UNIVERSITY OF
BIRMINGHAM

PHENOTYPING AND GENOTYPING OF PLATELET DEFECTS IN PATIENT POPULATIONS ENRICHED IN BLEEDING

by

GILLIAN CLARE LOWE

A thesis submitted to the University of Birmingham

for the degree of DOCTOR OF PHILOSOPHY

Cardiovascular Sciences, Institute of Biomedical Research

School of Clinical and Experimental Medicine

University of Birmingham

September 2015

ABSTRACT

Inherited platelet disorders vary in their associated bleeding risk. Individuals in families with known platelet gene mutations often have variable bleeding, suggesting that bleeding risk is multifactorial.

Inherited platelet disorders are difficult to diagnose due to the absence of a gold standard laboratory technique and their variable bleeding phenotype, which often only manifests after haemostatic challenges.

The work in this thesis furthers the previous studies in the genotyping and phenotyping of platelets project by significantly increasing the number of participants, allowing further characterisation of inherited platelet disorders in those with a normal platelet count. A bleeding assessment tool score was also recorded in all newly recruited adults.

Two specific groups of patients are also studied:

- Those with unexplained menorrhagia, in whom the hypothesis was that some have an undiagnosed platelet defect.
- Those with inherited thrombocytopenia, in whom I sought to develop an assay to assess platelet function, as bleeding risk can not be predicted by platelet count alone, suggesting that qualitative defects may contribute.

The genetic basis of platelet defects was investigated in many of the patients, leading to identification of mutations in genes known to be critical in platelet biology, and identification of several possible novel variants.

PUBLICATIONS ARISING FROM THIS WORK

i) Published Manuscripts

^ Selected marked publications are included in full text in Appendix 4.

a. Ben Johnson, **Gillian Lowe**, Jane Futterer, Marie Lordkipanidzé, David MacDonald, Michael Simpson, Isabel Sanchez-Guiu, Sian Drake, Danai Bem, Vincenzo Carlo Leo, Sarah Fletcher, Ban B Dawood, Jose Rivera, David Allsup, Tina Biss, Paula Bolton-Maggs, Peter W Collins, Nicola Curry, Charlotte Grimley, Beki James, Michael Makris, Jayashree Motwani, Sue Pavord, Katherine L Talks, Jecko Thachil, Jonathan T Wilde, Michael Williams, Paul Harrison, Paul Gissen, Stuart Mundell, Andrew D Mumford, Martina E. Daly, Stephen P. Watson, and Neil V Morgan on behalf of the UK-GAPP Study Group Whole exome sequencing identifies genetic variants in inherited thrombocytopenia with secondary qualitative function defects. Submitted to *Blood* 10th August 2015. [Previously presented as oral communication at International Society on Thrombosis and Haemostasis 2015 Congress (Toronto, Canada) – *Journal of Thrombosis and Haemostasis* 2015 **13** (Suppl 2). Abstr AS054. Selected in Congress "Highlights" (In top ten abstracts of over 2700 submissions)].

b. Sarah J. Fletcher, Ben Johnson, **Gillian C. Lowe**, Danai Bem, Sian Drake, Marie Lordkipanidzé, Isabel Sánchez Guiú, Ban Dawood, Michael A. Simpson, Martina E. Daly, Jayashree Motwani, Peter W. Collins, Steve P. Watson and Neil V. Morgan on behalf of the UK GAPP Study Group Consecutive *SLFN14* mutations in 3 unrelated families with an inherited bleeding disorder, thrombocytopenia and secretion defects. *Journal of Clinical Investigation* 2015 doi: 10.1172/JCI80347. [Epub ahead of print, Aug 17th]

c. Jones ML, Norman JE, Morgan NV, Mundell SJ, Lordkipanidzé M, **Lowe GC**, Daly ME, Simpson MA, Drake S, Watson SP and Mumford AD on behalf of the UK GAPP study group Diversity and impact of rare variants in genes encoding the platelet G protein-coupled receptors. *Thromb Haemost*. 2015 **113(4)**:826-37

d. Leo V, Morgan N, Bem D, Jones M, **Lowe G**, Lordkipanidzé M, Drake S, Simpson M, Gissen P, Mumford A, Watson S and Daly M on behalf of the UK GAPP Study Group Use of next generation sequencing and candidate gene analysis to identify underlying defects in patients with inherited platelet function disorders. *J Thromb Haemost*. 2014 **13(4):**643-50

e. Natalia Dovlatova, Marie Lordkipanidzé, **Gillian C. Lowe**, Ban Dawood, Jane May, Stan Heptinstall, Steve P. Watson and Susan C. Fox on behalf of the UK GAPP Study Group Evaluation of a whole blood remote platelet function test for the diagnosis of mild bleeding disorders. *Journal of Thrombosis and Haemostasis* 2014 **12(5)**:660-5

f. Yatin M. Patel, Marie Lordkipanidzé, **Gillian C. Lowe**, Shaista P. Nisar, Kathryn Garner, Jacqueline Stockley, Martina E. Daly, Mike Mitchell, Steve P Watson, Steve K. Austin and Stuart J Mundell A novel mutation in the P2Y12 receptor and a function-reducing polymorphism in PAR-1 in a patient with chronic bleeding. *Journal of Thrombosis and Haemostasis* 2014 **12(5)**:716-25

g. Martina E. Daly, Vincenzo C. Leo, **Gillian C. Lowe**, Steve P. Watson and Neil V. Morgan What is the role of genetic testing in the investigation of patients with suspected platelet function disorders? *British Journal of Haematology* 2014 **165(2)**:193-203

h. Lordkipanidzé M, **Lowe GC**, Kirkby NS, Chan MV, Lundberg MH, Morgan NV, Bem D, Nisar SP, Leo VC, Jones ML, Mundell SJ, Daly ME, Mumford AD, Warner TD and Watson SP Characterization of multiple platelet activation pathways in patients with bleeding as a high-throughput screening option: use of 96-well Optimul assay. *Blood* 2014 **123(8)**:e11-22

i. ^ Shaista P Nisar, Marie Lordkipanidze, Matthew L Jones, Ban B Dawood, Sherina Murden, Margaret R Cunningham, Andrew D Mumford, Jonathan T Wilde, Steve P Watson, Stuart J Mundell and **Gillian C Lowe** on behalf of the UK GAPP study group A novel thromboxane A2 receptor N42S variant results in reduced surface expression and platelet dysfunction. *Thrombosis and Haemostasis* 2014 **111(5)**:923-32

j. Marie Lordkipanidzé, **Gillian C Lowe** and Paul Harrison "Tests of platelet function". Submitted 24th September 2013 for publication in *Practical Haemostasis and Thrombosis 3rd edition*, Wiley-Blackwell, edited by Nigel Key, Michael Makris and David Lillicrap (Book chapter)

k. Stockley J*, Morgan NV*, Bem D, **Lowe GC**, Lordkipanidzé M, Dawood B, Simpson MA, Macfarlane K, Horner K, Leo VC, Talks K, Motwani J, Wilde JT, Collins PW, Makris M, Watson SP and Daly ME Enrichment of *FLI1* and *RUNX1* mutations in families with excessive bleeding and platelet dense granule secretion defects. *Blood* 2013 **122(25)**:4090-3 * *joint first authors*

l. **Gillian C. Lowe**, Marie Lordkipanidzé and Steve P. Watson on behalf of the UK GAPP study group Utility of the ISTH Bleeding Assessment Tool in predicting platelet defects in participants with suspected inherited platelet function disorders. *J Thromb Haemost* 2013 **11(9)**:1663-8

m. Lordkipanidzé M*, **Lowe GC***, Watson SP, on behalf of the UK GAPP study group. Simultaneous measurement of ATP release and LTA does not potentiate platelet aggregation to epinephrine. *Thromb Haemost* 2013 ; **110**(1):199-201 (* These authors contributed equally to this work and share first authorship)

n. Steve P. Watson, **Gillian C. Lowe**, Marie Lordkipanidzé and Neil V. Morgan Genotyping and phenotyping of platelet function disorders. *J Thromb Haemost* 2013 **11**, (Suppl s1) 351-63

o. ^ **Lowe GC**, Sánchez Guiu I, Chapman O, Rivera J, Lordkipanidzé M, Dovlatova N, Wilde J, Watson SP, Morgan NV on behalf of the UK GAPP collaborative. Microsatellite markers as a rapid approach for autozygosity mapping in Hermansky-Pudlak syndrome: Identification of the second HPS7 mutation in a patient presenting late in life. *Thromb Haemost* 2013; **109**(4):766-8

p. ^ Ban B Dawood, **Gillian C Lowe**, Marie Lordkipanidzé, Danai Bem, Martina E Daly, Mike Makris, Andrew Mumford, Jonathan T. Wilde and Steve P Watson Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. *Blood* 2012; **120** (25) 5041-9

ii) Published abstracts

a. Natalia Dovlatova, Marie Lordkipanidzé, **Gillian C. Lowe**, Ban Dawood, Jane May, Stan Heptinstall, Steve P. Watson and Sue Fox A reliable and simple diagnostic approach to detect platelet secretion defects in subjects with mucocutaneous bleeding. Accepted for presentation at International Society on Thrombosis and Haemostasis 2015 Congress (Toronto, Canada). *Journal of Thrombosis and Haemostasis* 2015 **13** (Suppl s2), abstr PO492-WED.

b. Neil Morgan**, Gillian Lowe**, Jayashree Motwani, Mike Williams, Michael Simpson, Gail Kirby, Eamonn Maher and Steve Watson A mutation in ANKRD18A is associated with a severe congenital thrombocytopenia. *Journal of Thrombosis and Haemostasis* 2013 **11** (Suppl s2), abstr AS 28.3

c. **Lowe GC**, Lordkipanidzé M, Dawood BB, Clark J, Lester W and Watson SP on behalf of the UK GAPP study Investigation of underlying platelet function defects in patients with unexplained menorrhagia. *Thrombosis Research* 2013 **131** (Suppl 1) S74 (abstract reference OC-13)

d. **Lowe GC**, Talks KL and Watson SP Expanding the GAPP project – feasibility of remote platelet phenotyping. *British Journal of Haematology*, 2012; **157** (Suppl. 1), 1–88, abstr 84

e. **Lowe GC**, Dawood BB, Lester WA, Wilde JT and Watson SP The relationship of light transmission aggregometry and flow cytometry to platelet count in platelet function testing. *Journal of Thrombosis and Haemostasis* 2011 **9** (Suppl s2), abstr P-WE-045

DEDICATION

This thesis is dedicated to the loving memory of my Dad, Doug Moore. He is sorely missed after his death aged 62 in November 2013 following a sudden, short and aggressive illness. He encouraged my interest in science and learning from a young age. He would have loved to have seen this thesis in its completed form, and I would have been very excited to share it with him.

ACKNOWLEDGEMENTS

Thank you to Professor Steve Watson for giving me the opportunity to work in the Birmingham Platelet Group and for encouraging me at all stages of my research fellowship from initial discussions about projects through to thesis writing. I have thoroughly enjoyed our discussions and all aspects of our work together, and am excited about collaborating on future projects.

I am grateful to the Wellcome Trust who funded my research fellowship under their Combined Training Programme scheme. Their idea of pairing clinicians and scientists in order to exchange ideas and optimise clinically orientated research was a fantastic one.

Special thanks also go to Dr Will Lester for research supervision and for encouragement within my chosen field of haemostasis and thrombosis, and also to Dr Jonathan Wilde for support and development over the last ten years.

GAPP is a large collaborative project and I am indebted to everyone else who works as part of the team and who made my research fellowship such an interesting and enjoyable experience. Special thanks to Neil Morgan and Marie Lordkipanidzé with whom I worked very closely and who have become great friends as well as colleagues, and to the GAPP Principal Investigators Martina Daly, Stuart Mundell, Paul Gissen and Andrew Mumford. I am grateful to Ban Dawood for the previous work she did in GAPP, and for her help and support, especially at the beginning of my studies.

I was fortunate to interact with many members of the UK and International Haemostasis and Thrombosis and Platelet Communities as part of my project, with whom I am looking forward to working over the coming years. I am particularly grateful to all the Haemophilia Centre Consultants, along with their multidisciplinary teams, who were interested in the project and referred patients into it. Thanks also go to Professor Justin Clark who was very helpful in supporting my studies of patients with menorrhagia and in identifying patients to recruit.

I am grateful to the National Institute of Health Research Non Malignant Haematology specialty group (chaired by Professor Peter Collins) and the Birmingham and the Black Country Comprehensive Local Research Network, who were instrumental in increasing the number of centres in the study, and who supported my interest in undertaking further clinical research in this area.

The project would not have been possible without the patients (and their families) who were willing to participate in the study, sometimes at considerable personal inconvenience. I hope that my findings will help them.

Thanks also to Gayle and Hannah who were great office mates, provided much needed administrative support and most importantly are fantastic friends. Neena and Alice also made rare coffee breaks fun, and I am very pleased to have got to know them.

I enjoyed meeting and working with all the other members of the Watson Lab who opened my eyes to the world of research, and who were enthusiastic about having a resident clinician in their midst, and patient when explaining things to me.

Thanks to my Mum, Maureen and brother, Andrew for their support and love over the years, and for always putting up with me always doing “just one more thing for work”.

Thanks to my husband Rob who has patiently and quietly loved, encouraged and supported me in everything (including all my work endeavours) over the last fifteen years, and at times has suffered from significant disruption and inconvenience due to my job. I feel very lucky to be able to have been able to develop a career that I love with his backing. I really enjoy discussing my research findings and statistical techniques with him in the rare uninterrupted moments that our one year old daughter Amy permits us to have.

Amy’s arrival timed with the end of my research fellowship, and she has brought and continues to bring us immeasurable joy. I am looking forward to more time to play together now that this work is completed.

LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ABI	Association of British Insurers
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
ARC	Arthrogryposis, renal tubular acidosis, and cholestasis
ATP	Adenosine triphosphate
BAT	Bleeding Assessment Tool
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
CAMT	Congenital amegakaryocytic thrombocytopenia
CD	Cluster of differentiation
CLEC-2	C-type lectin-like receptor-2
CLRN	Comprehensive Local Research Network
COX	Cyclooxygenase
CRP	Collagen-related peptide
CT	Closure time (PFA testing)
CT	Computed tomography (Radiologocal imaging)
dbSNP	Single Nucleotide Polymorphism Database
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FcR	Fc receptor
FEV1	Forced expiratory volume in 1 second
FITC	Fluorescein isothiocyanate
FPD / AML	Familial platelet disorder with propensity to acute myelogenous leukemia
FSC	Forward scatter
FVC	Forced vital capacity
FVIII:C	Factor VIII:C (activated)
GAPP	Genotyping and Phenotyping of Platelets
GDP	Guanosine diphosphate
GP	Glycoprotein
GPCR	G protein-coupled receptors
GPRP	Gly-Pro-Arg-Pro
GTP	Guanosine-5’-triphosphate
HPS	Hermansky Pudlak syndrome

Ig	Immunoglobulin
IQR	Interquartile range
IRAS	Integrated Research Application System
ISRCTN	International Standard Randomised Controlled Trial Number
ISTH	International Society on Thrombosis and Haemostasis
ITAM	Immunoreceptor tyrosine based activation motif
ITP	Immune thrombocytopenia purpura
JAK2	Janus kinase 2
KCO	Transfer coefficient
LTA	Light transmission aggregometry
MPV	Mean platelet volume
mRNA	Messenger ribonucleic acid
NGS	Next generation sequencing
NHLBI	National Heart, Lung, and Blood Institute
NHS	National Health Service
NIH	National Institutes of Health
PAF	Platelet activating factor
PAR1	Protease-activated receptor 1
PAR4	Protease-activated receptor 4
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PFA	Platelet function assay
PFD	Platelet function disorder
PPP	Platelet poor plasma
PRP	Platelet rich plasma
Ricof	Ristocetin co-factor assay
ROC	Receiver-operator curve
SNP	Single nucleotide polymorphism
SSC	Side scatter
TAR	Thrombocytopenia with absent radii
TEG	Thromboelastogram
THC2	Thrombocytopenia-2
TLC	Total lung capacity
TPO	Thrombopoietin
TxA_2	Thromboxane A_2
UK	United Kingdom
VWF	Von Willebrand Factor

CONTENTS

LIST OF FIGURES

LIST OF TABLES

List of Tables

CHAPTER ONE: GENERAL INTRODUCTION

1.1 Basics of platelet function, signalling and role of platelets in haemostasis and thrombosis

Human platelets are small, anucleated cells that circulate in blood and play a critical role in haemostasis and thrombosis. Their lifespan is approximately 10 days (1), and during this time they constantly survey the integrity of the vessel wall. Normal human platelets are small and discoid in shape (0.5 × 3.0 μm), have a mean volume of 7–11 fL, and circulate in relatively high numbers (between 150-400 × 10^9/L) (2-4). Their small disc shape enables the platelets to be marginated towards the edge of vessels so that the majority circulate adjacent to the vascular endothelial cells that line all blood vessels (5). Upon detection of vessel wall damage, they undergo rapid and controlled adhesion, activation, and aggregation to form a hemostatic plug and thus rapidly prevent blood loss (2, 3). They also provide a phospholipid surface for initiation of the coagulation cascade (6, 7). Endothelial cells produce a number of potent anti-platelet substances (e.g. nitric oxide, prostacyclin, and CD39) that normally inhibit vessel wall-platelet interactions (8-10). Vessel wall damage exposes highly adhesive substrates [e.g. P selectin, Von Willebrand factor (VWF), collagen, and many other extracellular matrix components], which overcome these inhibitory factors and result in a sequence of stepwise events resulting in the formation of a hemostatic plug (11, 12):

- Initial adhesion, transient rolling of platelets along the vessel wall, and slowing of the cells. Consequently, platelets are more likely to undergo stable adhesion.
- Platelet activation (if there is more extensive damage or stimuli-promoting platelet activation).

- Stable adhesion through additional receptor-ligand interactions and integrin activation.
- Platelet aggregation.
- Generation of platelet procoagulant activity and stabilization of the hemostatic plug via interaction with coagulation factors.
- Clot retraction

The process of platelet adhesion, activation and aggregation is depicted in **Figure 1.1.**.

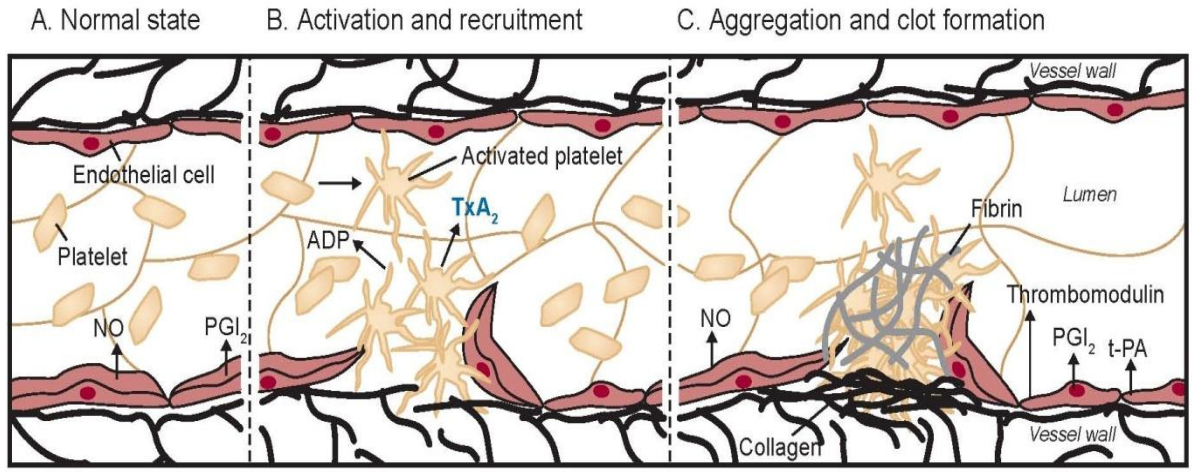


Figure 1.1 Platelet adhesion, activation, and aggregation. (A) Normal endothelium releases anti-aggregant molecules promoting haemostasis and nonthrombogenic state. (B) Injured endothelium exposes platelets to thrombogenic subendothelium. Activated platelets release pro-aggregant molecules. (C) Clot formation at site of injury. Endothelium releases factors that stabilize the clot and limit the haemostatic process to the site of injury. ADP, adenosine diphosphate; NO, nitric oxide; PGI_2, prostacyclin; t-PA, tissue plasminogen activator; TxA2, thromboxane A2. Figure reproduced with permission from (13).

The platelets interact with and sense the environment through many types of surface receptors (12). The net balance between activating or inhibitory stimuli thus controls whether platelets continue to circulate, begin to reversibly interact with the vessel wall, or become irreversibly adherent to either the vessel wall or to each other (2, 3).

During adhesion, platelets become activated through signal transduction pathways, which mediate shape change, degranulation and spreading upon areas of exposed subendothelium (11). Activated platelets recruit additional platelets into the growing platelet aggregate or thrombus via a number of positive feedback pathways, including release of dense granular adenosine diphosphate (ADP) and generation of thromboxane A_2 (11). Activated platelets also express negatively charged phospholipids on their surface and release microvesicles, facilitating the local generation of high amounts of thrombin, which not only further activates other platelets, but also stabilizes the platelet plug through fibrin formation via the coagulation cascade (6, 7). In this manner, platelets rapidly seal any areas of vessel wall damage and provide a catalytic surface for coagulation to occur, resulting in the formation of a stable hemostatic plug.

1.2 Platelet receptors, agonists and signalling pathways

Platelets interact with and sense the environment through numerous surface receptors. These receptors are activated by corresponding agonists (or ligands). Major platelet receptors and their agonists are summarized in **Table 1.1** overleaf.

AGONIST	RECEPTOR	EFFECT AND PHYSIOLOGICAL ROLE
Adhesion molecules		
Collagen	GP VI	Major signalling receptor for collagen
	α2β1	Supports adhesion by collagen
Fibrinogen	αIIbβ3	Aggregation, spreading and clot retraction
Fibronectin	α5β1, αIIbβ3	Adhesion through α5β1
Laminin	α6β1	Adhesion
von Willebrand factor	GP Ib-IX-V,	Platelet tethering (also fibrinogen)
	αIIbβ3	
Amines		
Adrenaline	α2	Positive feedback agonist
5-HT	5-HT2A	Mediates vasoconstriction, positive feedback
		agonist
Cytokines		
TPO	c-Mpl	Maturation of megakaryocytes, control of platelet
		number in circulation
Immune complexes		
Fc portion of antibodies	FcγRIIA	Immune- and bacteria-induced platelet activation
Lipids		
PAF	PAF	Positive feedback agonist
Prostacyclin (PGI2)	IP	Inhibition of platelet activation
Thromboxane A2	TP	Major positive feedback agonist
Prostaglandin E2 (PGE2)	EP3	Inhibition of platelet activation

AGONIST	RECEPTOR	EFFECT AND PHYSIOLOGICAL ROLE
Nucleotides		
Adenosine	A2a	Inhibition of platelet activation
ADP	$P2Y_1$	Early role in platelet activation
	$P2Y_{12}$	Major positive feedback receptor
ATP	$P2X_1$	Possible early role in platelet activation
Proteases		
Thrombin	PAR1, PAR4	Coagulation-dependent platelet activation
Surface molecules		
CD40 ligand	CD40 and αIIbβ3	Interaction with other blood cells, role in immune response and inflammation
P-selectin glycoprotein ligand 1 (PSGL-1)	P-selectin	Interaction with other blood cells
Tyrosine kinase receptors		
Podoplanin / possible other unknown ligand	CLEC-2	Role in foetal vascular development Possible role in platelet activation in atherosclerosis and cancer progression(?)
EphrinB1	EphA4 and EphB1	Late events in platelet activation(?)
Vitamin K-dependent		
Gas6	Sky, Axl and Mer	Supports platelet activation(?)

Table 1.1 Major platelet agonists and their surface receptors. Platelets express a remarkable number and variety of receptors for a wide range of ligands. For many of these receptor–ligand combinations, however, the effect on platelet activation is weak and of uncertain significance. (?) indicates a putative role /effect.
Originally published in (14). Adapted version reproduced from (4))

Platelet signalling is a complex process involving many biochemical pathways. Specific types of receptor and their signalling pathways are described below (15):

1.2.1 G protein-coupled receptors (GPCRs)

These are composed of seven transmembrane domains (and are therefore referred to as heptahelical). They activate heterotrimeric G proteins which are made up of α, β and γ subunits. There are four classes of α subunit ($G\alpha_s$, $G\alpha_q$, $G\alpha_i$ and $G\alpha_{12/13}$), each of which mediate different signalling effects. When these receptors are activated GDP dissociates from the α subunit and allows GTP to bind. This then allows the α subunit to dissociate from the remainder of the complex, allowing it to bind to effector proteins, such as adenylyl cyclase and phospholipase C β.

1.2.1.1 G_i proteins

G_i proteins are abundantly expressed in platelets, and are so named as they *i*nhibit adenylyl cyclase (in contrast, G_s proteins *s*timulate it). They also have several other effector proteins, such as phosphoinositide 3-kinase. The main G_i coupled receptors in platelets are the $P2Y_{12}$ ADP receptor and the α_2-adrenoceptor. Neither of these receptors can mediate activation of washed platelets on their own, and both require the presence of feedback mediators to fully activate platelets in platelet rich plasma. In synergy with calcium mobilising receptors they can mediate powerful platelet activation, and this effect renders ADP a key feedback agonist in regulating in vivo platelet activation.

The $P2Y_{12}$ receptor is the target for several drugs (eg ticlodipine, clopidogrel, prasugrel, ticagrelor) used in the treatment of cardiovascular disease. The receptor was cloned in 2001

by two separate groups working on human and rat isoforms (16, 17). Clopidogrel had been identified prior to this molecular cloning being reported (18). Mouse knockout models show evidence of increased bleeding (19), and there are now several cases reported in patients where increased clinical bleeding is accompanied by a $P2Y_{12}$ receptor mutation (20-22), as discussed in section 1.3.

Mice deficient in the α_2-adrenoceptor are observed to have an increase in tail bleeding time (23), however it is generally believed that the $P2Y_{12}$ receptor has a more significant role than the α_2-adrenoceptor in positive feedback activating the Gi family of G proteins in platelets (24).

1.2.1.2 G_q proteins

There are two forms of G_q protein, G_q and G_{11}. Platelets express only the G_q form. G_q deficient mice have been shown to have prolonged bleeding times and platelets that are unresponsive to multiple agonists (25). G_q-coupled receptors activate platelets via a pathway that regulates isoforms of phospholipase C β, generating inositol 1,4,5-trisphosphate and 1,2-diacylglycerol as secondary messengers. These compounds then mobilise intracellular calcium and activate protein kinase C. The G_q receptors in human platelets that are important in physiological platelet activation are the $P2Y_1$ ADP receptor, the thromboxane A_2 (TxA_2) receptor, and the two thrombin receptors, PAR1 and PAR4.

The $P2Y_1$ receptor synergises with the $P2Y_{12}$ receptor to mediate full aggregation of platelets exposed to ADP as an agonist (26). It is present in relatively low numbers on platelets (approximately 150 copies per platelet). The $P2Y_{12}$ receptor is also able to synergise with other calcium mobilising receptors. No patients have been reported with a $P2Y_1$ receptor

mutation that results in excessive bleeding. Work in mouse platelets suggests that this receptor may be important in thrombus formation (27), suggesting that pharmacological agents that antagonise this receptor may represent a novel form of antithrombotic agents (28).

The thromboxane A_2 receptor has two isoforms, although only the α isoform is reported to be expressed on the platelet surface (29). The receptor is activated by thromboxane A_2 which is a metabolite of arachidonic acid. Arachidonic acid is liberated by cytosolic phospholipase A_2, which is activated by elevation of intracellular calcium downstream of G_q coupled receptors and tyrosine kinase linked surface receptors. The thromboxane receptor is a powerful platelet activator in synergy with the $P2Y_{12}$ receptor, and as such is a critical feedback receptor in the control of platelet activation. Aspirin targets this pathway, by inhbiting platelet cyclooxygenase-1 and thereby preventing thromboxane formation. This is of very high clinical relevance as aspirin is one of the most commonly taken drugs worldwide, and has a very important role in treating and preventing cardiovascular disease. Half of all adults aged 45-75 in the United States are reported to take low dose aspirin as a preventative measure (30). A number of patients with bleeding tendencies have been reported to have thromboxane receptor mutations (31-34), and studies show prolonged bleeding times in mice who do not express the receptor (35).

The PAR1 and PAR4 thrombin receptors are activated by thrombin, a soluble serine protease. The PAR1 receptor plays the major role in activation (36). Mouse platelets do not express PAR1, but do express PAR4 and in addition PAR3. Thrombin cleaves the N-terminal of the PAR1 and PAR4 receptors to expose a tethered peptide ligand. Receptor activation can be replicated by using short thrombin receptor activating peptides, which activate the receptor without the need for cleavage. These peptides can be used to induce aggregation in platelet

rich plasma, but thrombin can not (in the absence of GPRP to block fibrin polymerisation) as it cleaves fibrinogen to form fibrin and consequently forms a clot rich in platelets. Thrombin is a very powerful agonist and plays a critical role in platelet activation and also in positive feedback in the coagulation cascade which takes place on the platelet's phospholipid surface. Once the PAR1 and PAR4 receptors have been cleaved by thrombin they activate G_q and G_{13} proteins, leading to regulation of phospholipase C β and Rho kinase. There are no reports of patients with mutations in the PAR1 or PAR4 receptors leading to excessive bleeding, but there is evidence that PAR1 and PAR4 polymorphisms alter platelet responses to thrombin (37-40).

Figure 1.2 (overleaf) shows the signalling pathways of GPCR coupled receptors in platelets.

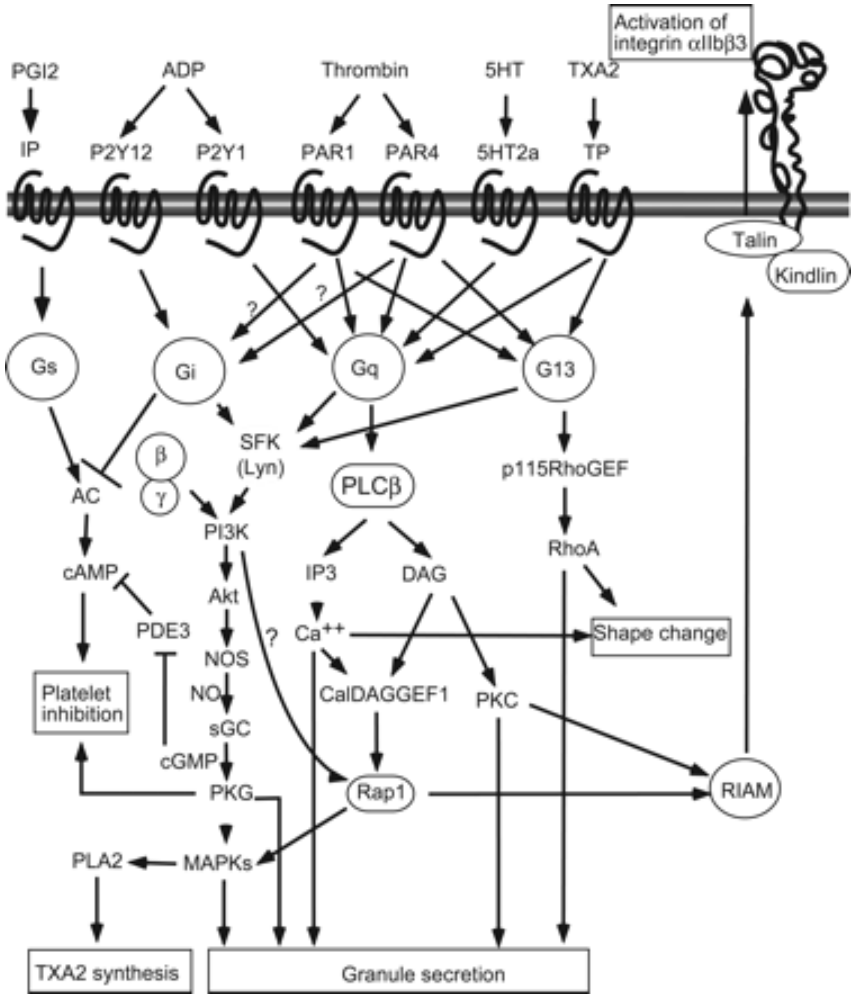


Figure 1.2 GPCR-coupled platelet activation signalling. PDE3, phosphodiesterase 3; p115RhoGEF, p115 Rho guanine nucleotide exchange factor. Figure and legend reproduced with permission from (41)

Additional abbreviations: 5HT=5-hydroxytryptamine, AC=Adenylyl cyclase, ADP= Adenosine diphosphate, Akt= Serine–threonine kinase, also termed protein kinase B [PKB], cAMP= cyclic adenosine monophosphate, cGMP= cyclic guanosine monophosphate, DAG=diacylglycerol, IP= Inositol phosphate, IP3=Inositol Trisphosphate, MAPKs= Mitogen-activated protein kinases, NO=nitric oxide, NOS=nitric oxide synthase, PAR= Protease-activated receptor, PGI2= Prostaglandin I_2, PI3K = Phosphoinositide 3-kinase, PKC=Protein Kinase C, PKG= Protein Kinase G (cGMP-dependent protein kinase), PLA2= Phospholipase A_2, PLCβ= Phospholipase Cβ, Rap1= Ras-related protein 1, RhoA= Ras homolog gene family A, RIAM= Rap1-GTP-interacting adaptor molecule, SFK=Src family kinases, sGC= soluble guanylyl cyclase, TP= Thromboxane receptor, TXA2= Thromboxane A_2.

1.2.2 Glycoprotein receptors

Numerous platelet membrane receptors are glycoproteins. Amongst these are the GPVI-FcR γ chain complex which is the major collagen signalling receptor, GPIb-IX-V which is the tethering receptor for von Willebrand Factor (VWF), and αIIbβ3 which is a critical platelet integrin and a receptor for a number of proteins including fibrinogen and VWF.

GPVI was recognised as the major platelet collagen receptor after it was demonstrated to associate with the FcR γ chain in the platelet membrane (42). An earlier report had shown that a case of autoimmune thrombocytopenia was due to a GPVI autoantibody which diminished platelet aggregatory response to collagen (43). The FcRγ chain contains an immunotyrosine based activation motif (ITAM) that is essential in the signalling process after GPVI clusters following collagen stimulation(44). Src-kinase mediated phosphorylation of this ITAM allows binding of Syk and initiation of a pathway which results in powerful activation of phospholipase C γ2. GPVI is a member of the immunoglobulin (Ig) family of surface receptors and has two extracellular Ig domains (45). GPVI levels on the platelet surface are believed to be tightly regulated (46). The interaction between collagen and the GPVI receptor is strengthened by the integrin α2β1 in a two step process(47). The synthetic collagen, CRP can activate GPVI independently of the association with α2β1(48). Both GPVI and FcRγ chain deficient mice demonstrate an increase in tail bleeding time (49, 50), and mice treated with an anti-GPVI antibody are protected from lethal thrombogenic injections of collagen and adrenaline (51). A small number of mutations in the GPVI receptor in humans associated with a bleeding phenotype have been described (52-54).

The VWF receptor GPIb-IX-V is highly expressed on the platelet surface, and is composed of two units each of GPIbα, GP1bβ and GPIX and one subunit of GPV. It tethers platelets to VWF at intermediate and high shear rates. Additionally, the GP1bα subunit binds to thrombin, allowing it to activate the PAR1 and PAR4 receptors. When VWF binds to GPIb-IX-V, weak intracellular signalling events lead to activation of the integrin αIIbβ3. Patients with reductions in or qualitative abnormalities in GPIb (both subunits) and GPIX suffer from Bernard Soulier syndrome, a macrothrombocytopenia associated with relatively severe bleeding. A variety of mutations are responsible for this disorder. (55, 56).

The integrin αIIbβ3 is very highly expressed on platelets, being the most abundant protein on them. It binds to several proteins including VWF and fibrinogen. These proteins mediate formation of a platelet aggregate by cross linking platelets. αIIbβ3 activation at the platelet surface is referred to as inside-out signalling, as opposed to outside-in signalling which results from αIIbβ3 clustering and supports several components in platelet activation. Patients with mutations in either of the two subunits suffer from Glanzmann's thrombasthenia, which is characterised by severe bleeding with a normal platelet count. In a similar fashion to Bernard Soulier disease, this condition is also associated with numerous mutations (57, 58).

Figure 1.3 (overleaf) shows the signalling pathways of glycoprotein receptors in platelets.

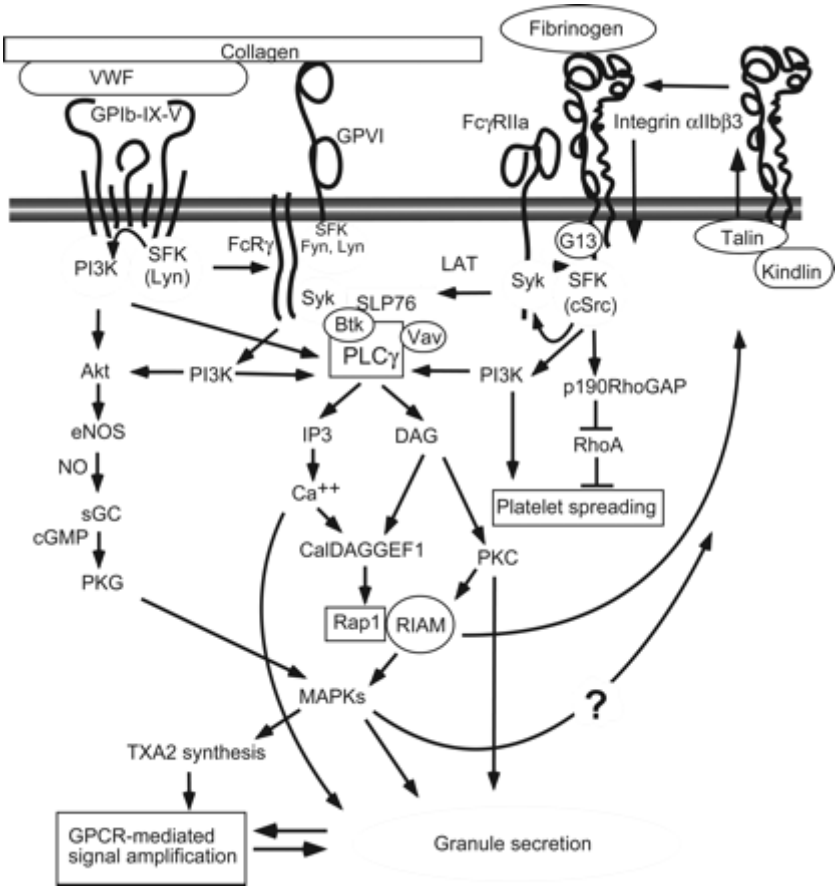


Figure 1.3. Signalling pathways of three major platelet adhesion receptors. sGC, soluble guanylyl cyclase; eNOS, endothelial NO synthase. Figure and legend reproduced with permission from (41)

Additional abbreviations: Akt= Serine–threonine kinase, also termed protein kinase B [PKB], Btk= Bruton's tyrosine kinase, cAMP=, DAG=diacylglycerol, FcγRIIa= FcγRIIa receptor, GPVI=Glycoprotein VI, GPCR= G protein-coupled receptors, IP= Inositol phosphate, IP3=Inositol Trisphosphate, LAT=Linker for activation of T cells, MAPKs= Mitogen-activated protein kinases, NO=nitric oxide, NOS=nitric oxide synthase, PI3K = Phosphoinositide 3-kinase, PKC=Protein Kinase C, , PLCγ= Phospholipase Cγ, Rap1= Ras-related protein 1, RhoA= Ras homolog gene family A, RIAM= Rap1-GTP-interacting adaptor molecule, SFK=Src family kinases, SLP76=adapter protein SLP76, Vav=Vav family proteins, VWF=von Willebrand Factor.

