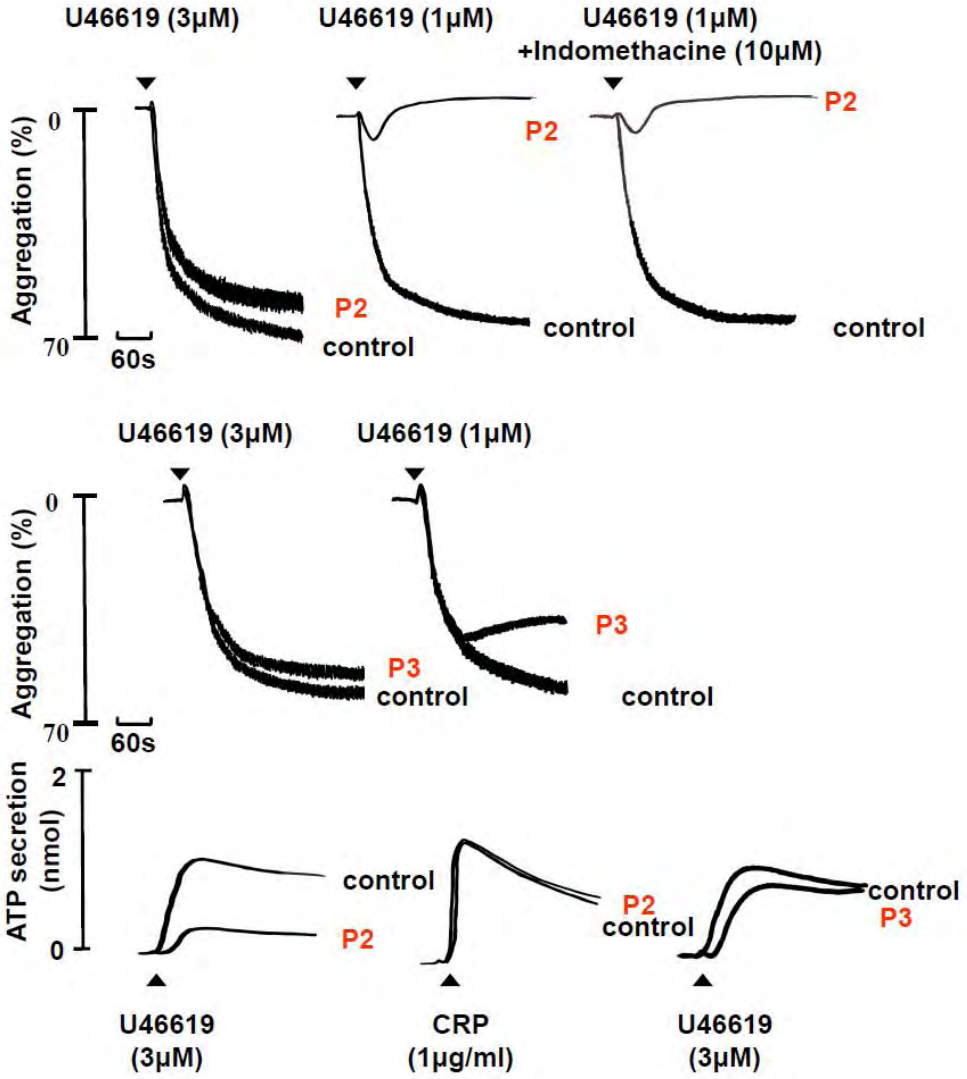
Binding of [3H]-SQ29548 to CHO cells transfected with either wild-type (WT) or D304N variant TxA2R was measured at a range of ligand concentrations and is expressed as DPM per mg of total CHO cell protein.

Work done by Dr. Stuart Mundell / University of Bristol

5.3.5 Investigation of relatives of Birm-MW-030408

Two other members of the family of patient Birm-MW-030408 donated samples for investigation, including the father (P2) and the mother (P3). Although the father has no history of excessive bleeding, his platelets exhibited a partial defect in aggregation and ATP secretion in response to arachidonic acid and U46619 which was similar to that seen in his son (figure 5.15). Furthermore, DNA sequencing identified the same heterozygous mutation as that in the patient (D304N). On the other hand, the mutation was not found in the mother (P3) even though she has a history of heavy menstrual periods. Further, aggregation and secretion responses to U46619 were similar in the mother to that of the control (Figure 5.15).

Figure 5.15
The aggregation and secretion responses in the father (P2) and mother (P3) of the patient with the TP receptor defect



Aggregation and maximal levels of ATP secretion in PRP from a healthy volunteer (control) and the father (P2) and mother (P3) of the patient with the TP receptor defect.

5.4 Discussion

In this study, as in several previously conducted studies, patients with suspected defects within the TxA_2 signaling pathway have been identified. I estimate that 11 (i.e. 14%) of the patients that I have studied have a 'COX-like' defect making this one of the most common causes of mild bleeding alongside secretion-like (21%) and a Gi signaling (19%) defects. The majority (10 out of 11) of patients with TxA_2 signaling pathway were categorized as having a defect in arachidonate metabolism, while the other patient was identified to have a defect at the TP receptor level. Aspirin-like defects in platelets have been described for over 40 years and defective platelet cyclooxygenase activity has been proposed as a cause of excessive bleeding by several groups but a genetic defect has never been reported.

Patients with a defect in arachidonate metabolism showed a markedly reduced aggregation in response to arachidonic acid, a metabolic precursor of TxA_2, but not to the stable thromboxane mimetic, U46619. Measurement of TxB_2 formation from exogenous arachidonic acid confirmed a defect in arachidonate metabolism in a sub-group of these patients (4) and is a powerful means of confirming the initial diagnosis. Interestingly, however, in the one patient from this group who was thoroughly investigated, we have been unable to find a change in the level of expression or a mutation in the coding region of the two enzymes that convert arachidonate acid to TxA_2, namely COX-1 or TXAS, thereby indicating an alternative cause. Importantly, this patient has consistently denied taking a known COX inhibitor such as aspirin thereby raising the interesting possibility that the defect is in an unknown accessory protein that supports the conversion of arachidonate acid to TxA_2. To my knowledge,

there are no obvious candidates for such a protein to direct further western blotting and sequencing studies and thus further work on this patient will have to wait until the costs of sequencing and bioinformatics analysis of whole genomes reaches a level that gives 'value-for-money' in order to identify candidate gene defects. In the future, the application of whole genome sequencing to the study of patients like this case has the potential to reveal new unexpected findings in regard to the mechanisms that give rise to platelet activation and thereby potential new targets for development of antithrombotics.

On the other hand, a heterozygous molecular defect in the one patient who exhibited a reduced aggregation and secretion response to both arachidonic acid and agonist U44619 was identified in the 7^{th} TM region of the TP receptor, namely an aspartic acid to asparagine at position 304 (D304N). This mutation is in an evolutionary highly conserved region in 7 transmembrane receptors, namely a NPXXY motif, which maintains hydrogen-bond networks between adjacent transmembrane domains, although paradoxically in the case of the TP receptor, the mutation is from an aspartic acid (D) back to the evolutionary conserved amino acid, asparagine (N). Mutations in this region are known to disturb the receptor function, a result that was also found in the case of the D304N mutation by expression of the mutant TP receptor in an immortalized cell line model by Dr Stuart Mundell in Bristol. Importantly, this is only the second mutation in the thromboxane receptor to be described in the literature and the first in which a bleeding defect has been linked to a heterozygous mutation.