1.2.3 Other platelet receptors

There are a large number of other receptors that are expressed on platelets. In some cases their physiological role is not defined. Examples of additional receptors include CLEC-2, P2X1 and c-Mpl as outlined in Table 1.1. Mutations in c-Mpl and its signalling molecule JAK2 cause congenital amegakaryocytic thrombocytopenia and essential thrombocythaemia (a myeloproliferative disorder) respectively (59, 60). JAK2 inhibitors are currently being researched as a treatment option in essential thrombocythaemia (61).

1.3 Platelet-based bleeding disorders

The study of patients with bleeding problems is a powerful approach in determining the function and regulation of important proteins in human platelets. The identification of patients with defects in the major platelet integrin, αIIbβ3 (57, 62), the leucine rich receptor, the GPIb-IX-V complex (56, 63, 64) and the immunoglobulin receptor, GPVI (65), played a pivotal role in their identification as platelet receptors for fibrinogen, VWF and collagen respectively. More recently identification of patients with defects in kindlin-3 (66) and the ADP $P2Y_{12}$ receptors (67) have contributed to our understanding of their roles in platelet regulation. A brief reference guide to platelet disorders is outlined in **Table 1.2** overleaf.

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

	Thromboxane A_2 receptor	autosomal recessive (mild phenotype in heterozygote)	*TBXA2R* (19p13.3)	Platelet count normal Platelet aggregation reduced in heterozygotes to arachidonic acid and U44619 and presumed absent in homozygotes
	GPIIbIIIa (αIIbβ3) (Glanzmann's thrombasthenia)	autosomal recessive	*ITGA2B* (17q21.32) *ITGB3* (17q21.32)	Normal platelet count, size and morphology Presents with severe bleeding symptoms in early life Absent platelet aggregation with all agonists; agglutination to ristocetin is normal. Flow cytometry with CD41 and CD61 antibodies may show reduced levels of either GPIIb or GPIIIa
Platelet secretion	Hermansky-Pudlak syndrome	autosomal recessive	*HPS1* (10q24.2), *HPS2/AP3B1* (5q14.1), *HPS3* (3q24), *HPS4 (22q12.1)*, *HPS5* (11p14), *HPS6* (10q24.32), *HPS7/dysbindin* (6p22.3), *HPS8* (19q13.32), *HPS9* (15q21.1)	Platelet count normal Skin and hair hypopigmentation Reduced/absent δ-granules on electron microscopy Lumiaggregometry: reduced/absent ATP release

	Chediak-Higashi syndrome	autosomal recessive	*CHS1/LYST* (1q42)	Platelet count normal Skin and hair hypopigmentation Immunodeficiency Giant inclusions in granulocytes and their precursors, reduced or irregular α-granules
	Grey platelet syndrome	autosomal recessive	*NBEAL2* (3p21.31)	Thrombocytopenia Increased MPV with platelet anisocytosis Platelets grey in colour on blood film Absent α-granules
	X-linked dyserythropoietic anemia and thrombocytopenia	X-linked	*GATA1* (Xp11.23)	Thrombocytopenia with increased MPV Reduced α-granules Anaemia
	Arthrogryposis, Renal dysfunction and Cholestasis (ARC) syndrome	autosomal recessive	*VPS33B* (15q26.1) *VIPAS39* (14q24.3)	Thrombocytopenia with increased MPV Severe multisystem syndrome, leading to fatal complications very early in life Absent α-granules
	Paris-Trousseau/Jacobsen syndrome	autosomal dominant	*FLI1* (11q24.1-24.3)	Thrombocytopenia with increased MPV Developmental delay and facial abnormalities
	Quebec platelet disorder	autosomal dominant	*PLAU* (10q22.2)	Platelet count at low end of normal range α granule protein degradation Increased urokinase-type plasminogen activator storage in platelets

Platelet cytoskeleton	MYH9-related disorders (May-Hegglin anomaly, Sebastian, Fechtner, Epstein syndrome)	autosomal dominant	*MYH9* (22q12-13)	Thrombocytopenia with increased MPV Döhle-like inclusions in neutrophils Nephritis, hearing loss and cataracts in some forms
	Wiskott-Aldrich syndrome (WAS) / X linked thrombocytopenia	X-linked	*WAS* (Xp11.23)	Thrombocytopenia with small platelets Immune deficiency and eczema (in WAS)
	Filamin A disorders (Periventricular nodular heretotopia/ Otopalatodigital syndrome)	X-linked	*FLNa* (Xq28)	Thrombocytopenia with raised MPV and abnormal platelet morphology Abnormal distribution of platelet filamin on confocal microscopy
	Actin disorders	autosomal dominant	*ACTN1*(14q24.1)	Thrombocytopenia with raised MCV and platelet anisocytosis Patients either have moderate bleeding tendency or may be asymptomatic
Platelet procoagulant activity	Scott syndrome	autosomal recessive	*TMEM16F* (12q12-13.11)	Platelet count normal Anomalies in flippases translocating negatively charged phospholipids on the plasma membranes. Impaired annexin A5 binding with flow cytometry

Other thrombocytopenias	Congenital amegakaryocytic thrombocytopenia (CAMT)	autosomal recessive	*MPL* (1p34)	Severe thrombocytopenia Pancytopenia Absent megakaryocytes in bone marrow Increased plasma thrombopoietin levels
	Thrombocytopenia with absent radius (TAR) syndrome	Co-inheritance of 2 mutations (behaves as autosomal recessive)	*RBM8A* (1q21.1)	Severe thrombocytopenia Normal platelet morphology Shortened/absent radii in forearm
	THC2	autosomal dominant	*MASTL, ACBD5, ANKRD26* (all 10p12.1)	Mild to moderate thrombocytopenia with normal MPV Platelets deficient in GPIa (subunit of collagen $\alpha_2\beta_1$ receptor) and α-granules Possible dysmegakaryopoiesis Platelet aggregation normal
	Familial platelet disorder with predisposition to acute myelogenous leukaemia (FPD / AML)	autosomal dominant	*RUNX1* (21q22.12)	Mild thrombocytopenia, with possible raised MPV Abnormal aggregation to multiple agonists
	GFI1B mutation	autosomal dominant	*GFI1B* (single nucleotide insertion in exon 7, c880-881insC – results in frameshift mutation)	Red cell anisopoikilocytosis Moderate thrombocytopenia, raised MPV Decrease in platelet alpha granules Absent aggregation with collagen, +/- other agonists

Table 1.2: Brief reference guide to inherited platelet disorders. Adapted from (68). ADP: adenosine diphosphate; ATP: adenosine triphosphate; GP: glycoprotein; MPV: mean platelet volume; VWF: von Willebrand Factor

Megakaryocytes transcribe many thousands of genes which suggests that there are many more genes that could potentially be involved in the pathogenesis of bleeding disorders. Surprisingly few inherited mutations have been identified in genes encoding proteins that are known to play important roles in regulating platelet activation, especially in patients with mild bleeding disorders. Six patients with function disrupting mutations in the stimulatory receptor for collagen, GPVI, have been described (52-54). Two of these are compound heterozygotes, and the remaining four unrelated individuals are homozygotes for a gene insertion resulting in a premature stop codon. A small number of inherited mutations in the Gq-coupled thromboxane receptor have been reported, all of which are heterozygous for the mutation (31-34). The number of patients with mutations in the Gi-coupled $P2Y_{12}$ ADP receptor is now into double figures (20-22, 40), with several also being heterozygous. There are no patients with mutations in the two PAR receptors, PAR1 and PAR4. Many of the patients with excessive bleeding and heterozygous mutations in the thromboxane and $P2Y_{12}$ receptors are likely to have either a second abnormality or the presence of modifying genetic defects, as some of their relatives who also carry the heterozygous change do not have a history of bleeding. Further, inhibitors of these two pathways are used widely as prophylaxis and treatment for arterial thrombosis but only induce excessive bleeding in a minority of treated patients (69). It should also be borne in mind that the second abnormality may not necessarily be a platelet defect as illustrated by the presence of heterozygous $P2Y_{12}$ mutations in patients with type 1 von Willebrand disease (70).

Platelet function disorders are difficult to diagnose and are likely to be under diagnosed (71) and therefore under researched. Several factors have contributed to this including:

- The absence of a 'gold standard' point of care assay of platelet function.
- The generally asymptomatic presentation of patients with mild defects in platelet function who only exhibit symptoms of excessive bleeding when subject to an appropriate challenge such as surgery or severe injury. Women often tend to present earlier due to heavy periods (menorrhagia) or excessive bleeding associated with childbirth.
- The considerable redundancy in the pathways underlying platelet activation such that a single gene defect may not be sufficient to give rise to excessive bleeding (but may become clinically important under challenging conditions e.g. administration of an antiplatelet agent).
- The very limited amount of mRNA that can be recovered from the anucleate platelet which severely hampers many standard approaches in molecular biology including use of microarrays because of concerns over contamination.
- The lack of standardisation and quality assurance in platelet function testing, (although this has been addressed in some recent publications (72, 73)), in addition to the fact that platelet function testing requires fresh blood samples and is labour intensive.

Platelet disorders that give rise to abnormal bleeding may be of genetic origin or may be acquired in situations such as ingestion of an antiplatelet agent (e.g. aspirin), liver or renal failure or immune dysregulation such as in immune thrombocytopenia purpura. Diagnosis is supported by the nature of the bleeding (with bleeding from mucous membranes being

particularly common), by a defect in platelet function (often aggregation), and by the absence of abnormal findings in basic coagulation testing (typically prothrombin time and activated partial thromboplastin time) and lack of evidence for von Willebrand disease, which is caused by a quantitative or qualitative defect in von Willebrand Factor. In some cases, however, the diagnosis of a platelet disorder is based on clinical judgement. As a result, insight into the pathophysiological mechanisms underlying most mild platelet disorders remains limited.

1.4 Diagnosis of inherited platelet function disorders

While the first suspicion of a platelet function disorder (PFD) is made on the clinical history, a definitive diagnosis requires demonstration of a laboratory defect in platelet function. Historically, the most widely used test was the bleeding time, but not only is this invasive, it is poorly reproducible and no longer recommended (72, 74). The modern day 'gold standard' test for monitoring platelet activation is light transmission aggregometry (LTA), which was first described by Born (75). The basic principle has not changed in over 50 years, even though modern day aggregometers are much smaller and easier to use (76). The test uses anticoagulated (usually citrated) platelet-rich plasma (PRP) or washed platelets and monitors the change in light transmission upon agonist addition. The response can consist of several components, including an initial increase in optical density brought about by a change in platelet shape, followed by a primary, biphasic, sustained or reversible increase in light transmission, which is normally mediated by αIIbβ3-dependent aggregation (77). The nature of the response is dependent on the agonist, agonist concentration and the feedback action of ADP and thromboxane A_2 (TxA_2). LTA does require an experienced operator and does not pick up many aspects of platelet activation including procoagulant activity and platelet spreading.

A variation of LTA is the addition of luciferin-luciferase reagent to simultaneously monitor dense granule secretion (78). The combination of LTA with assessment of secretion by this method is often called lumiaggregometry, and an example trace is shown in **Figure 1.4.**.

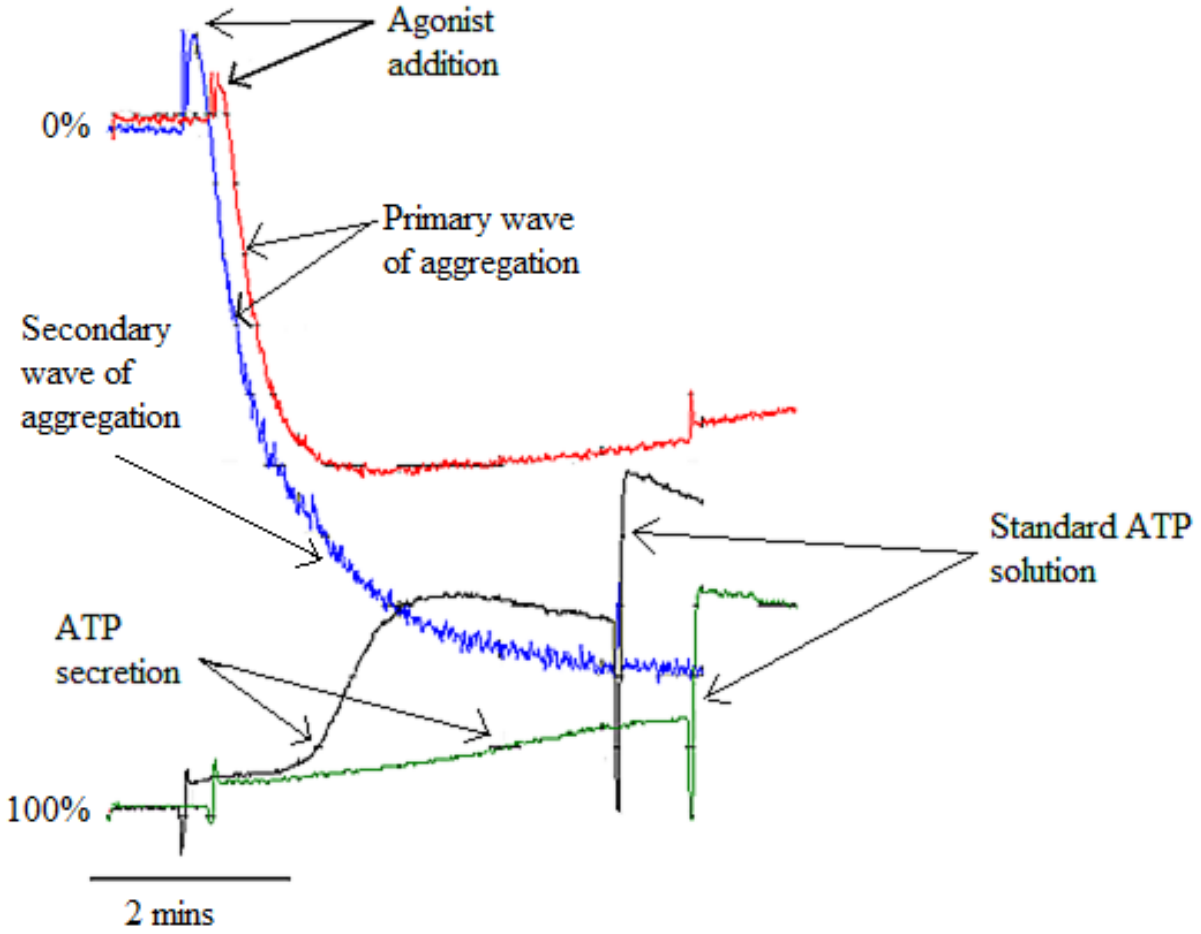


Figure 1.4: Example trace of lumiaggregometry**.** Lumiaggregometry traces from a patient with a heterozygous ADP $P2Y_{12}$ receptor mutation in the DRY motif predicted to be function disrupting (R122H) and from a healthy volunteer (control). The agonist used in this trace was a high concentration of ADP (30μM). Control aggregation is shown in blue, patient aggregation is shown in red. Control ATP secretion is shown in black, patient ATP secretion is shown in green. Secondary wave of aggregation is seen in the healthy volunteer and is labelled. The patient shows minimal ATP secretion and no secondary wave of aggregation, with deaggregation noted after initial primary wave formation. Addition of the ATP standard to allow calculation of secretion to normalized platelet count is also labelled.

Diagnosis of Hermansky Pudlak Syndrome and other dense granule secretory disorders cannot be achieved on the basis of aggregation alone as all of the commonly used platelet agonists, including ADP and adrenaline, can elicit full aggregation in the absence of secretion (72, 79). It should also be borne in mind that a defect in ATP secretion could reflect either abnormal granule formation or defective trafficking of granules. Additional tests such as electron microscopy, serotonin uptake, and total ATP content, are therefore required to distinguish between these two possibilities.

A wide range of other platelet function tests are also available, some of which focus on responses to specific agonists and some of which measure a global output of platelet activation. Each places a differing emphasis on the role of primary and secondary aggregation and feedback pathways, which explains the subtle distinctions in their results. The PFA-200® is a modern update of the original PFA-100® device first released in the mid 1990's, based on the prototype instrument developed by Kratzer and Born (80). All test components are within disposable cartridges that are loaded into the instrument at the start of the test. Citrated whole blood is pipetted into the cartridge and, after a short incubation period, exposed to high shear (5000–6000/s) through a capillary tube before encountering a membrane with a central aperture of 150 μm diameter. The instrument monitors the drop in flow rate as platelets form a haemostatic plug that seals the aperture and stops blood flow. This parameter is recorded as the closure time (CT). A number of studies suggest that the PFA-100/200® is a potential in vitro replacement of the bleeding time (80). The disadvantages are that, like the in vivo bleeding time, the test is sensitive to both the platelet count and hematocrit, and it is therefore crucial that a full blood count is performed to help

interpret abnormal results (81). False negative results are sometimes obtained in milder inherited platelet disorders; for example, in patients with Hermansky-Pudlak syndrome and the Quebec platelet disorder (80). Diagnosis of these disorders could therefore be missed if relying solely on the PFA-100® as a screening test (72). In patients with apparently normal platelet function, the instrument has also been shown to occasionally give false-positive results, which then require further detailed testing. Guidelines on the utility and practice of using the PFA-100/200® for clinical assessment of platelet disorders have been provided by various international and national organizations (72, 80, 82). The advantages of the test are that it is simple, rapid, and does not require specialist training (apart from training in the manipulation of blood samples). It is a potential screening tool for assessing patients with many types of platelet abnormalities. Given the high shear conditions to which platelets are exposed during the test, the test is highly VWF-dependent and is therefore abnormal in patients with von Willebrand disease (83, 84). The instrument thus provides laboratories with a limited but optional screening tool that can give rapid and reliable data with a relatively high negative predictive value (72). However, further confirmatory testing with light transmission aggregation or a von Willebrand profile would be required, and clinicians and researchers often opt to proceed directly to these assays (72).

Whole blood aggregometry measures the change in resistance or impedance between two electrodes as platelets adhere and aggregate in response to classical agonists (85). The significant advantage of this assay is that anticoagulated blood does not require further processing for analysis. Commercial multichannel impedance-based aggregometers, such as the Chronolog® or Multiplate® aggregometers, provide the ability to characterize several pathways of platelet activation in parallel. The use of these analyzers has seen a rise in

popularity in the last decade, but is held back by their dependency on platelet count. They are better established in the monitoring of anti-platelet therapy with some reported associations between laboratory findings and clinical outcomes, although these are not robust at present and require further investigation (86-88). At present, clinical guidelines advise against the use of platelet function testing for routine monitoring of antiplatelet therapy (89-91). The use of whole blood aggregometry analyzers in the diagnosis of inherited platelet defects is limited and is seen in specialized laboratories. Specific guidelines for the use of whole blood aggregometry for the diagnosis of platelet disorders are required before this assay can be widely implemented.

A number of platelet function assays have been developed over the years to be used at the patient's bedside (85). Most of these assays were specifically developed for monitoring of antiplatelet therapy, and the experience with these assays in identifying platelet defects is limited. The VerifyNow® device, a fully automated and near patient testing aggregation system, is available solely for the monitoring of the three major classes of anti-platelet drugs (e.g. GPIIb/IIIa inhibitors, aspirin, and $P2Y_{12}$ receptor inhibitors/antagonists) using specific cartridges (92). Plateletworks®, a standardized platelet counting ratio technique, based upon comparing platelet counts within a control EDTA tube and after aggregation with platelet agonists within citrated tubes, could theoretically also be applied to the diagnosis of platelet disorders, although experience with this assay is limited (93). Thromboelastography® (TEG) provides various data relating to clot formation and fibrinolysis (the lag time before the clot starts to form, the rate at which clotting occurs, the maximal amplitude of the trace or clot strength and the extent and rate of amplitude reduction) (94). With the PlateletMapping™ system, arachidonic acid and ADP can be used as agonists to pre-activate platelets within the

TEG system, thus making the assay theoretically suitable to monitor antiplatelet drugs, although the test lacks sensitivity to detect moderate changes in platelet function (95). Its use is currently mostly limited to the monitoring of surgical haemostasis. A recent study within the GAPP group examined the utility of the 96 well plate aggregation assay (known as Optimul®) in diagnosing platelet defects and found it to have good correlation with lumiaggregometry (96). Further studies within the group have looked at the feasibility of a flow cytometric whole blood platelet function test which can be performed remotely (after samples are fixed and posted to a central laboratory), and concluded that it may be a helpful screening test in determining who should go on to have extensive lumiaggregometry studies (97).

1.5 Genetic sequencing of patients with platelet disorders

The Birmingham Platelet Group has previously identified several patients with function-disrupting mutations in the $P2Y_{12}$ ADP receptor and the TxA_2 receptor (98). Strikingly, three of these mutations have been shown to be co-inherited with the most common forms of bleeding disorder, type 1 von Willebrand's disease, which is caused by a quantitative defect in von Willebrand factor (70). Thus, mild bleeding may often be multifactorial and should be considered to be a complex trait, drawing comparisons with the multifactorial aetiology of thrombosis (**Figure 1.5**).

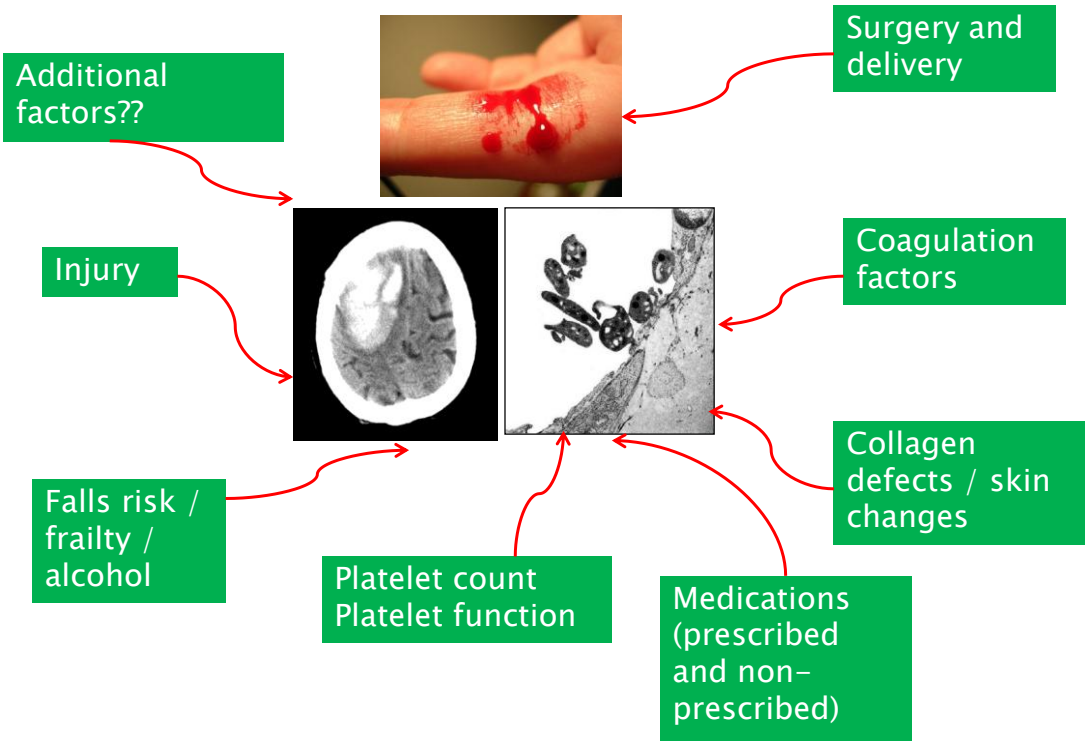


Figure 1.5 – The multifactorial aetiology of bleeding, encompassing genetic, acquired and environmental factors

The success in identifying mutations was initially achieved by targetted sequencing of candidate genes identified by investigation of light transmission aggregation and ATP secretion, together with other testing methods such as flow cytometry and electron microscopy. Sequencing of the relevant genes in a population enriched for bleeding (i.e. von Willebrand disease) was also used (70). More recently, whole exome sequencing has been used to identify genes associated with an abnormal platelet phenotype (99-101). This approach to gene mapping of platelet disorders based on initial evaluation of clinical and laboratory phenotypes of patients with clinically diagnosed bleeding disorders and subsequent

targeted gene sequencing is referred to as the GAPP [genotyping and phenotyping of platelets (102)] approach and is summarised in **Figure 1.6**.

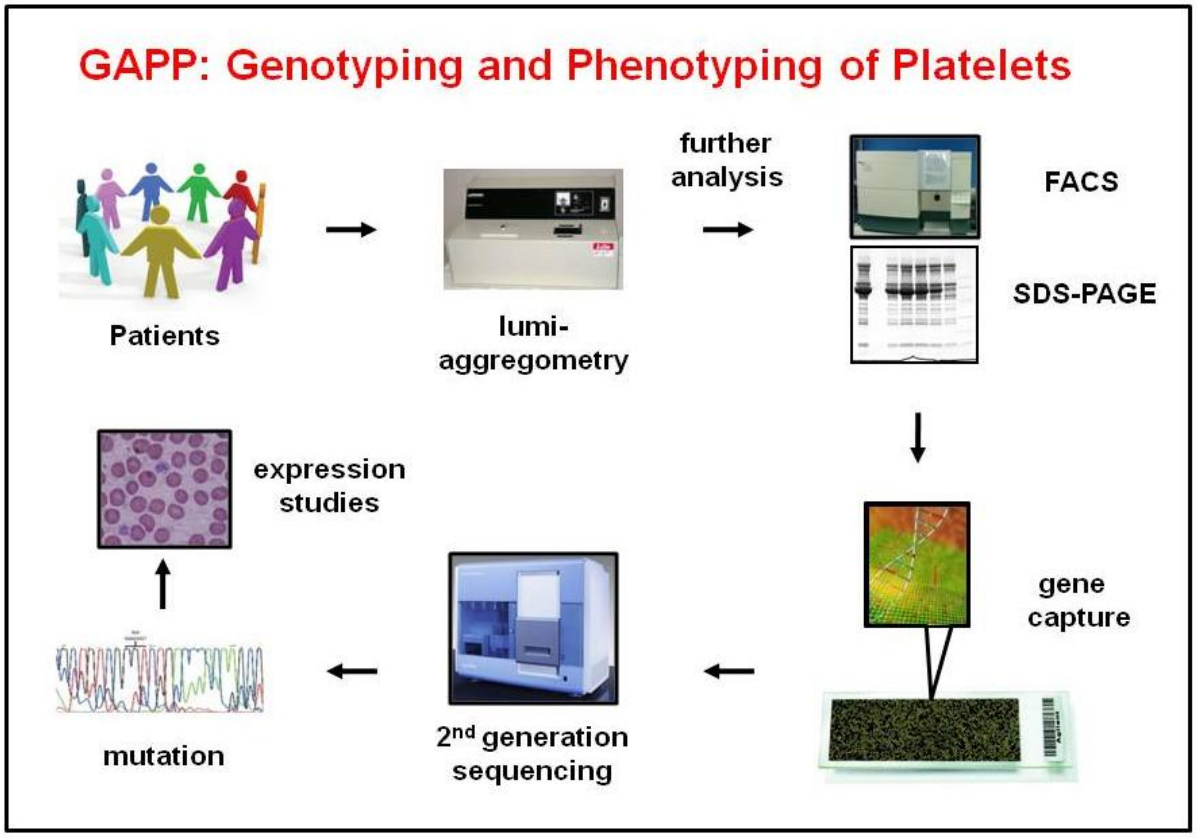


Figure 1.6 – GAPP approach to investigating patients with platelet disorders showing initial sample collection and lumiaggregometry studies, in addition to examples of further testing methods. Modified from (98). Figure courtesy of Professor Steve Watson.

In cases where the location of the underlying mutation is strongly suspected direct Sanger sequencing of candidate genes can be used to identify the defect. This is most applicable in the situation where there is selective loss of response to one agonist, suggesting an abnormality in its cell surface receptor. However, the fact that some defects are partial (function disrupting rather than ablating) and the complicated feedback pathways in platelet signalling often complicate this interpretation. Examples of mutations identified in this way

within GAPP include discovering a homozygous mutation in the ADP $P2Y_{12}$ receptor (where deaggregation to high dose ADP was seen) (103) and a heterozygous function disrupting point mutation in the thromboxane receptor in a patient who had markedly reduced responses on testing with arachidonic acid and U46619, a direct stimulant of the thromboxane receptor (33).

Next generation DNA sequencing (NGS) has led to major advances in the ability to sequence large amounts of genetic material (104), and has made sequencing of whole exomes financially viable (68). However, expert interpretation of the data generated is required as huge amounts of information are returned, and comparison to datasets containing known candidate function disrupting mutations is required (105). Confirmatory Sanger sequencing of the patient with the bleeding disorder and both affected and unaffected family members further supports genotype-phenotype association. Limitations of this approach include the fact that several candidate disrupting mutations may be present, family members may be unavailable or unwilling to be tested, the sequencing methodology may not be fully optimised and reference databases may themselves not be completely contemporaneous (106). In addition, mutations outside the exome (such as in regulatory regions) will not be identified, and GC rich regions can lead to incomplete coverage and failure to capture some mutations.

When a candidate mutation is identified the role of the implicated protein in platelet function may not be known, and cell line expression models to evaluate its role are required. This is important to attribute pathogenesis to the identified mutation, as healthy volunteers are known to be heterozygous for several function disrupting mutations which are not associated with any disease state (107). However, even if a functional defect can be related to the mutant

protein, it may be that the combination of additional, possibly heterozygous, mutations that give rise to the overall bleeding pathogenesis.

In genetically related individuals, autozygosity mapping is one technique where NGS can be used to identify autosomal recessive mutations. The basis of this approach is that affected family members have common regions of autozygosity encompassing the disease-causing mutation. Restricting analysis of nonsense and missense single nucleotide polymorphisms to regions of autozygosity increases the speed of identifying causative genes in families with consanguineous marriages, which are commonly found amongst patients with inherited bleeding disorders. Patients in these families will share thousands of nonsense and missense SNPs.

This methodology has previously been used in identifying Hermansky Pudlak syndrome 8 (108). In cases with more than one candidate gene such as Hermansky Pudlak syndrome, use of microsatellite markers can further aid mapping of the causative mutation (109). Autozygosity mapping has also led to identification of *ANKRD18A* and *NBEAL2* as causative genes in a families with thrombocytopenia associated with severely reduced platelet counts and platelet dysfunction, and grey platelet syndrome respectively (110-113).

Next generation sequencing has also been used in investigating the mutations responsible for TAR (thrombocytopenia and absent radius) syndrome. Patients with this condition have abnormal forearm development due to the absence of a radial bone in addition to a mild bleeding disorder with thrombocytopenia. Gene mapping revealed a heterozygous microdeletion on the long arm of chromosome 1 in the gene *RBM8A* (114), but some family members with this mutation did not have the disorder. NGS was used to identify a second mutation in the regulatory region of *RBM8A* in individuals with this disease (115). This

mutation in the second allele was found in 53 out of 55 cases and was associated with reduced expression of the protein.

NGS can be used in families with bleeding disorders suspected to be inherited in an autosomal dominant manner by sequencing affected and unaffected family members and examining novel variants which co-segregate with those individuals with excessive bleeding. There are relatively few autosomally dominant inherited bleeding disorders.

The application of NGS to unrelated individuals is a challenge and requires large numbers of patients with similar clinical characteristics. This is currently under study in both national and international studies (101, 116, 117). The ability to clearly phenotype unrelated patients should assist in the search for a causative gene, as it narrows the number of possible causative candidate genes. A similar approach has been successfully used in identifying mutations in *GATA-2* as being responsible for causing an inherited immunodeficiency syndrome with predisposition to leukaemic transformation. In this case four unrelated individuals were studied using whole exome sequencing (118).

Clear phenotyping would also allow the use of targeted arrays in identifying the responsible gene, such as an array of 200 candidate genes for defective platelet activation that was generated within the GAPP project (119). This was used to identify a novel HPS4 mutation in a patient with clinically suspected HPS and supporting laboratory changes in phenotype that demonstrated lack of dense granule secretion (119). However, as whole exome sequencing comes down in cost this may be a more attractive option than using targeted arrays for initial screening, with confirmatory Sanger sequencing and use of cell line studies for further investigation of candidate mutations.

1.6 Aims of PhD study

1. To create a multi-centre clinical network with coverage of haemophilia centres across the United Kingdom (three participating centres were registered at the start of my research fellowship in 2010). The increase in centre numbers was necessary to allow the study of inherited platelet disorders, which are rare diseases with only small numbers of patients registered at each centre.
2. To better define the normal range of platelet aggregation responses in healthy volunteers, including assessment of whether measuring secretion using Chronolume® during lumiaggregometry effects aggregation measurements.
3. To rationalise lumiaggregometry laboratory testing in platelet disorders with normal platelet number, producing a recommended testing algorithm for investigation of patients with a suspected platelet disorder.
4. To classify platelet defects in patients with a normal platelet count included in the GAPP study.
5. To assess bleeding scores in patients with and without platelet disorders to establish whether a raised score is indicative of an underlying defect.
6. To undertake genotyping of selected patients found to have an abnormal phenotype to uncover genes involved in platelet function and megakaryopoiesis (using Sanger and second generation sequencing technology).
7. To establish whether there is a significant prevalence of undiagnosed platelet function defects in patients with unexplained menorrhagia.
8. To develop a new assay to allow for testing platelet function (ie presence of qualitative defects) in patients with thrombocytopenia.

CHAPTER TWO: METHODS

2.1 NHS Permissions and patient recruitment

2.1.1 NHS Permissions

Retrospective approval for incorporation of the GAPP project into the National Institute of Health Research Portfolio (Non-Malignant Haematology subgroup, ID 9858) was obtained in February 2011, based on the fact that the study met a relevant clinical need. This approval enabled access to support from the Comprehensive Local Research Networks (CLRNs), and Birmingham and the Black Country CLRN was elected as lead network for the study. NHS Permissions were then obtained at over fifteen new centres using the Integrated Research Application System (IRAS), allowing coverage across most of the United Kingdom. Testing continued to be performed at the three central laboratories in Birmingham, Bristol and Sheffield, with blood samples being sent by courier to the central laboratory in Birmingham for recruits from the newly approved centres.

For centres at a travelling distance of more than four hours, recruitment and investigation was done in the local haemophilia unit and laboratory using on site testing, as the results obtained couriering blood may have been affected by the delay between venepuncture and PRP preparation.

A secure portal was devised to allow data sharing across the three sites where the grant principal investigators were based (Birmingham, Bristol and Sheffield) using the National Institute of Health Research database.

Numerous revisions to the study protocol, patient information sheets and consent forms were undertaken to allow for expansion in the scope of GAPP, all of which were reviewed and approved by the West Midlands Research Ethics Committee (reference 06/MRE07/36). The

study was also registered with ISRCTN (reference 77951167). An example patient information sheet and consent form are shown in **Appendix 1**. Study reports containing a summary of bleeding history, laboratory findings and a conclusion on the platelet phenotype were produced on every recruited patient (as per the study protocol) and were returned to the referring consultant.

2.1.2 Patient recruitment

This section describes the patient recruitment procedure for patients with a normal platelet count and a suspected inherited platelet disorder. Recruitment processes for patients with menorrhagia and inherited thrombocytopenia are discussed in Chapters 8 and 9 respectively.

Potential participants with suspected platelet function defects were referred from UK Comprehensive Care Haemophilia Centres and were invited to participate if they satisfied all the following inclusion criteria:

a. Aged 0 – 85 years
b. Abnormal bleeding symptoms compatible with a possible inherited platelet function disorder (ranging from spontaneous bruising and easy bleeding to life threatening bleeding episodes requiring transfusion)
c. Platelet count within the local laboratory reference range
d. Results from coagulation factor tests within local laboratory reference intervals (minimum panel of prothrombin time, activated thromboplastin time, Clauss fibrinogen activity, von Willebrand factor antigen level, Ristocetin cofactor activity and FVIII:C activity)
e. Willing to participate and able to provide informed consent

Exclusion criteria were as listed below:

a. Patients with existing diagnoses of Glanzmann's thrombasthenia, Bernard-Soulier syndrome, May Hegglin anomaly or Hermansky-Pudlak syndrome
b. Patients taking drugs that are known to influence platelet function, including nonsteroidal anti-inflammatory drugs, aspirin, clopidogrel and dipyridamole within 7 days of enrolment
c. Patients having undergone a major surgical procedure within 1 month of enrolment.
d. Patients with chronic renal failure requiring dialysis
e. Patients with severe anaemia (Haemoglobin < 80g/l)

After informed written consent (in accordance with the declaration of Helsinki), participants were interviewed to determine the distribution, frequency and severity of bleeding episodes.

Healthy volunteers aged 18 years or older were also included in the study. Participants were considered healthy if they did not have a history of bleeding symptoms and did not require long-term medical therapy. All healthy volunteers denied taking drugs known to influence platelet function in the 2 weeks prior to recruitment.

The ISTH bleeding assessment tool (Appendix 2) was used for objective assessment of the participants' bleeding history. This score can range from 0-56 in women and 0-48 in men, based on a maximal score of 4 in fourteen separate categories assessing different types of bleeding.

The questionnaire was carried out in both patients and in healthy volunteers, and was conducted by a small number of key trained personnel (of which I was one) who regularly took bleeding histories in study participants. The bleeding assessment scores were compiled prior to undertaking platelet function testing so that investigators were not influenced by lumiaggregometry results when assessing and quantifying the bleeding history.

2.2 Platelet preparation and assessment of platelet function

2.2.1 Platelet preparation

Blood samples from participants and a simultaneous healthy volunteer (control, recruited from the same site as the patient) were taken into trisodium citrate (3.2-3.8%) with a trisodium citrate:whole blood ratio of 1:9. Total volume of blood taken ranged from 15-60ml dependent on the age of the patient, their clinical status and the discretion of the referring clinician. Blood drawing was performed using either a syringe or an evacuated test tube system (either Vacutainer or Sarstedt), and was undertaken with caution to avoid prolonged venous stasis, using a large-bore needle (between 19 and 21 gauge).

The collected samples were then gently inverted to ensure complete mixing of the content. They were transported at ambient temperature (~20˚C) to the laboratory, using a courier service (DHL) for centres outside of the Midlands. For centres in Newcastle and Edinburgh testing was performed in the local haematology laboratory due to the prohibitive travelling distance.

Platelet rich plasma was prepared by transferring whole blood samples to 5ml polypropylene tubes using a Pasteur pipette, with subsequent centrifugation of whole blood at 200*g* for 20 min. Autologous platelet-poor plasma was obtained by further centrifugation at 1000g for 10

min. Buffy coat samples were aliquoted after removal of platelet poor plasma and were stored at -80ºC to allow for future genetic work. The platelet count in PRP and mean platelet volume were assessed with a Coulter Z_2 analyser (Beckman Coulter (UK) Ltd).

2.2.2 Assessment of platelet function

Platelet aggregation and ATP secretion were measured in PRP using a dual Chronolog lumiaggregometer (Model 460 VS, Havertown, PA, USA). Autologous platelet poor plasma (PPP) was used to set the aggregation scale prior to each study according to the manufacturer's instruction. All experimentation was performed within six hours of preparation of the PRP. ADP and adrenaline responses were always tested first as their responses are the most time dependent. PRP samples (400µl) were pre-warmed in siliconized glass cuvettes (Ladmedics, Manchester, UK) at 37˚C for 120s and stirred at 1200rpm for 60s prior to agonist addition. Recording of aggregatory responses was undertaken for five minutes after agonist addition, with the exception of adrenaline responses which were recorded for up to ten minutes after agonist addition. When a chart recorder was used, percentage aggregation was calculated by the observed change in optical density compared to the full scale deflection as set by the PPP control. Latterly, responses were assessed using the AggroLink computer interface (ChronoLog / LabMedics UK) with the AggroLink for Windows software (version 5.2.3). Parameters assessed using this software were maximal aggregation, final aggregation (to assess whether deaggregation occurred), and area under the aggregatory curve (to assess overall magnitude of aggregation response).

Reagents used and their sources, stock and final concentrations are listed in **Table 2.1**.

Agonist	*Stock concentration*	*Final concentrations tested (in cuvette)*	*Source*
ADP	10 mM	3µM, 10µM, 30µM	Sigma-Aldrich A2754
Adrenaline	100 mM	3µM. 10µM and 30µM	Sigma-Aldrich E-4375
Arachidonic acid	15 mM	0.5mM, 1mM and 1.5mM	Cayman Chemical 10006607
PAR1 peptide (TRAP - SFLLRN)	10 mM	30µM and 100µM	Commercial synthesis, e.g. Alta Biosciences, Birmingham
PAR4 peptide (TRAP - AYPGKF)	50 mM	250µM and 500µM	Commercial synthesis, e.g. Richard Farndale, Cambridge
Ristocetin	50 mg/ml	1.5mg/ml	Helena Laboratories 5372
U46619	1 mM	1µM and 3µM	Cayman Chemical CAY16450-5 mg
Collagen	1 mg/ml	3µg/ml and 10µg/ml	Nycomed- Austria NYAR 1130630
Collagen-related peptide	3 mg/ml	1µg/ml and 3µg/ml	Commercial synthesis, e.g. Richard Farndale, Cambridge

Table 2.1 Agonists used in platelet function testing, with their stock concentrations, final concentrations and source listed.

All stock agonists apart from collagen and collagen-related peptide were kept at -80˚C and diluted with phosphate buffered saline. Collagen and collagen related peptide were kept at 4˚C and diluted with their specific diluents. Reagents were kept on ice throughout the aggregation testing, and were diluted from stock concentrations during the platelet preparation time. Each patient would not always have all concentrations of each agonist tested, dependent on available sample volume and responses to the lowest concentrations of each agonist. In rare circumstances, with lack of response at the highest agonist concentration listed in the table, a higher concentration again was tried in order to attempt to elicit a response.

Measurement of secretion was carried out by adding 30µl of Chronolume® (Chronolog corporation, Havertown, PA, USA / Labmedics, Manchester, UK - containing 0.2 mg luciferin, 22,000 units d-luciferase plus magnesium sulphate, human serum albumin, stabilizers and buffer) to the highest concentration of each agonist tested. Chronolume® was diluted according to the manufacturer's instructions and added to the cuvette during the prewarming and stirring process (120s before agonist addition). The ATP secreted was recorded using the Chronolog aggregometer (model 460VS), and was quantified by adding a known amount (4nmol) of ATP standard (Chronolog corporation, Havertown, PA, USA / Labmedics, Machester, UK). Calculated secretion was normalised and expressed as nmol/1x10^8 platelets. Latterly, secretion responses were assessed using the AggroLink computer interface (ChronoLog / LabMedics UK) with the AggroLink for Windows software (version 5.2.3). Parameters assessed using this software were time to secretion and standardised secretion.

2.3 Statistical analyses

Statistical analyses was carried out using Microsoft Excel 2007, GraphPad Prism 4 and IBM SPSS Statistics 19, with testing methodologies as specified in figure legends (dependent upon the data being interrogated). P-values were considered to be significant if they were ≤ 0.05.

2.4 Genetic and receptor expression studies

Genetic and receptor expression studies were carried out by Neil Morgan and Stuart Mundell respectively (along with their laboratory teams), and are outlined in publications which they and I have co-authored (34, 40, 99, 109, 117).

CHAPTER THREE: CREATION OF A MULTI-CENTRE NATIONAL STUDY ENABLING FURTHER GENOTYPING AND PHENOTYPING OF PLATELETS

3.1 Introduction

Prior to commencement of my research fellowship, the GAPP project had recruited 126 participants (patients and healthy volunteers) over a four year period. These recruits had come from three registered Haemophilia Comprehensive Care Centres in Birmingham, Bristol and Sheffield. The target recruitment for the study was 600 participants, with a planned end date of December 2015.

Several publications had arisen from this work, examining normal ranges for aggregation and secretion and describing phenotypic and novel genetic findings in recruited patients (15, 33, 77).

As inherited platelet disorders are rare, collaboration with more United Kingdom (UK) Haemophilia Centres was recognised as being critical to obtain sufficient numbers of patients. In addition, having good geographical coverage of Haemophilia Centres across the UK would ensure that the study was accessible to as many patients and clinicians as possible.

3.2 Aim

To create a multi-centre clinical network with coverage of haemophilia centres across the United Kingdom, allowing improved recruitment to the GAPP study.

3.3 Results

Figure 3.1 shows the fifteen additional centres where NHS Permissions were received as a result of the multi centre expansion, with good geographical coverage of the United Kingdom achieved. **Figure 3.2** shows the improved accrual rates to the study as a result of this expansion, with actual recruitment exceeding projected target recruitment by September 2012. Participating centres (as of 2013) along with their local Principal Investigators are listed in **Table 3.1**.

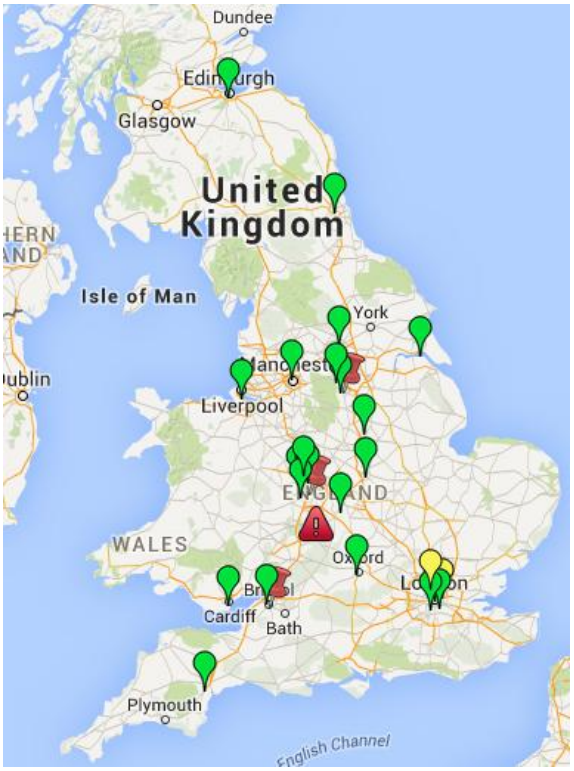


Figure 3.1 Map showing GAPP project collaborating centres (as of October 2013). Red triangle with exclamation mark shows coordinating CLRN (Birmingham and the Black Country), and red map pins show central laboratories for phenotyping (Birmingham Platelet Group, Royal Hallamshire Hospital [Sheffield] and Bristol Royal Infirmary). Green markers show sites with approved NHS Permissions, and yellow markers show sites with NHS Permissions submitted but final approval pending.

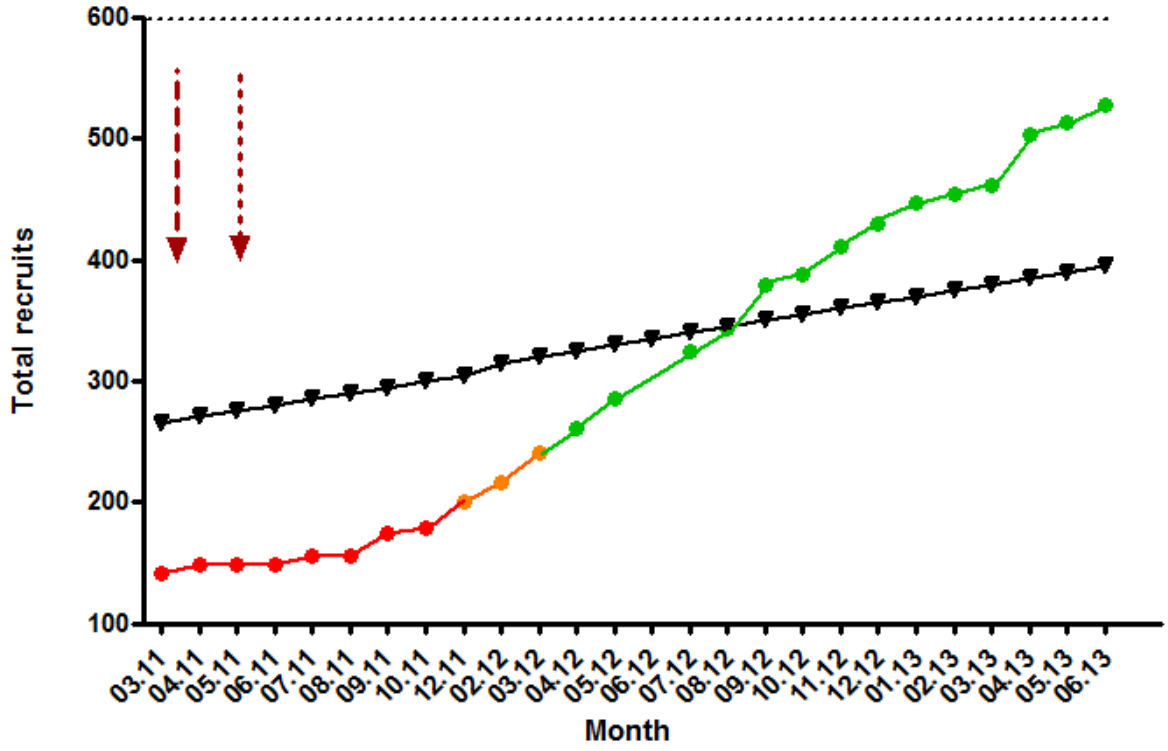


Figure 3.2 Recruitment to the GAPP study over time. Black triangles show projected or target accrual. Red / amber / green line shows actual accrual, coded according to whether recruitment fell within the NIHR red (less than 65% target), amber (65-80% target) or green (over 80% target) zones. As of September 2012 the project was recruiting to over 100% of its time projected targets. Dotted black line shows target total accrual (600 participants). Dashed burgundy arrow shows time of incorporation into the National Institute of Health Research portfolio (Non Malignant Haematology Specialty Group) and dotted burgundy arrow shows time of adoption of the study into a lead network model by Birmingham and the Black Country CLRN.

Participating Centre	Principal Investigator
Birmingham Children's Hospital NHS Foundation Trust	Dr Mike Williams
Birmingham Women's NHS Foundation Trust	Dr Will Lester
Cardiff and Vale University Health Board	Professor Peter Collins
Central Manchester University Hospitals NHS Foundation Trust	Dr Jecko Thachil
Great Ormond Street Hospital for Children NHS Foundation Trust	Dr Paul Gissen
Guys' and St Thomas' NHS Foundation Trust	Dr Steve Austin
Hull and East Yorkshire Hospitals NHS Trust	Dr David Allsup
Imperial Healthcare NHS Trust (Hammersmith Hospital)*	Dr Nichola Cooper
Leeds Teaching Hospitals NHS Trust	Dr Beki James
Midlands Research Practices (General Practice) Consortium (MidReC)	Professor David Fitzmaurice
Newcastle Hospitals NHS Foundation Trust	Dr Kate Talks
NHS Lothian (Royal Infirmary Edinburgh and Royal Hospital for Sick Children)	Dr Angela Thomas
Nottingham University Hospitals NHS Trust	Dr Gerry Dolan
Oxford University Hospitals NHS Foundation Trust	Dr David Keeling
Royal Devon and Exeter NHS Foundation Trust	Dr Jason Coppell
Royal Liverpool and Broadgreen University Hospitals NHS Foundation Trust	Professor Cheng Hock Toh
Sheffield Children's NHS Foundation Trust	Dr Jeanette Payne
Sheffield Teaching Hospitals NHS Foundation Trust	Professor Mike Makris
University College London Hospitals NHS Foundation Trust	Dr Elaine Murphy
University Hospital Birmingham NHS Foundation Trust	Dr Jonathan Wilde
University Hospitals Bristol NHS Foundation Trust	Dr Andrew Mumford
University Hospitals Coventry and Warwickshire NHS Trust	Dr Peter Rose
University Hospitals of Leicester NHS Trust	Dr Sue Pavord

Table 3.1 Centres participating in the GAPP study and their local principal investigators, as of October 2013. Centres marked with a * had pending NHS Permissions, with the relevant documents submitted to the Trust Research and Development Departments.

3.4 Discussion

The approach and resultant increase in recruitment reported in this chapter highlight the need to study rare diseases using a multi-centre approach, using national research networks to identify valuable support (120). Sponsorship of the project by the Birmingham and the Black Country Comprehensive Local Research Network (as a "lead network") aided the process of gaining NHS Permissions at new sites, and meant that the study was accessible to both patients and clinicians across the UK. The processes described here and significant improvement in accrual rates led to the GAPP study being identified as "Study of the Month" in the October 2012 edition of "Specialty Group News", a national publication circulated by the NIHR describing research in all clinical specialties (121).

CHAPTER FOUR: ASSESSMENT OF PLATELET AGGREGATION AND SECRETION IN HEALTHY VOLUNTEERS

4.1 Introduction

A major challenge in the use of lumiaggregometry in the investigation of PFDs is the wide variation observed in responses of healthy volunteers (77, 122). Thus, the common practice of comparing results in the patient to a healthy volunteer analysed at the same time can often be misleading in the absence of locally determined normal ranges (72). Other factors that can influence the response include the platelet count, although previous work in GAPP has shown that this is not significant in PRP over the range 1.5–6.0x10^6/ml (77). This is in line with recent literature suggesting the use of native PRP to be superior to PRP diluted to a standard platelet count (123, 124). However, the level of ATP secretion is platelet count-dependent, thus making normalisation to platelet count necessary (103). Below a platelet count of 1.5x10^8/ml platelet function is hard to assess by aggregometry, and there is a paucity of data surrounding responses of control individuals at these platelet counts.

Previous work in GAPP compiled response data to a minimum of three concentrations of 9 platelet agonists and established normal ranges with cut-offs. Readings for these ranges were obtained by using a chart recorder and manually measuring and calculating responses (77, 103)) .

4.2 Aims

- To define normal platelet aggregation and secretion responses in a new cohort of healthy volunteers recruited to the GAPP study using Aggrolink® software.
- To establish whether distance travelled by the sample has an impact upon aggregation findings in healthy volunteers.

4.3 Results (125)

The platelet aggregation and secretion responses [using the rationalized agonist panel with a minimum of twelve independent dose points (103)] of 68 healthy volunteers recruited to the GAPP study between December 2011 and March 2013 were characterized. For further information on the rationalised agonist panel development and validation, see **Chapter 5**. All participants had had their aggregation and secretion responses recorded using Aggrolink® software. 35 participants (51%) were female, 19 (28%) were male, and sex was not stated on the consent form for the remaining 14 (21%). Age was available on 19 participants, with a median of 32 years and interquartile range of 3 years.

The 5^{th} percentile for platelet count in PRP was $200x10^9$/l, with the 95^{th} percentile lying at $556x10^9$/l. **(Figure 4.1)**. Mean platelet volume ranged from 8.2 to 10.3fl (5^{th} to 95^{th} percentile), as shown in **Figure 4.2.**

In general, higher concentrations of agonists resulted in narrow reference intervals, but lower agonist concentrations were significantly more variable, especially for ADP **(Figure 4.3)**, Collagen **(Figure 4.4)** and Arachidonic Acid **(Figure 4.5)**. Approximately 10% of participants did not show secondary wave aggregation to Adrenaline **(Figure 4.6)**. A lack of

full response to ristocetin was seen in 6/61 individuals (10%), with a biphasic response in 24/61 individuals (39%), as detailed in **Table 4.6**. The impact of distance travelled by the sample on aggregation was examined, and transport over 100 miles did not alter platelet aggregation responses to the most variable dose points – ADP 3µM, adrenaline 10µM, collagen 1µg/ml and PAR-1 peptide 30µM. There was no effect of distance travelled by the sample on deaggregation to ADP 3µM **(Figure 4.7)**.

The data obtained is summarised in numerical form in **Tables 4.1** (ADP responses), **4.2** (Adrenaline responses), **4.3** (Arachidonic Acid responses), **4.4** (PAR-1 peptide responses), **4.5** (collagen responses) and **4.6** (ristocetin responses).

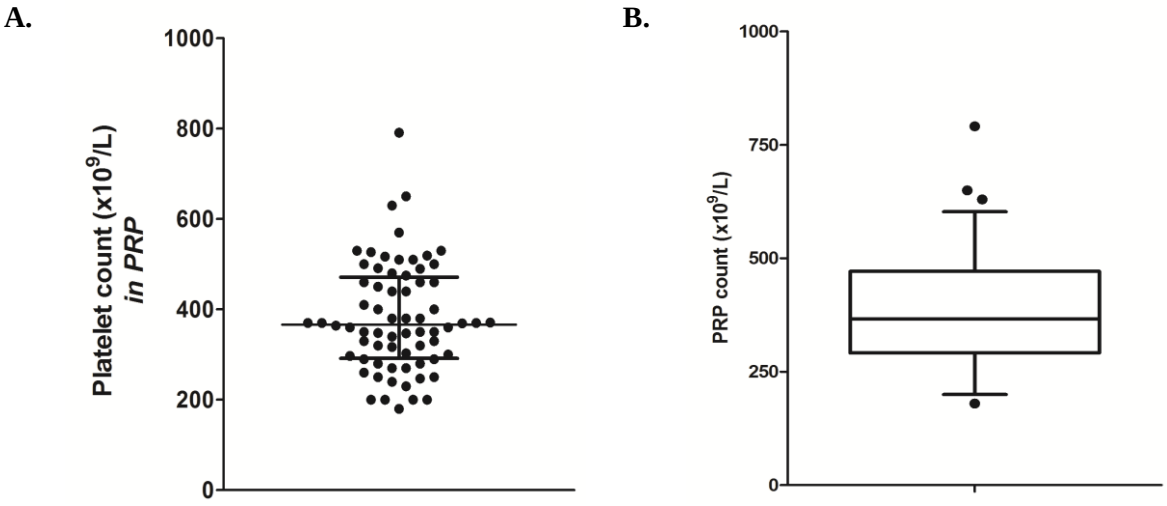


Figure 4.1 Platelet count in PRP. For panel A, bars represent median and interquartile range. For panel B, box represents median and interquartile range, whiskers represent 5th and 95th percentiles, outlying points represented by dots.

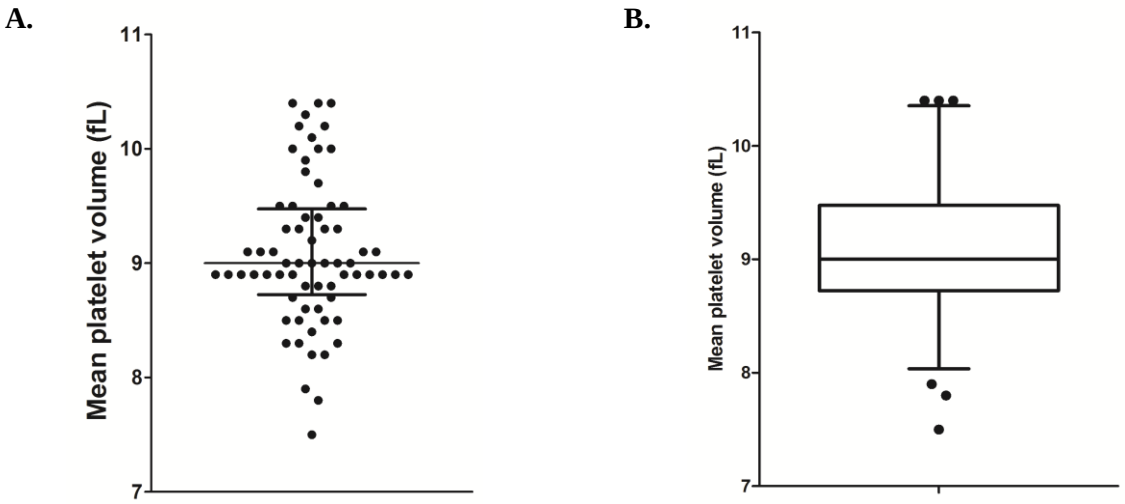


Figure 4.2 Mean platelet volume. For panel A, bars represent median and interquartile range. For panel B, box represents median and interquartile range, whiskers represent 5th and 95th percentiles, outlying points represented by dots.

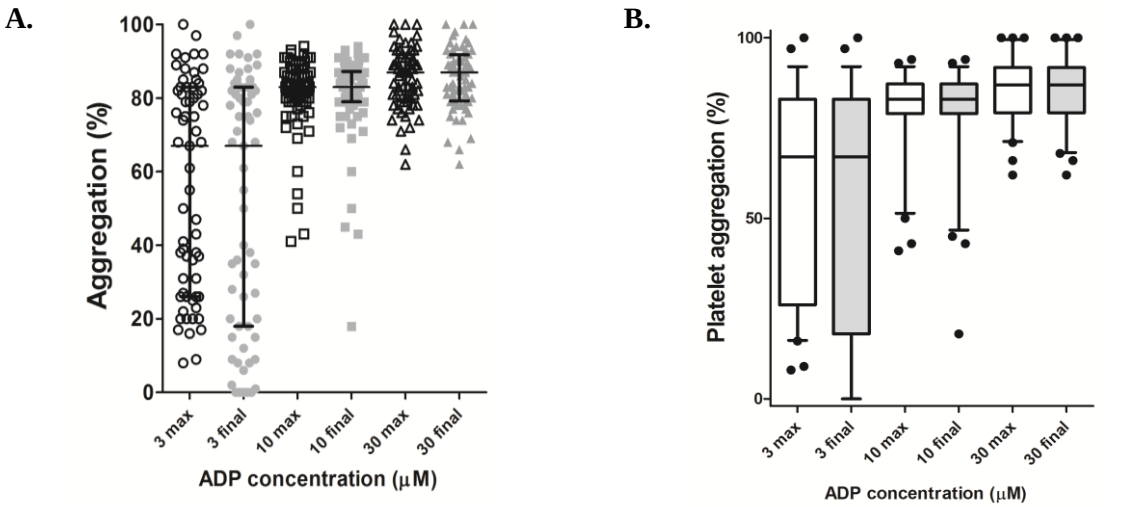


Figure 4.3 ADP responses. For panel A, bars represent median and interquartile range. Black symbols represent maximal aggregation and grey symbols represent final aggregation 5 minutes after agonist addition. For panel B, boxes represent median and interquartile range, whiskers represent 5th and 95th percentiles and outlying points are represented by dots. Black symbols represent maximal aggregation, grey symbols represent final aggregation 5 mins after agonist addition.

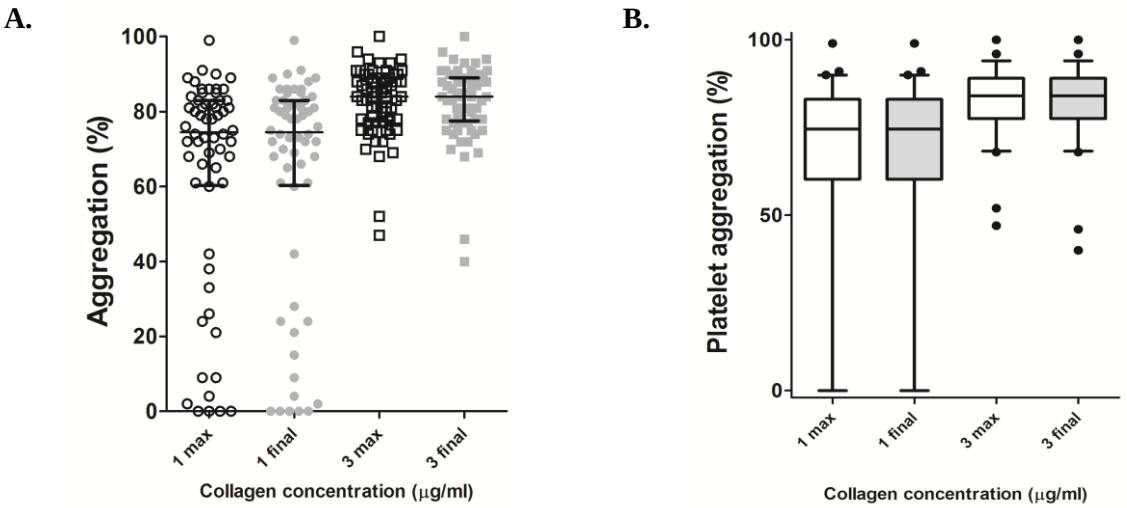


Figure 4.4 Collagen responses. For panel A, bars represent median and interquartile range. Black symbols represent maximal aggregation and grey symbols represent final aggregation 5 minutes after agonist addition. For panel B, boxes represent median and interquartile range, whiskers represent 5th and 95th percentiles and outlying points are represented by dots. Black symbols represent maximal aggregation, grey symbols represent final aggregation 5 mins after agonist addition.

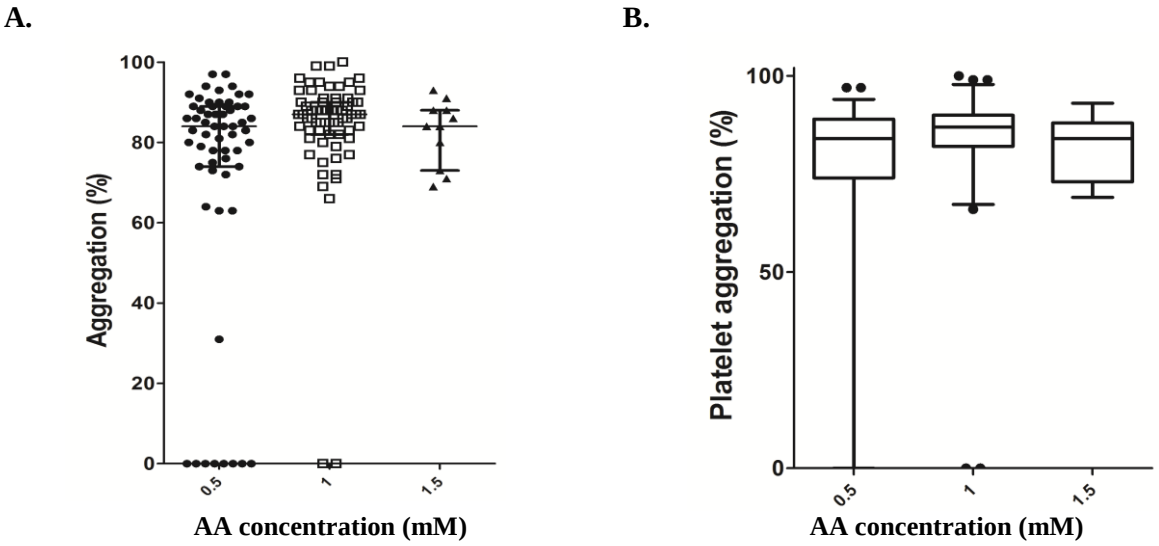


Figure 4.5 Arachidonic acid responses. For panel A, bars represent median and interquartile range. For panel B, boxes represent median and interquartile range, whiskers represent 5th and 95th percentiles and outlying points are represented by dots. Maximal aggregation only is shown (as no healthy volunteers showed deaggregation to arachidonic acid).

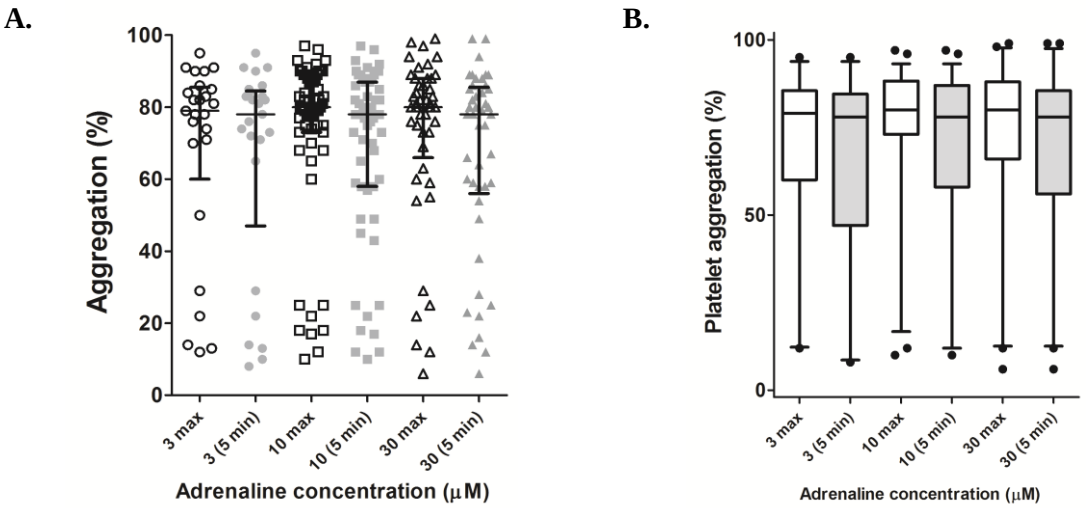


Figure 4.6 Adrenaline responses. For panel A, bars represent median and interquartile range. Black symbols represent maximal aggregation and grey symbols represent aggregation 5 minutes after agonist addition (aggregation was monitored for ten minutes in total for adrenaline). For panel B, boxes represent median and interquartile range, whiskers represent 5th and 95th percentiles and outlying points are represented by dots. Black symbols represent maximal aggregation and grey symbols represent aggregation 5 minutes after agonist addition (aggregation was monitored for ten minutes in total for adrenaline).

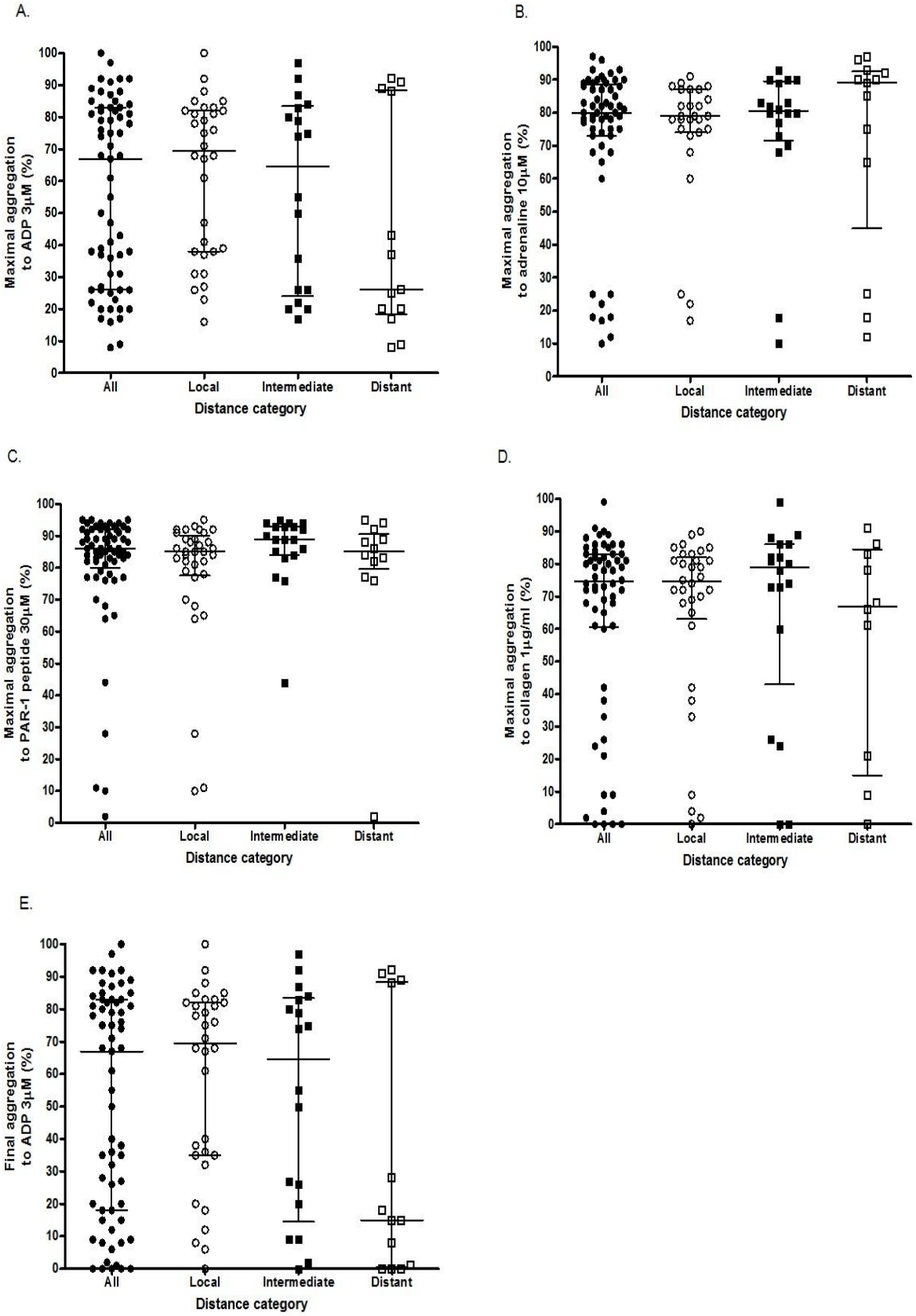

A.
Maximal aggregation to ADP 3µM (%)
All
Local
Intermediate
Distant
Distance category
B.
Maximal aggregation to adrenaline 10µM (%)
All
Local
Intermediate
Distant
Distance category
C.
Maximal aggregation to PAR-1 peptide 30µM (%)
All
Local
Intermediate
Distant
Distance category
D.
Maximal aggregation to collagen 1µg/ml (%)
All
Local
Intermediate
Distant
Distance category
E.
Final aggregation to ADP 3µM (%)
All
Local
Intermediate
Distant
Distance category

Figure 4.7 Effect of distance travelled by sample. X axis for all panels shows all responses, and subcategories of local (testing and venepuncture performed in same city), intermediate (tested from a city less than 100 miles away) and distant (tested from a city 100 miles or more away) testing. Bars represent median and interquartile range. Panel A shows maximal aggregation to ADP 3µM, Panel B shows maximal aggregation to adrenaline 10µM, Panel C shows maximal aggregation to PAR-1 peptide 30µM, Panel D shows maximal aggregation to collagen 1µg/ml, and Panel E shows final aggregation at 5 minutes post agonist addition (allowing for deaggregation) to ADP 3µM. For all panels $p > 0.05$ using Kruskal Wallis test to compare local, intermediate and distant groups. For all panels no p values ≤ 0.05 were obtained using Dunn's multiple comparisons test.

	ADP 3µM	ADP 10µM	ADP 30µM
Maximal aggregation (%)	17-92	56-92	72-98
Final aggregation (%)	0-92	53-92	70-98
Area under the curve (%.s)	12.9-361	234-365	274-395
Time to secretion (s)			15-120
Standardised secretion (nmol ATP/1x10^9 platelets)			0.28-1.93

Table 4.1 Normal responses to ADP. Secretion data shown for highest concentration tested only, as secretion was not measured at lower concentrations. Values stated represent 5th percentile to 95th percentile. Final aggregation measured at 5 minutes post agonist addition.

	Adrenaline 3µM	Adrenaline 10µM	Adrenaline 30µM
Maximal aggregation (%)	13-91	18-93	16-98
Aggregation at 5 minutes (%)	11-91	16-92	14-93
Area under the curve (%.s)	40-305	45-302	30-302
Time to secretion (s)		45-240	60-240
Standardised secretion (nmol ATP/1x10^9 platelets)		0.47-2.19	0.32-2.16

Table 4.2 Normal responses to Adrenaline. Secretion data shown for highest concentrations tested only, as secretion was not measured at lowest concentrations. Values stated represent 5th percentile to 95th percentile. Aggregation to adrenaline was measured for ten minutes post agonist addition. Maximal aggregation reflects the highest percentage aggregation attained during this time.

	Arachidonic acid 0.5mM	Arachidonic acid 1mM	Arachidonic acid 1.5mM
Maximal aggregation (%)	0-94	70-96	70-922
Final aggregation (%)	0-94	70-96	70-92
Area under the curve (%.s)	0-372	235-382	218-362
Time to secretion (s)		0-30	0-53
Standardised secretion (nmol ATP/1x10^9 platelets)		0.30-1.99	0.41-1.82

Table 4.3 Normal responses to Arachidonic Acid. Secretion data shown for highest concentrations tested only, as secretion was not measured at lower concentration. Values stated represent 5th percentile to 95th percentile. Final aggregation measured at 5 minutes post agonist addition.

	PAR-1 peptide 30 µM	PAR-1 peptide 100 µM
Maximal aggregation (%)	31-94	77-98
Final aggregation (%)	10-94	77-98
Area under the curve (%.s)	51-409	298-414
Time to secretion (s)		0-0
Standardised secretion (nmol ATP/1x10^9 platelets)		0.85-3.02

Table 4.4 Normal responses to PAR-1 peptide. Secretion data shown for highest concentration tested only, as secretion was not measured at lowest concentration. Values stated represent 5th percentile to 95th percentile. Final aggregation measured at 5 minutes post agonist addition.

	Collagen 1µg/ml	Collagen 3µg/ml
Maximal aggregation (%)	0-89	69-94
Final aggregation (%)	0-89	69-94
Area under the curve (%.s)	0-302	162-325
Time to secretion (s)		30-60
Standardised secretion (nmol ATP/1x10^9 platelets)		0.37-2.09

Table 4.5 Normal responses to Collagen. Secretion data shown for highest concentration tested only, as secretion was not measured at lowest concentration. Values stated represent 5th percentile to 95th percentile. Final aggregation measured at 5 minutes post agonist addition.

	Ristocetin 1.5mg/ml
Maximal agglutination (%)	25-100
Agglutination at 5 minutes (%)	16-100
Area under the curve (%.s)	60-424

Table 4.6 Normal responses to Ristocetin. Values stated represent 5th percentile to 95th percentile.

4.4 Discussion

The work presented in this chapter defined normal responses to a panel of platelet agonists by lumiaggregometry in 68 healthy volunteers recruited to the GAPP study. Use of Aggrolink® software allowed accurate characterisation of various parameters detailing aggregation and secretion responses. This work builds on previously published data (77, 103) addressing normal ranges in lumiaggregometry. Overall this is an under-researched field, with lack of guidelines on what constitutes a normal response.

Whilst published guidelines recommend testing a parallel control sample when assessing platelet function in a patient (72), they do not comment on the range of normal responses expected in the control sample, thus complicating the interpretation of platelet lumiaggregometry studies in diagnosing a platelet function defect.

My results show that higher concentrations of agonists resulted in narrow reference intervals, but lower agonist concentrations (including some routinely used in current laboratory practice, such as ADP 3 μM and collagen 1 μg/ml) were significantly more variable.

Transporting samples over 100 miles was not found to alter platelet aggregation responses. This is supported by previous work within the GAPP study which found that storage of citrated blood at room temperature for up to 6 hours prior to platelet preparation had a minimal effect on responses to all agonists except for ADP, where a small reduction in aggregation (approximately 20%) was observed (77). This finding is important from a practical viewpoint, as it supports the potential practice of undertaking lumiaggregometry in a relatively small number of specialised haemostasis laboratories, and validates multicentre recruitment to research studies in which lumiaggregometry is assessed.