Interestingly, the heterozygous D304N mutation was also identified in the patient's father even though he does not have a history of excessive bleeding. Nevertheless, functional studies revealed a partial defect in aggregation and ATP

secretion in response to arachidonic acid and U46619 that was similar to that seen in his son. This therefore indicates that this mutation alone is insufficient to explain the bleeding diathesis and that the son has second defect that, presumably in combination with the TP receptor mutant, gives rise to the mild bleeding diathesis. It is therefore of interest that his mother has a history of heavy periods that could be taken as evidence for a very mild bleeding defect. This study therefore emphasizes that a TP receptor defect should be investigated when an impairment in response to arachidonic acid is observed.

CHAPTER 6

OVERVIEW OF STUDIES ON PATIENTS WITH MILD PLATELET-BASED BLEEDING DISORDERS

6.1 Summary

Mild platelet-based bleeding disorders are a heterogeneous group of disorders with a variety of causes and functional consequences. Diagnosis is challenging to both clinicians and research laboratories due to the absence of a gold standard test. This chapter summarises the results of patients who have been investigated over the course of nearly 4 years using aggregation and secretion studies along with other specialized tests.

6.2 Introduction

The presence of adequate numbers of normally functioning platelets is essential in arresting haemorrhage from an injured blood vessel. Excessive bleeding following a haemostatic challenge is the hallmark feature of platelet dysfunction, which can arise through defects in surface receptors, signaling proteins, granules contents/release, cytoskeletal proteins and platelet procoagulant function. The prevalence of platelet disorders is uncertain due to the difficulties in diagnosis with only the more severe being recognised (Hayward, 2008).

In Chapter 3, I described a series of reference curves for aggregation and ATP secretion to nine platelet agonists in controls, together with the effect of antagonists / inhibitors of the major two feedback pathways on these curves. The reference curves and representative aggregation traces have proven invaluable in the assessment of the patient's platelets and also in confirming that each control, measured on the same day as the patient sample, was within the normal range. Further, with available resources in

the Birmingham Platelet Group, it has been possible to perform additional, specialist tests alongside the aggregation and secretion studies as part of the initial evaluation of each patient sample.

Using this approach, patients have been classified on the basis of the platelet phenotype as summarized in this chapter. Strikingly, however, a defect in approximately one third of patients has either not been identified or subdivided into a specific group (Table 6.1).

Table 6.1
Classification of patients with mild platelet-based bleeding defects

Type of platelet defect	Number of patients	Percentage of patients	Number of novel mutations
Gi-like defect (including $P2Y_{12}$ receptor defect)	15	19 %	2
TxA_2 formation/function defect (including both COX-like and TxA_2 receptor defects)	11	14 %	1
GPVI-like defect	4	5 %	
Gq-like defect	1	1%	
Secretion like defect (including HPS)	17	21 %	1
Complex	1	1%	
Unknown	31	39%	
Total	80	100 %	4

6.3 Results

6.3.1 Overall classification of the platelet defects

Over the course of nearly 4 years, aggregation and ATP secretion were monitored in 80 index patients with a bleeding tendency who are judged clinically to have a defect in platelet function. According to the patterns of abnormalities in aggregation and secretion, these patients were classified by the nature of their platelet defect into 5 main groups as outlined in Table 6.1. Approximately two thirds of these patients in this study were found to have a platelet defect. The most common defects were those associated with dense granule secretion (21%), Gi signalling (19%) and TxA_2 formation/function (14%). The phenotype of patients with defects in Gi signaling and TxA_2 formation/function were described in Chapters 4 and 5. The pattern of response in the remaining groups of patients is summarized below.

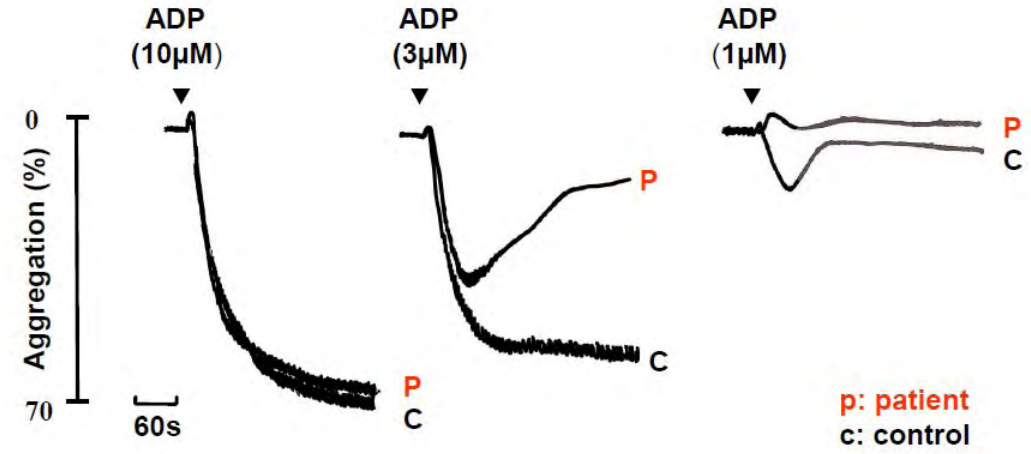
6.3.2 Secretion-like defect

ATP secretion from platelet dense granules, measured using the luciferin/luciferase firely system, was analysed in all patients in order to investigate a possible storage pool defect. Accordingly, 21% of patients were found to have low or absent ATP secretion when compared with controls, a result that is consistent with the fact that storage pool disease is recognized to be a major cause of mild bleeding of platelet origin. Importantly, in all of these cases, the secretion defect was associated with a reduction in aggregation to low concentrations of all agonists, most notably collagen (not shown),

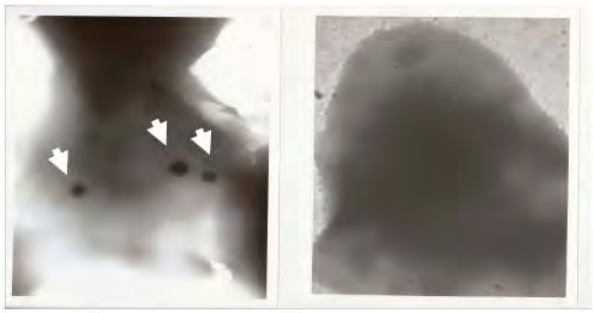
but with the exception of ADP as illustrated in a patient diagnosed with a new form of Hermansky-Pudlak syndrome, HPS-8, caused by mutation in the gene BLOC1-S3/reduced pigmentation (Figure 6.1A). This novel variant of HPS was identified by the group of Prof Eamonn Maher in a Birmingham family of Pakistan origin with a high level of consanguinity through autozygosity mapping (Morgan et al., 2006). Confirmation of the absence of dense granules in this patient was shown using transmission electron microscopy (Figure 6.1B). Figure 6.2 shows the level of secretion observed to high concentrations PAR1 peptide and to CRP in the majority of patients diagnosed with a secretion disorder relative to controls. In all cases, a significant decrease in secretion was seen to both the PAR1 peptide and to CRP, although the degree of the secretion defect varied between patients from approximately 50% to complete abolition.

Figure 6.1
Aggregation traces to ADP and electron microscopy images in a patient with Hermansky-Pudlak syndrome (HPS)

(A) Aggregation traces



(B) Transmission electron microscopy (TEM) images

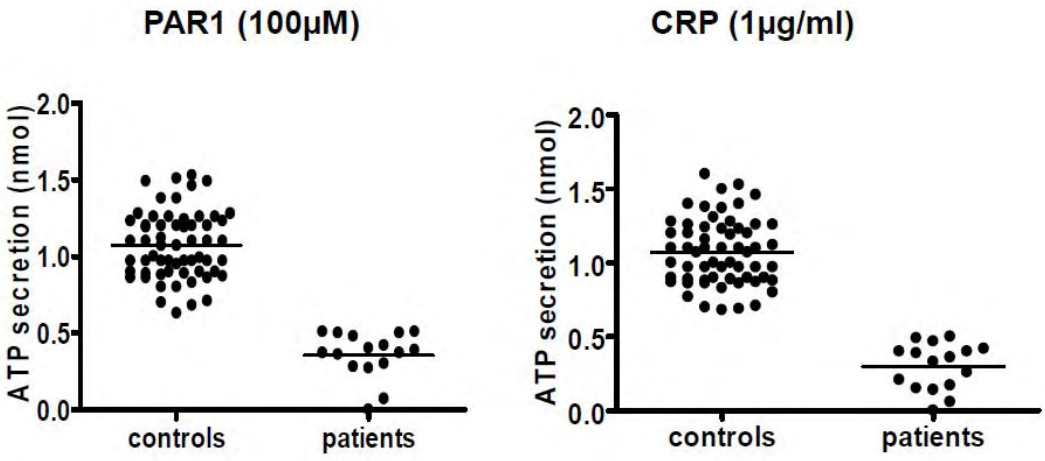


Dense granules (arrowed) are missing in the patient with HPS

control **patient** **X 25000**

A) Aggregation of platelets in citrated PRP from a healthy volunteer (control) and from a patient with HPS in response to the indicated concentrations of ADP.
B) TEM images showing the lack of dense granules in the patient with HPS in comparison with the control.

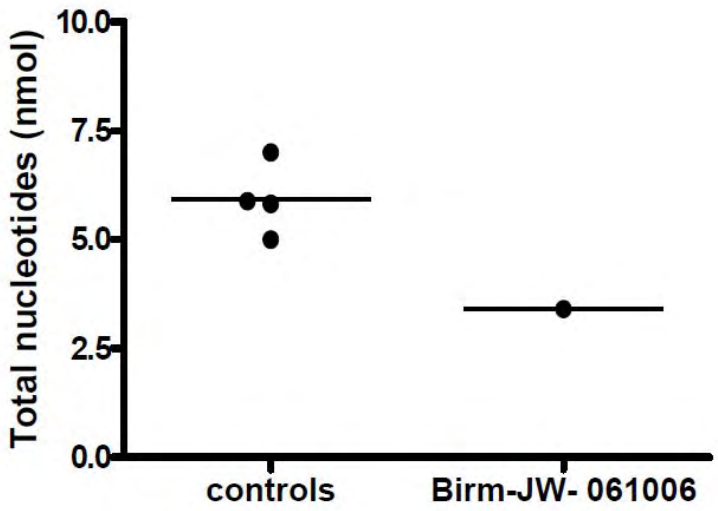
Figure 6.2
Levels of ATP secretion in response to PAR1 peptide and CRP in patients with secretion-like defect



Maximal levels of ATP secretion in PRP from healthy volunteers (controls) and patients who have secretion-like defect induced by a PAR1 peptide (100µM) and the GPVI receptor agonist, CRP (1µg/ml)

It is important to emphasis that a reduction in ATP secretion could be due to dense granule content deficiency (Holmsen and Weiss, 1972) or impaired secretion (Weiss and Rogers, 1972) which is considerably more common and could be caused by a defect in the signal transduction process or in the release machinery. These two conditions can be distinguished by measurement of platelet nucleotides, which is an assay that is performed in a limited number of platelet testing laboratories. I measured nucleotides in two patients, one of them (Birm-JW-061006) had a reduction in the level of nucleotides (Figure 6.3) while the other was within the normal range. However, measurement of nucleotides was not performed as a routine part of my investigations as the major goal of this British Heart Foundation funded research was to identify patients with defects in the $P2Y_{12}$ ADP receptor.

Figure 6.3
Total amount of nucleotides in a patient with secretion-like defect (Birm-JW- 061006) and 4 controls



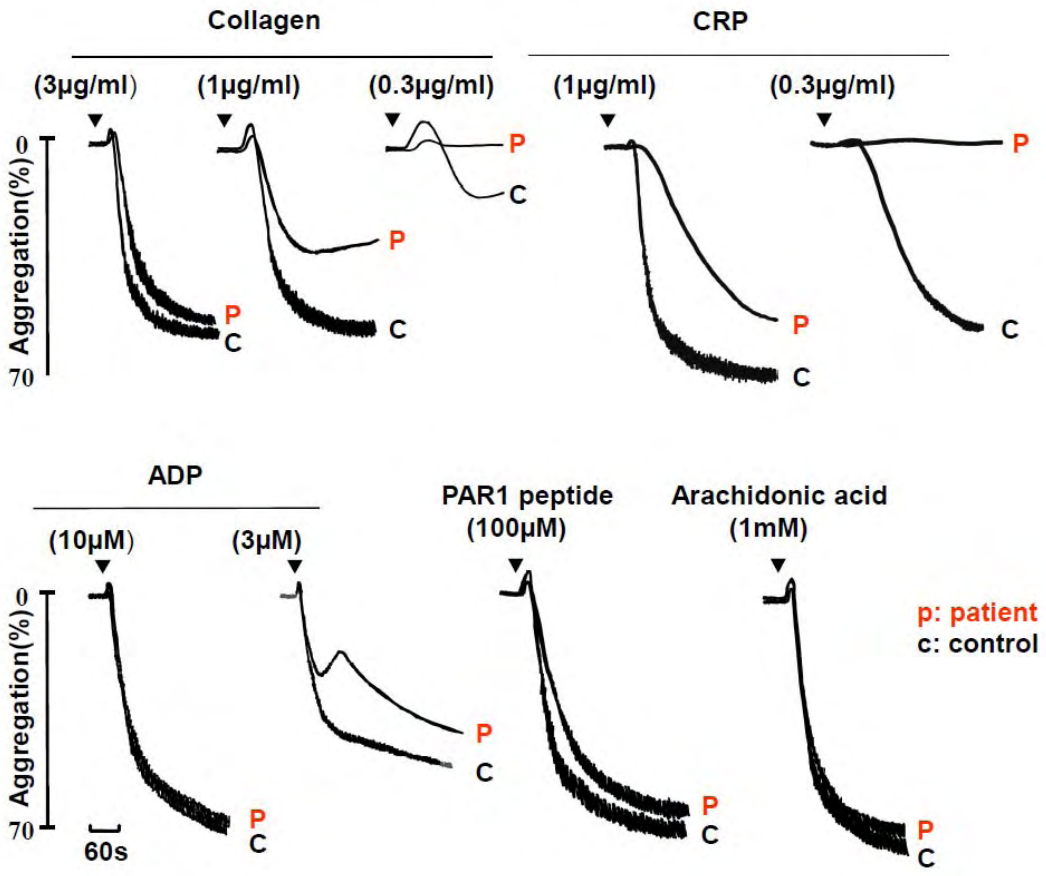
The total amount of both ATP and ADP nucleotides is shown for the patient Birm-JW-061006 alongside four controls. The amount of ATP was determined from a standard curve of ATP concentrations constructed using serial dilutions of ATP standard. The ADP in the samples was converted to ATP via phosphoenol pyruvate/pyruvate kinase reaction as stated in the methods.

Storage pool disease refers to defects in secretion of dense or α-granules, or both sets of granules. Defects in α-granule secretion, a condition known as Grey (or Gray) platelet syndrome, were not monitored in this study, as these would have been diagnosed by the testing laboratory through the characteristic 'grey' appearance of platelets on a blood smear as a consequence of loss of content and alteration in morphology. However, α-granule defects that are not associated with this 'grey' appearance would have therefore been missed.