The findings presented here are relevant in improving the interpretation of lumiaggregometry and in better defining the presence of a platelet defect in patients with excessive bleeding. Based on the pattern of responses observed in healthy volunteers and described here, caution should be exercised in labelling a platelet defect based on an isolated reduced response to a low dose of agonist.

CHAPTER FIVE: COMPILATION AND VALIDATION OF A RATIONALISED AGONIST PANEL FOR LUMIAGGREGOMETRY TESTING (103)

5.1 Introduction

Platelet function testing has historically been poorly standardised, although this has been addressed in some recent publications (72, 73). In addition, it is not subject to quality assurance schemes, is labour intensive and is time critical due to the need to work with fresh samples. Recent documents (72, 73) make suggestions about the agonists and concentrations that should be used when performing aggregometry, but these are discrepant between studies and use concentrations of some agonists that I found to have a wide variation in response amongst healthy volunteers, such as ADP 2.0-2.5µM.

We therefore compiled a "streamlined agonist panel" **(Table 5.1)**, choosing dose points from previous work on healthy volunteers in GAPP including my work as described in **Chapter 4** (77, 125). I compiled this panel along with Marie Lordkipanidzé and Ban Dawood. The dose points included were chosen based on their ability to discriminate between normal and abnormal responses, meaning that dose points with a tight distribution of results in healthy volunteers were preferable, such as ADP 10µM rather than ADP 3µM, which displays a wide range of responses in healthy individuals **(Figure 4.3)**.

Agonist	Concentration	Comments / further guidance
ADP	10µM	Maximal, sustained aggregation expected at this concentration. If reversible aggregation seen use 30µM.
Adrenaline	10µM	Biphasic aggregation expected. If aggregation absent or reduced use 30µM.
Arachidonic acid	1mM	Maximal, sustained aggregation expected. If aggregation reduced or reversible use U46619 3µM.
PAR-1 receptor specific peptide (SFLLRN)	100µM	Maximal, sustained aggregation expected. If aggregation reduced use PAR-4 receptor specific peptide (AYPGKF; 500µM).
Collagen	1µg/ml	If aggregation reduced use 3µg/ml. If this concentration shows reduced aggregation, use collagen related peptide (CRP) 3 µg/ml.
Ristocetin	1.5mg/ml	Maximal, sustained agglutination (often biphasic) expected. If reduced consider vWF profile and GPIb levels by flow cytometry.

Table 5.1 A recommended streamlined panel of agonists for diagnosing platelet function defects. ATP secretion from dense granules should be measured with ADP 30µM, adrenaline 10µM, arachidonic acid 1mM, PAR1 – specific peptide 100µM and collagen 3µg/ml

5.2 Aim

We sought to develop and validate the streamlined agonist panel for platelet function testing by comparing it to an expanded (or original) agonist panel, which included a wider range of agonists and dose points, and had historically been used in the GAPP project.

This work was carried out with Marie Lordkipanidzé and Ban Dawood.

5.3 Methods

Platelet function testing methods are outlined in **Section 2.2.2**. For the purpose of validation of the streamlined agonist panel I was one of two independent experts (along with Marie Lordkipanidzé) who were blinded to previous diagnoses and reviewed data that would have been created using the streamlined agonist panel. A mutually acceptable consensus was reached following discussion for the cases where the independent experts had a difference of opinion. The diagnoses from this process were then compared to the previous historical diagnoses which had been reached using expanded agonist testing.

5.4 Results

Data were available for 107 historical cases, out of which 94 were suitable for full analysis. Inter observer variation was minimal with an agreement of over 90% (kappa statistic 0.829, p value for significance <0.001). There was a significant level of agreement between historical diagnoses and diagnoses reached via the streamlined agonist panel with a Cohen's kappa statistic for agreement of 0.721 (p value for significance < 0.001). In addition, the sensitivity (87%, 95% Confidence Interval (CI) 74%-94%), specificity (86%, 95% CI 71% to 94%), negative predictive value (84%, 95% CI 67% to 93%) and positive predictive value (88%, 95% CI 75% to 88%) of the streamlined agonist panel were all good **(Table 5.2)**. By way of comparison, published sensitivity and specificity values for the bleeding time in patients with congenital platelet disorders are 37% and 88% respectively (126). Estimation of the sensitivity and specificity of the PFA-100 in diagnosing congenital platelet disorders varies widely, with sensitivity reports ranging from 24% to over 80% depending upon the population studied and the severity of the platelet defects that they suffer from (80).

	Expanded agonist panel positive	**Expanded agonist panel negative**	
Streamlined agonist panel positive	45	6	**Positive predictive value 88%**
Streamlined agonist panel negative	7	36	**Negative predictive value 84%**
	Sensitivity 87%	**Specificity 86%**	

Table 5.2 A comparison between the expanded agonist panel and streamlined agonist panel in diagnosing platelet function defects. The data shows a comparison between diagnoses of platelet function defect using the expanded agonist panel and a recommended streamlined agonist panel, displaying sensitivity, specificity, negative predictive value and positive predictive value. Cohen's kappa statistic for agreement was 0.721 ($p<0.001$). 95% Confidence Intervals for Sensitivity, Specificity, Negative Predictive Value and Positive Predictive Value were 74-94%, 71-94%, 67-93% and 75-88% respectively.

This data led to adoption of this panel for prospective platelet function testing in the GAPP study.

5.5 Discussion

In the results presented in this chapter, the use of a rationalised panel of six platelet agonists **(Table 5.1)** has been validated by comparing the diagnoses that would have been created from this panel with the original extended agonist testing profile. The rationalised agonist panel could be used in nearly all clinical testing centres and would serve to guide further sub-typing of inherited platelet function disorders by a specialist laboratory or a clinical research study through functional investigations and targeted genotyping or analysis of whole exome sequencing data.

This panel was devised based on discriminatory platelet aggregation responses in healthy volunteers (77, 103, 125), and may contribute to the published guidelines (72, 73) in assisting clinical laboratories who perform platelet function testing in patients.

Additional functional tests following the rationalised agonist panel application could include an expanded range of platelet agonists and more specialised tests, such as measurement of second messengers (cAMP and Ca^{2+} elevation), TxA_2 formation, shear-based assays of platelet adhesion and aggregation, and flow cytometric measurements of α-granule secretion and glycoprotein receptor levels.

The rationalised agonist panel would also identify nearly all of the 40% of patients in whom a platelet defect was not identifiable by extended agonist testing and where additional testing would appear to have limited value. Identifying patients in whom additional time consuming testing is not of benefit is an important area, and has been further addressed in studies within GAPP looking at the use of 96 well plate testing and remote flow cytometric based testing in identifying which patients should go on to have formal assessment of platelet function by lumiaggregometry (96, 97). Coupling laboratory testing with clinical bleeding history,

including assessment of a bleeding score such as the ISTH BAT score, is clinically important in identifying which patients should have further investigation (127).

CHAPTER SIX: CLASSIFICATION OF PLATELET DEFECTS IN PATIENTS WITH A NORMAL PLATELET COUNT

6.1 Introduction

Even when locally determined normal ranges are available, diagnosing a defect in platelet function often remains a challenge because of overlap with the response of healthy volunteers. There is also the further challenge of interpreting the likely mechanism of the defect because of the feedback effects of ADP and TxA_2 and the fact that many PFDs are associated with a partial rather than a complete loss of a particular receptor or signalling pathway (103). Assessment of the overall pattern of response by an experienced investigator is therefore essential in concluding whether there is a defect in platelet activation, in addition to looking at responses to several agonists. Interpreting defects based on responses to a single agonist (especially those with wide variation in response in the normal population, such as ADP or adrenaline) may lead to an over diagnosis of platelet abnormalities.

Table 6.1 describes typical aggregation and secretion findings in the main categories of inherited platelet disorders.

DISORDER	AGGREGATION FINDINGS	SECRETION FINDINGS
Bernard–Soulier syndrome	Absence of agglutination to high dose ristocetin with preserved responses to other platelet agonists	Unaffected
Type IIB VWD / Platelet-type von Willebrand's disease	Presence of agglutination to low dose ristocetin with preserved responses to other platelet agonists. Exact diagnosis can be established by platelet and plasma mixing studies.	Unaffected
Glanzmann's thrombasthenia	Absence of sustained aggregation to all agonists except ristocetin Shape change and primary wave of aggregation can be seen	Can be reduced as a result of reduced positive feedback from GPIIbIIIa outside-in signalling
Secretion defects	Commonly, absence of secondary wave aggregation to most agonists used in low concentrations, and epinephrine at all concentrations	Significantly reduced levels of ATP secretion, when normalized for platelet count
Thromboxane pathway defects	Absence/severe reduction of aggregation in response to arachidonic acid while response to thromboxane mimetics is preserved (for cyclooxygenase defects) or absence of aggregation response to both arachidonic acid and thromboxane mimetics (for thromboxane receptor defects) Commonly, absence of secondary wave aggregation to most agonists used in low concentrations, and epinephrine at all concentrations	Unaffected
Gi-coupled receptor defects	Reduced aggregation to ADP with notable deaggregation even at high concentrations of agonist Reduced primary wave response to epinephrine and absence of secondary wave	Unaffected

Table 6.1 Typical lumi-aggregometry findings in commonly encountered platelet defects. Reproduced from (4)

At the start of my research fellowship 80 patients had been studied in the GAPP project, of whom 17 (21%) were found to have a secretion defect, 15 (19%) were found to have a G_i-coupled receptor defect, 11 (14%) were found to have a thromboxane pathway defect, and 6 (7%) had other categories of defects including GPVI receptor defects and complex defects. 31 patients (39%) did not have a platelet function defect based on the testing performed.

6.2 Aim

To recruit, test and classify patients with a suspected platelet function defect and normal platelet count within the GAPP project.

6.3 Results

Between October 2010 and March 2013 I tested and reported 88 patients from 10 centres with a normal platelet count and clinical suspicion of an inherited platelet disorder, in addition to testing many other patients whose reports were compiled by other members of the GAPP team. The 88 patients that I tested and reported were enrolled into the "clinically suspected platelet disorder" arm of the GAPP study. Amongst the 88 patients tested, 39 (44%) were not found to have a platelet defect using the modalities available for testing. Of the 49 (56%) who were found to have a defect, most (43/49, 88%) could be classified as having either a dense granule secretion defect, an abnormality in Gi pathway signalling or an abnormality in the cyclooxygenase pathway. The distribution of defects amongst these patients is shown in **Figure 6.1**. By means of comparison, the distribution of defects amongst 111 patients

recruited to the same arm of GAPP had previously been analysed and described (68) and is shown in **Figure 6.2.** Overall this showed very similar findings, with 42% not having a demonstrable platelet defect, and the patients with a defect mostly falling into the categories of dense granule secretion defect, Gi pathway signalling defect and cyclooxygenase pathway defect.

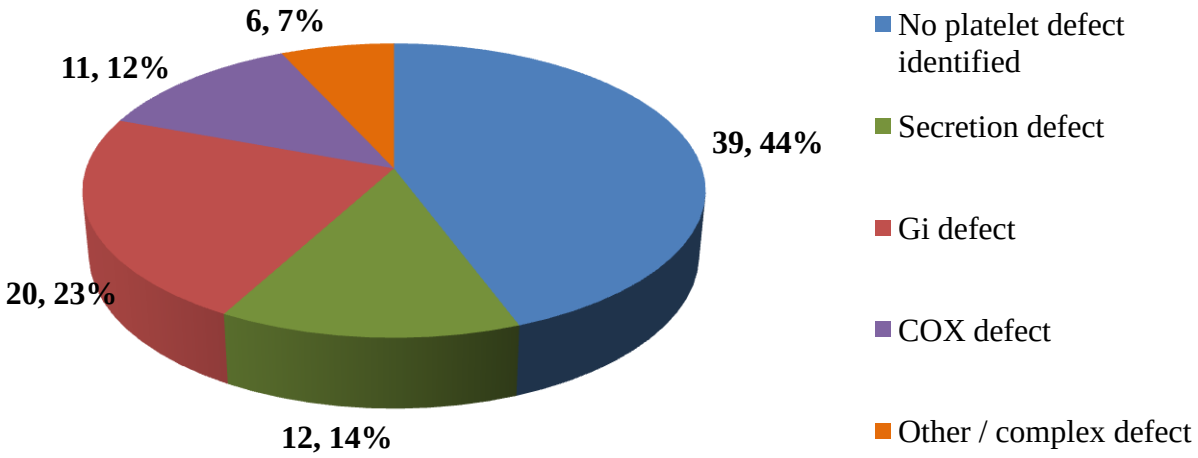


Figure 6.1 Distribution of platelet defects amongst 88 patients I recruited into the "clinically diagnosed platelet disorder" arm of the GAPP project. Segment labels show (absolute number, percentage). COX = cyclooxygenase, Gi = Gi signalling pathway.

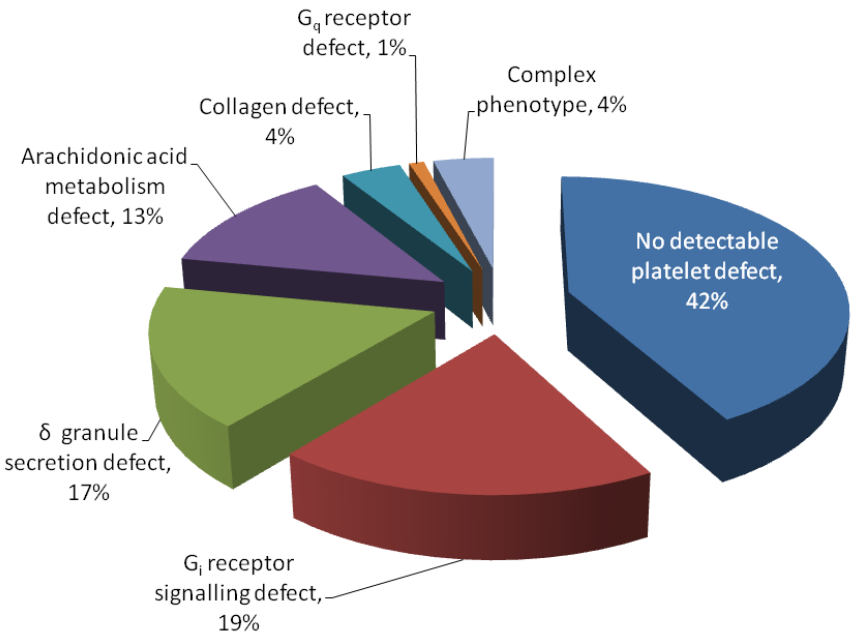


Figure 6.2 Distribution of platelet defects amongst 111 previously studied patients recruited into the "clinically diagnosed platelet disorder" arm of the GAPP project. . Reproduced from (103). δ granule = platelet dense granules.

Example traces from patients found to have the three main categories of defects are shown in **Figures 6.3** (dense granule secretion defect)**, 6.4** (Gi signalling defect) and **6.5** (cyclooxygenase pathway defect).

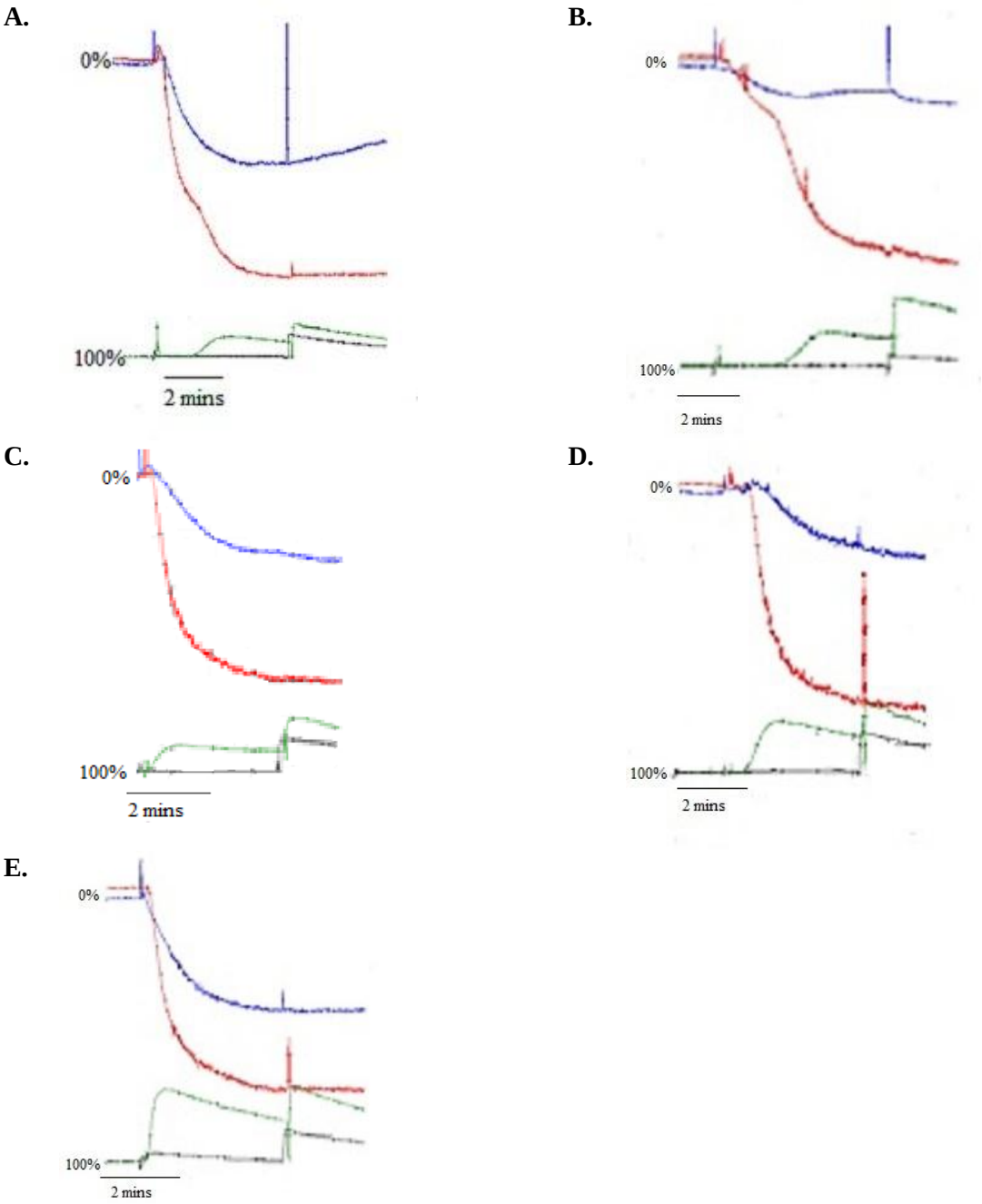


Figure 6.3 Representative images from a patient with a dense granule secretion defect. Horizontal bars represent 2 minutes time. Y axes show percentage aggregation. For all traces patient aggregation is shown in blue, healthy volunteer aggregation is shown in red, patient secretion is shown in black and healthy volunteer secretion is shown in green. Panel A shows response to ADP 30µM, Panel B shows response to adrenaline 30µM, Panel C shows response to arachidonic acid 1.5mM, Panel D shows response to collagen 10µg/ml and Panel E shows response to PAR-1 peptide 100µM. Responses to all agonists are reduced, with deaggregation to high dose ADP and no secondary wave of aggregation seen with high dose adrenaline. Normalised secretion to PAR-1 peptide is 0.34nmol/1x10^8 platelets (5^{th} centile in healthy volunteers = 0.84 nmol/1x10^8 platelets)

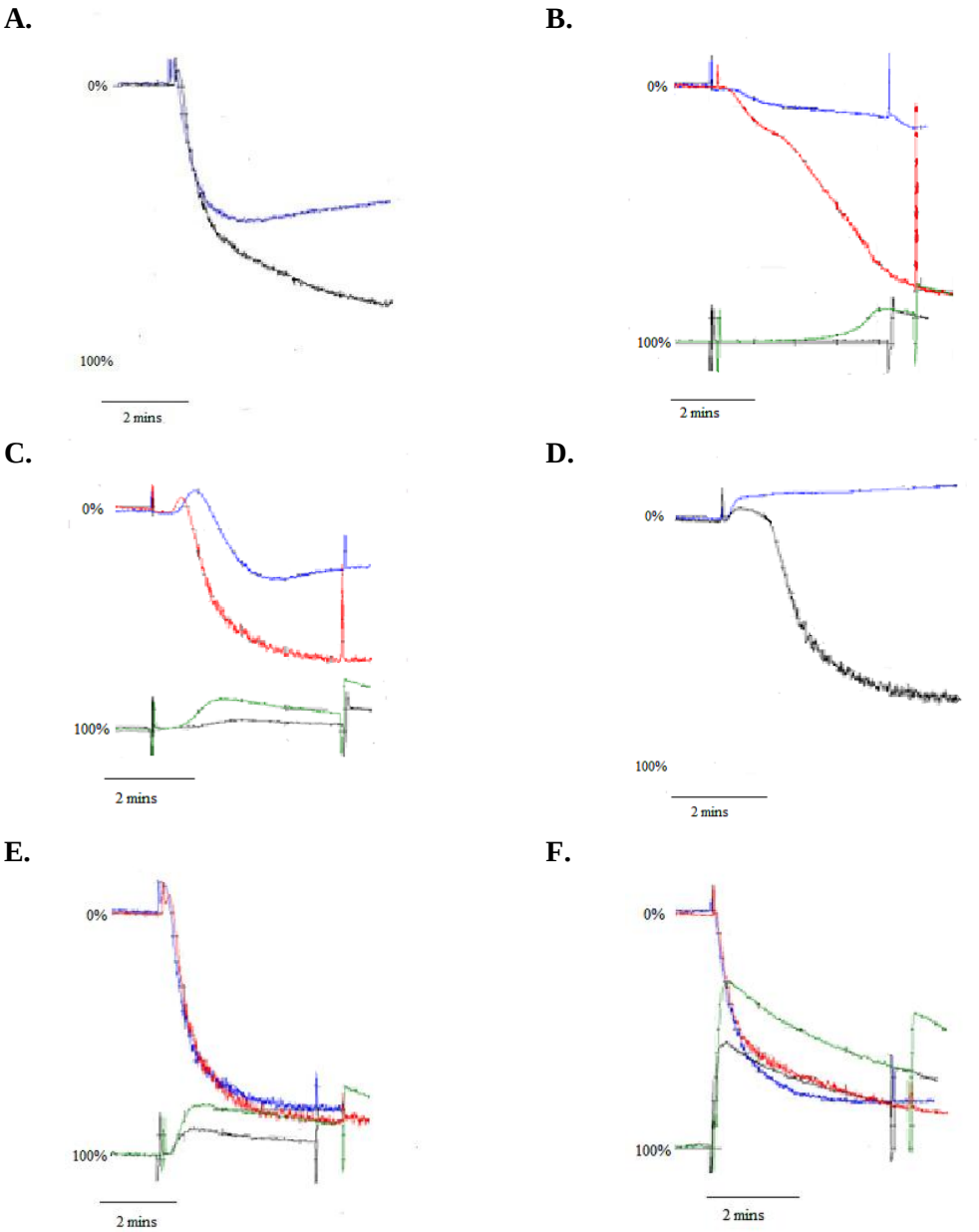


Figure 6.4 Representative images from a patient with a G_i signalling defect. Horizontal bars represent 2 minutes time. Y axes show percentage aggregation. For panels B, C, E and F patient aggregation is shown in blue, healthy volunteer aggregation is shown in red, patient secretion is shown in black and healthy volunteer secretion is shown in green. For panels A and D patient aggregation is shown in blue and healthy volunteer aggregation is shown in black. Panel A shows response to ADP 10µM, Panel B shows response to adrenaline 30µM, Panel C shows response to collagen 3µg/ml, Panel D shows response to arachidonic acid 0.5mM, Panel E shows response to arachidonic acid 1mM and Panel F shows response to PAR-1 peptide 100µM. Deaggregation is seen to high dose ADP, with no secondary wave of aggregation with high dose adrenaline. Response to high dose collagen is reduced with some deaggregation. No aggregation is seen with low dose arachidonic acid, although this normalises at a higher concentration. Response to high dose PAR-1 peptide is normal. Normalised secretion to PAR-1 peptide is within the normal range at 1.40nmol/1x10^8 platelets (5^{th} centile in healthy volunteers = 0.84 nmol/1x10^8 platelets)

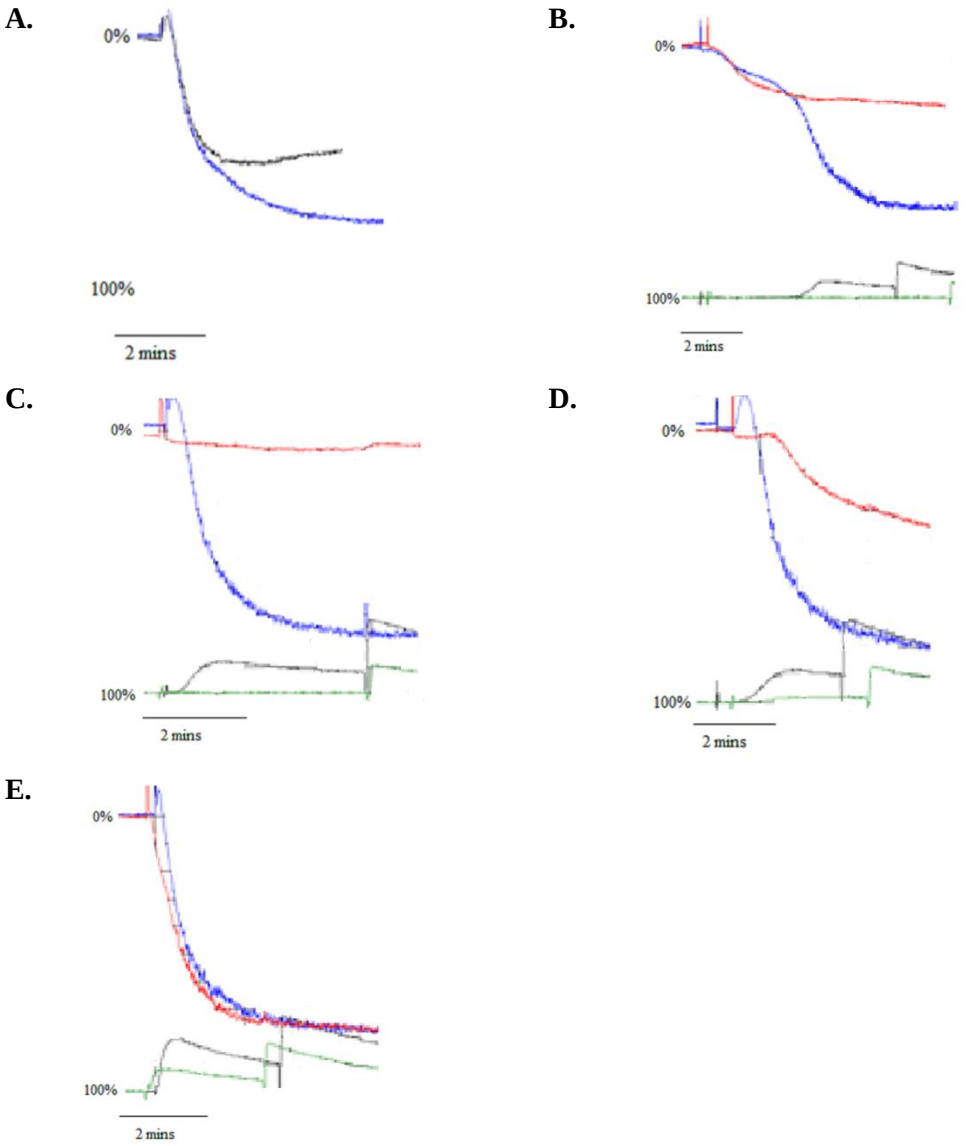


Figure 6.5 Representative images from a patient with a cyclooxygenase pathway defect. Horizontal bars represent 2 minutes time. Y axes show percentage aggregation. For panels B, C, D and E patient aggregation is shown in red, healthy volunteer aggregation is shown in blue, patient secretion is shown in green and healthy volunteer secretion is shown in black. For panels A patient aggregation is shown in black and healthy volunteer aggregation is shown in blue. Panel A shows response to ADP 10µM, Panel B shows response to adrenaline 30µM, Panel C shows response to arachidonic acid 1mM, Panel D shows response to collagen 3µg/ml and Panel F shows response to PAR-1 peptide 100µM. Deaggregation is seen to high dose ADP, with no secondary wave of aggregation with high dose adrenaline. Response to high dose collagen is reduced. No aggregation is seen with high dose arachidonic acid, and this was confirmed on a repeat experiment using arachidonic acid 1.5mM (data not shown). Response to high dose PAR-1 peptide is normal. Normalised secretion to PAR-1 peptide is within the normal range at 0.86nmol/1x10^8 platelets (5^{th} centile in healthy volunteers = 0.84 nmol/1x10^8 platelets). Response to U46619 was not tested by lumiaggregometry due to sample constraints, but was tested using the 96 well plate Optimul assay (96) and this demonstrated a normal dose response curve, suggesting that the defect lies above the level of the thromboxane A_2 receptor.

6.4 Discussion

My work examining patients with a suspected platelet function disorder and a normal platelet count found similar results to those that had previously been studied within the GAPP project (103). In both datasets a defect could not be identified in approximately 40% of recruited patients. In the patients where a defect was seen, three main categories of defect were observed – Gi defect, dense granule secretion defect and cyclooxygenase pathway defect, constituting 41%, 24% and 22% of patients with an identified platelet defect respectively. These categories of defect and their basic laboratory features are also included in national guidelines for the laboratory investigation of suspected platelet function disorders (72). Whilst accurate classification of an inherited platelet function defect does not necessarily change haemostatic management, correct diagnosis *per se* of an inherited platelet function defect enables appropriate surveillance and management of periods of heightened bleeding risk, and ensures appropriate family counselling.

It is interesting to consider the reason for excessive bleeding in the 40% of patients in whom a platelet defect was not identified. It is possible that the testing undertaken within GAPP is not comprehensive enough to capture all inherited platelet defects – for example, defects in procoagulant activity or alpha granule function may not be detected. The work presented in **Chapter 5** suggests that a small number of patients may be labelled with a platelet defect when using extended lumiaggregometry agonist testing who would not have been diagnosed using the rationalised agonist method (7/52 [13%] in our experience).

It is also possible that the patients in whom a platelet defect was not identified have an abnormality in another part of the haemostatic process which is difficult to test, such as vessel wall function and collagen exposure, or fibrinolysis. Clinical noting of excessive bleeding in

patients with connective tissue disorders is well reported, with an example being increased incidence of menorrhagia in patients with Ehlers-Danlos syndrome (128). As bleeding is likely to be a multifactorial trait **(Figure 1.5)**, genetic variants in several parts of the haemostatic cascade may also contribute to an individual's overall bleeding risk when exposed to suitable challenges.

CHAPTER SEVEN: ASSESSMENT OF WHETHER SIMULATANEOUS MEASUREMENT OF LIGHT TRANSMISSION AGGREGATION AND SECRETION POTENTIATES ADRENALINE RESPONSES (129)

7.1 Introduction

A previous report (130) concluded that using lumiaggregometry for simultaneous measurement of LTA and platelet secretion / ATP release (as was our standard practice within GAPP) could potentiate platelet responses to adrenaline. This resulted in the presence of a secondary wave of aggregation to adrenaline in subjects in whom it would not have been expected (such as those with Quebec platelet disorder), and was reported to have a possible impact on incorrect classification of whether a patient has a platelet defect, and on labelling the category of defect.

7.2 Aim

To establish whether simultaneous assessment of secretion using Chronolume® during lumiaggregometry effects aggregation measurements.

7.3 Methods

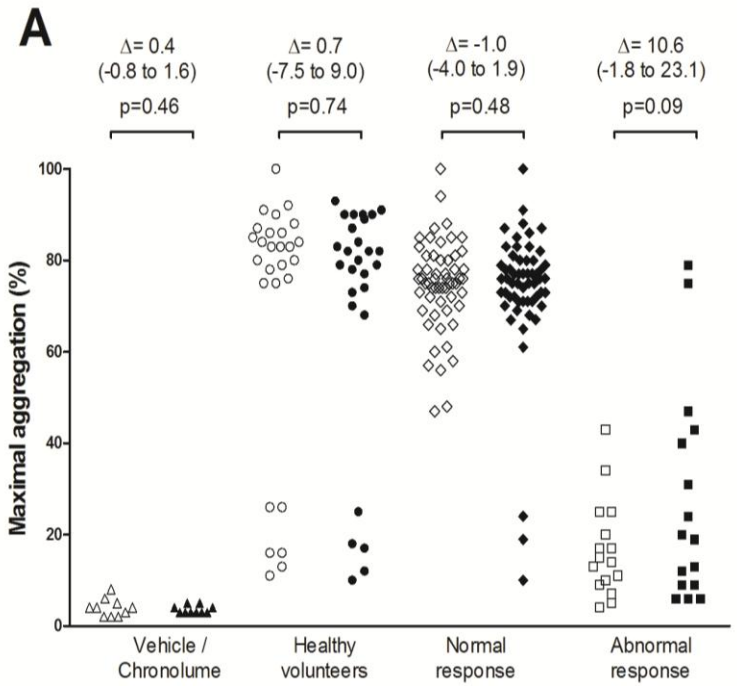
Platelet aggregation measurement without and with Chronolume® was carried out simultaneously in response to adrenaline 10 µM with recording left for up to 10 min. The effect of addition of vehicle (Phosphate buffered saline, PBS) or Chronolume® alone in inducing aggregation was also measured. General methods were as described in **Section 2.2.2**. This work was carried out in conjunction with Marie Lordkipanidzé.

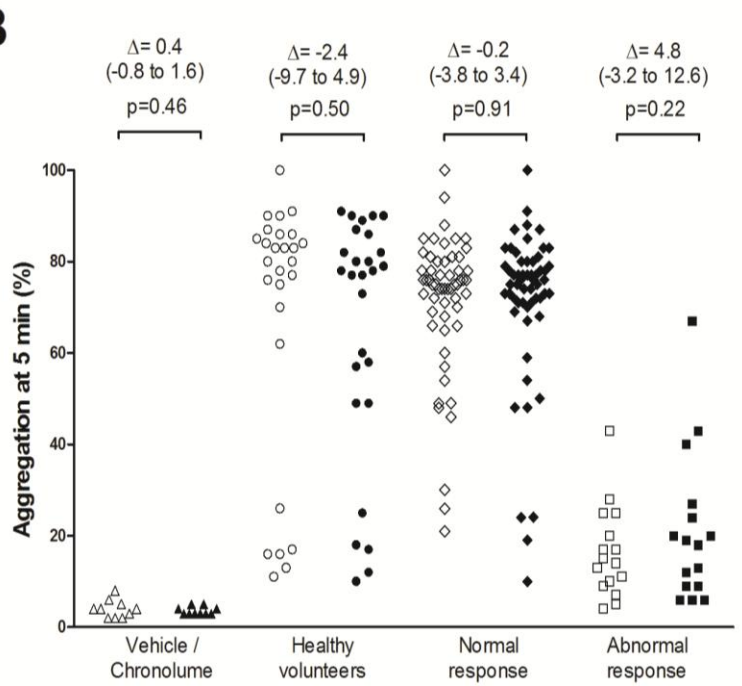
7.4 Results

100 participants were studied, of whom 27 were healthy volunteers and 73 were patients suspected to have a platelet function defect. The median age of healthy volunteers and participants with bleeding symptoms was 29 (interquartile range from 28 to 30 years) and 38 years (interquartile range from 12 to 47 years), respectively. The majority of recruited participants were female (75% among participants with suspected platelet function defects and 74% among healthy volunteers).

A platelet defect was found on platelet function testing in 38% of participants with bleeding symptoms (62% had no demonstrable platelet defect, 16% had a G_i-like defect, 11% had a dense granule secretion defect, 4% had a defect in the thromboxane pathway and 7% had a complex phenotype). As shown in **Figure 7.1**, addition of the vehicle (PBS) or Chronolume® alone did not induce aggregation in 10 healthy volunteers. Moreover, addition of Chronolume® did not result in a significant increase in maximal platelet aggregation or platelet aggregation 5 minutes after addition of adrenaline in healthy volunteers (n=27) or in participants with bleeding symptoms (n=57, 78%) who had a sustained and biphasic response to adrenaline 10 µM. In 16 (22%) participants with bleeding symptoms, there was no secondary wave in response to adrenaline 10 µM. Addition of Chronolume® in this group had no significant effect on maximal platelet aggregation in response to adrenaline 10 µM (mean difference between aggregation levels with vs. without Chronolume® = 10.6%, 95%CI -1.8% to 23.1%, p=0.09). Addition of Chronolume® induced a secondary wave of aggregation in 4 out of 16 participants who had no secondary wave in response to adrenaline in the absence of Chronolume®. However, addition of Chronolume® resulted in abrogation of a secondary wave in 3 out of 57 participants with biphasic aggregation. Overall, the McNemar test

showed no impact of adding Chronolume® on induction of a secondary wave in response to adrenaline 10 µM (**Figure 7.1**, p=0.625 in healthy volunteers and p=1.0 in patients with bleeding symptoms).





C

	No Chronolume ®		
With Chronolume ®		No secondary wave	Biphasic
No secondary wave		4	1
Biphasic		3	19

D

	No Chronolume®		
With Chronolume®		No secondary wave	Biphasic
No secondary wave		12	3
Biphasic		4	54

Figure 7.1 Effect of adding Chronolume® on platelet responses to adrenaline 10 µM in healthy volunteers (n=27), in participants with bleeding symptoms who had a sustained and biphasic response to adrenaline 10 µM (n=57, "normal response"), and in participants with bleeding symptoms who displayed no secondary wave in response to adrenaline 10 µM (n=16, "abnormal response"). A. Maximal aggregation. Open symbols without Chronolume®, closed symbols with Chronolume®. Δ (95% CI) represents the mean difference between aggregation levels with vs. without Chronolume®. B. Aggregation at 5 minutes after addition of adrenaline 10 µM. Open symbols without Chronolume®, closed symbols with Chronolume®. Δ (95% CI) represents the mean difference between aggregation levels with vs. without Chronolume®. C. Cross-comparison of the presence of a secondary wave in response to adrenaline 10 µM in healthy volunteers. McNemar test, p=0.625 D. Cross-comparison of the presence of a secondary wave in response to adrenaline 10 µM in participants with bleeding symptoms. McNemar test, p=1.0

7.5 Discussion

The findings reported above do not support the previously reported observation that Chronolume® consistently induces a secondary wave of aggregation in response to adrenaline 10 µM in patients with an aberrant response to this agonist in the absence of Chronolume® (130). This is in agreement with other findings where no effect of Chronolume® on human platelets was demonstrated, whereas it was shown to potentiate aggregation in response to ADP and collagen in canine, feline, bovine and equine platelets (131). Potentiation of platelet aggregation by Chronolume® had been suggested by previous studies which used a slightly different formulation of the reagent (132, 133); however, absence of effect and even the opposite effect (i.e. inhibition of platelet responses when luciferin:luciferase-containing reagents [including Chronolume®] were used) have also been reported (134, 135).