6.3.3 GPVI-like defect

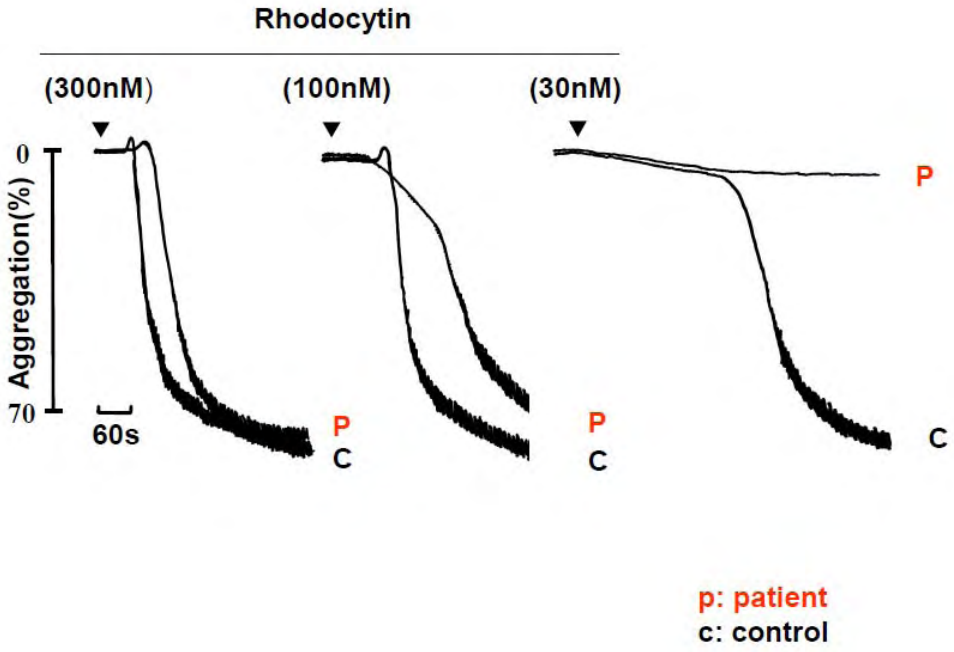
Four of the eighty index cases were diagnosed with a GPVI-like defect on the basis of a selective defect in aggregation to the GPVI agonists, collagen and CRP, whereas the response to ADP, arachidonic acid and PAR1 and PAR4 peptides were within the normal range (Figure 6.4). In view of the selective reduction in response to the two GPVI receptor agonists, I compared the ability of the snake venom toxin rhodocytin, which activates platelets through a similar pathway to that used by GPVI receptor but via a distinct receptor, CLEC-2 (Suzuki-Inoue et al., 2006), to activate the platelets. In three of these cases, a marked defect in response to rhodocytin was also observed as illustrated in Figure 6.5 suggesting a defect in the tyrosine kinase signaling cascade used by the two receptors. In the other patient, described below, a selective loss of response to CRP and collagen was seen which is indicative of a defect at the level of the GPVI-FcR γ-chain complex.

Figure 6.4
Aggregation traces to different agonists in a patient with GPVI-like defect



Aggregation of platelets in citrated PRP from a healthy volunteer (control) and from a patient with GPVI-like defect in response to the indicated concentrations of Collagen, CRP, ADP, PAR1 peptide and arachidonic acid. A similar profile was seen in the other 3 patients with GPVI-like defect enrolled in the study

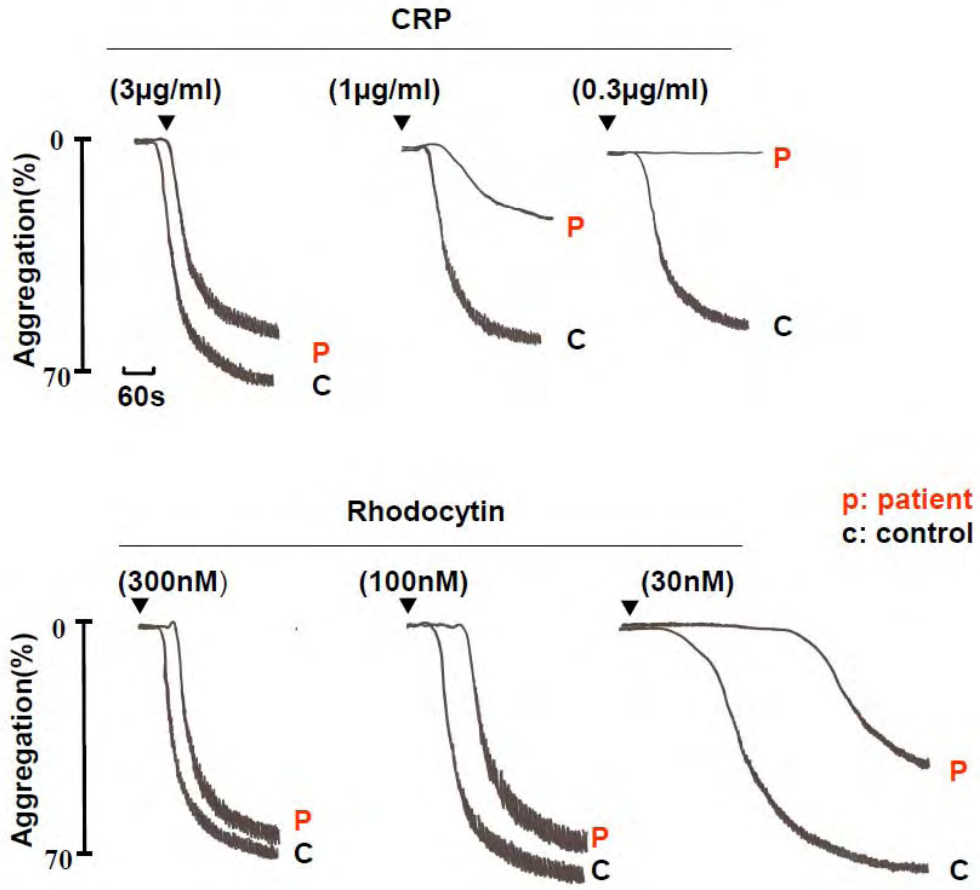
Figure 6.5
Aggregation traces to Rhodocytin in a patient (Birm-JW-060308) with a GPVI-like defect



Aggregation of platelets in citrated PRP from a healthy volunteer (control) and from the (Birm-JW-060308) patient with GPVI-like defect in response to the indicated concentrations of rhodocytin. Similar profile was found in the other 2 patients of this group.