Closer examination of the patients whose results were discrepant in the presence of Chronolume® in the findings in this chapter revealed that the final diagnosis of the presence of a platelet function defect would not have been affected by using Chronolume® to simultaneously assess ATP secretion alongside aggregation to adrenaline 10µM. In the cases where a secondary wave of adrenaline was induced in the presence of Chronolume®, it was either significantly delayed (>300 sec, which represents the 95th percentile of locally-derived normal ranges) or was accompanied by abnormalities in ADP-induced aggregation (also dependent on appropriate activity of the G_i-coupled $P2Y_{12}$ receptor) (136). The opposite effect, i.e. abrogation of a secondary wave when Chronolume® was added, was seen in one participant labelled as having a G_i-like defect. The interpretation was not affected as abnormalities in ADP-induced aggregation were also noted in the same participant, alongside a shift in dose-response to adrenaline. It is noteworthy that similar differences in the presence

of Chronolume® were seen in healthy volunteers, with either induction or abrogation of a secondary wave in response to adrenaline 10 µM, which suggests that the variation seen in the patients with bleeding symptoms is within the variability of the assay in the normal population, consistent with previous literature (72).

Overall, the results presented above suggest that the presence of Chronolume® may occasionally induce or abrogate the secondary wave of aggregation in response to adrenaline 10µM, but that this effect does not have an impact on either diagnosis of an underlying platelet function defect, or classification of such a defect in participants with a history of excessive bleeding. This supports the practice of using adrenaline in the presence of Chronolume® in the assessment of lumiaggregometry as part of a rationalised agonist panel, as detailed previously (136).

CHAPTER EIGHT: STUDY OF THE ISTH BLEEDING ASSESSMENT TOOL SCORE IN HEALTHY VOLUNTEERS AND IN PATIENTS SUSPECTED TO HAVE A PLATELET DISORDER

8.1 Introduction

Assessing and recording mild bleeding symptoms can be difficult, and several bleeding assessment tools (BATs) have been developed for this purpose, with the aim of standardising the documentation of symptoms of excessive bleeding in patients, allowing comparison between patients and selecting patients in whom further investigations are appropriate (137). This is further compounded by the fact that mucocutaneous bleeding symptoms are reported in a significant proportion of the healthy population, which makes determination of the threshold that constitutes excessive bleeding difficult (138). Initially BATs focussed on patients with von Willebrand disease (139, 140) as it is the commonest inherited bleeding disorder, and later scoring systems were designed for specific patient populations on some occasions, such as children (141-143) or women with menorrhagia (144, 145). The International Society on Thrombosis and Haemostasis Bleeding Assessment Tool was developed specifically to also detect recurrent milder bleeding symptoms (146), and is therefore applicable to patients with some types of inherited platelet disorder. The full questionnaire is included in **Appendix 2**. It is designed to be conducted by a physician, and takes approximately fifteen to twenty minutes to complete. It has been shown to be helpful in assessing children and parents in routine clinical practice, and found to be equally discriminative as a BAT specifically designed for paediatric patients with regard to the prevalence of an underlying bleeding disorder (147). Data is currently being collected into an

ISTH registry to examine bleeding scores using this assessment tool in patients with inherited platelet disorders (127).

8.2 Aims

To assess ISTH bleeding assessment tool scores in patients with and without platelet disorders, and to establish whether a raised score is indicative of an underlying defect.

This work was carried out in conjuction with Marie Lordkipanidzé.

8.3 Results

ISTH Bleeding Assessment Tool (BAT) scores were analysed in 100 participants in GAPP (148).

5.3.1 Participant characteristics

Of the 100 participants studied, 21 were healthy volunteers and 79 were recruited from UK Comprehensive Care Haemophilia Centres with suspected inherited platelet function disorders. The median age of participants with bleeding symptoms was 39 years (Interquartile range [IQR] from 31 to 53 years), while it was 32 years (IQR from 30 to 35 years) in healthy volunteers. The majority of recruited participants were female (81% among participants with bleeding symptoms and 67% among healthy volunteers).

5.3.2 Association of scores with platelet function testing results

A platelet defect was found on lumiaggregometry in 52% of participants with bleeding symptoms, in accordance with our previously published findings (103). The score obtained through the ISTH BAT in participants referred by UK Comprehensive Care Haemophilia Centres with bleeding symptoms (12; IQR 8 to 16) was significantly higher than in healthy volunteers (0; IQR 0 to 0), consistent with the clinical diagnosis of a bleeding disorder. However, there was no significant difference between participants in whom a platelet defect was detected by lumiaggregometry (11; IQR 8 to 16) and in whom platelet function was deemed normal (12; IQR 8 to 14) **(Figure 8.1)**.

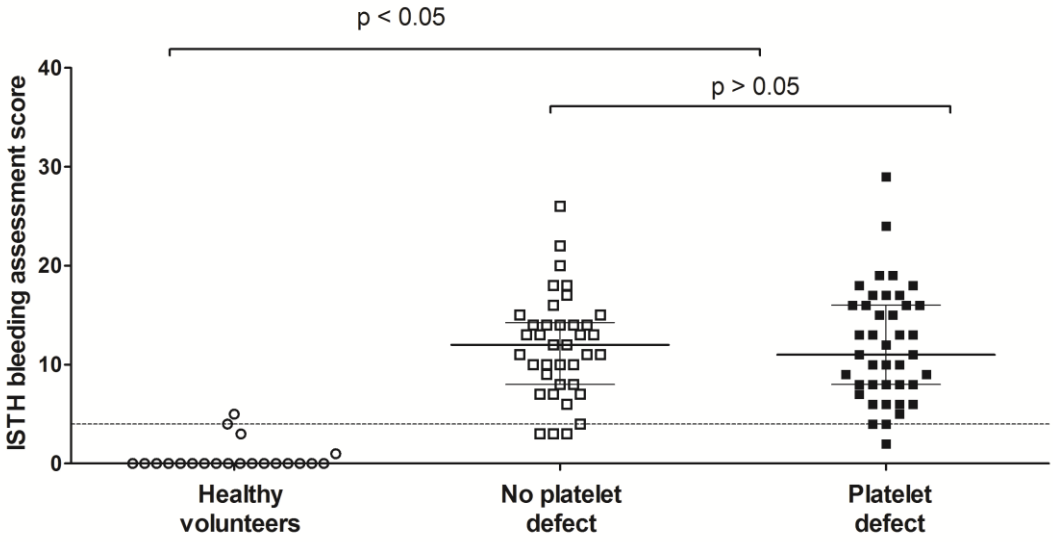


Figure 8.1 Association between presence of a platelet function defect on lumiaggregometry and the ISTH bleeding assessment tool score 95th percentile (score of 4) calculated from healthy volunteers and represented by horizontal dotted line. For the data points the line represents the median and the whiskers represent the interquartile range. Statistical analysis performed by the non-parametric Kruskall-Wallis test, with Dunn's adjustment for multiple comparisons.

There was no association between the type of platelet defect detected by lumiaggregometry (as defined in (103)) and the ISTH BAT score, although the numbers of patients in some diagnostic classification subgroups was relatively small **(Figure 8.2)**.

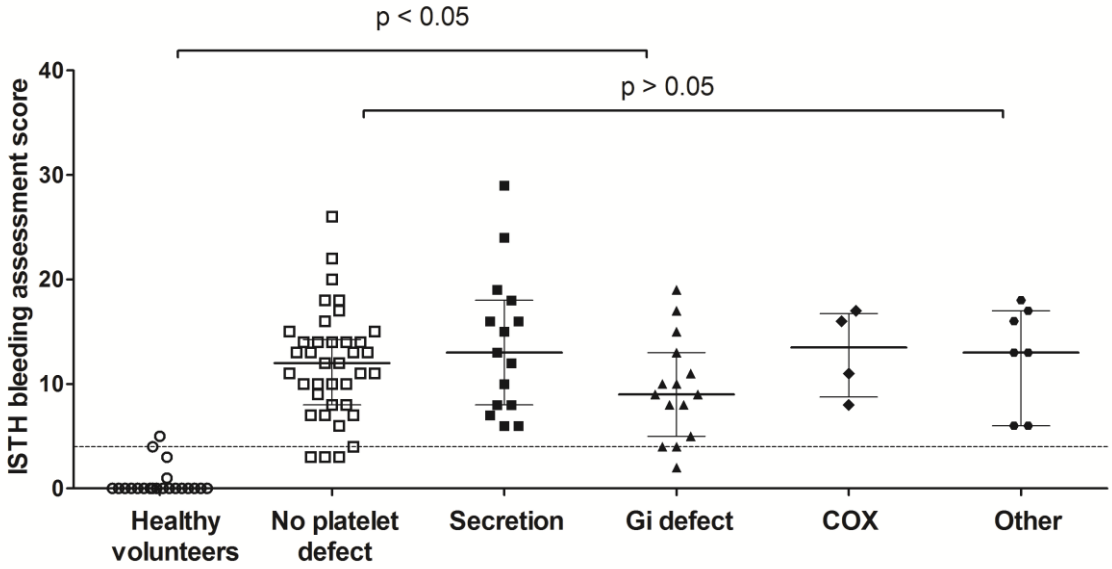


Figure 8.2 Association between type of platelet function defect on lumiaggregometry and the ISTH bleeding assessment tool score 95th percentile (score of 4) calculated from healthy volunteers and represented by horizontal dotted line. For the data points the line represents the median and the whiskers represent the interquartile range. Statistical analysis performed by the non-parametric Kruskall-Wallis test, with Dunn's adjustment for multiple comparisons. COX: cyclooxygenase. For description of types of defects, refer to (103).

A receiver-operator curve (ROC) analysis was carried out to evaluate whether the ISTH bleeding assessment tool could discriminate between patients with and without a demonstrable platelet defect on lumiaggregometry **(Figure 8.3)**. The area under the curve was 0.50 (95% CI 0.37 to 0.63, p=0.98) thus demonstrating a lack of discriminative ability. Moreover, the ISTH BAT score was not associated with a demonstrable platelet defect on platelet function testing in a simple logistic regression model (OR 1.01 [95% CI 0.93 – 1.09], p=0.91).

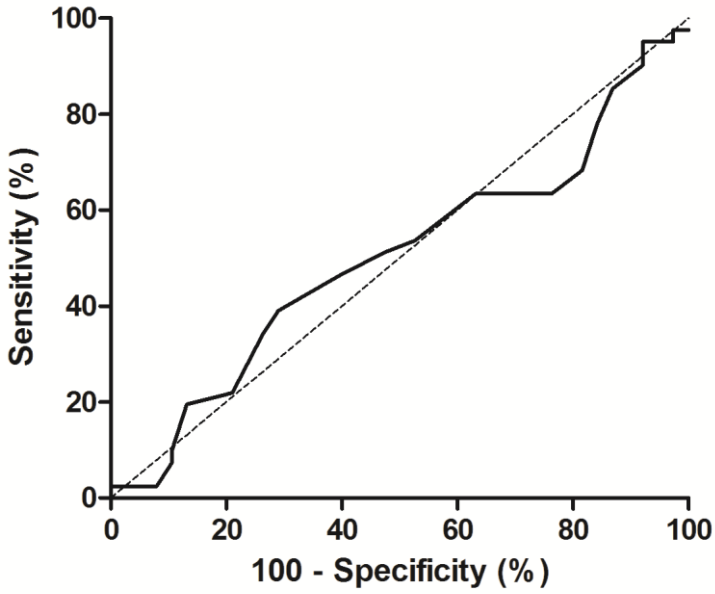


Figure 8.3 Receiver-operator curve for the presence of a platelet function defect on lumiaggregometry and the ISTH bleeding assessment tool score. Area under the curve: 0.50 (95% CI 0.372 to 0.63), p=0.98

Subgroup analyses were performed to examine whether certain bleeding symptoms were predictive of the presence of a platelet defect on lumiaggregometry. ISTH BAT scores for epistaxis, cutaneous bleeding, post operative (including post dental work) bleeding and menorrhagia in women were assessed **(Table 8.1)**. No statistical significance was found for any of these subgroups.

Bleeding symptom	Patients with platelet defect (Median, Interquartile range)	Patients without platelet defect (Median, Interquartile range)	p value
Epistaxis	1, 0-3	1, 0-2	0.67
Cutaneous bleeding	1, 0-2	1, 0-1	0.17
Post operative bleeding (including post dental work)	4, 2-6	4, 2-6	0.89
Menorrhagia	3, 1-4	3, 2-4	0.27

Table 8.1 Subgroup analyses for ISTH bleeding assessment tool score in patients with and without a platelet defect**.** Symptoms assessed were epistaxis (maximum score 4), cutaneous bleeding (maximum score 4), post operative bleeding (including post dental work, maximum score 8) and menorrhagia (in female participants only, maximal score 4). Statistical analyses performed by the non-parametric Mann Whitney U test.

Sensitivity, specificity, positive predictive value and negative predictive value of the ISTH BAT in predicting the presence of a platelet defect on lumiaggregometry was assessed **(Table 8.2)**. A cut off value of 12 was used for the ISTH BAT score as this represented the 50th centile of ISTH BAT score in all patients. This analysis confirmed the absence of association between a high ISTH BAT score and the likelihood of having a platelet defect (κ=-0.04, [95% CI -0.26 to 0.18]).

	Defect present on lumiaggregometry	Defect absent on lumiaggregometry	
BAT score ≥ 12	20	20	**Positive predictive value = 50%**
BAT score < 12	21	18	**Negative predictive value = 54%**
	Sensitivity = 49%	**Specificity = 53%**	

Table 8.2 2x2 analysis of the predictive value of a ISTH BAT score of 12 and over in predicting the presence of a platelet defect on lumiaggregometry. ISTH BAT score of 12 represents the 50th centile in all patients studied. Sensitivity, specificity, positive predictive value and negative predictive value are shown. Kappa statistic for concordance = -0.04 (95% CI -0.26 to 0.18), demonstrating a lack of significance.

8.4 Discussion

The findings presented in this chapter confirm that the ISTH BAT score is raised in patients with clinical evidence of excessive bleeding and a suspected platelet function disorder. The results also show that healthy volunteers have a low score (≤4) in keeping with the absence of a history of bleeding episodes. However, the score is unable to discriminate between patients with clinically diagnosed bleeding disorders subsequently shown to have a platelet defect on lumiaggregometry and those in whom an underlying platelet defect was not detected. This suggests that the ISTH BAT is powerful in documenting bleeding symptoms, but that its level *per se* is not predictive of the likelihood of diagnosing an inherited platelet disorder in patients with excessive bleeding.

It is possible that some patients classified as not having a platelet defect do have a mild defect which is below the limit of sensitivity of our current testing assays, or have a defect in other aspects of platelet function (such as procoagulant activity) which was not tested for in the GAPP project. Given the history of excessive bleeding in all patients, abnormalities in other untested parts of the haemostatic pathway may also account for the clinical presentation of a proportion of the patients in whom a platelet defect was not detected.

The healthy volunteer group included in this study is small (n=21). The 95th centile for BAT score in this group is 4, although defining a more precise upper limit of normal range for the BAT score requires larger studies that ideally examine heterogenous populations. In addition, the median age in the healthy volunteer group was slightly lower than in the patient group. This may have reduced the BAT score in healthy volunteers due to fewer cumulative challenges to the haemostatic system such as surgery or pregnancy and childbirth in women. Previous work has shown that platelet aggregability slightly increases with age (149),

meaning that the aggregation ranges observed in the healthy volunteer group may be lower than that expected in the patients if platelet function was normal. However, this would have biased the results in the patient group towards increased platelet reactivity and therefore should not have led to mislabeling of patients with normal responses as having a platelet defect.

The documentation of bleeding risk in mild bleeding disorders is challenging. Mucocutaneous bleeding symptoms are reported in a significant proportion of the healthy population, which makes determination of the threshold that constitutes excessive bleeding difficult (138). Mild bleeding disorders are prevalent in the general population, reaching up to 1%, but both the clinical diagnosis and laboratory characterization remain complicated (150). The presence of a laboratory phenotype does not necessarily lead to bleeding especially in the absence of a hemostatic challenge and as a consequence most mild bleeding disorders, particularly inherited platelet disorders, remain clinically and biochemically ill defined (150, 151). There is an important clinical need for an assessment tool that would allow clinical characterisation of this population in order to stratify which patients require further investigation.

Numerous scores for bleeding assessment have been proposed over the years (reviewed in (137, 151)). The strength of the ISTH BAT tool is to specifically capture the frequency of mild bleeding symptoms, which are often missed in traditional BATs (152). As such, the ISTH BAT is particularly relevant to patients with recurrent and mild bleeding symptoms, which are a key feature of inherited platelet disorders (150, 151).

There is little prior experience with this tool in mild bleeding disorders. Using a previous version of a BAT based on the European MCMDM-1 VWD study (140) the BAT's clinical

utility in 215 patients with mild bleeding disorders was analysed (153). The findings supported the use of the BAT instrument, as a low bleeding score could reasonably exclude the presence of a mild bleeding disorder in an unselected population, and the utility increased when the BAT score was used in conjunction with laboratory testing (153). The results presented in this chapter agree with these findings, as patients with clinical evidence of excessive bleeding in our study did have significantly higher ISTH BAT scores than healthy volunteers. A further study looked at the clinical utility of the ISTH BAT questionnaire in a routine pediatric setting (147). In this study on 100 children presenting with a mild bleeding diathesis, a low ISTH BAT score made a bleeding disorder diagnosis unlikely, consistent with findings in this study and those of others (147, 153). However, only 5 children were diagnosed with a possible platelet disorder in this study, thus reducing the generalisability to a large cohort of patients with suspected inherited platelet function disorders. Also, given that the ISTH BAT assesses responses to haemostatic challenges, including menorrhagia, childbirth, dental procedures and surgery, the applicability of the scores obtained in children to an adult population is uncertain, as many children will not yet have been exposed to these challenges.

The findings presented here were the first to report on the utility of the ISTH BAT in a large cohort of patients with suspected inherited platelet function disorders. The results supported the use of the ISTH BAT to document recurrent bleeding symptoms in this population (148). However, in this work, the ISTH BAT score was not found to be predictive of which patients have a platelet function defect by laboratory investigation using lumiaggregometry.

CHAPTER NINE: GENETIC TESTING OF PATIENTS IDENTIFIED TO HAVE A PLATELET DEFECT

9.1 Introduction

As outlined in Section 1.5, previous work in the GAPP project has identified the genetic cause of some of the platelet defects phenotyped in patients recruited to the study (33, 103). Identification of the underlying genetic defect responsible for a change in platelet phenotype and a bleeding tendency in the patient helps with both appropriate diagnosis and possible screening of other family members. Use of newer sequencing techniques such as next generation sequencing and whole exome sequencing approaches has increased the opportunities available to identify these underlying defects (as compared to direct Sanger sequencing of possible candidate genes) (154). One of the fundamental aims of GAPP was to identify genetic defects responsible for inherited platelet disorders, and examples of this approach in two patients recruited to the project are outlined here.

9.2 Aim

To undertake genotyping of selected patients found to have an abnormal phenotype to uncover genes involved in platelet function and megakaryopoiesis (using Sanger and second generation sequencing technology).

9.3 Results

9.3.1 Hermansky Pudlak syndrome – identification of second novel mutation in HPS7 gene (109)

A 77 year old Caucasian female was referred to the bleeding disorder clinic with a lifelong bleeding tendency (family tree shown in **Figure 9.1A**). There was no history of a bleeding disorder in this participant's half siblings, parents or children. Her parents were first cousins. She had pale skin and hair and had reduced visual acuity and nystagmus throughout life. She also had spontaneous epistaxes as a teenager which lasted for several hours and required medical attention, and several episodes of prolonged bleeding from minor injuries that required surgical haemostasis. She bled for several days after dental extractions in her twenties and thirties and required packing of the tooth sockets to control the bleeding. She had menorrhagia from menarche which eventually necessitated a hysterectomy at the age of 37. She experienced heavy post partum bleeding after all of her three vaginal deliveries. Surgical procedures in this patient, including surgery for an ectopic pregnancy in the 1960s, abdominal hysterectomy and salpingo-oophrectomy in 1971 and excision of a lipoma from her back in 2002, were followed by prolonged bleeding requiring blood product transfusion. She had two episodes of severe per-rectal bleeding. The first one occurred in 1979 and was attributed to a rectal polyp which was surgically removed. She bled significantly after this procedure and required a platelet transfusion. A further episode occurred in 2009 which was attributed to Crohn's disease. During this episode the patient required red cell and platelet transfusions and tranexamic acid and was being considered for a right hemicolectomy at the time of her haematology referral.

Colonic biopsies showed florid granulomatous inflammation with no co-existing infection (**Figure 9.1B**). Acid fast bacilli were absent. She was reviewed by a respiratory physician in 2010 and had no evidence of any respiratory disease, with normal lung function tests (FEV1 100% predicted, FVC 95% predicted, TLC 80% predicted and KCO 105% predicted, all within normal ranges). A chest X ray showed no active lung disease, and a CT thorax with contrast showed no convincing features of fibrosis.

I recruited the patient to the GAPP study, and performed platelet function testing by lumiaggregometry using Chronolume® to assess secretion. The responses to intermediate concentrations of PAR-1 peptide (30 μM) and collagen (1 μg/ml) were reduced relative to the control tested on the day and also a panel of over 70 healthy volunteers. Normal aggregation was observed at higher concentrations of these agonists (not shown). A lack of dense granule secretion with the entire panel of agonists tested (rationalised agonist panel described in (103)) was noted, as illustrated for PAR-1 and PAR-4 peptides (**Figures 9.1C and 9.1D**). The lack of platelet ATP secretion was consistent with an absence of platelet dense granules, and in combination with the patient's clinical features was diagnostic of Hermansky Pudlak syndrome (HPS).

DNA was extracted from peripheral blood and genetic studies were undertaken by Neil Morgan and Isabel Sanchez Guiu. As the patient's parents were related by blood, they were able to apply autozygosity linkage mapping by genotyping several microsatellite markers flanking all of the known human HPS genes. This was carried out to prioritize a limited number of HPS genes for direct sequencing. Strikingly the only HPS locus that displayed autozygosity for both flanking markers and over an extended region of genetic distance was the HPS7 locus. Therefore the most likely candidate HPS gene was *HPS7/Dysbindin* on

chromosome 6p22.3. The 10 coding exons of *DTNBP1* (encoding dysbindin) including exon-intron boundaries were PCR-amplified and sequenced. Sequencing of *DTNBP1* revealed a homozygous nonsense mutation in exon 4 (c.177 G>A; p.Trp59Stop) confirming a diagnosis of HPS type 7 (**Figure 9.1E**).

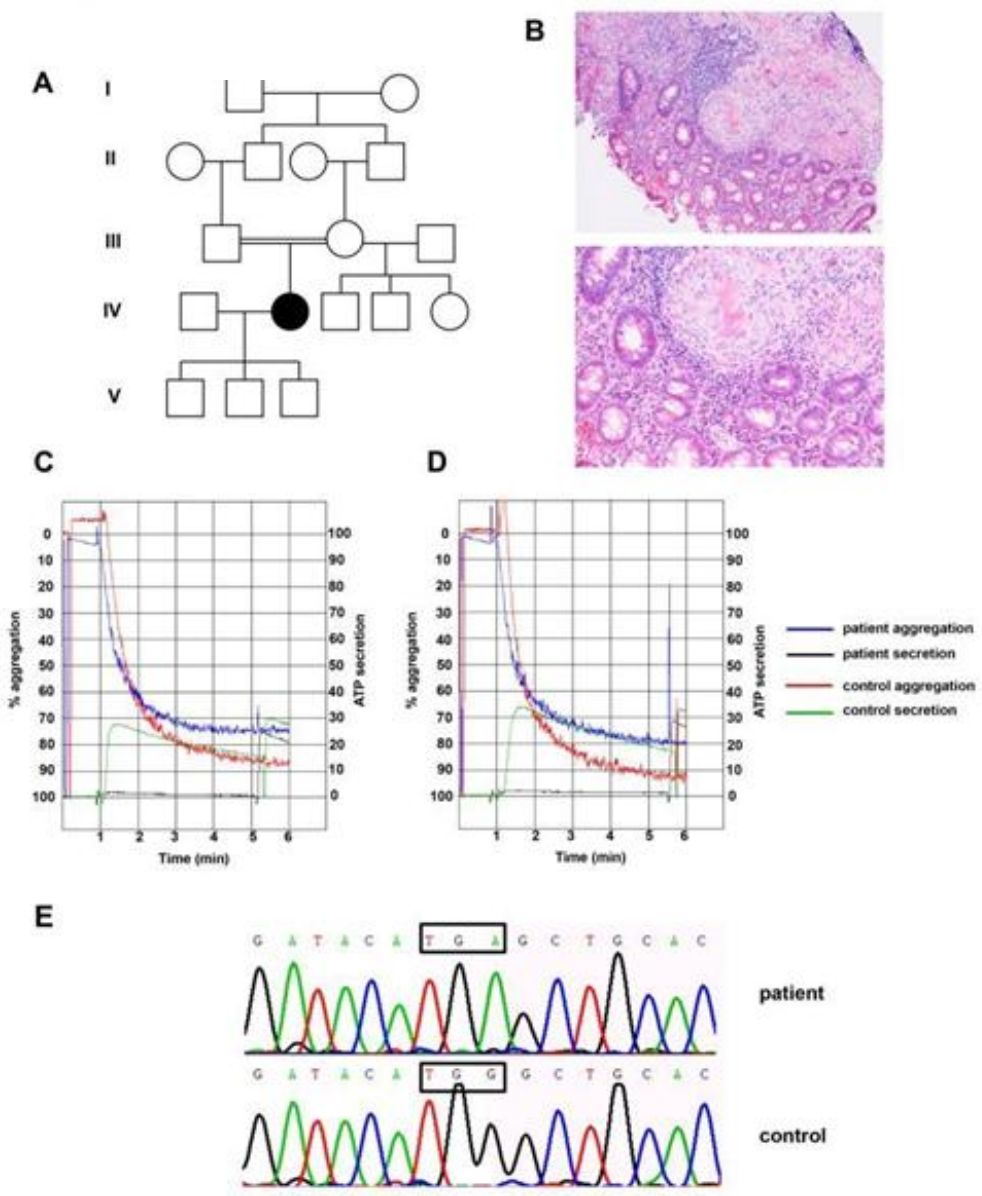


Figure 9.1 Identification of the second HPS7 mutation in a patient with Hermansky-Pudlak syndrome. (Panel A) Pedigree of a consanguineous family with Hermansky-Pudlak syndrome. The affected individual is represented by a solid symbol. (Panel B) Images of a colonic biopsy from the patient in low power (upper panel) and high power (lower panel). Both images show inflammatory infiltrates and granulomata with numerous giant cells, alongside normal bowel mucinous glands. No caseous necrosis is seen, and special stains showed no evidence of micro-organisms (including mycobacteria). (Panels C and D) Absence of secretion (black trace) to high doses of PAR-1 peptide 100µM (Panel C) and PAR-4 peptide 500µM (Panel D) in this patient. Left sided Y axis depicts percentage aggregation, and right sided Y axis represents platelet ATP secretion assessed using Chronolume®. 1.6nmol of ATP standard were added to each cuvette in order to calculate absolute secretion and secretion normalised to platelet count in PRP for both patient and control.
(Panel E) Identification of a homozygous single base substitution (c.177 G>A) in Dysbindin leading to a premature stop codon (p.Trp59Stop). Sanger sequencing showing wild-type and mutant *DTNBP1* sequence traces. The position of the mutation is indicated by the boxed regions.

9.3.2 Identification of a PAR1 receptor polymorphism associated with reduced surface receptor expression in combination with an ADP $P2Y_{12}$ receptor mutation in a family with a history of excessive bleeding (40)

A 44 year old woman (P1) was investigated in the GAPP project. In order to allow her to be recruited I had liaised with her treating physicians and sought NHS Permissions for her treating comprehensive care haemophilia centre. She had a lifelong history of haemorrhagic symptoms dominated by spontaneous epistaxis and recurrent limb bruising, in addition to haemorrhage after surgical challenges and menorrhagia. She had not experienced any life-threatening bleeding episodes and she had no co-morbidities. Her ISTH bleeding assessment tool score was high at 17 (upper limit of normal in healthy volunteers 4). The patient had two sisters with a similar bleeding history. Initial laboratory assessment in her referring hospital revealed a normal platelet count and normal basic coagulation parameters (prothrombin time and activated partial thromboplastin time).

Aggregation testing in this patient was undertaken by Marie Lordkipanidzé. I contributed to interpretation of the aggregation findings, production of the patient's report and reports of other family members, planning further investigation and compiling the findings for publication. Aggregation responses to ADP (**Figures 9.2A** and **9.2D**), adrenaline and PAR1 peptide (**Figures 9.2C** and **9.2D**) were significantly reduced whilst aggregation responses to arachidonic acid (**Figures 9.2B** and **9.2D**), PAR-4 peptide, collagen, collagen-related peptide and ristocetin were normal. Only shape change was seen in response to ADP, even at supra-physiological concentrations as high as 300 µM, which was accompanied by an absence of aggregation in response to a high concentration of adrenaline (30 µM). These results were

indicative of a loss of ADP $P2Y_{12}$ receptor function, but with more a pronounced reduction in PAR-1 peptide aggregation than expected for this type of defect.

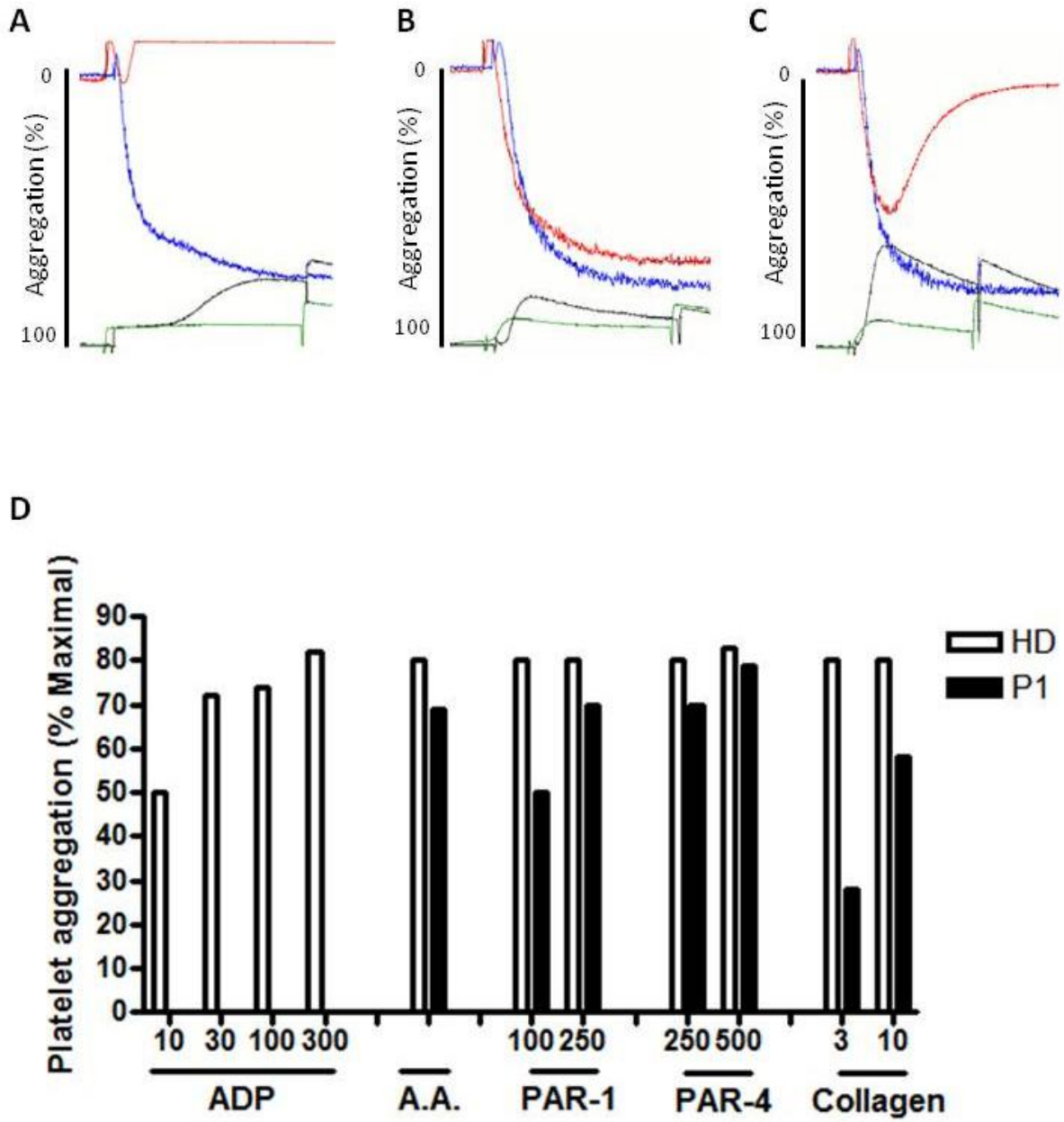


Figure 9.2 Agonist-induced platelet activation in homozygous carrier of the $P2Y_{12}$ R122C mutation. (**A-C**) Representative traces showing platelet aggregation in response to (A) ADP (100 µM) (B) Arachidonic acid (1 mM) and (C) PAR-1 peptide (100 µM) (**D**) Maximal platelet aggregation in response to ADP (10, 30, 100 and 300 µM), arachidonic acid (A.A; 1 mM), PAR-1 peptide (100 and 250 µM), PAR-4 peptide (250 and 500 µM) and collagen (3 and 10 µg/ml). All experiments were in citrated PRP from a healthy volunteer (HD) and from the homozygous carrier of the $P2Y_{12}$ R122C mutation (P1) and are representative of two independent experiments.

Genetic analysis was subsequently undertaken by Neil Morgan to examine both the $P2Y_{12}$ and PAR1 genes. All 3 exons of *P2RY12* were PCR amplified and sequenced, covering the entire 342 amino acid coding region. The patient was found to be homozygous for a novel missense mutation p.Arg122Cys, the result of a c.364C>T substitution. The p.Arg122Cys mutation was found at the start of the 2nd intracellular loop of the receptor found within the DRY motif of this receptor. The exonic and flanking intronic sequences of the *F2R* gene encoding for PAR-1 were amplified from genomic DNA and sequenced on an automated ABI 3730 DNA capillary sequencer. Sequencing of all coding exons and flanking regions did not reveal any novel variations in any of the participants. However the patient was homozygous for the rs168753 single nucleotide polymorphism (SNP). The rs168753 polymorphism is an A>T transversion located in the intervening sequence, 14 nucleotides upstream of the exon 2 start site (IVSn – 14 A/T). The T allele (allelic frequency of 0.14) has previously been associated with decreased expression of PAR1 receptor on platelets, lower aggregation and secretion response to the PAR1 activating peptide, and decreased procoagulant activity (38).

Platelets from three carriers of the R122C mutation, P1 and her sons P2 and P3 were subsequently tested (**Figure 9.3**). Both P2 and P3 were heterozygous for the novel missense mutation p.Arg122Cys in their *P2RY12* gene. Surprisingly, while ADP response was reduced in P3, it was within the normal range in P2 when high ADP concentrations were used (**Figure 9.3B**). At a lower concentration of ADP (10 µM) however, aggregation responses were clearly biphasic (data not shown), whereas this concentration induces maximal sustained aggregation of platelets in control subjects studied previously (as shown in healthy volunteers results section) and is therefore believed to be discriminatory for the presence of an ADP defect. All participants also showed reduced responsiveness to PAR1 peptide (100 µM;

Figure 9.3A), which was disproportionate to their impairment of ADP-induced aggregation (particularly in P2 who had a preserved ADP response). Further genetic analysis by Neil Morgan revealed that all participants were carriers of the rs168753 polymorphism which likely explained their reduced PAR1 receptor responsiveness. This data is summarized in Figure **9.3C**.

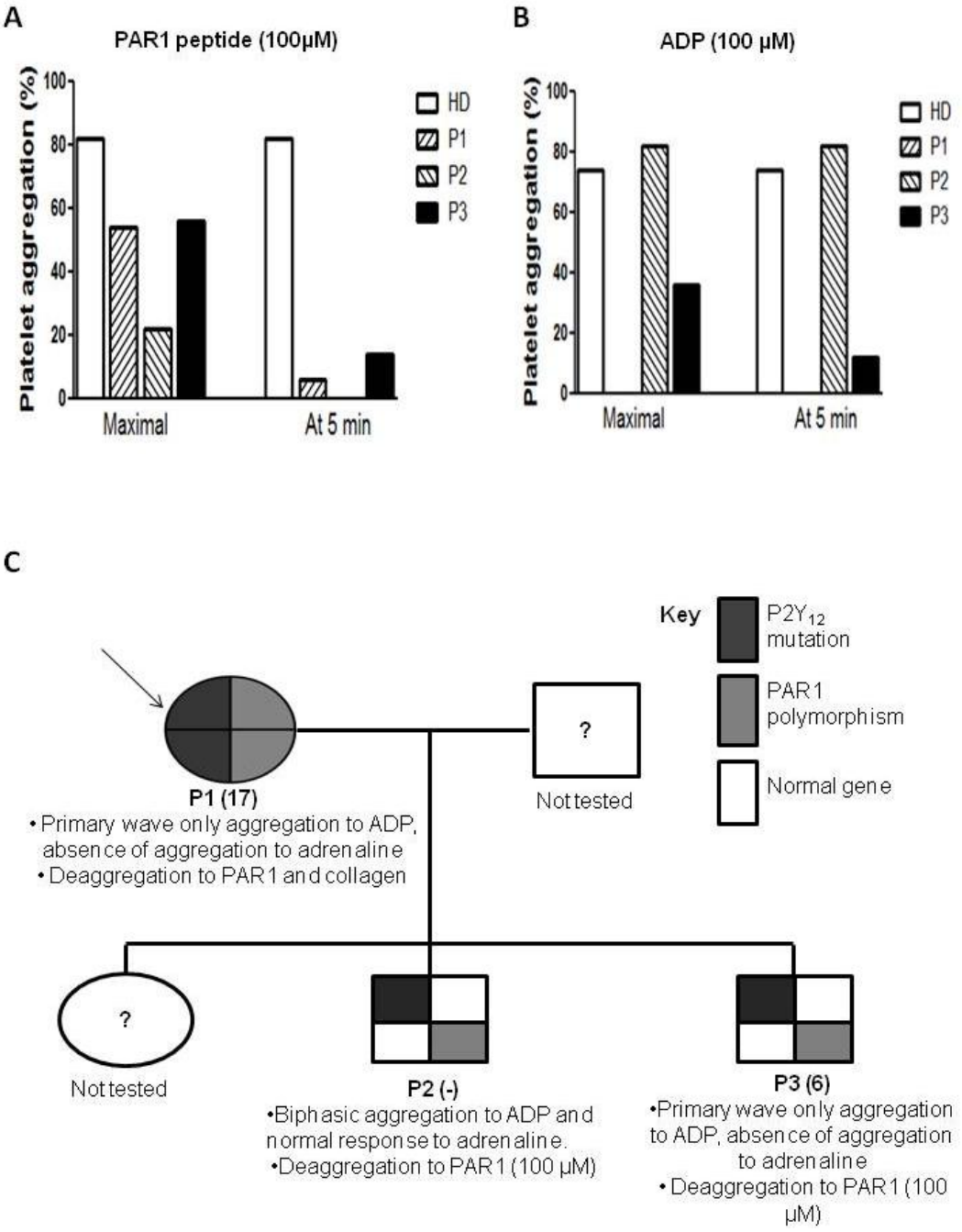


Figure 9.3 Agonist-induced platelet activation in index case and family members. Aggregation of platelets in PRP from a healthy volunteer (HD), index case (P1) or family members (P2 and P3) in response to (A) PAR-1 peptide (100 μM) or (B) ADP (100 μM). Data are expressed as maximal platelet aggregation and aggregation after 5 minutes agonist- stimulation. (C) Inheritance of ADP P2Y12 receptor mutation and PAR-1 defects in family of index case P1. Males are represented by squares and females by circles. The index case is arrowed with summary of platelet phenotyping for P1-P3. Homozygosity for the *P2RY12* 364C>T (R122C) mutation is indicated by black filling on the left side of the symbols, while shading of the right indicates homozygosity for the rs168753 (SNP) mutation in the PAR-1 gene. Patient ISTH bleeding assessment tool scores, if available, are in brackets.