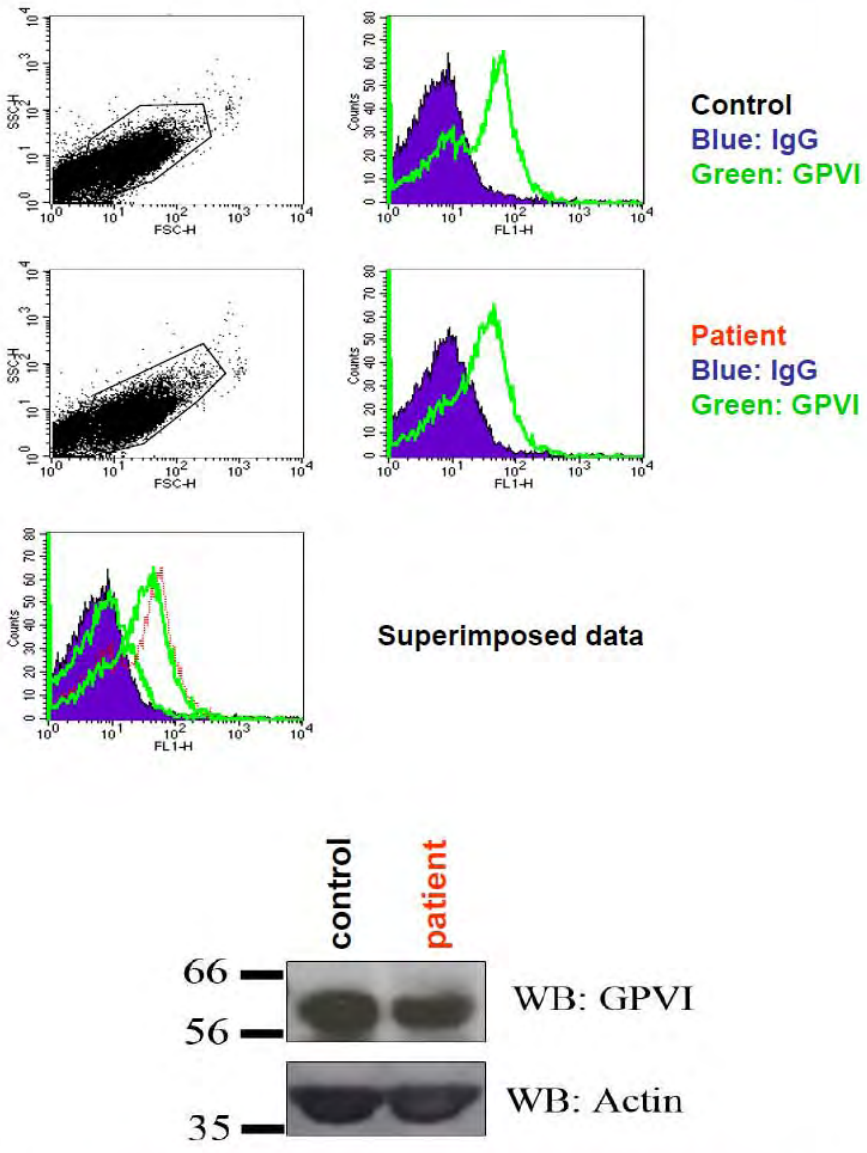
The patient referred to above is a 62 yr old female, Sheff-MM-181207-01, who had a history of severe nosebleeds, one of which required hospital admission, easy bruising, excessive bleeding following dental extraction and heavy menstrual periods around menopause. Aggregation and secretion studies were performed and showed a right shift in the DRCs in response to CRP in comparison to the control, although full aggregation was elicited at a high concentration (Figure 6.6). In contrast, the rhodocytin response was similar, albeit slightly delayed, to the control at high and intermediate concentrations suggesting a possible defect at the GPVI receptor level. A slightly reduced level of GPVI expression was detected in the patient relative to the control by western blotting where as similar levels were observed by flow cytometry (Figure 6.7). These results although ruled out a quantitative defect in the GPVI receptor, they could not exclude dysfunctional defect in the receptor. It is currently waiting to be sequenced.

Figure 6.6
Aggregation traces to CRP and rhodocytin in a patient (Sheff-MM-181207) with GPVI-like defect



Aggregation of platelets in citrated PRP from a healthy volunteer (control) and from the patient (Sheff-MM-181207) with GPVI-like defect in response to the indicated concentrations of CRP and rhodocytin.

Figure 6.7
Expression levels of GPVI using flow cytometry and western blotting in a patient (Sheff-MM-181207) with GPVI-like defect



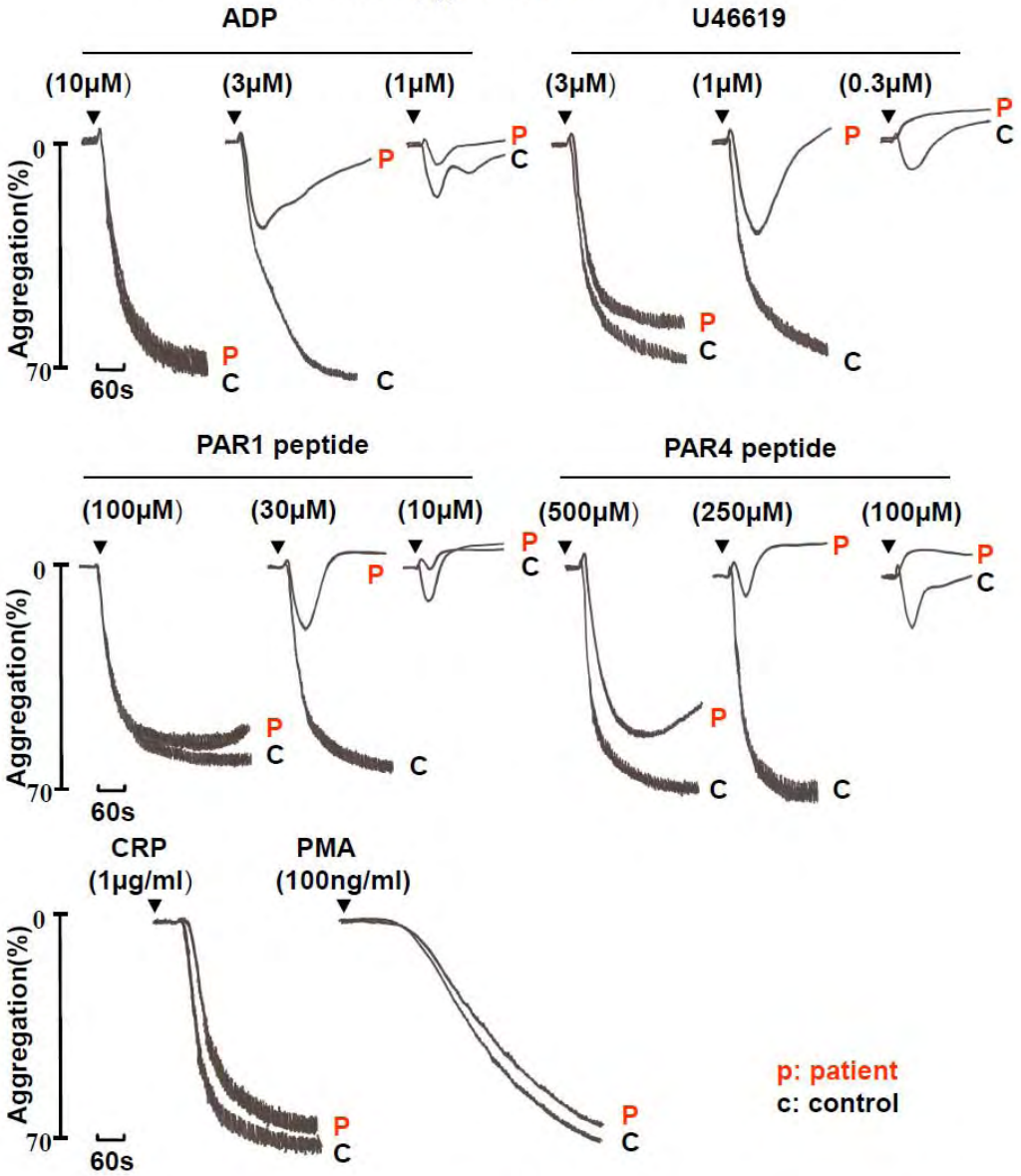
GPVI expression levels by Flow cytometry (above) and Western Blot (below) of patient and control.

6.3.4 $Gq/_{13}$-like defect

Two related patients (therefore one index case), brother and sister, from a non-consanguineous relationship, were observed to have a partial defect in aggregation and secretion to agonists that signal through Gq and G_{13} heterotrimeric G proteins, namely PAR1 and PAR4 peptides and the TxA_2 analogue U46619 (Figure 6.8). In contrast, the responses to ADP and CRP were only marginally inhibited arguing against a general defect in platelet activation. This is further supported by the similar nature of the response to the phorbol ester PMA in the patient's platelets (Figure 6.8). Potentially, the defect could be at the level of Gq or G_{13} although studies in mutant mice have shown that a defect in Gq gives rise to more pronounced inhibition of response similar to that observed in the two patients (Offermanns et al., 1997, Weig et al., 2008).

The brother, 25 yrs old, had a history of excessive bleeding from childhood following tooth extraction (3 wisdom teeth) and with infrequent nose bleeds. The sister used to have heavy painful menstrual periods and more recently frequent nasal bleeding. Their mother also has a history of bleeding but was not available for investigation. Thus, it seems that that the two patients have inherited a dominant signalling defect in the G_q / G_{13}-coupled receptor pathway, although this was not manifest at the level of protein kinase C as aggregation to phorbol myristate acetate (PMA), which directly activates protein kinase C, was similar to the control (Figure 6.8). Further tests including calcium measurement will be essential to localise the defect.

Figure 6.8
Aggregation traces to different agonists in a patient with $Gq/_{13}$-like defect



Aggregation of platelets in citrated PRP from a healthy volunteer (control) and from the patient with $Gq/_{13}$ like-defect in response to the indicated concentrations of ADP, U46619, PAR peptides, CRP and PMA

6.3.5 Patients with a complex defect

Two related patients (a father and a son) showed a wide range of platelet defects from early childhood indicating a dominant pattern of inheritence. Defects included a marked decrease in the level of dense granules, reversible aggregation to ADP, loss of primary wave to adrenaline, loss of aggregation to GPVI, impaired aggregation to PAR4 and mild thrombocytopenia (not shown). It seems unlikely that all of these defects are brought about by a mutation in a single signalling protein suggesting a defect at the level of a transcription factor. One candidate is RUNX1, which is also associated with acute myeloid leukaemia (Ho et al., 1996, Sun et al., 2007). The family declined the request to sequence the transcription factor.

6.3.6 Patients with unknown defects

In spite of a bleeding history and a clinical diagnosis of platelet dysfunction, no defect was detected in approximately one third of patients when investigated using lumiaggregometry. It is possible that these patients do not have a defect in platelet function or that they have a defect that cannot be detected using this approach. Possible defects that could have been missed include a defect in procoagulant activity (Scott syndrome), defects in actin polymerization that only become manifest under shear, or defects in response to other platelet receptors such as CLEC-2. The use of novel agents such as rhodocytin and further, more specialist tests are required in these patients to aid diagnosis.

6.4 Discussion

The classification of platelet disorders on the basis of lumiaggregation results is a convenient way to subdivide patients and direct future gene sequencing projects. Over the course of this thesis, I have had the opportunity to study 80 index cases of patients who have been clinically diagnosed with a platelet disorder. This is a reasonable number on which to subdivide patients on the basis of their phenotype. Strikingly, the most common defects were in granule secretion, Gi signaling and thromboxane function/formation, all of which are involved in the feedback activation of platelets. In addition, four index cases were observed to have a GPVI-like defect and one a $Gq/_{13}$-like defect. The low frequency of the latter was surprising and may reflect the fact that most defects in this pathway cause more severe phenotypes in other tissues that cannot be tolerated.

Interestingly, we were still unable to observe a defect in approximately one third of candidate patients using lumiaggregometry. Additional platelet tests are required to investigate a possible defect in platelet activation by these agonists including the use of further agonists, measurement of aggregation under arteriolar rates of shear and procoagulant exposure. It is also possible that the defect is not platelet in origin despite the clinical history being indicative of a platelet defect (e.g. it may be at the level of the vessel wall) or in the most mild of these cases, that the patients do not have a bleeding disorder. Interestingly, we have not detected defects in the two PAR receptors throughout the course of this work despite the routine use of two PAR-specific peptides thereby leaving open the question of whether a defect in one or both receptors will give rise to mild bleeding.

Overall, I believe that further development and refinement in aggregation testing and coanalysis of ATP secretion in combination with the measurement of other responses will be necessary to increase diagnosis of patients with mild, platelet-based bleeding disorders. This increase in testing will require extra resources, but in the long term will benefit both the patient and the clinic.

CHAPTER 7

GENERAL DISCUSSION

7.1 How should we test for patients with mild platelet-based bleeding disorder?

A patient with a suspected platelet disorder is subject to a battery of preliminary tests which includes a full blood count, blood smear and assessment of platelet function. These tests will establish whether the platelet size, appearance and count are within the normal range. The absence of a gold-standard functional test means that platelet function studies vary between Institutions. Although there is a growing consensus against the bleeding time test, as it is invasive and poorly reproducible, it is still used in many centres today. In other cases, this has been replaced by a point-of-care test, such as the PFA-100, which measures time to occlusion following flow of whole blood at high shear over a cartridge of collagen/ADP or collagen/adrenaline. This test is not definitive for a platelet disorder, however, and in particular is influenced by the level of VWF which varies 2–3 fold in the normal population, and is reduced in patients with type 1 VWD, the most common form of bleeding disorder. As such, the value of the PFA-100 as an initial screen remains unproven (Hayward et al., 2006a).

The most widely used test for platelet function is that of Born aggregometry which monitors light transmission through a suspension of platelets as aggregation proceeds. Among the advantages of this test its relative simplicity, the monitoring of responses to individual agonists and (in our experience) its reproducibility. Further, it can be combined with real-time monitoring of ATP secretion in a lumiaggregometer. The drawbacks of aggregation include the time taken to perform the assays and the fact that many investigators consider it to be unreliable and operator-dependent. The interpretation of aggregation traces is also complex due to the feedback effects of ADP and TxA_2, although this applies to nearly all tests of platelet function. These limitations

in current testing procedures can be minimised by standardisation of the light transmission aggregation and secretion assays as demonstrated by our work. Further, in a recent study of 229 patients, light transmission aggregometry was standardized and validated to detect patients with platelet dysfunction (Hayward et al., 2009). The likelihood of diagnosing a bleeding disorder was significantly increased when maximal aggregation is reduced with two or more agonists (Hayward et al., 2009).

Our approach of first defining the platelet phenotype through detailed laboratory analysis of the dose response relationships for nine platelet agonists provides important information on the defective pathway(s), and in some cases the defective protein, thereby allowing targetted genetic analysis. It is not practical for the clinical laboratories to follow this procedure because of the time taken for the analysis and also the relatively low number of patients that are referred to each Centre. Nevertheless, a more limited analysis of platelet aggregation and secretion using one or two concentrations of the 'standard' platelets agonists (ADP, adrenaline, AA, collagen and ristocetin) is extremely valuable in aiding diagnosis as illustrated in the recent study by Hayward and colleagues (Hayward et al., 2009). Once a defect in platelet function is observed, referral to a specialized platelet laboratory is recommended to further aid diagnosis and thereby targetted genetic analysis. The further testing can include an increased range of platelets agonists and more specialist tests, such as a measurement of second messengers (cAMP formation and Ca^{2+} elevation), shear-based assay of platelet aggregation and flow cytometric measurements such as α-granule secretion.