Further work by Stuart Mundell and colleagues in Bristol (a central GAPP laboratory) examined if there were any significant changes in receptor expression as a result of the R122C mutation (40). These results are shown in **Appendix 3**. This shows reduced cell surface expression of the mutant R122C receptor, with resultant intracellular pooling and impaired ability to recycle to the cell surface. As a result of this, the R122C receptor was found to be associated with lysosomal markers, and is therefore rendered a target for degradation.

9.4 Discussion

The cases discussed in this chapter show the potential applicability of autozygosity mapping and phenotype guided direct gene sequencing in determining the genetic cause of platelet defects in patients recruited to the GAPP study. Whole exome sequencing has also been used to look for genetic defects in recruited patients, as discussed in **Section 11.4.3** and published elsewhere (68, 99, 155).

In the first patient discussed in this chapter, a novel mutation in the *HPS7* (Dysbindin) gene causing a premature stop codon was rapidly identified following autozygosity mapping using microsatellite markers. This high-speed technique provided a rapid approach to identify candidate genes for Sanger sequencing. An alternative method to identify the genetic defect in this patient would have been to utilise second generation sequencing strategies such as whole exome sequencing or custom built arrays; however, this would have been far more costly and time consuming given the consanguinity in this family which we exploited to narrow down the culprit gene. Despite having had numerous hospital visits and lifelong excessive bleeding, the cause of this patient's bleeding was not elucidated until she was aged in her eighth decade. Mild inherited platelet disorders should therefore be considered in patients presenting with excessive bleeding later in life.

When mutations have been identified verification of their effect is necessary to establish their functional consequence and to demonstrate causality. An example of this work by Stuart Mundell and colleagues (in the patient discussed in this chapter with an ADP $P2Y_{12}$ mutation) is shown in **Appendix 3**, and shows reduced cell surface expression of the mutant receptor, with impaired recycling to the cell surface and a resultant increase in lysosomal association, increasing the chance of receptor degradation. The co-inheritance of the *F2R* (PAR1)

rs168753 single nucleotide polymorphism (SNP) in this patient and her recruited relatives may explain her disproportionate deaggregation with high dose PAR1 peptide and could contribute to her overall bleeding risk, in keeping with the multifactorial theory of bleeding **(Figure 1.5)**.

Both cases presented here demonstrate the importance of using information obtained from taking a clinical history and from laboratory assessment of platelet phenotype in guiding genetic investigation. Establishing the underlying genetic cause of the increased bleeding seen in the patients described is important in ensuring correct treatment is given for any future periods of high risk of bleeding (such as peri-operatively or after injury) and in family counselling, particularly in families where consanguinuity is common.

CHAPTER TEN: STUDY OF PATIENTS WITH UNEXPLAINED MENORRHAGIA AND INVESTIGATION OF THE CONTRIBUTION OF AN UNDERLYING PLATELET DEFECT

10.1 Introduction

Diagnosing an underlying platelet disorder may be important is those with unexplained menorrhagia (heavy menstrual bleeding), and may significantly change medical management. Menorrhagia is defined as menstrual bleeding lasting more than seven days or with a total blood loss of greater than eighty millilitres (156). Initial secondary care investigations include a coagulation and von Willebrand factor screen and an ultrasound scan of the uterus to exclude fibroids (which are very common and increase in prevalence with age (157)) or other anatomical abnormalities. In a significant number of patients this testing does not reveal an underlying cause.

The World Health Organisation estimates that 18 million women worldwide suffer from menorrhagia (158) and it is perceived as a public health challenge (159). It is common in women of reproductive age, and is most prevalent in those aged 30-49. One in every twenty women in this age group will consult their general practitioner each year due to excessive menstrual bleeding (160). Iron deficiency anaemia is very common in women with menorrhagia and leads to the need for oral iron replacement and reduced quality of life due to lethargy (161). A reduction in work productivity and ability to conduct activities of daily living has also been documented in patients with heavy menstrual bleeding (162).

Assessment of menorrhagia is difficult as blood loss is hard to quantify. The alkaline haematin method of assessing blood loss (156) is impractical and an alternative is now assessment of blood loss using pictorial charts such as the Pictorial Blood Assessment Chart (144). Menorrhagia is often a taboo subject for discussion, especially in some cultural

groups, and patients can therefore present after many months or years of troublesome symptoms. Many women prefer to consult a female doctor with regard to menorrhagia, and in some areas and practices this may not always be an option.

Abnormalities of haemostasis have emerged as an important contributor to the pathology of menorrhagia over the last ten to fifteen years. There is a very wide variation in the estimate of the prevalence of menorrhagia in women with inherited bleeding disorders with an estimate of 5-98% of women with known platelet disorders developing this complication (163). A reduction in quality of life due to menorrhagia is found in these patients (164). Over two thirds of adolescents with known platelet function disorders experience menorrhagia which can result in hospitalisation and blood transfusion (165).

Undiagnosed abnormalities of haemostasis can be an underlying cause in a significant proportion of women with menorrhagia. Amongst newly diagnosed patients. 11-15% are believed to have von Willebrand disease, the commonest inherited bleeding disorder (166). When examining adolescents with menorrhagia the proportion of underlying haemostatic abnormalities may be higher, with one study demonstrating an underlying haemostatic abnormality in over half of young females referred for assessment (167). The original description of von Willebrand disease came from a family where the index case died due to excessive and unstoppable menstrual bleeding (translated and discussed in (168)).

Some studies suggest that as high as 80% of patients have impaired platelet aggregation as a contributing pathology (169). There is a huge variation on the estimated prevalence of abnormal platelet function in this group which may be explained by the fact that studies have taken place across many centres using variable techniques and non standardised classification of what constitutes a platelet defect. Significant variation between hospital sites and racial

origin of patients have been described within studies (170). Menorrhagia has been documented as the presenting symptom in several patients subsequently found to have an inherited platelet disorder (171-173). When focussing on adolescents with menorrhagia, laboratory platelet function abnormalities have been shown in a significant proportion, although the difficulty of non standardised methodology rendering comparison difficult persists (174, 175).

There are no formal guidelines addressing the utility of platelet function testing in this condition and it is therefore not routinely undertaken in investigating patients, although some recent publications have suggested that investigating a possible platelet defect in patients with unexplained menorrhagia may be worthwhile (176, 177). Therefore, the contribution of mild platelet disorders to the pathophysiology of menorrhagia remains unclear. The recommended therapy for women who have failed hormonal treatment of this condition is hysterectomy, which can sometimes take place at a relatively young age (178). In 2012-13, 30 000 hysterectomies were carried out in England, most commonly in women aged between 40 and 50 (179).

A better understanding of the implication of platelet function disorders in the aetiology of menorrhagia and the potential development of platelet targetted therapies may offer an important therapeutic avenue in the treatment of this disease, and could prevent unnecessary early surgical intervention with its associated comorbidities.

10.2 Aim

1. To establish whether there is a significant prevalence of undiagnosed platelet function defects in patients with unexplained menorrhagia.

10.3 Methods

Patients with menorrhagia were studied from two distinct referral pathways:

1. Those who had seen a tertiary gynaecology service (at Birmingham Women's Hospital) due to menorrhagia and had no structural uterine abnormality or coagulopathy on local testing (referred to as having "primary menorrhagia", n=20).

2. Those who had excessive clinical bleeding with menorrhagia as a predominant feature and were referred to UK Comprehensive Care Haemophilia Centres for further investigation (referred to as having "clinically diagnosed" menorrhagia, n=21). Post menopausal women were included in this group when menorrhagia had been their historical predominant bleeding feature.

Inclusion and exclusion criteria are listed below

Inclusion criteria

- Female adult patients, aged ≥ 16 years
- Willing to participate and able to provide informed consent
- Diagnosed with menorrhagia and reviewed in secondary care
- Platelet count within the local laboratory reference range.
- No evidence of structural abnormalities such as fibroids on ultrasound scanning of the pelvis (for primary menorrhagia group)

Exclusion criteria

- Patients taking drugs that are known to influence platelet function, including nonsteroidal anti-inflammatory drugs, aspirin, clopidogrel and dipyridamole within 7 days of enrolment
- Patients known to have a platelet disorder, coagulopathy or von Willebrand disease
- Patients having undergone a major surgical procedure within 1 month of enrolment
- Patients with chronic renal failure requiring dialysis
- Patients with severe anaemia (Haemoglobin < 80g/l)

As some of the patients recruited to the primary menorrhagia arm did not require clinical follow up, recruitment into the trial and blood sampling within patients' homes was offered as an alternative. A risk assessment pertaining to this was completed and assessed by the ethics committee. This option significantly improved accrual to the study following its introduction.

Local coagulopathy screening comprised basic coagulation screens (prothrombin time and activated partial thromboplastin time) and a von Willebrand profile in the clinically diagnosed group (von Willebrand factor levels, von Willebrand factor activity – ristocetin co-factor assay (Ricof) and FVIII:C levels). Von Willebrand profiles were not pre-tested but were checked in the primary menorrhagia group as part of my studies (testing carried out by haemostasis laboratory, University Hospitals Birmingham). A ferritin level was also measured in the local biochemistry laboratory in all patients from the primary menorrhagia group.

An ISTH bleeding assessment tool score was recorded in participants (patients only), and a sample from a healthy volunteer (recruited from the same site as the patient) was studied alongside each patient sample. Further information about ISTH bleeding assessment tool analysis and healthy volunteer recruitment is given in **Section 2.1.2**.

Platelet preparation and platelet function testing was carried out as detailed in **Section 2.2.2**, using the rationalised agonist panel described in **Chapter 5** and detailed in **Table 5.1**.

Statistical analyses was carried out using Microsoft Excel 2007 and GraphPad Prism 4, with testing methodologies as specified in figure legends (dependent upon the data being interrogated). P-values were considered to be significant if they were ≤ 0.05.

Genetic studies were carried out by Neil Morgan and his laboratory team, as detailed in **Section 2.4**.

10.4 Results

10.4.1 Patient cohort

Median age in the primary menorrhagia group was 44 years, with an interquartile range of 38 to 47 years. In the clinically diagnosed group median age was 44.5 years, with an interquartile range of 33 to 56 years (p=0.62 for age difference between the two groups using Mann Whitney U test). There was a wider spread of ages in the clinically diagnosed group as some women were referred later in life, in some cases when they were post menopausal, but gave a clear history of troublesome menorrhagia throughout their reproductive years. The spread of ages in both groups is shown in **Figure 10.1.**

In the primary menorrhagia referral group 19/20 (95%) of patients reported significant impairment in activities of daily living (such as work, housework, exercise and social activities) with most menses. In addition, 6/16 (38%) who had previously had normal vaginal deliveries had experienced post partum haemorrhage. One participant in this group was suspected to have Type 1 von Willebrand's disease, although on repeat testing her von Willebrand profile results were within the normal range. The prevalence of von Willebrand's disease in this group was therefore much lower than that previously reported in the literature (166).

ISTH Bleeding Assessment Tool (BAT) scores were recorded in both groups. The local 95th centile score in healthy volunteers was 4. In patients with primary menorrhagia, the median score was 4.5, with an interquartile range of 3.75 to 7.25. This group had a median score of 3 for the segment of the ISTH BAT specifically relating to menorrhagia (maximum score 4), with an interquartile range of 2.75-3.25. In patients with clinically diagnosed menorrhagia, the median score was 13 with an interquartile range of 10 to 16 (**Figure 10.2**). The higher score in the clinically diagnosed group was statistically significant ($p<0.0001$ using Mann Whitney U test), and was explained by the presence of other bleeding symptoms in this group.

The ISTH BAT score was not predictive of the presence of an underlying platelet defect in either of the referral groups (**Figure 10.3A and 10.3B**, $p=0.54$ and 0.23 for primary menorrhagia and clinically diagnosed groups respectively when comparing BAT score in those with and without a platelet defect, using Mann Whitney U test).

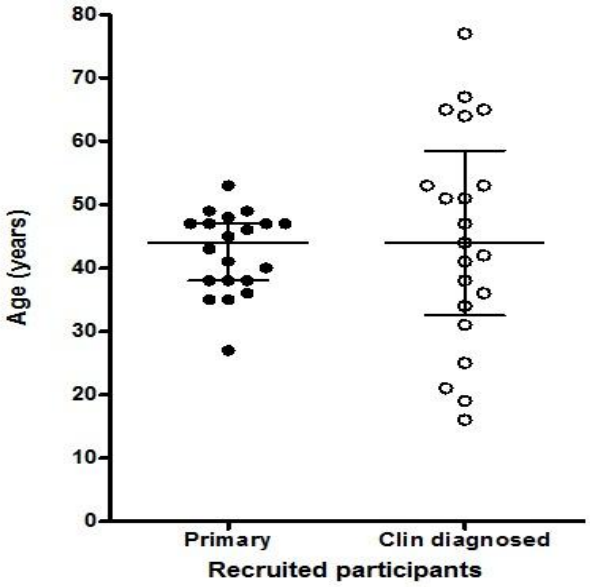


Figure 10.1 Age of patients with menorrhagia by referral group. Bars represent median and interquartile range. $p=0.62$ using Mann Whitney U test.

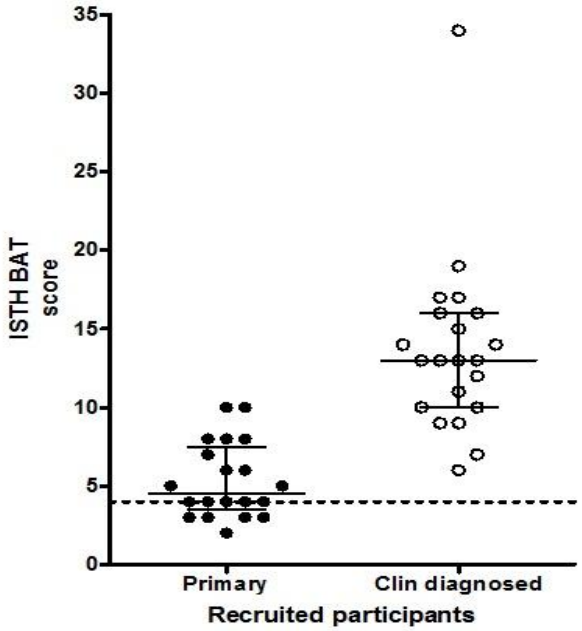


Figure 10.2 ISTH Bleeding Assessment Tool score by referral group. Bars represent median and interquartile range. Horizontal dotted line represents 95th centile of normal BAT score (score of 4) in healthy volunteers. p<0.0001 using Mann Whitney U test.

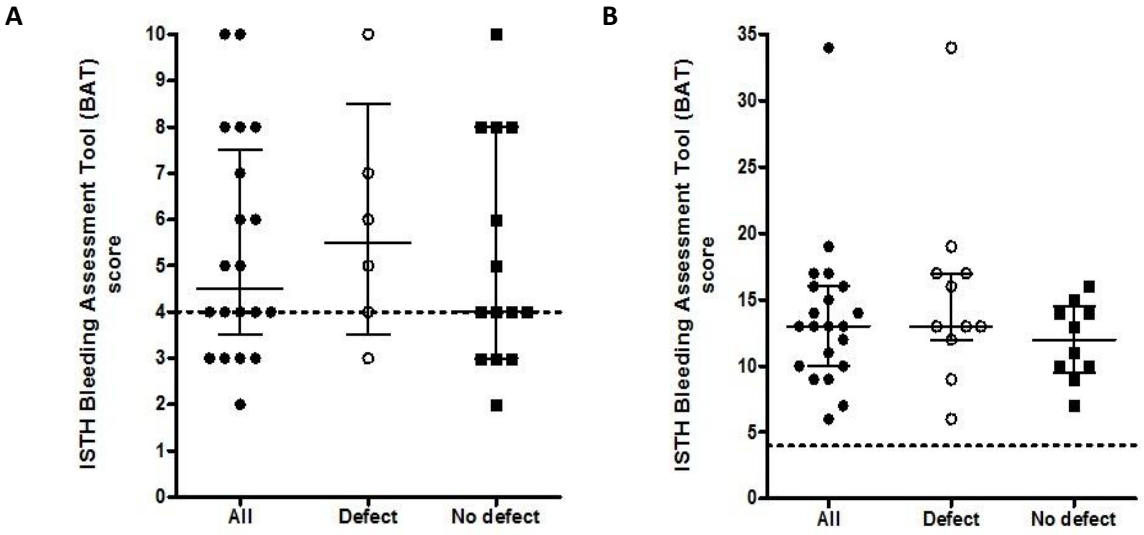


Figure 10.3 ISTH Bleeding Assessment Tool scores in patients with and without a platelet defect on laboratory testing. Panel A shows patients with primary menorrhagia, Panel B shows patients with clinically diagnosed menorrhagia. Bars represent median and interquartile range. Horizontal dotted line represents 95th centile of normal BAT score (score of 4) in healthy volunteers. p<0.0001 using Mann Whitney U test. p=0.54 for Panel A and 0.23 for Panel B when comparing groups with and without a platelet defect using the Mann Whitney U test.

10.4.2 Lumiaggregometry responses

6 participants (30%) in the primary menorrhagia group were found to have platelet defects – one with a dense granule secretion defect, and five with a defect in Gi pathway signalling (with predominantly reduced responses in ADP and adrenaline mediated aggregation). Their aggregation responses are quantified and compared to normal ranges in healthy volunteers in **Tables 10.1, 10.2, 10.3** and **10.4**.

By contrast, 11 participants in the clinically diagnosed menorrhagia group were found to have a platelet defect (52%). These comprised 4 dense granule secretion defects, 4 defects in Gi pathway signalling and 3 cyclooxygenase pathway defects. Their aggregation responses are quantified and compared to normal ranges in healthy volunteers in **Tables 10.5, 10.6, 10.7, 10.8** and **10.9**.

Example clinical histories and laboratory findings from two recruited patients are shown in Figures **10.4** and **10.5**. The patient described in **Figure 10.4** was recruited to the primary menorrhagia arm of the study, and was diagnosed with a G_i defect based on deaggregation to ADP 10μM (resulting in a right shift in the ADP dose response curve) and the presence of only a primary wave of aggregation to adrenaline 30μM. The patient described in **Figure 10.5** was recruited to the clinically diagnosed arm of the study and was found to have deaggregation to ADP 10μM, a small amount of deaggregation to ADP 30μM, primary wave only aggregation to adrenaline 10μM and deaggregation with intermediate dose (30μM) PAR-1 peptide. On the basis of this she was diagnosed with a G_i defect, and genetic sequencing revealed a *P2Y*$_{12}$ polymorphism located in exon 3A (c.36T>G [GGT>GGG], G12G) in this patient and her recruited relatives, with a minor allele frequency of 0.11. No variants were

identified in *F2R* (thrombin receptor gene) in this participant or any of her recruited family members.

The difference in defect rate between the primary menorrhagia and clinically diagnosed menorrhagia groups was statistically significant ($p=0.003$) using a χ^2 test.

Patient code	ADP 3µM max agg (%)	ADP 3µM final agg (%)	ADP 3µM AUC (%.s)	ADP 10µM max agg (%)	ADP 10µM final agg (%)	ADP 10µM AUC (%.s)	ADP 30µM max agg (%)	ADP 30µM final agg (%)	ADP 30µM AUC (%.s)	ADP 30µM time to secretion (s)	ADP 30µM standardised secretion (nmol ATP/1x10^9 platelets)
Normal range (n=68 healthy volunteers, 5th– 95th centile)	**17-92**	**0-92**	**12.9-361**	**56-92**	**53-92**	**234-365**	**72-98**	**70-98**	**274-395**	**15-120**	**0.28-1.93**
Birm-JC-140512	60	60	238	81	81	307	72	72	**219**	60	0.69
Birm-JC-170512	60	60	226	78	78	291	86	86	322	60	0.51
Birm-JC-151012	45	42	189	74	74	297	**69**	**69**	285	105	0.31
Birm-JC-280113	**9**	9	29	58	58	212	73	73	235	90	0.98
Birm-JC-040313	38	32	152	**48**	**45**	**199**	76	76	283	120	0.43

Table 10.1 Gi defects in patients with primary menorrhagia – ADP response. Abnormal values are depicted in **red and bold** text. Agg = aggregation, AUC = area under the curve

Patient code	Adr 3µM max agg (%)	Adr 3µM final agg (%)	Adr 3µM AUC (%.s)	Adr 10µM max agg (%)	Adr 10µM final agg (%)	Adr 10µM AUC (%.s)	Adr 30µM max agg (%)	Adr 30µM final agg (%)	Adr 30µM AUC (%.s)	Adr 10µM time to sec. (s)	Adr 10µM stand. sec. (nmol ATP/ $1x10^9$ platelets)	Adr 30µM time to sec. (s)	Adr 30µM stand. sec. (nmol ATP/ $1x10^9$ platelets)
Normal range (n=68 healthy volunteers, 5^{th} – 95^{th} centile)	**13-91**	**11-91**	**40-305**	**18-93**	**16-92**	**45-302**	**16-98**	**14-93**	**30-302**	**45-240**	**0.47-2.19**	**60-240**	**0.32-2.16**
Birm-JC-140512	Not done	Not done	Not done	**11**	**11**	**38.9**	69	75	142	**No sec.**	**No sec.**	180	0.90
Birm-JC-170512	Not done	Not done	Not done	**7**	**7**	**21.3**	**7**	**7**	**26.2**	**No sec.**	**No sec.**	**No sec.**	**No sec.**
Birm-JC-151012	Not done	Not done	Not done	35	23	69.5	20	17	59.2	**No sec.**	**No sec.**	**No sec.**	**No sec.**
Birm-JC-280113	Not done	Not done	Not done	32	32	82.6	63	55	111	**No sec.**	**No sec.**	210	0.59
Birm-JC-040313	Not done	Not done	Not done	**16**	**16**	52	19	19	61.6	**No sec.**	**No sec.**	**No sec.**	**No sec.**

Table 10.2 Gi defects in patients with primary menorrhagia – Adrenaline response. Abnormal values are depicted in **red and bold** text. Agg = aggregation, AUC = area under the curve, Sec. = secretion, Stand. Sec = standardised secretion.

Patient code	PAR-1 30µM max aggregation (%)	PAR-1 30µM final aggregation (%)	PAR-1 30µM AUC (%.s)	PAR-1 100µM max aggregation (%)	PAR-1 100µM final aggregation (%)	PAR-1 100µM AUC (%.s)	PAR-1 100µM time to secretion (s)	PAR-1 100µM standardised secretion (nmol ATP/1x10^9 platelets)
Normal range (n=68 healthy volunteers, 5th centile – 95th centile)	**31-94**	**10-94**	**51-409**	**77-98**	**77-98**	**298-414**	**0-0**	**0.85-3.02**
Birm-JC-211212	62	62	278	**66**	**66**	**272**	0	**0.71**

Table 10.3 Dense granule secretion defects in patients with primary menorrhagia – PAR-1 peptide response. Abnormal values are depicted in **red and bold** text. AUC = area under the curve.

Patient code	ADP 10µM max aggregation (%)	ADP 10µM final aggregation (%)	ADP 10µM AUC (%.s)	Adr 10µM max aggregation (%)	Adr 10µM final aggregation (%)	Adr 10µM AUC (%.s)	Coll 3µg/ml max aggregation (%)	Coll 3µg/ml final aggregation (%)	Coll 3µg/ml AUC (%.s)
Normal range (n=68 healthy volunteers, 5th centile – 95th centile)	**56-92**	**53-92**	**234-365**	**18-93**	**16-92**	**45-302**	**69-94**	**69-94**	**162-325**
Birm-JC-211212	64	64	236	**13**	**13**	**42.6**	**61**	**61**	**157**

Table 10.4 Dense granule secretion defects in patients with primary menorrhagia –other agonist responses. Abnormal values are depicted in **red and bold** text. AUC = area under the curve.

Patient code	ADP 3µM max agg (%)	ADP 3µM final agg (%)	ADP 3µM AUC (%.s)	ADP 10µM max agg (%)	ADP 10µM final agg (%)	ADP 10µM AUC (%.s)	ADP 30µM max agg (%)	ADP 30µM final agg (%)	ADP 30µM AUC (%.s)	ADP 30µM time to sec. (s)	ADP 30µM stand. sec. (nmol ATP/1x10^9 platelets)
Normal range (n=68 healthy volunteers, 5th centile – 95th centile)	**17-92**	**0-92**	**12.9-361**	**56-92**	**53-92**	**234-365**	**72-98**	**70-98**	**274-395**	**15-120**	**0.28-1.93**
Leic-SP-210512.1	Not done	Not done	Not done	**45**	**42**	**179**	73	73	280	120	**0.15**
Newc-KT-030812.1*	68	68	264	80	80	319	**70**	70	263	120	0.47
Hull-DA-061212.2	36	28	141	**50**	**45**	**191**	**59**	**59**	**245**	**No sec.**	**No sec.**
Hull-DA-220313.1	**12**	8	21	**28**	**16**	**90**	**57**	**55**	**241**	**No sec.**	**No sec.**

Table 10.5 Gi defects in patients with clinically diagnosed bleeding and menorrhagia – ADP response. Abnormal values are depicted in **red and bold** text. Agg = aggregation, AUC = area under the curve, Sec. = secretion, Stand. Sec = standardised secretion. Incomplete data set for Newc-KT-030812.1 as analysis performed on chart recorder and PAP-8E (during remote testing in Newcastle) with no Aggrolink software.

Patient code	Adr 3µM max agg (%)	Adr 3µM final agg (%)	Adr 3µM AUC (%.s)	Adr 10µM max agg (%)	Adr 10µM final agg (%)	Adr 10µM AUC (%.s)	Adr 30µM max agg (%)	Adr 30µM final agg (%)	Adr 30µM AUC (%.s)	Adr 10µM time to sec. (s)	Adr 10µM stand. sec. (nmol ATP/ $1x10^9$ platelets)	Adr 30µM time to sec. (s)	Adr 30µM stand. sec. (nmol ATP/ $1x10^9$ platelets)
Normal range (n=68 healthy volunteers, 5^{th} centile – 95^{th} centile)	**13-91**	**11-91**	**40-305**	**18-93**	**16-92**	**45-302**	**16-98**	**14-93**	**30-302**	**45-240**	**0.47-2.19**	**60-240**	**0.32-2.16**
Leic-SP-210512.1	Not done	Not done	Not done	**10**	**10**	**32**	**13**	**13**	41	**No sec.**	**No sec.**	**No sec.**	**No sec.**
Newc-KT-030812.1*	Not done	Not done	Not done	25	25	Not available	55	55	Not available	**No sec.**	**No sec.**	**No sec.**	**No sec.**
Hull-DA-061212.2	Not done	Not done	Not done	24	24	84	23	23	83	**No sec.**	**No sec.**	**No sec.**	**No sec.**
Hull-DA-220313.1	Not done	Not done	Not done	29	29	91	50	43	121	**No sec.**	**No sec.**	**No sec.**	**No sec.**

Table 10.6 Gi defects in patients with clinically diagnosed bleeding and menorrhagia – Adrenaline response. Abnormal values are depicted in **red and bold** text. Agg = aggregation, AUC = area under the curve, Sec. = secretion, Stand. Sec = standardised secretion.

*Incomplete data set for Newc-KT-030812.1 as analysis performed on chart recorder and PAP-8E (during remote testing in Newcastle) with no Aggrolink software.

Patient code	PAR-1 30µM max aggregation (%)	PAR-1 30µM final aggregation (%)	PAR-1 30µM AUC (%.s)	PAR-1 100µM max aggregation (%)	PAR-1 100µM final aggregation (%)	PAR-1 100µM AUC (%.s)	PAR-1 100µM time to secretion (s)	PAR-1 100µM standardised secretion (nmol ATP/1x10^9 platelets)
Normal range (n=68 healthy volunteers, 5th centile – 95th centile)	**31-94**	**10-94**	**51-409**	**77-98**	**77-98**	**298-414**	**0-0**	**0.85-3.02**
Card-PC-100112.1	84	84	364	89	89	387	0	**0.57**
Leic-SP-240112	84	84	368	92	92	401	0	**0.57**
Birm-JW-050712	53	46	240	**74**	**74**	327	**No secretion**	**0**
Nott-CG-250313.3	64	30	201	88	88	332	0	**0.44**

Table 10.7 Dense granule secretion defects in patients with clinically diagnosed bleeding and menorrhagia – PAR-1 peptide response. Abnormal values are depicted in **red and bold** text. AUC = area under the curve.

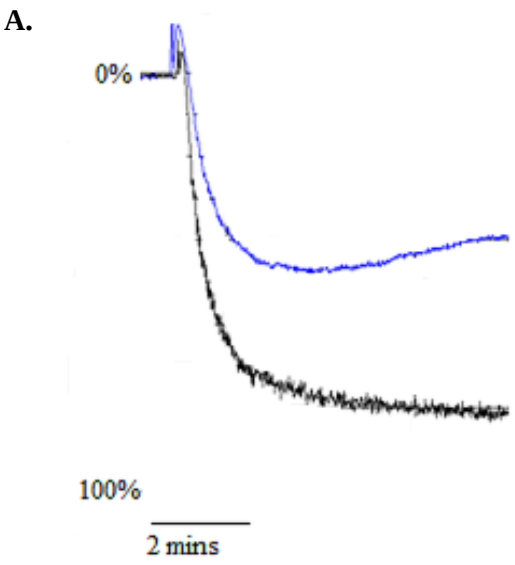
Patient code	ADP 10µM max agg (%)	ADP 10µM final agg (%)	ADP 10µM AUC (%.s)	Adr 10µM max agg (%)	Adr 10µM final agg (%)	Adr 10µM AUC (%.s)	Coll 3µg/ml max agg (%)	Coll 3µg/ml final agg (%)	Coll 3µg/ml AUC (%.s)
Normal range (n=68 healthy volunteers, 5th centile – 95th centile)	**56-92**	**53-92**	**234-365**	**18-93**	**16-92**	**45-302**	**69-94**	**69-94**	**162-325**
Card-PC-100112.1	**49**	**49**	271	65	40	114	82	82	217
Leic-SP-240112	82	82	320	85	78	159	89	89	340
Birm-JW-050712	73	73	303	72	72	258	**65**	**65**	243
Nott-CG-250313.3	71	71	305	20	20	76	**63**	**63**	220

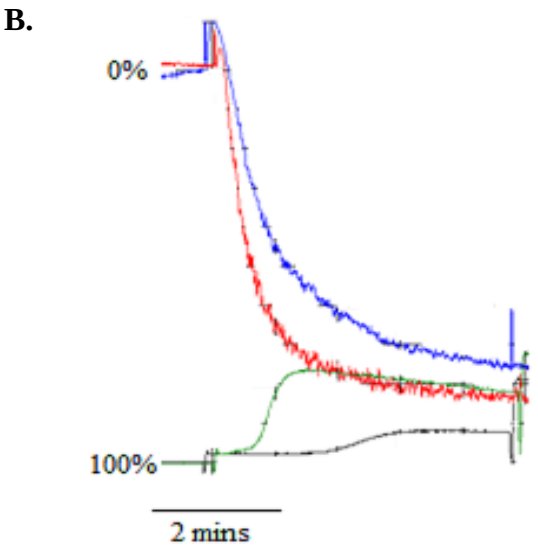
Table 10.8 Dense granule secretion defects in patients with clinically diagnosed bleeding and menorrhagia –other agonist responses. Abnormal values are depicted in **red and bold** text. Agg = aggregation, AUC = area under the curve.

Patient code	AA 0.5mM max agg (%)	AA 0.5mM final agg (%)	AA 0.5mM AUC (%.s)	AA 1mM max agg (%)	AA 1mM final agg (%)	AA 1mM AUC (%.s)	AA 1.5mM max agg (%)	AA 1.5mM final agg (%)	AA 1.5mM AUC (%.s)	AA 1mM time to sec. (s)	AA 1mM stand. sec. (nmol ATP/1x10^9 platelets)
Normal range (n=68 healthy volunteers, 5th centile – 95th centile)	**0-94**	**0-94**	**0-372**	**70-96**	**70-96**	**235-382**	**70-92**	**70-92**	**218-362**	**0-30**	**0.30-1.99**
Nott-CG-291012.1	0	0	0	**68**	**68**	300	Not done	Not done	Not done	**45**	0.36
Birm-JW-180210	Not done	Not done	Not done	**1**	**1**	**6.1**	**2**	**2**	**6.7**	**No secretion**	**No secretion**
Nott-CG-250313.1	Not done	Not done	Not done	**1**	**1**	**49.4**	77	77	321	**No secretion**	**No secretion**

Table 10.9 Cyclooxygenase defects in patients with clinically diagnosed bleeding and menorrhagia. Abnormal values are depicted in **red and bold** text. Agg = aggregation, AUC = area under the curve, Sec. = secretion, Stand. Sec = standardised secretion.

Clinical History: Patient aged 38. She had a history of easy skin bruising and oral cavity bleeding. She bruised easily after venepuncture and had previously experienced ovulation bleeding.
She had experienced menorrhagia for approximately six years at the time of entry to the study (since the delivery of her first child). She had been treated with the oral contraceptive pill and subsequently the levonorgestrel releasing intrauterine device in addition to oral iron therapy She had no family history of excessive bruising and bleeding.





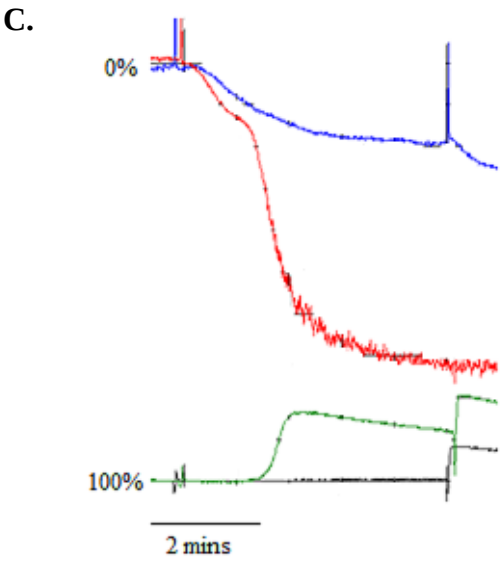
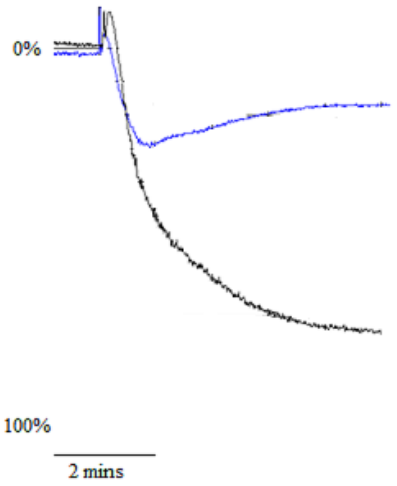


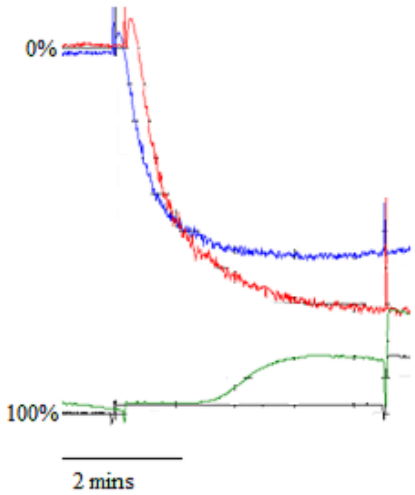
Figure 10.4 Clinical history and lumiaggregometry findings from a patient (Birm-JC-040313) recruited to the primary menorrhagia arm of the study. Horizontal bars represent 2 minutes time. Y axes show percentage aggregation. For Panel A patient aggregation is shown in blue and healthy volunteer aggregation is shown in black. For panels B and C patient aggregation is shown in blue, healthy volunteer aggregation is shown in red, patient secretion is shown in black and healthy volunteer secretion is shown in green. Panel A shows response to ADP 10µM, Panel B shows response to ADP 30µM and Panel C shows response to adrenaline 30µM. Responses to PAR-1 peptide 100µM, collagen 3µg/ml, arachidonic acid 1mM and ristocetin 1.5mg/ml were all normal (data not shown). Normalised secretion to PAR-1 peptide was normal at 1.15 nmol/1x10^8 platelets (5^{th} centile in healthy volunteers = 0.84 nmol/1x10^8 platelets). The patient was diagnosed with a G_i defect based on a right shift in the ADP dose response curve with deaggregation to ADP 10µM, and lack of secondary wave aggregation to adrenaline at both 10µM and 30µM (10µM data not shown here).

Clinical History: Patient aged 65. She had a history of epistaxis, easy bruising, oral cavity bleeding and prolonged bleeding from minor wounds since birth. She required resuturing, packing, and treatment with antifibrinolytics and desmopressin for excessive bleeding after a dental procedure, and experienced severe post-operative bleeding requiring antifibrinolytic treatment, wound packing and blood transfusion. She suffered from menorrhagia since menarche, requiring iron and hormonal therapy, and had emergency hospital admissions on 2 occasions, eventually necessitating hysterectomy. She had post partum haemorrhage after all of her three deliveries necessitating blood product treatment.
Two of her daughters had excessive bleeding and were also recruited to the GAPP study.

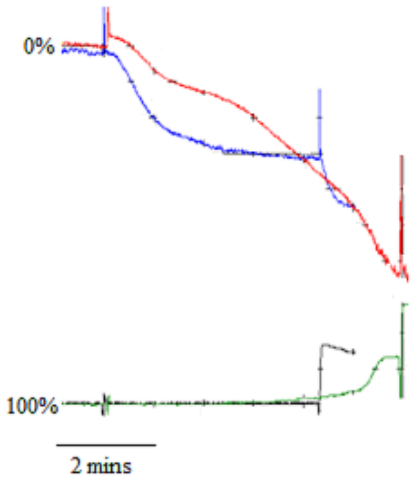
A.



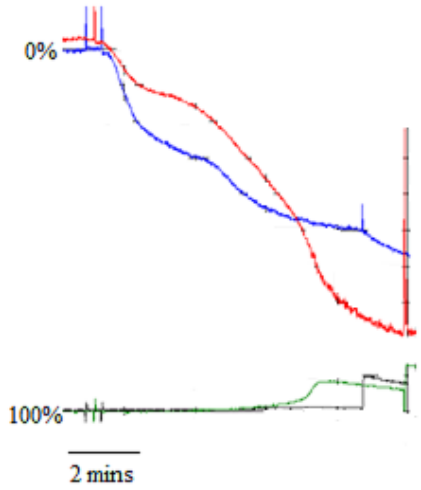
B.



C.



D.



E.