The sequencing of the genome and use of mass spectrometry in protein identification has lead recently to attempts to map the platelet transcriptosome and proteome,

respectively. Neither of these goals however can be readily achieved because of the low level mRNA that is present in platelets, making contamination from other cells a major concern, and the very wide range of levels of protein expression found in platelets combined and the difficulty in identifying proteins that are expressed at low level (Watson et al., 2005). Further, neither of these 'cataloguing' approaches provides information on the functional roles of the platelet-expressed proteins. An alternative approach to study gene and protein function that has been used by European Union funded Bloodomics consortium, headed by Dr Willem Ouwehand in Cambridge, is to perform genome-wide sequencing studies on patients with vascular disease. These studies have concentrated on arterial thrombotic disorders because of their frequency rather than on platelet bleeding disorders which are both relatively rare and difficult to diagnose. Further, by their very nature, these studies have not focused solely on platelet-expressed genes. To date, a significant association has only been mapped to a single intronic sequence on chromosome 9 but the functional significance of this region of the chromosome is unknown (Consortium, 2007). This surprisingly low success rate is believed to reflect the multifactorial nature of cardiovascular disease which is influenced by many factors governing life-style and diet.

The use of genetic sequencing studies for identification of inherited mutations that give rise to mild platelet bleeding is in its infancy. Whole genome sequencing is extremely expensive even though costs are rapidly coming down. More importantly, the interpretation of whole genomes sequences requires sophisticated bioinformatics and patient controls (of the same ethnic population) to account for the large number of polymorphisms. It is only in the case of sequence deletions or frame-shift mutations in platelet proteins of known function that a reasonable prediction of cause and effect be

made from whole genome sequencing. Thus, at the present time, targeted sequencing as directed by platelet function studies offers the best way to identify mutations that give rise to platelet dysfunction. This approach can either focus on a single candidate gene, as exemplified by my studies that lead to the identification of the homozygous base-pair shift mutation in the $P2Y_{12}$ patient and the heterozygous mutation in the thromboxane receptor, or in the future, by focusing of groups of genes using second generation sequencers. The latter approach will be essential for investigation of patients with suspected defects in signaling pathways due to the high number of candidate genes/proteins involved.

7.2 Are platelet-based bleeding disorders under-recognised and multifactorial?

Patients with a history of mild bleeding that is consistent with a platelet defect are considered to be extremely rare. It is my belief that this low frequency is in part due to limitations in diagnosis and the available methods of testing. Further, a mild platelet-based bleeding disorder may often go undiagnosed for years, even to early adulthood, as the patient may not have received a sufficient number of haemostatic challenges. Indeed, many individuals with a mild platelet-based bleeding disorder will have a relatively normal life and may not even be aware of their increased bleeding tendency. A history of heavy bruising and frequent nose bleeds from childhood, on their own, are not sufficient for diagnosis as many unaffected individuals experience similar symptoms. Indeed, suspicion of a mild platelet disorder may not occur until a major trauma has occurred, such as invasive surgery (e.g. tooth removal) or childbirth, and the need for a blood transfusion. Diagnosis is further supported by the nature of the bleeding, with bleeding from mucous membranes being particularly common in

platelet-based defects and by negative results for coagulation defects and VWD. A definitive diagnose is made through identification of a clear platelet functional defect in a functional study usually Born aggregometry. However, in many cases, diagnosis of a platelet disorder is based entirely on a clinical judgment and laboratory exclusion of other disorders.

The complex signaling and feedback events that underlie platelet activation, and their apparent redundancy with each other, is one reason as to why investigation of aggregation may be insufficient to diagnose a platelet disorder. However, an additional explanation is that many platelet-based bleeding disorders are likely to be multifactorial and therefore caused by two or more inherited / acquired platelet defects. This is consistent with the fact that two of the genetic defects that have been found in patients studied during the course of this work, namely the K174E mutation in the $P2Y_{12}$ receptor, and the thromboxane receptor defect, are heterozygous. Further, four of the ten reported mutations in the $P2Y_{12}$ receptor are heterozygous even though heterozygous family members exist alongside without an apparent history of bleeding. Moreover, the $P2Y_{12}$-receptor antagonist clopidogrel is routinely prescribed to patients considered at risk of thrombosis with only a very low number experiencing an increased risk of bleeding. A similar story also exists for the cyclooxygenase / thromboxane pathway. The mutation in the thromboxane receptor that I have reported is only the second to be identified and yet there are literally tens of millions of people worldwide who have taken non-steroidal anti-inflammatory agents such as aspirin without an apparent defect in bleeding and moreover the father of the patient, who has the same mutation, does not gave a history of bleeding. Thus, it seems very likely that

the patients with the heterozygous defects in the $P2Y_{12}$ and thromboxane receptors will have one or more further defects that underscore the increase in bleeding. Indeed, in the case of the first $P2Y_{12}$ patient, we believe this to be the case in that, for reasons unknown, only the nonfunctional allele of the $P2Y_{12}$ receptor is expressed on the platelet surface (Hollopeter et al., 2001). Furthermore, the identification of the K174E mutation in a patient diagnosed with type 1 VWD is also consistent with the concept of the multifactorial nature of platelet-based bleeding disorders. Indeed, using the same approach that led to the identification of the K174E mutation through analysis of the EU funded MCMDM-1VWD cohort, we have identified two other heterozygous mutations in patients with type 1 VWDs, namely a P341A mutation in the $P2Y_{12}$ ADP receptor and a L355F in the thrombin receptor. The former mutation has been shown to interfere with receptor trafficking (Daly ME, 2009) while, as yet, we do not know the functional consequence of the thrombin receptor mutation. The multifactorial model for platelet bleeding has important implications for patients who experience life-threatening bleeding episodes while taking antiplatelet prophylactic medication for arterial thrombosis.

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APPENDICES

Appendix 1:

CRP Cross-Linking

Monomeric CRP (purchased from Dr. Richard Farndale (Cambridge University, UK) was dissolved in deionized water (11.1 mg/ml final concentration), and dialyzed using two dialyzing cells in 4 L deionized water at 4°C over night. In the following day, the CRP solutions was transferred from the dialyzing cells into 1.5 ml tubes and mixed with a prewarmed 1 M $NaHCO_3$. Then 50 mM of the cross-linking reagent SPDP (succinimidyl 3-(-2-pyridyldithio)-propionate) that has been dissolved in a dry ethanol and prewarmed to 60 $^{\circ}$C was added, vortexed hard and blew off air with nitrogen gas then incubated at room temprature for almost exactly 1 hr. Finally, the CRP solution was dialysed twice, for 2 hr each, in 2 L of 0.01 M acetic acid per sample and stored at 4°C. The activity of the XL-CRP were tested the next day in comparison with an old CRP batch as a control. A stock of 1.4 mg/ml of CRP was obtained.

Appendix 2:

Sequencing of *P2RY12* gene:

$P2Y_{12}$ is encoded by sequence located entirely within exon 3 of *P2RY12*. The polymerase chain reaction (PCR) was used to amplify exon 3 (1480 bp), which includes 229 bp of 3' untranslated region, and 137 bp of flanking intron 2 sequence, as three overlapping fragments (A, B and C) from the DNA of the index case. To

facilitate sequencing of amplified fragments with universal M13 primers, oligonucleotide primers were designed to incorporate universal M13-tails at the 5' end. PCRs contained 30 ng of genomic DNA in a final volume of 15 µl of 1 x ReddyMix™ Master Mix (ABgene Ltd, Surrey, UK) containing 3 pmoles of each primer and either 1.5 mM (fragments A and B) or 3.0 mM (fragment C) $MgCl_2$. Samples were denatured at 94°C for 7 minutes and then subjected to 35 cycles of denaturation at 94°C for 1 minute, annealing for 1 minute at different temperatures depending on the fragment being amplified and extension at 72°C for 1 minute, followed by a final extension step at 72°C for 7 minutes. The amplified products were purified using ExoSAP-IT for PCR Product Clean Up (GE Healthcare, Amersham, UK) and sequenced on an automated ABI 3730 DNA capillary sequencer (ABI, Warrington, UK). DNA sequences were visualised using FinchTV software.

Appendix 3:

Binding of [^{3}H]2MeS-ADP to platelets from a heterozygous carriers of the K174E mutation:

The defect identified causes substitution of a positively charged lysine residue by a negatively charged glutamic acid residue at position 174 in the second extracellular loop of $P2Y_{12}$, a region predicted to be required for ADP binding. The substitution of lysine 174 was therefore likely to cause inhibition of ADP binding (Costanzi et al., 2004). Consistent with this, there was an up to 50% reduction in binding of [^{3}H]2MeS-ADP to the $P2Y_{12}$ receptor (measured in the presence of the $P2Y_{12}$ receptor antagonist, AR-C69931MX) on platelets from carriers of the K174E mutation, whereas binding to

$P2Y_1$ (measured in the presence of the $P2Y_1$ receptor antagonist, A3P5P) was similar to that observed on control platelets. This 50% reduction in binding of ligand to $P2Y_{12}$ on platelets from a heterozygous carrier of the K174E defect is consistent with a complete abolition of binding to the mutated receptor.

Appendix 4:

Heterologous expression of wild-type and variant $P2Y_{12}$ receptors:

The partial $P2Y_{12}$ defect associated with the K174E substitution was further investigated after introduction of the mutation into a HA-tagged $P2Y_{12}$ expression construct, and stable expression of the wild-type and mutated $P2Y_{12}$ receptor in CHO cells. The ability of the wild-type and K174E $P2Y_{12}$ receptors to negatively couple to adenylyl cyclase was assessed by measurement of inhibition of forskolin-stimulated adenylyl cyclase activity. Forskolin-stimulated adenylyl cyclase activity was inhibited at all concentrations of ADP tested in cells stably expressing the wild-type receptor, with a maximal level of inhibition of 75%. In contrast, cells expressing the K174E variant showed defective ADP-dependent inhibition of forskolin-stimulated adenylyl cyclase activity with less than 20% inhibition observed at 10 μM ADP, a finding supporting a role for K174 in determining the ligand binding capacity of $P2Y_{12}$. Direct confirmation of the disruption in ligand binding to the K174E $P2Y_{12}$ receptor stably expressed in CHO cells was demonstrated using [^{3}H]2MeS-ADP. Binding to cells expressing the wild-type receptor was saturable and maximal at a ligand concentration of 1μM and the Kd was 0.31 ± 0.05 μM. In contrast, the K174E variant displayed approximately 30% of the ligand binding observed with the wild-type receptor using

concentrations of ligand up to 10μM indicating a defect in binding of ligand to the K174E variant, and explaining the absence of agonist-induced signalling in cells expressing the K174E variant.

Appendix 5:

RNA extraction and Reverse transcription of WBC RNA

The buffy coats were extracted from centrifuged blood samples during platelet preparation for subsequent use in sequencing studies. 15ml of 1x RBC lysis buffer (1.5M NH_4Cl, 10mM $KHCO_3$, 1mM EDTA) was added and the sample rotated at 0.12g for 15minutes on Stuart rotator (model SB3, Jencons PLS, UK.) The sample was then centrifuged at 380g for 5 minutes and the resulting red, transparent supernatant aspirated. Lysis steps were then repeated until a clean, white pellet was obtained with no obvious red blood cells present. The RNA was purified via chloroform extraction (200μl chloroform per 1ml Trizol) followed by isopropanol precipitation with incubation at -20^{o}C for at least 1 hr. Following centrifugation at 18000g for 15 minutes at 4^{o}C, the pellet was washed in ice-cold 75% v/v ethanol and dried at 50^{o}C to allow all residual alcohol to evaporate. Finally, the pellet was resuspended in 20μl DEPC-treated nanopure water (dH_2O- 0.1% DEPC), mixed and left to stand at room temperature (RT) for 10 minutes. Quantification of the RNA product was conducted using a Nanodrop spectrophotometer (model ND-1000, Labtech International, East Sussex, UK) to ensure sufficient yield had been obtained for use in subsequent assays.

Synthesis of complementary DNA (cDNA) from white blood cell messenger RNA (mRNA) was performed using the First strand cDNA synthesis kit (Roche, UK)

according to the manufacturer's protocol. The reaction mix comprised 2μg RNA, 3.2μg oligo (dT) primer, 40 units AMV reverse transcriptase, 100 units RNAse inhibitor, 1mM dNTPs, 5mM MgCl, 1X reaction buffer (final concentration 10mM Tris HCl, 50mM KCl, pH 8.3.) A negative control experiment was conducted in parallel with the RNA sample, where RNA was replaced by DEPC-treated nanopure water. The PCR reaction was conducted using peQlab PCR machine (advanced primus 25 model, Geneflow, UK) under the following conditions: 22^{o}C for 10 minutes, 42^{o}C for 60 minutes, 90^{o}C for 10 minutes and held at 4^{o}C on completion. The resulting cDNA product was stored at –20^{o}C.

Appendix 6:

Sequencing of *TXA_2R* gene

Genomic DNA was purified from venous blood using the Nucleon DNA isolation kit (Tepnel Life Sciences, Manchester, UK). The entire coding sequence and splice donor and acceptor sites of *TBXA2R* (including α and β isoforms) were then amplified by PCR. Sequenced with a model 3700 DNA analyser using an ABI PRISM Big Dye V2 reaction kit (Applied Biosystems, Foster City, CA, USA) and sequence variations were identified by comparison with the *TBXA2R* cDNA reference sequences NM_001060 and NM_201636. The presence of the *TBXA2R* c. 910G>C transversion was confirmed by restriction analysis using *Ava*II.

Appendix 7:

Binding of [^{3}H]-SQ29548 to platelets from a heterozygous carriers of the D304N mutation:

Platelets were isolated from PRP by centrifugation for 10 minutes at 1000g in the presence of 0.02 U/ml apyrase and prostaglandin E_1 (140 nM). The pellet was resuspended in a modified Tyrode's-HEPES buffer (145 mM NaCl, 2.9 mM KCl, 10mM HEPES, 1 mM Mg Cl_2 and 5mM glucose, pH 7.3) and fixed with 4% formaldehyde. Platelets were then isolated by centrifugation and resuspended in binding buffer (20mM HEPES and 1mM $MgCl_2$). Aliquots of platelet suspension were incubated with [^{3}H]-SQ29548 (3 Ci/mmol, 0.01 to 0.1 μM) and specific binding was determined either in the presence or absence of unlabelled ligand (10 μM). After incubation for 20 minutes at room temperature, reactions were terminated by the addition of ice-cold binding buffer and rapid filtration through Whatman GF/C glass fiber filters under vacuum. Radioactivity bound to the filters was measured by scintillation counting .

Appendix 8:

TXA_2R expression constructs, cell culture and transfections

A pcDNA3.1 hygromycin expression vector containing the wild-type $TXA_2R\alpha$ cDNA fused at the N-terminus with the FLAG epitope tag was generated by standard cloning techniques. The variant p.D304N expression construct was then generated from this template by site-directed mutagenesis using a QuikChange® Site-directed Mutagenesis

Kit (Stratagene, Cambridge, UK) in accordance with the manufacturer's instructions. The wild type and D304N expression constructs were then stably transfected into CHO-K1 cells.