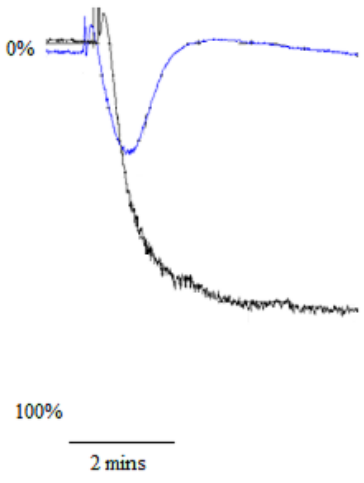


Figure 10.5 Clinical history and lumiaggregometry findings from a patient (Hull-DA-220313.1) recruited to the clinically diagnosed arm of the study. Horizontal bars represent 2 minutes time. Y axes show percentage aggregation. For Panels A and E patient aggregation is shown in blue and healthy volunteer aggregation is shown in black. For panels B, C and D patient aggregation is shown in blue, healthy volunteer aggregation is shown in red, patient secretion is shown in black and healthy volunteer secretion is shown in green. Panel A shows response to ADP 10µM, Panel B shows response to ADP 30µM, Panel C shows response to adrenaline 10µM, Panel D shows response to adrenaline 30µM and Panel E shows response to PAR-1 peptide 30µM. Responses to PAR-1 peptide 100µM, collagen 3µg/ml and arachidonic acid 1mM were all normal. Partial agglutination was seen with ristocetin 1.5mg/ml (data not shown). Normalised secretion to PAR-1 peptide was normal at 0.94 nmol/1x10^8 platelets (5^{th} centile in healthy volunteers = 0.84 nmol/1x10^8 platelets). The patient was diagnosed with a G_i defect based on deaggregation and absence of secretion in response to ADP (up to 30 µM), absence of a secondary wave and secretion in response to adrenaline 10 µM, a delayed secondary wave with absent secretion in response to adrenaline 30 µM and complete deaggregation to PAR-1 peptide 30 µM. Genetic sequencing (by Neil Morgan) revealed a *$P2Y_{12}$* polymorphism located in exon 3A (c.36T>G [GGT>GGG], G12G) in this patient and her recruited relatives, with a minor allele frequency of 0.11. No variants were identified in *F2R* (thrombin receptor gene) in this participant or any of her recruited family members.

10.4.3 Other laboratory assay findings

Values for ferritin, von Willebrand factor antigen, von Willebrand factor activity (Ricof) and FVIII:C activity in the patients with primary menorrhagia are shown in **Figure 10.6**. For all of these parameters no statistically significant difference was seen when comparing patients with and without a platelet defect on laboratory testing. 5/16 (31%) of the patients in the primary menorrhagia group were noted to have iron deficiency.

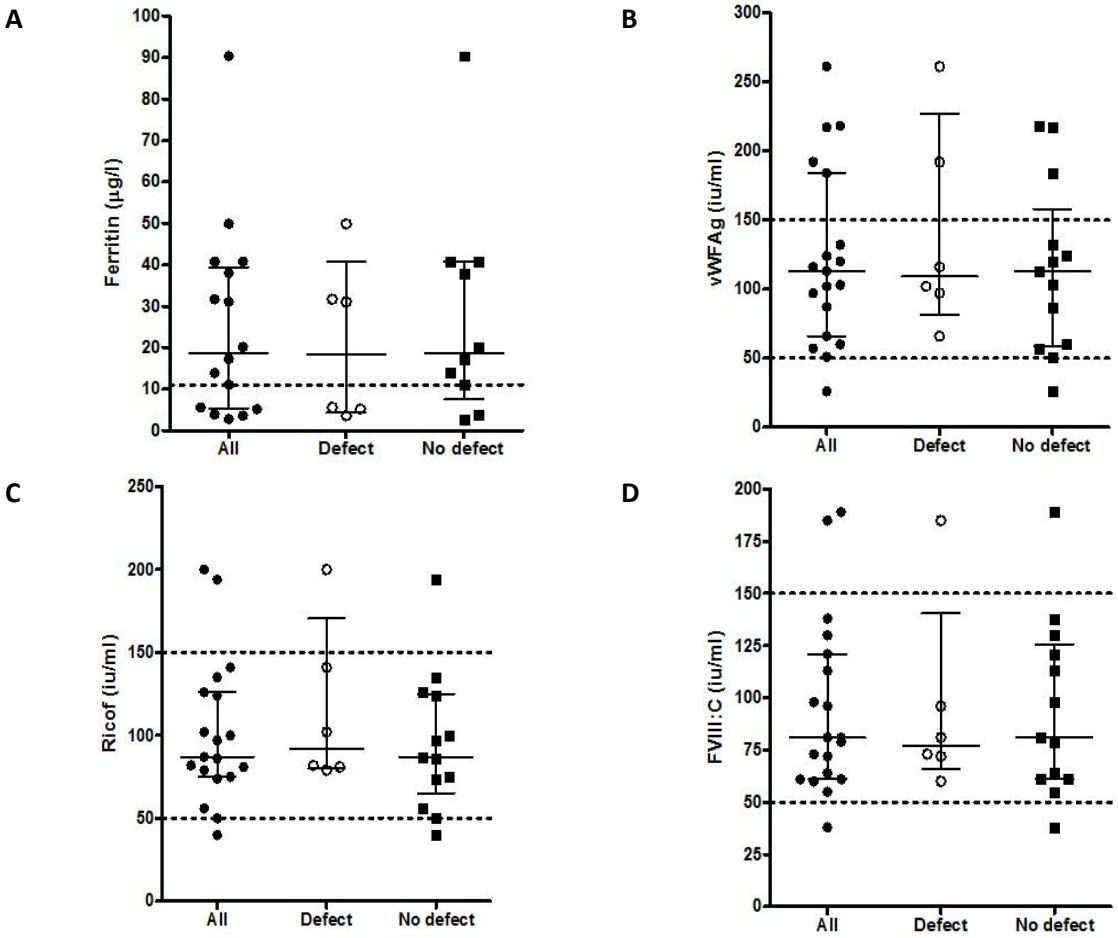


Figure 10.6 Ferritin (Panel A), von Willebrand factor antigen (Panel B), von Willebrand factor activity (Panel C) and FVIII:C (Panel D) values for patients in the primary menorrhagia group. Bars represent median and interquartile range, and horizontal dotted lines represent upper and lower limits of values in healthy volunteers for the laboratory where testing was performed (lower limit only displayed for ferritin). For all parameters no statistically significant difference was seen between patients with and without a platelet defect on laboratory testing (p values 0.72, 0.63, 0.41 and 0.93 for Panels A, B, C and D respectively when using Mann Whitney U test). Five participants were noted to have ferritin values below the lower limit of normal.

10.5 Discussion

The work presented in this chapter supports the hypothesis that a proportion of patients with unexplained menorrhagia have an underlying platelet defect when assessed with lumiaggregometry. In keeping with the previously discussed multifactorial nature of bleeding **(Figure 1.5)**, these defects are likely to contribute to an individual's overall bleeding risk and may be partly responsible for their symptoms.

The prevalence of platelet defects is higher in patients who have other symptoms of excessive bleeding in addition to menorrhagia, and the likelihood of an underlying platelet defect can not be predicted by the ISTH Bleeding Assessment Tool score, supporting the data discussed in **Chapter 8**. Almost all the patients in the primary menorrhagia group had experienced a significant impact on their activities of daily living as a result of their symptoms, highlighting the morbidity associated with heavy menstrual bleeding.

These findings have important clinical implications and reinforce the previously published recommendations that platelet function testing should be part of the investigation pathway for patients with unexplained menorrhagia (176, 177). An update to the clinical guidelines on investigation and treatment of menorrhagia (178) to incorporate this could potentially prevent unnecessary hysterectomy in a small but significant number of patients. Due to the time consuming nature of platelet function testing it would need to be considered after other more basic investigations (such as uterine ultrasound and initial laboratory coagulation screening) had been performed. This potential investigative pathway may avoid the peri- and postoperative complications associated with hysterectomy (180) and could prevent longer term problems such as an adverse effect on quality of life and sexual function in some patients who undergo this procedure (181). Non-surgical management of menorrhagia in patients with

bleeding disorders is the subject of current research, and haemostatic treatment options are available (182).

The low prevalence of von Willebrand's disease in the primary menorrhagia group is an interesting finding and out of keeping with previous reports (166). This may imply that, in those patients where mildly reduced von Willebrand levels and activity have historically been causally attributed to menorrhagia, additional unexplained haemostatic defects may also be present.

One of the limitations of the work I undertook was that the age of patients recruited to both the primary menorrhagia and the clinically diagnosed groups was relatively high. A small number of post menopausal women were included in the clinically diagnosed group, as they gave a clear history of menorrhagia throughout their reproductive years that had often not had an appropriate explanation and for which they had not always sought timely medical attention. It could be hypothesised that younger patients who have consistently suffered from menorrhagia since menarche may have a higher chance of having an underlying platelet disorder, although larger studies would be required to confirm this.

In summary, the work presented in this chapter shows that undiagnosed platelet defects may contribute to the pathology of menorrhagia not explained by conventional investigation, and supports the possibility of including platelet function testing in prospective investigation of this patient group. Identifying an underlying platelet defect in these patients could have significant implications on the treatments offered to them, and would also ensure that they were appropriately managed for any future episodes with a high risk of bleeding, such as surgery or childbirth.

CHAPTER ELEVEN: STUDY OF PATIENTS WITH INHERITED THROMBOCYTOPENIA: DEVELOPMENT OF AN ASSAY TO MEASURE QUALITATIVE PLATELET FUNCTION, AND APPLICATION OF THIS ASSAY TO CHARACTERISE BLEEDING PHENOTYPE IN PATIENTS WITH INHERITED THROMBOCYTOPENIA

11.1 Introduction

Haemophilia centres manage and treat patients with bleeding who have a lifelong inherited low platelet count (thrombocytopenia) with no attributable genetic diagnosis. Inherited thrombocytopenias ranging from moderate to severe are relatively rare but there are a number of patients and families enlisted at UK Haemophilia Centres classified as having these disorders. In 2013-14, 1768 patients were registered as having a platelet defect not attributable to Glanzmann's thrombasthenia or Bernard-Soulier syndrome, but this figure includes those with normal platelet counts and those with thrombocytopenia (183). The number of patients with inherited thrombocytopenia is likely to be underestimated as some will be misdiagnosed as having immune thrombocytopenia purpura. Misdiagnosis of conditions such as *MYH9* disorders occurs in up to half of all cases (184). This misdiagnosis may lead to unnecessary treatments such as steroids or splenectomy which pose their own unpleasant and occasionally potentially life threatening side effects. Underestimation of the numbers in this patient group is also compounded by the fact that some will not be formally registered via a haemophilia centre. In cases other than Bernard Soulier syndrome and the group of *MYH9* disorders (caused by a mutation in myosin-II), the genetic cause is not normally known and a precise diagnosis can therefore not be made. Furthermore, lack of knowledge of the genetic cause means that identification of the defect in family members has

to rely on platelet count and bleeding history.

Much of the available data related to bleeding and platelet count has come from studies on patients with temporary chemotherapy induced thrombocytopenia or acquired autoimmune thrombocytopenia. In many cases, the severity of bleeding cannot be predicted by the reduction in platelet count alone (185) suggesting additional qualitative platelet function defects as important contributors to bleeding in this patient group. However, the assessment of platelet function in this population suffers from the limitation that the current functional testing procedures (namely light transmission aggregometry) do not work with such low platelet counts. Percentage maximal aggregation falls as platelet counts drop below the normal (15, 186). As a consequence, the understanding of the contribution of a functional defect in patients with low platelet numbers is limited, leaving the reason for the wide variability in individuals' platelet counts at which bleeding is observed unexplained.

Various aspects of platelet function can be tested using flow cytometry. This is an attractive technique in thrombocytopenia, as it is independent of platelet count. The principle of it is that the cytometer detects scattered fluorescent light (via use of fluorescently tagged antibodies) emitted by each individual platelet, with signal intensity being directly proportional to antigen density or the size/granularity of the platelet (4). This technique is routinely used in the diagnosis of Bernard-Soulier syndrome and Glanzmann thrombasthenia to confirm absence of platelet surface glycoproteins (187-189). Diagnostic assays are also available for quantifying copy number of any major glycoprotein, studying granular defects, diagnosing heparin-induced thrombocytopenia, and examining defects in platelet aggregation, secretion, or procoagulant activity. When looking for activation markers it is important to choose a molecule not normally present on resting platelets such as CD62P (or P-selectin)

(expressed on the platelet surface following fusion of alpha granules upon stimulation). Some applications of flow cytometry are currently limited to a research context**,** such as its use in monitoring antiplatelet therapy, but could become integrated into clinical practice in the future (190-192).

Whole blood flow cytometry has previously been applied in a research context to patients with autoimmune thrombocytopenia (ITP) and patients with myelodysplasia to compare platelet function at similar levels of thrombocytopenia (193). Platelet activation and microparticle formation have also been assessed using flow cytometry in patients with ITP, myelodysplasia and myeloproliferative disorders in addition to healthy volunteers (194). There is no standardisation on the use of flow cytometry to examine platelet function in patients with inherited thrombocytopenia.

Identification of patients with a qualitative platelet defect as well as a low platelet count could lead to a reduction in the number of prophylactic platelet transfusions in patients with normal platelet function despite a low platelet count, which would therefore limit the risks associated with transfusions in this patient population. When compared with red cell transfusion there is approximately a four fold increased risk of adverse reactions with platelet transfusion. These reactions can include transmission of life threatening infections and development of alloantibodies which can complicate and limit further options for platelet transfusion (195). On a health economic point, reduction in unnecessary platelet transfusion would also save money as platelet units are currently priced around £200 per unit (196).

Current practice is to give prophylactic platelet transfusion to patients with chemotherapy induced thrombocytopenia below a platelet count of 10-20x10^9/l as the risk of life threatening spontaneous bleeding increases below this threshold (197, 198). A multicentre clinical trial

recently concluded that platelet transfusion prophylaxis was still indicated in patients with haematological malignancy, as more bleeding was demonstrated in the no prophylaxis arm that they studied (199). Previous smaller studies looking at omitting prophylactic transfusion during induction chemotherapy for acute myeloid leukaemia and autologous bone marrow transplantation had demonstrated lack of prophylaxis to be a feasible approach (200, 201). In patients with immune thrombocytopenia purpura current guidelines suggest platelet transfusion should be restricted to emergency situations, such as when major active bleeding is present, or when a surgical procedure is imminent (202, 203). In patients with inherited platelet disorders published guidelines recommend platelet transfusion in severe disorders and where other agents have failed, but caution that a clear indication should be present because of the risks of allergy, transfusion transmitted infection and alloantibody formation (71).

Patients with both impaired platelet function and thrombocytopenia may require closer clinical follow up and should be cautioned appropriately about the heightened risk of bleeding, with relevant lifestyle advice such as avoiding close contact sports or activities with a high risk of head injury. Additional treatment options such as thrombopoietin mimetics could then be considered for groups at higher risk. Evidence of patients who have reduced platelet numbers and function and have mild bleeding clinically would also support the idea that bleeding is a complex trait and is multifactorial.

11.2 Aim

To develop a new assay to allow for testing platelet function (ie presence of qualitative defects) in patients with thrombocytopenia.

11.3 Methods

Patients with inherited thrombocytopenia were recruited from Haemophila Centres around the United Kingdom. Inclusion and exclusion criteria are listed below:

Inclusion criteria

- Adult patients, aged ≥ 18 years
- Willing to participate and able to provide informed consent
- Long term thrombocytopenia (platelet count less than $150x10^9$/l)

Exclusion criteria

- Patients with a known additional platelet or coagulation disorder
- Patients previously given treatment for immune thrombocytopenia purpura
- Patients taking drugs that are known to influence platelet function, including nonsteroidal anti-inflammatory drugs, aspirin, clopidogrel, and dipyridamole within 7 days of enrolment.
- Patients having undergone a major surgical procedure within 1 month of enrolment
- Patients with chronic renal failure requiring dialysis
- Patients with severe anaemia (Haemoglobin <80g/l)

An ISTH bleeding assessment tool score was recorded in participants (patients only), and a sample from a healthy volunteer (recruited from the same site as the patient) was studied alongside each patient sample. Further information about ISTH bleeding assessment tool analysis and healthy volunteer recruitment is given in **Section 2.1.2**.

11.3.1 Assay development

Initial experiments focussed on assessing platelet response using flow cytometry to platelet P-selectin (CD62P, marker of platelet α-granules) to a range of agonists. P-selectin was chosen as it is stably expressed in platelets over time. This was assessed by the following method:

10ml of blood from a healthy volunteer (n=10) was taken into 10% by volume 3.2-3.8% trisodium citrate. Platelet rich plasma was prepared as described in **Section 2.2.1** then count adjusted platelets to produce $2x10^7$/ml were made by dilution with phosphate buffered saline (PBS). This was then left for 30 minutes and 45µl of PRP was tested with a panel of agonists as shown overleaf **(Table 11.1)**. The PRP with added agonist was placed in a waterbath at 37ºC for 2 minutes. The reaction was terminated with 250µl ice cold PBS (a five-fold excess of PBS when compared with the total experimental reaction volume).

Agonist	***Conc.***	***Conc.***	***Conc.***	***Conc.***
0 (control)	Nil (PBS vehicle)			
Adenosine diphosphate (ADP)	100µM	30µM	10µM	3µM
Protease activated receptor (PAR)-1 peptide Sequence Ser-Phe-Leu-Leu-Arg-Asn	100µM	30µM	10µM	3µM
Protease activated receptor (PAR)-4 peptide Sequence Ser-Phe-Leu-Leu-Arg-Asn	300µM	100µM	30µM	
U46619	10µM	3µM	1µM	0.3µM
Adrenaline	30µM			
Arachidonic acid	1.5mM	1mM	0.5mM	0.1mM
Collagen related peptide (CRP)	3µg/ml	1µ/ml	0.3µg/ml	0.1µg/ml

Table 11.1 – Agonist concentrations used in initial platelet flow cytometry experiments. Conc. = concentration

5µl of P-selectin (mouse anti-human CD62 antibody, BD Pharmingen) was then added to 25 FACS tubes. 300µl from each reaction volume (the total reaction volume) was added to a FACS tube and incubated in the dark for 45 minutes. Flow cytometric analysis (Becton Dickinson FACS Calibur machine) was then undertaken to establish baselines and gating on the platelet population to record a total of 10000 events. Geometric mean was recorded for each agonist and concentration.

This method was subsequently modified to assess response at three dilutions of PRP (n=6) in the same way as described previously. The concentrations of PRP assessed were neat (undiluted), and 1 in 3 and 1 in 10 diluted with PBS. The reason for assessing several concentrations was to assess if dilution reduced the geometric mean fluorescence intensity. For each dilution the agonist concentrations listed below were assessed:

ADP	0, 3, 10 and 30µM
CRP	0, 0.3, 1 and 3 µg/ml
PAR-1 peptide	0, 10, 30 and 100µM

Responses to these agonists were chosen for assessment as they signal in different ways – CRP predominantly via tyrosine kinases, ADP predominantly via the $P2Y_{12}$ receptor and G_i pathway, and PAR-1 peptide predominantly via the G_q pathway.

At this stage a new Accuri C6 flow cytometer was obtained for future work and was dedicated solely to use with platelets. Due to the nature of this instrument (digital with no logarithmic amplifiers), it was not possible to record geometric mean fluorescence intensity and median

fluorescence intensity was the most appropriate substitute measure. However, it was still possible to compare trends of response between the two instruments.

The PRP dilution experiments (n=4) outlined above were re-conducted using this equipment to check for reproducibility of results. The agonists tested were the same as in the previous PRP dilution experiments. An isotype control (mouse anti human-IgG1κ, BD Pharmingen) was also investigated alongside.

The effects of inhibitors were investigated (n=5) by preparing PRP with DMSO (0.1% final concentration), indomethacin (cyclooxygenase inhibitor, Sigma-Aldrich, Poole, UK) in DMSO (10μM, 0.1% DMSO final concentration) and cangrelor (ADP $P2Y_{12}$ receptor inhibitor, the Medicines Company, Abingdon, United Kingdom) in DMSO (1μM, 0.1% DMSO final concentration). Each of these batches was tested neat and diluted 1 in 3 and 1 in 10 with the agonists as per the original dilution experiments.

This approach was extended to PAC-1 (activated glycoprotein $\alpha II_b\beta_3$, Becton Dickinson) and fluorescent fibrinogen (DAKO cytomation polyclonal rabbit anti human fibrinogen-FITC). For each dilution 4 groups were studied:

- PAC-1 added pre agonist
- PAC-1 added post agonist
- Fluorescent fibrinogen added pre-agonist
- Fluorescent fibrinogen added post agonist

The agonists studied for each dilution and group were ADP 30μM and PAR-1 100μM.

11.3.2 Investigation of patients with inherited thrombocytopenia

10ml of blood from recruited patients was taken into 10% by volume 3.2-3.8% trisodium citrate, along with blood samples from a healthy volunteer recruited at the same site. Platelet rich plasma was prepared as described in **Section 2.2.1**.

11.3.2.1 Resting expression levels

Surface receptor expression levels of CD42b (BD Pharmingen, FITC mouse anti-human CD42b) CD41 (DAKO, FITC mouse anti-human CD41) and GPVI (Gift from Elizabeth Gardiner, Monash University, Australia, PE conjugated) were assessed by incubating 45µl of PRP with 5µl of antibody and allowing to develop in the dark for 45 minutes. A zero value using 5µl of PBS alone and an isotype control (mouse anti human-IgG1κ, BD Pharmingen) were also recorded. Reactions were terminated with 250µl ice cold PBS. Flow cytometric analysis (Accuri C6 machine) was then undertaken to establish baselines and gating on the platelet population to record a total of 10000 events. Median fluorescence intensity was recorded for each incubation.

11.3.2.2 P selectin responses

P selectin responses were then assessed by testing aliquots of 45µl of PRP from both the recruited patient and healthy volunteer with a panel of agonists as listed below:

Zero value	PBS alone
Isotype control	PBS alone
ADP	3µM and 30µM
CRP	0.3µg/ml and 3µg/ml
PAR-1 peptide	10µM and 100µM

The PRP with added agonist was placed in a waterbath at 37ºC for 2 minutes. The reaction was terminated with 250µl ice cold PBS (a five-fold excess of PBS when compared with the total experimental reaction volume).

5µl of P-selectin (mouse anti-human CD62 antibody, BD Pharmingen) was then added to FACS tubes. 300µl from each reaction volume (the total reaction volume) was added to a FACS tube and incubated in the dark for 45 minutes. Flow cytometric analysis (Accuri C6 machine) was then undertaken to establish baselines and gating on the platelet population to record a total of 10000 events. Median fluorescence intensity was recorded for each agonist and concentration. 5µl of isotype control antibody (mouse anti human-IgG1κ, BD Pharmingen) was used for the isotype control assessment (in place of anti-CD62P).

Median fluorescence intensities obtained were then compared to the platelet count equivalent healthy volunteers dilution experiments results in order to determine whether a possible qualitative platelet defect was present.

11.3.2.3 Fluorescent fibrinogen responses

Fluorescent fibrinogen responses were assessed by testing aliquots of 45µl of PRP diluted 1 in 10 with PBS from both the recruited patient and healthy volunteer with the same panel of agonists as listed for the P selectin response testing. Isotype control values were not recorded for fluorescent fibrinogen. As per the pilot experiment data, 5µl of fluorescent fibrinogen (DAKO cytomation polyclonal rabbit anti human fibrinogen-FITC) was added to the diluted PRP prior to agonist addition. The diluted PRP with added fluorescent fibrinogen and agonist was placed in a waterbath at 37ºC for 2 minutes. The reaction was terminated with 250µl ice cold PBS (a five-fold excess of PBS when compared with the total experimental reaction volume). The reaction volumes were incubated in the dark for 45 minutes. Flow cytometric analysis (Accuri C6 machine) was then undertaken to establish baselines and gating on the platelet population to record a total of 10000 events. Median fluorescence intensity was recorded for each agonist and concentration. Median fluorescence intensities obtained were then compared to data accrued from all healthy volunteers in order to determine whether a possible qualitative platelet defect was present.

11.3.2.4 Statistical analyses and genetic testing

Statistical analyses was carried out using Microsoft Excel 2007, GraphPad Prism 4 and IBM SPSS Statistics 19, with testing methodologies as specified in figure legends (dependent upon the data being interrogated). P-values were considered to be significant if they were ≤ 0.05.

Genetic studies were carried out by Neil Morgan and his laboratory team, as detailed in **Section 2.4**.

11.4 Results

11.4.1 Assay development

11.4.1.1 Assessement of platelet rich plasma response to a variety of agonists using flow cytometry to P-selectin (mouse anti human-CD62P)

A total of 10 healthy volunteers were assessed using methods as described above. Results are shown in **Figure 11.1.**

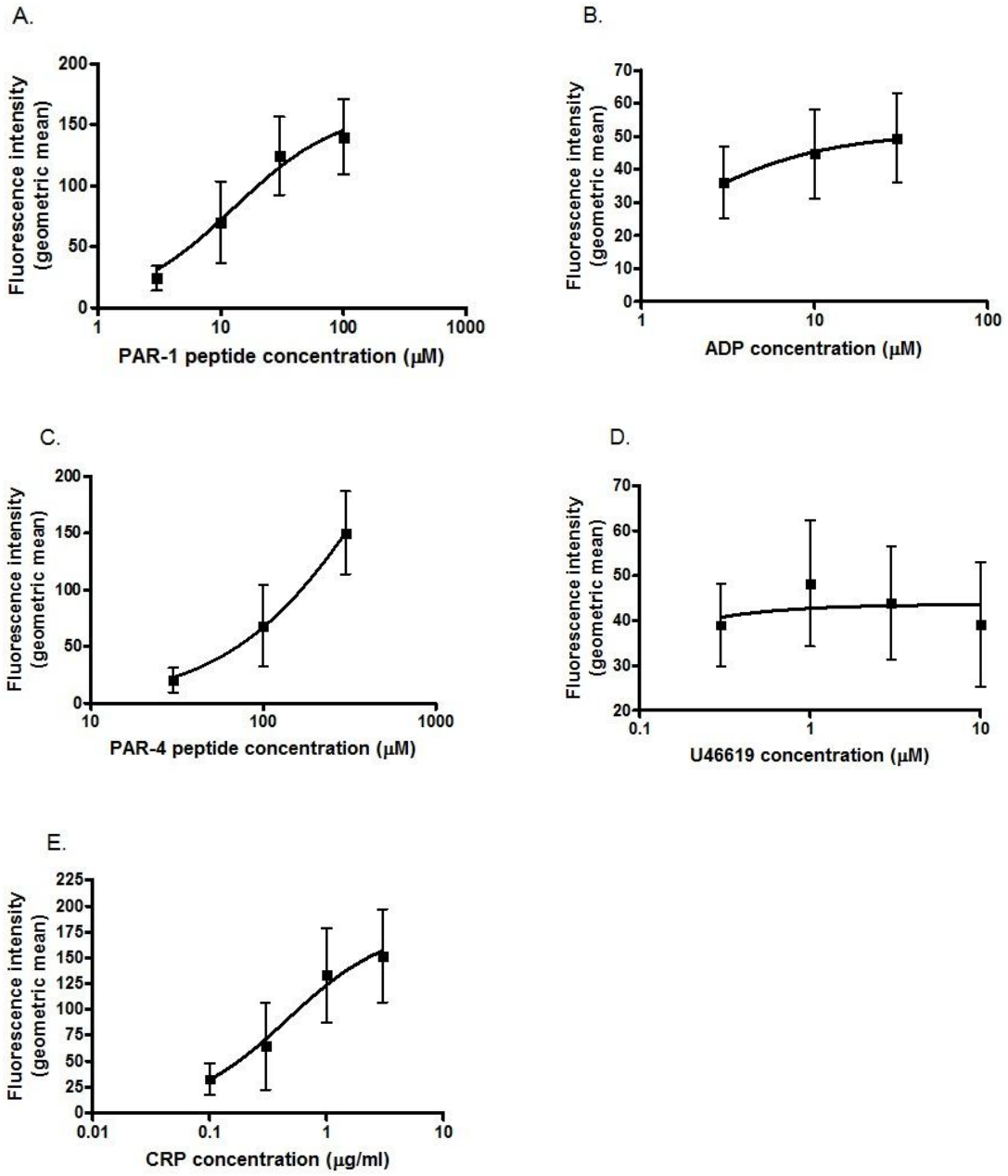


Figure 11.1 Assessment of platelet rich plasma response using flow cytometry to P-selectin (mouse anti human-CD62P) on BD Facs Calibur machine. Assessed at a platelet rich plasma count of $2x10^7$/ml. Results expressed as mean ± 1 standard deviation. A = PAR-1 peptide 3-100μmol, B= ADP 3-30μmol, C= PAR-4 peptide 30-300μmol, D= U46619 0.1-10μmol, E= CRP 0.1-3μg/ml. Mean PRP count prior to dilution = 3.5 $x10^8$/ml, standard deviation $1.26x10^8$/ml. Basal fluorescence intensity value was 15.8 (mean), with a standard deviation of 6.12.

Dose response curves were demonstrated to PAR-1 peptide, ADP, PAR-4 peptide and CRP. U46619 demonstrated a maximal (probable saturating) response at 1µM with overlapping standard deviations at all concentrations.

Examples of dose response curves for individual participants are shown in **Figure 11.2**:

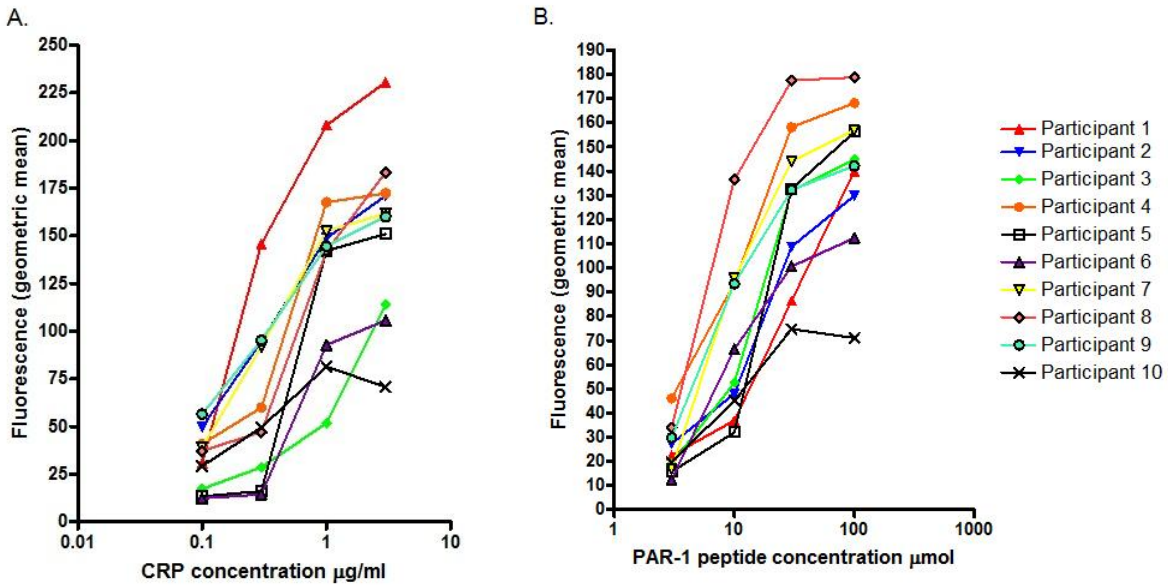
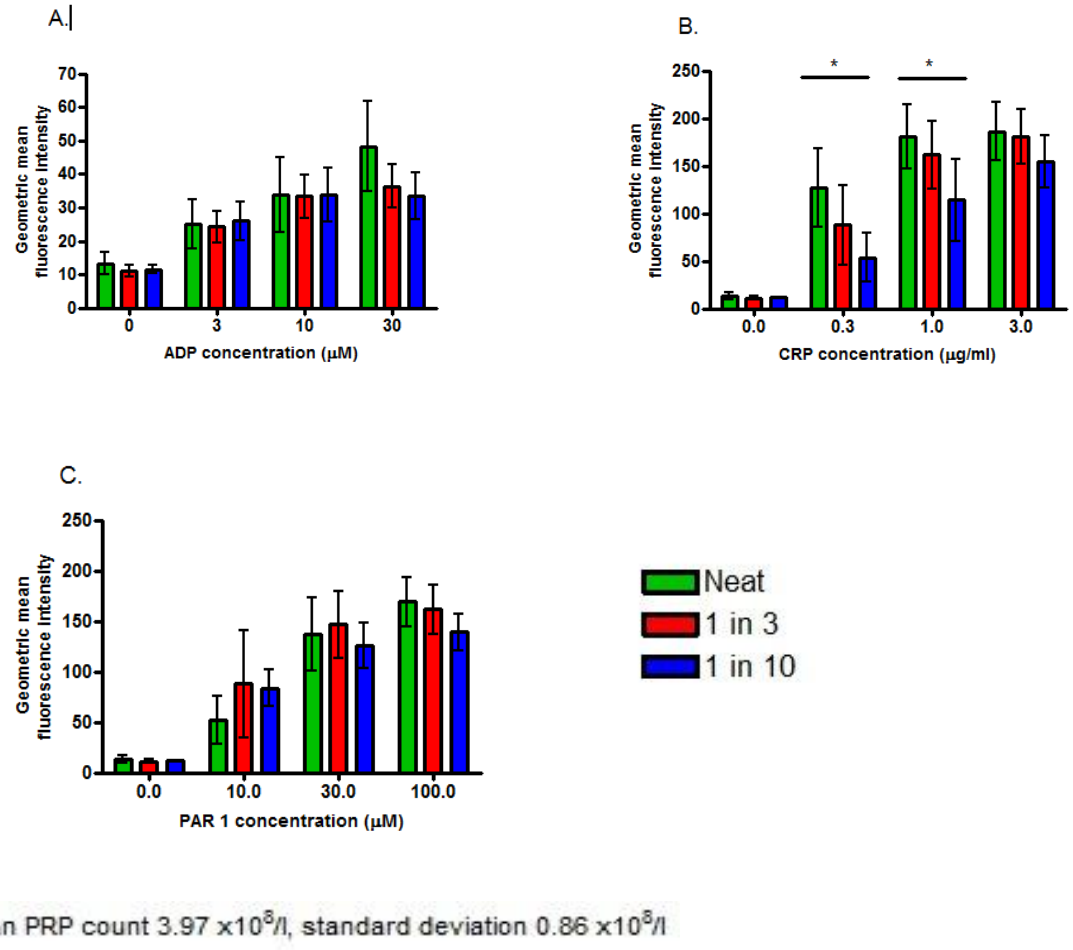


Figure 11.2 Assessment of individual participant's platelet rich plasma responses using flow cytometry to P-selectin (mouse anti human -CD62P) on BD Facs Calibur machine. Assessed at a platelet rich plasma count of $2x10^7$/ml. A= Response to CRP 0.1-3µg/ml, B = Response to PAR-1 peptide 3-100µM. Mean PRP count prior to dilution = $3.5 x10^8$/ml, standard deviation $1.26x10^8$/ml

As expected, there were significant differences in the magnitude of response between participants demonstrating a wide variety within the normal range.

11.4.1.2 Assessment of platelet rich plasma response at three dilutions (neat, 1 in 3 and 1 in 10) to ADP, CRP and PAR-1 peptide

A total of six healthy volunteers were assessed using the BD Facs Calibur machine and responses are depicted in **Figure 11.3**:



Mean PRP count 3.97 x10^8/l, standard deviation 0.86 x10^8/l

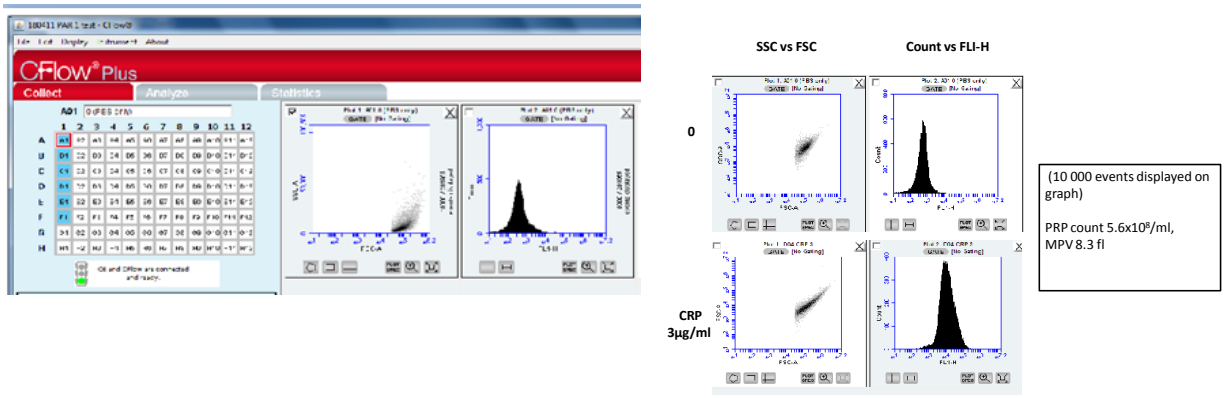
Figure 11.3 Assessment of platelet rich plasma response at 3 dilutions (neat, 1 in 3 diluted with PBS and 1 in 10 diluted with PBS) using flow cytometry to P-selectin (mouse anti human -CD62P) on BD Facs Calibur. Agonists tested – panel A = ADP 3-30µM, panel B=CRP 0.3-3µg/ml, panel C = PAR-1 peptide 10-100µM. Results expressed as mean ± 1 standard deviation. n=5-6.

For each graph, one way ANOVA was conducted for each concentration of agonist represented. The dependent variable was median fluorescence intensity and the varying dilutions of PRP were the independent variables. For statistical significance bars, * = $p \leq 0.05$

These results show that for ADP and PAR-1 peptide measurement at 1 in 3 and 1 in 10 PRP dilutions show similar results to neat PRP. The platelet count in these dilutions is similar to

the count we would expect in the PRP of patients with inherited thrombocytopenia and this technique is therefore potentially feasible as a way of assessing platelet function in this group of patients. For CRP at low and intermediate concentrations, there is a significant difference in the values obtained with differing dilutions. This may be because dilution also reduces the amount of important feedback mediators such as thromboxane A_2. This effect seems to be overcome at the highest CRP concentration.

Initial work was then done to calibrate and establish familiarity with the Accuri C6 machine relative to the Becton Dickinson Flow Cytometer and examples of this are shown in **Figure 11.4**:

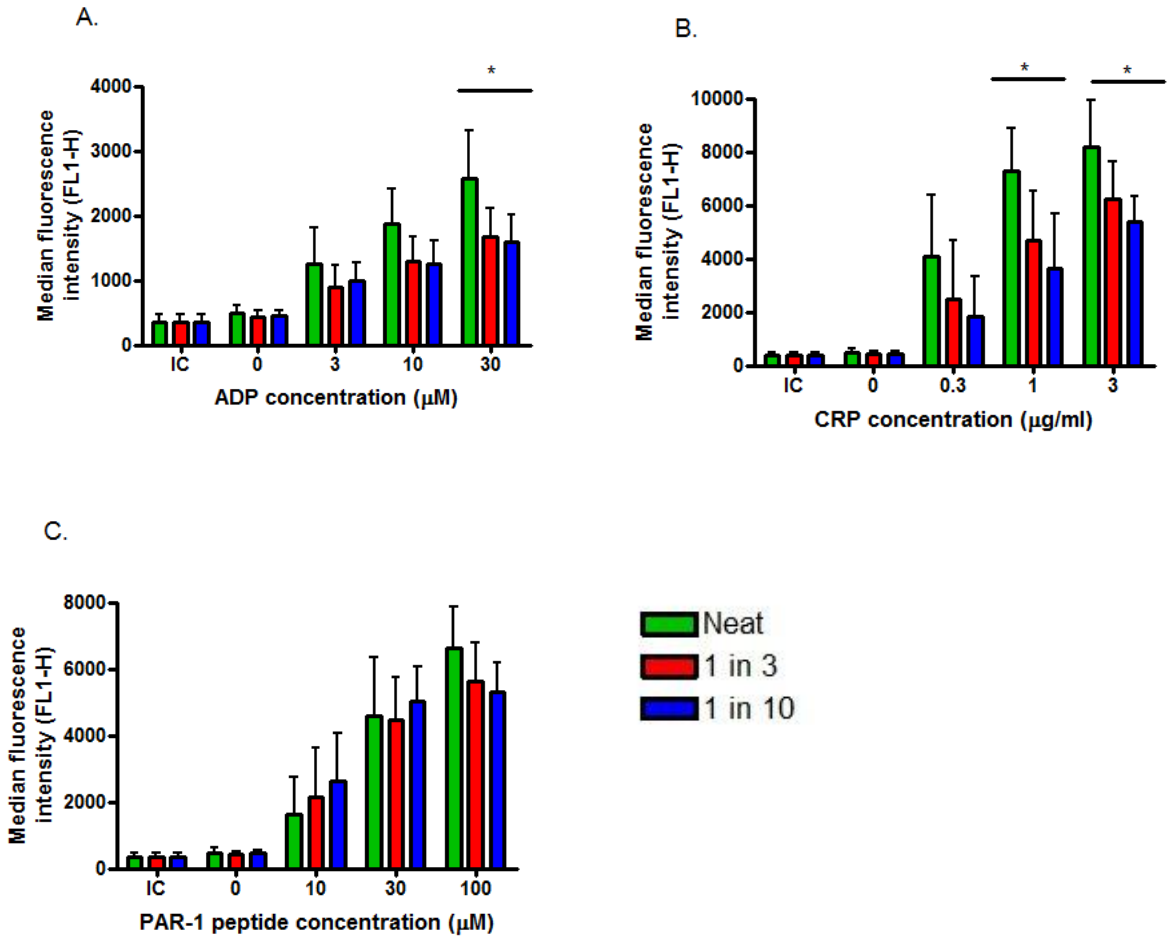


A. B.

Figure 11.4 Setting up Accuri C6. Panel A shows a screen shot from the data collection and analysis software, and panel B shows example SSC vs FSC and count vs fluorescence plots for basal PRP samples and those activated with CRP 3µg/ml using an antibody to P selectin (mouse anti-human CD62P)

A change in the shape of the SSC vs FSC plot was clearly visible on stimulation with high concentration CRP, as was a right hand shift in count vs FL1 plot indicating an increase in fluorescence and platelet activation.

A total of nine healthy volunteers were then assessed and results are depicted in **Figure 11.5**.



Mean PRP count $4.6x10^8$/l, standard deviation $1.26x10^8$/l
Mean platelet volume 8.84fl, standard deviation 0.58fl

Figure 11.5 Assessment of platelet rich plasma response at 3 dilutions (neat, 1 in 3 diluted with PBS and 1 in 10 diluted with PBS) using flow cytometry to P-selectin (mouse anti human -CD62P) on Accuri C6. Agonists tested – panel A = ADP 3-30µM, panel B = CRP 0.3-3µg/ml, panel C = PAR-1 peptide 10-100µM. Results expressed as mean ± 1 standard deviation. n=9. For each graph, one way ANOVA was conducted for each concentration of agonist represented. The dependent variable was median fluorescence intensity and the varying dilutions of PRP were the independent variables. For statistical significance bars, * = $p \leq 0.05$

These results were not directly comparable to those obtained on the BD Facs Calibur because of the differing modes of analysis, however they were broadly in agreement with similar trends and feasibility of this technique at the 1 in 10 PRP dilution was shown again. These results show that for all concentrations of ADP except the highest and for all concentrations of

PAR-1 peptide measurement at 1 in 3 and 1 in 10 PRP dilutions show similar results to neat PRP. For CRP at intermediate and high concentrations, there is a significant difference in the values obtained with differing dilutions.

Further analysis of these results including spread of data across individual participants and response according to PRP count in each dilution is shown in **Figure 11.6** below. As expected for any biological test, there is significant inter-individual variability within the normal range.

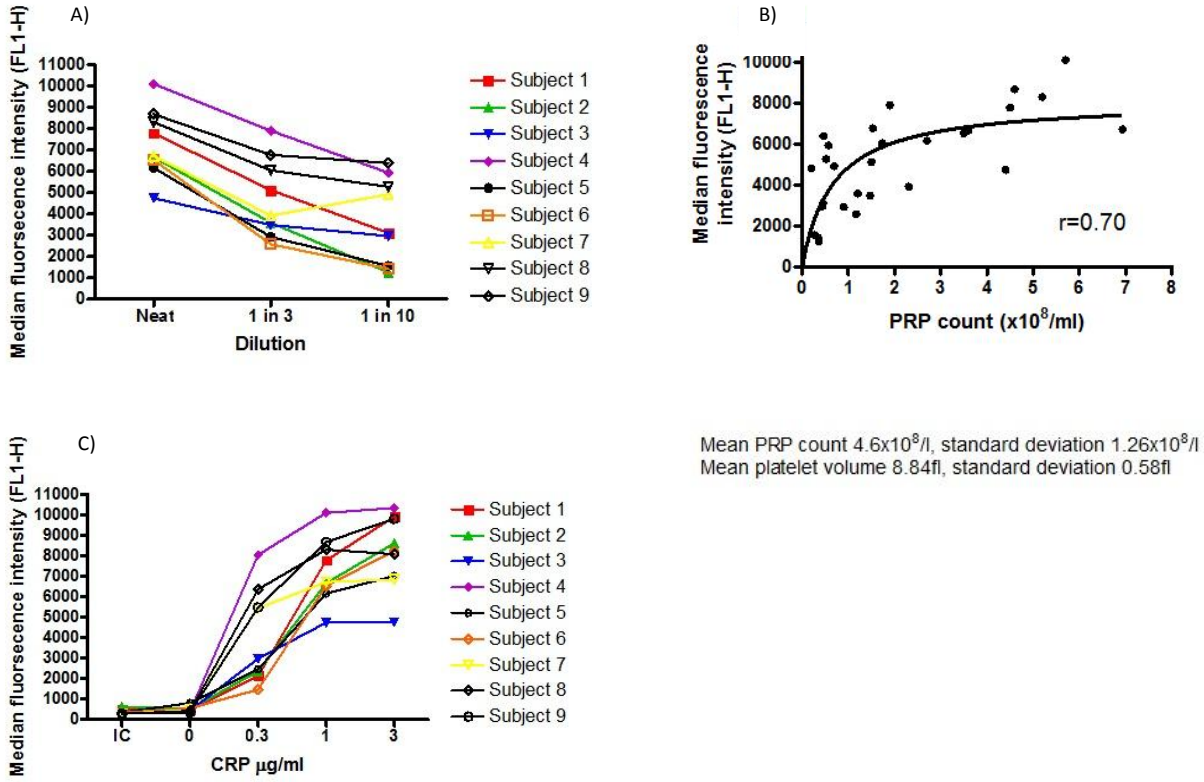


Figure 11.6 Assessment of platelet rich plasma response at 3 dilutions using flow cytometry to P-selectin (mouse anti human -CD62P) assessed on Accuri C6 machine. Panel A uses an example of response to intermediate concentration CRP (1µg/ml) and shows the spread of response over individual participants. Panel B shows a scatter plot of platelet count per dilution and response to intermediate concentration CRP (1µg/ml) with a non-linear regression line of best fit. Panel C shows the dose response curves for CRP in neat PRP in individual subjects. IC = isotype control. n=9.
r value displayed in Panel B represents the calculated correlation coefficient.
Panels A and C highlight the variability observed between individuals within the normal range in assessing response at various dilutions and at increasing concentrations of CRP respectively. Panel B shows a line of best fit (non-linear regression) demonstrating the response between PRP count and response to CRP 1µg/ml.

11.4.1.3 Assessment of platelet rich plasma response dilutions to ADP, CRP and PAR-1 peptide using inhibitors.

Five healthy volunteers were assessed yielding the results shown in **Figure 11.7**: The effect of blockade of the major feedback pathways of platelet activation, namely thromboxane formation and ADP secretion, on the expression of P-selectin was investigated using the cyclooxygenase inhibitor, indomethacin, and $P2Y_{12}$ receptor antagonist, cangrelor, respectively.

Complete inhibition of the ADP response was demonstrated at all PRP dilutions (neat, 1 in 3 and 1 in 10) and analysis of the ADP results shows a statistically significant difference in mean fluorescence intensity at all PRP dilutions with addition of inhibitors. A significant difference on addition of inhibitors was only shown at the lowest concentrations of CRP and PAR-1 peptide.

Post-hoc Tukey analysis (using IBM SPSS Statistics 19) showed the statistical significance on addition of inhibitors to be dependent on cangrelor. For all dilutions of PRP tested with ADP 30µM p values were less than 0.05 for comparison of no inhibitor versus cangrelor and indomethacin versus cangrelor. P values for other comparisons did not show statistical significance.

For neat PRP tested with CRP 3µg/ml the statistical significance on ANOVA was due to the difference between no inhibitor and the addition of cangrelor ($p=0.045$) with other comparisons not yielding statistical significance.

Lastly, for neat PRP tested with PAR-1 peptide 100µM the statistical significance on ANOVA was due to the differences between no inhibitor versus cangrelor (p=0.001) and indomethacin versus cangrelor (p=0.021). Comparisons of other pairs within this group did not reach statistical significance.

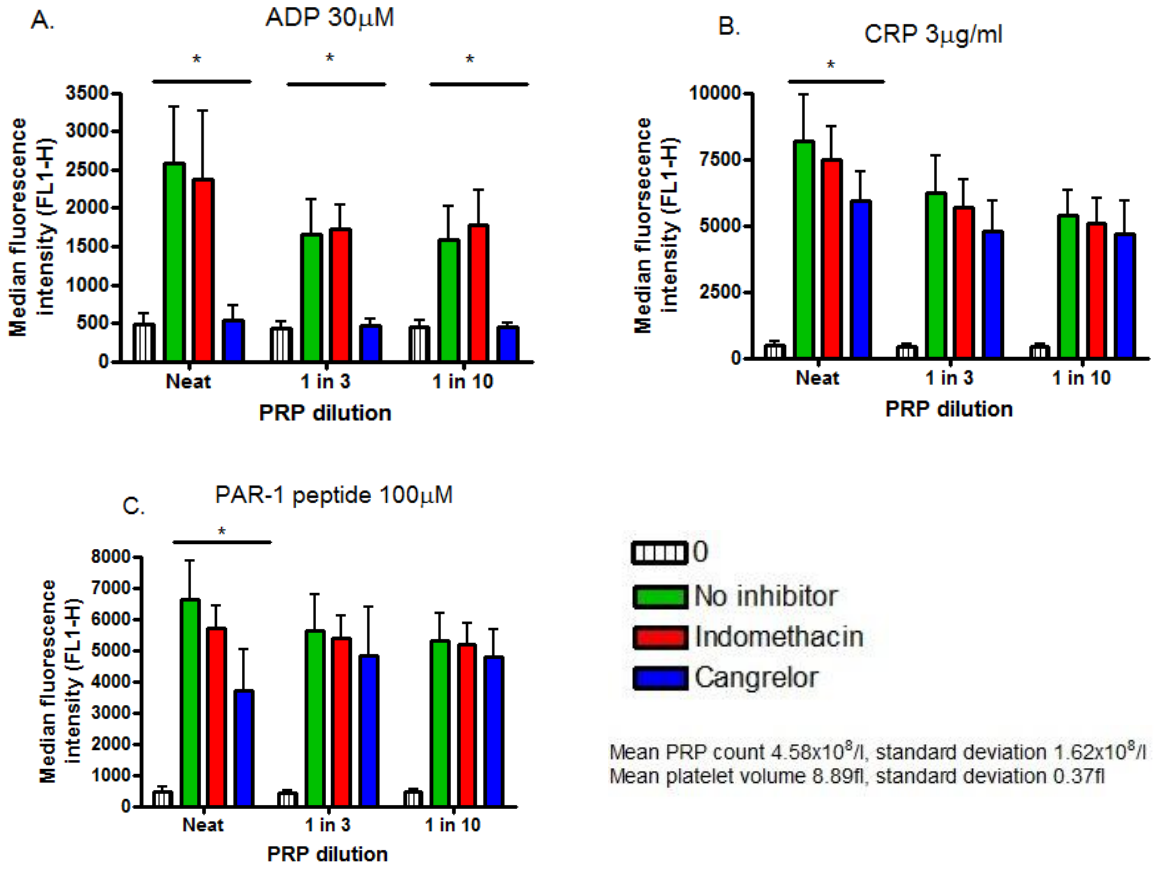


Figure 11.7 Assessment of PRP response at 3 dilutions (neat, 1 in 3 diluted with PBS and 1 in 10 diluted with PBS) for high concentrations of ADP (Panel A), CRP (Panel B) and PAR-1 peptide (Panel C) using flow cytometry to P-selectin (mouse anti human -CD62P) and inhibitors, indomethacin 10µM final concentration and cangrelor 1µM final concentration. Results expressed as mean $\pm$ 1 standard deviation. X-axes represent PRP dilutions. Y axes represent median fluorescence intensity as assessed on Accuri C6 machine. n=5. For each graph, one way ANOVA was conducted for each PRP dilution represented. The dependent variable was median fluorescence intensity and the inhibitor added was the independent variable. Basal values were excluded from the ANOVA analyses (each analysis examined data for no inhibitor, indomethacin and cangrelor). For statistical significance bars, * = $p \leq 0.05$

11.4.1.4 Pilot experiment assessing response to PAC-1 (activated glycoprotein II_bIII_a) and fluorescent fibrinogen

A single experiment was carried out to determine which of these targets should be the next step in constructing standard curves for PRP response using flow cytometry and whether the flow cytometric active component (PAC-1 antibody or fluorescent fibrinogen) should be added prior to or after agonists. Results are shown in Figure **11.8**.

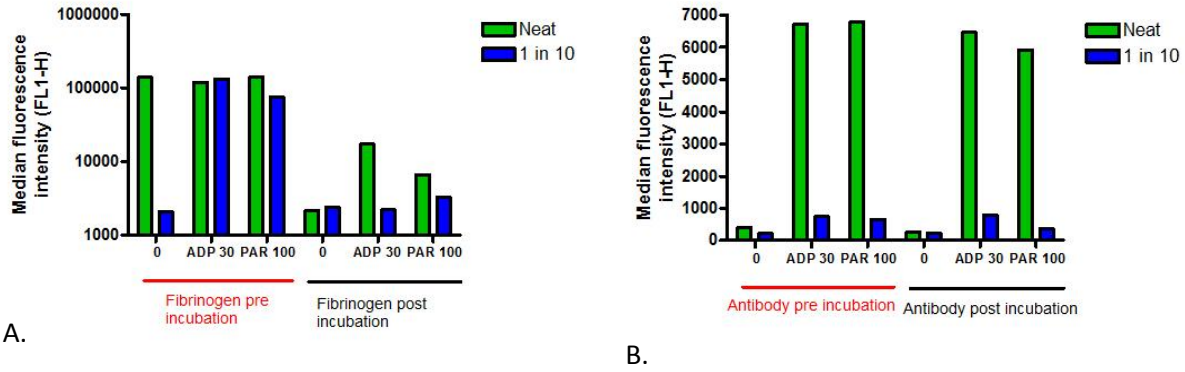


Figure 11.8 Assessment of platelet rich plasma response (neat and 1 in10 dilutions) using flow cytometry to fluorescent fibrinogen (panel A) and PAC-1 (mouse anti-human activated glycoprotein II_bIII_a) (panel B). X-axes agonist and concentration (0, ADP 30µmol and PAR-1 peptide 100µmol). **Please note logarithmic Y axis in panel i).** Y axes represent median fluorescence intensity as assessed on Accuri C6 machine.

Using fibrinogen added prior to agonists at a 1 in 10 PRP dilution showed a dose response curve, which was not observed with neat PRP and fibrinogen added prior to incubation, or with fibrinogen added post incubation to either neat or 1 in 10 diluted PRP. No dose response curve was seen with all combinations of neat and 1 in 10 diiluted PRP and PAC-1 added pre or post incubation. On the basis of this, assessment of fibrinogen binding (adding fluorescent fibrinogen prior to incubation) using 1 in 10 diluted PRP was selected to be studied further and applied to investigation of patients.

11.4.2 Application to patients with inherited thrombocytopenia

42 patients and 24 healthy volunteers were recruited to the inherited thrombocytopenia arm of the GAPP project between October 2010 and March 2013. Analysis was performed on 37 patients, as the remaining five were tested using a different flow cytometer and software and therefore did not have directly comparable results. Amongst the 37 patients analysed, 13 were male (35%). Median age in the patient group was 32 years, with an interquartile range (IQR) of 16 to 51 years.

Patient's responses were classified as normal (no qualitative defect) or abnormal (qualitative defect present) based upon responses to the assays outlined in the methods section. 23/36 (64%) were classified as abnormal (qualitative defect present) based on this assay. One patient (3%) had insufficient platelets in the sample received for the assay to be performed, and we suspect this to be an artefactual phenomenon related to venepuncture or sample processing.

Median platelet count in platelet rich plasma (PRP) in the patients was $1.2x10^8$/ml ($120x10^9$/l), with an IQR of 0.7-$1.9x10^8$/ml (70-190 $x10^9$/l). In the healthy volunteers median platelet count in PRP was $3.65x10^8$/ml ($365x10^9$/l), with an IQR of 2.85-$4.9x10^8$/ml (285-$490x10^9$/l). No significant difference was seen in PRP count between patients with and without a qualitative defect on laboratory testing (**Figure 11.9A**).

Median mean platelet volume (MPV) in the patients was 9.3fl with an IQR of 8.6-10.7fl. In the healthy volunteers median MPV was 9.0fl with an IQR of 8.6-9.3fl. No significant difference was seen in MPV between healthy volunteers and patients, or in patients with and without a qualitative defect on laboratory testing (**Figure 11.9B**).

ISTH bleeding assessment tool score was available on 26 patients (it was not conducted in children), with a median score of 8, IQR 5-14. No significant difference in score was seen between patients with and without a qualitative defect (**Figure 11.9C**). The previously defined 95th centile of the score in healthy volunteers was 4.

Surface glycoprotein levels

Surface levels of CD41 (**Figure 11.10A**), CD42b (**Figure 11.10B**) and GPVI (**Figure 11.10C**) were studied. For CD41 and GPVI, patients with a qualitative defect were found to have significantly lower levels of surface receptors ($p \leq 0.05$), however no significant difference was seen in CD42b expression. For GPVI, data were available on 14 patients and 14 healthy volunteers due to restricted availability of the PE-conjugated anti human GPVI antibody used.

P selectin dose response curves

Dose response curves assessed using FITC conjugated anti human P selectin antibody are shown in **Figures 11.11** and **11.12**. No significant difference was found between responses of patients with and without a qualitative defect and healthy volunteers for baseline and isotype control values, but at all other dose points a significant ($p \leq 0.05$) difference was seen between patients with and without a qualitative defect.

Fibrinogen dose response curves

Dose response curves using FITC conjugated fibrinogen are shown in **Figures 11.13** and **11.14**. No significant difference was found between responses of patients with and without a qualitative defect and healthy volunteers for baseline. Some dose points (ADP 3µM and

PAR-1 peptide 10μM) were non-discriminatory and did not show a significant difference ($p>0.05$) between all groups and specifically between patients with and without a qualitative defect. However, high concentrations of each agonist did show a significant difference both overall amongst all groups and between patients with and without a qualitative defect. Low dose C reactive peptide showed a non significant difference overall ($p=0.06$), but the difference between patients with and without a defect was significant ($p<0.05$).

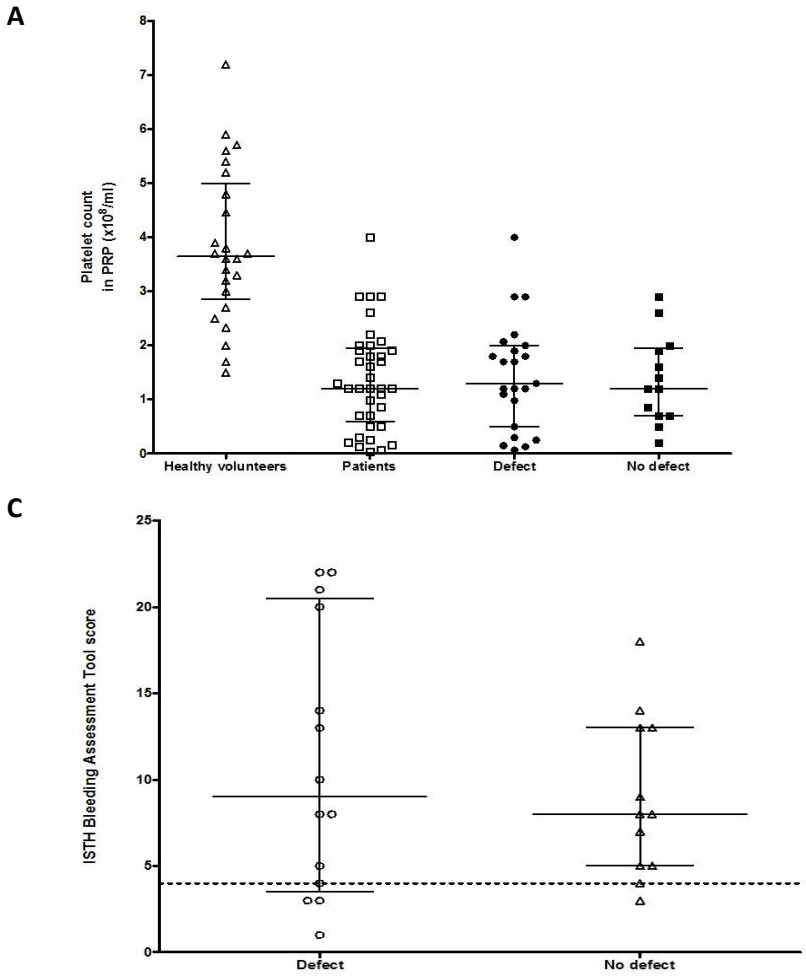


Figure 11.9 Platelet count in PRP (Panel A), Mean Platelet Volume (Panel B) and ISTH Bleeding Assessment Tool Score (Panel C) in patients and healthy volunteers.. In all panels, bars represent median and interquartile range. For Panels A and B, X axis shows categories representing healthy volunteers, all patients, patients with a qualitative defect and patients without a qualitative defect. For Panel C data for patients only is shown, split into those with and without qualitative defects. The horizontal dotted line represents the 95th centile (score of 4) in previously studied healthy volunteers. For Panel A, $p<0.0001$ when comparing groups using Kruskal Wallis test with Dunn's adjustment for multiple comparisons. Significant differences ($p\leq0.05$) were found for healthy volunteers versus patients, but no statistical significance ($p>0.05$) was seen between patients with and without a qualitative defect. For Panel B $p=0.06$ when comparing groups using Kruskal Wallis test with Dunn's adjustment for multiple comparisons. Comparison between pairs of groups was non significant ($p>0.05$) in all cases. For Panel C, $p=0.42$ using Mann Whitney U test.

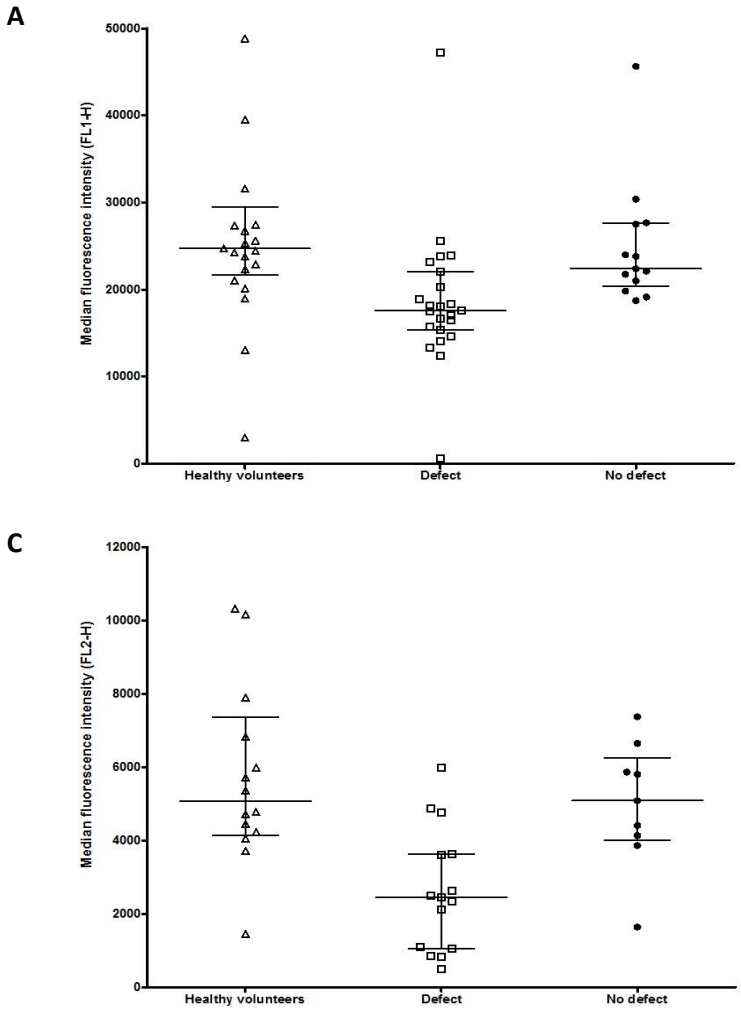


Figure 11.10 Surface receptor expression of CD41 (Panel A), CD42b (Panel B) and GPVI (Panel C) in healthy volunteers and patients with and without a qualitative defect on laboratory testing. In all panels, bars represent median and interquartile range. X axis shows categories representing healthy volunteers, patients with a qualitative defect and patients without a qualitative defect. For Panel A, surface expression was assessed using a FITC conjugated anti-human CD41 antibody. p=0.0003 when comparing groups using Kruskal Wallis test with Dunn's adjustment for multiple comparisons. For Panel B surface expression was assessed using a FITC conjugated anti-human CD42b antibody. p=0.93 when comparing groups using Kruskal Wallis test with Dunn's adjustment for multiple comparisons. For Panel C, surface expression was assessed using a PE conjugated anti-human GPVI antibody. p=0.0014 when comparing groups using Kruskal Wallis test with Dunn's adjustment for multiple comparisons.

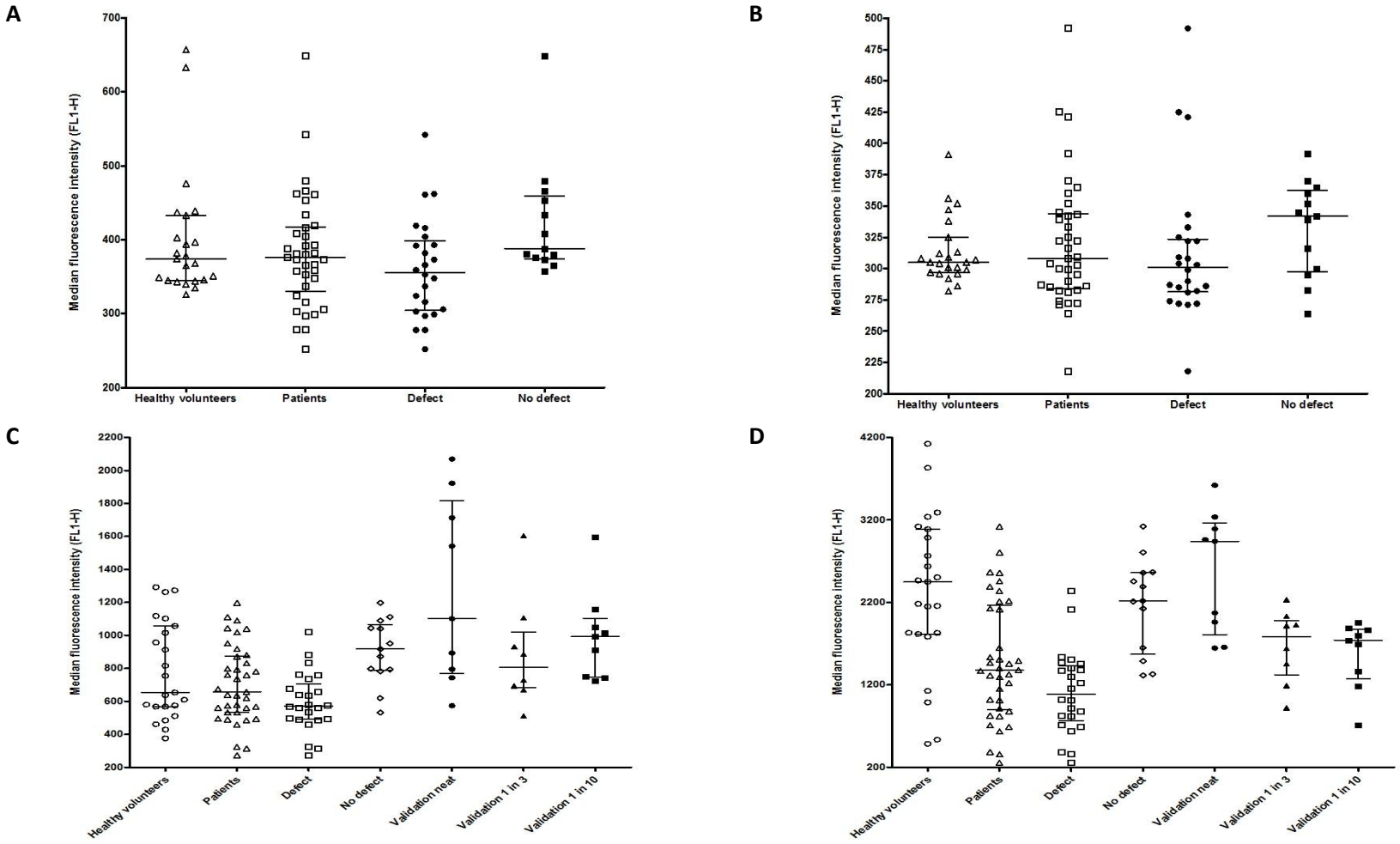

A
Median fluorescence intensity (FL1-H)
700
600
500
400
300
200
Healthy volunteers
Patients
Defect
No defect
B
Median fluorescence intensity (FL1-H)
500
475
450
425
400
375
350
325
300
275
250
225
200
Healthy volunteers
Patients
Defect
No defect
C
Median fluorescence intensity (FL1-H)
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
Healthy volunteers
Patients
Defect
No defect
Validation neat
Validation 1 in 3
Validation 1 in 10
D
Median fluorescence intensity (FL1-H)
4200
3200
2200
1200
200
Healthy volunteers
Patients
Defect
No defect
Validation neat
Validation 1 in 3
Validation 1 in 10

Figure 11.11 Dose response curves assess using FITC conjugated anti-human P selectin antibody. Baseline values (Panel A), IgGκ mouse anti-human isotype control antibody (Panel B), ADP 3µM (Panel C) and ADP 30µM (Panel D). Bars represent median and interquartile ranges. All X axes show responses for healthy volunteers, all patients, patients with a qualitative defect and patients without a qualitative defect. Panels C and D also show data from the initial validation cohort relating to assay development. For the validation cohort neat = neat platelet rich plasma (PRP), 1 in 3 = PRP diluted 1 in 3 with phosphate buffered saline, 1 in 10 = PRP diluted 1 in 10 with phosphate buffered saline. For panels A and B, $p > 0.05$ (0.08 and 0.25 respectively) when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons. Comparison between groups was non significant ($p>0.05$) in all cases. For panels C and D, $p<0.001$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons. $p < 0.01$ when comparing patients with and without a qualitative defect.

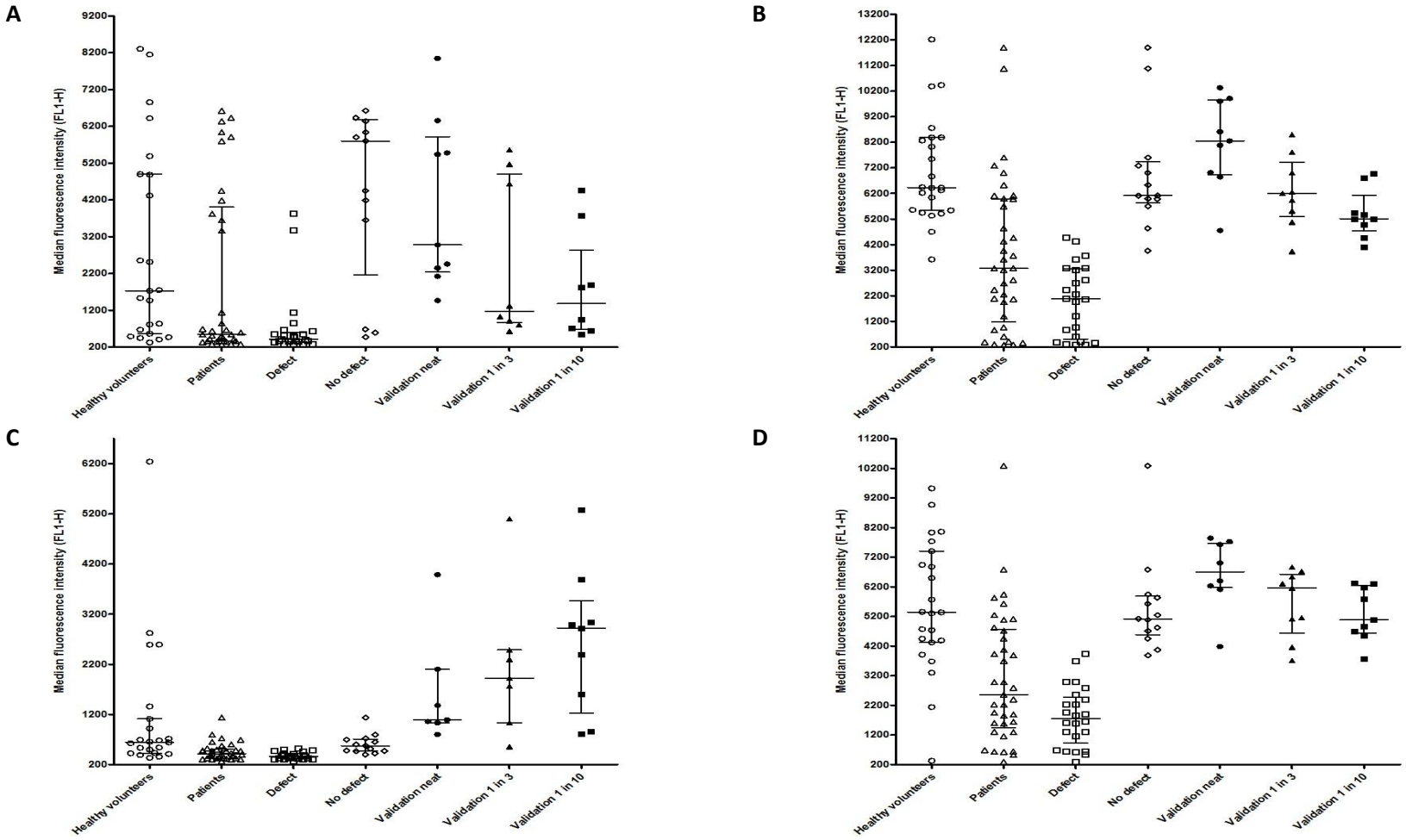

A
B
C
D
Median fluorescence intensity (FL1-H)
Healthy volunteers
Patients
Defect
No defect
Validation neat
Validation 1 in 3
Validation 1 in 10

Figure 11.12 Dose response curves assess using FITC conjugated anti-human P selectin antibody. CRP 0.3µg/ml (Panel A), CRP 3µg/ml (Panel B), PAR-1 peptide 10µM (Panel C) and PAR-1 peptide 100µM (Panel D). Bars represent median and interquartile ranges. All X axes show responses for healthy volunteers, all patients, patients with a qualitative defect and patients without a qualitative defect. Data from the initial validation cohort relating to assay development is also shown. For the validation cohort neat = neat platelet rich plasma (PRP), 1 in 3 = PRP diluted 1 in 3 with phosphate buffered saline, 1 in 10 = PRP diluted 1 in 10 with phosphate buffered saline. For panels A, B and D, $p<0.0001$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons, $p < 0.001$ when comparing patients with and without a qualitative defect. For panel C, $p<0.0001$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons, $p < 0.05$ when comparing patients with and without a qualitative defect.

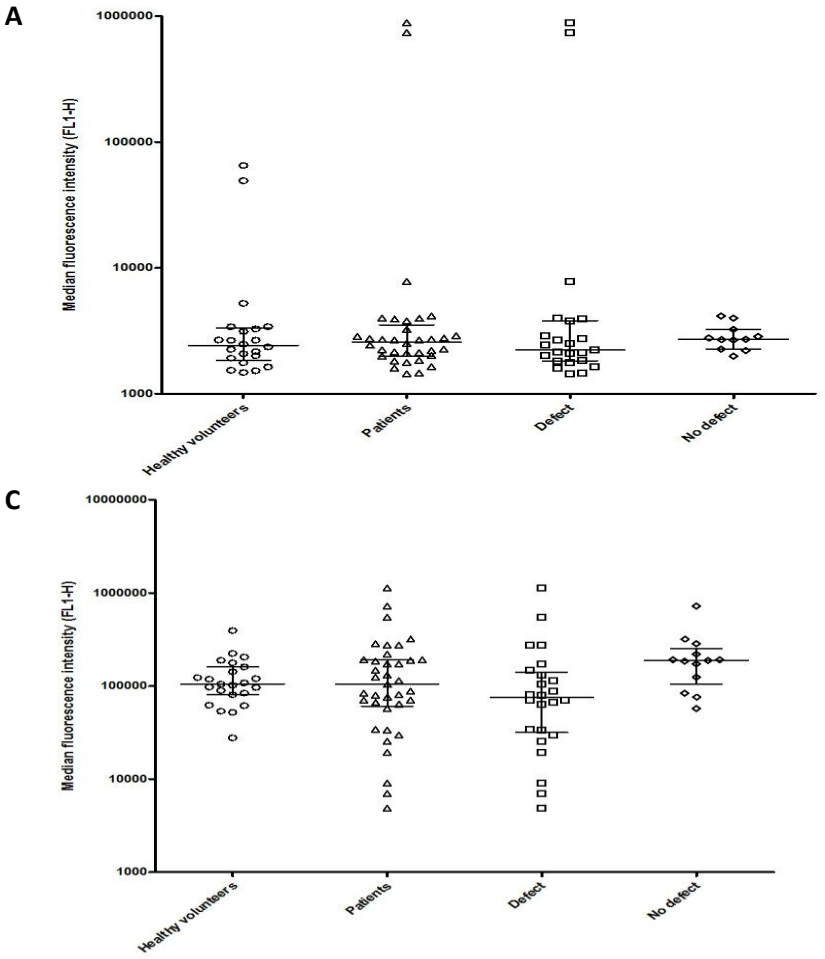


Figure 11.13 Dose response curves assess using FITC conjugated fibrinogen. Baseline values (Panel A), ADP 3µM (Panel B) and ADP 30µM (Panel C) Bars represent median and interquartile ranges. All X axes show responses for healthy volunteers, all patients, patients with a qualitative defect and patients without a qualitative defect. PRP was diluted 1 in 10 with phosphate buffered saline prior to running the assay, as outlined in methods section. For panels A and B, $p>0.05$ (0.56 and 0.11 respectively) when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons. Comparison between pairs of groups was non significant ($p>0.05$) in all cases. For panel C, $p=0.047$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons, $p < 0.05$ when comparing patients with and without a qualitative defect.

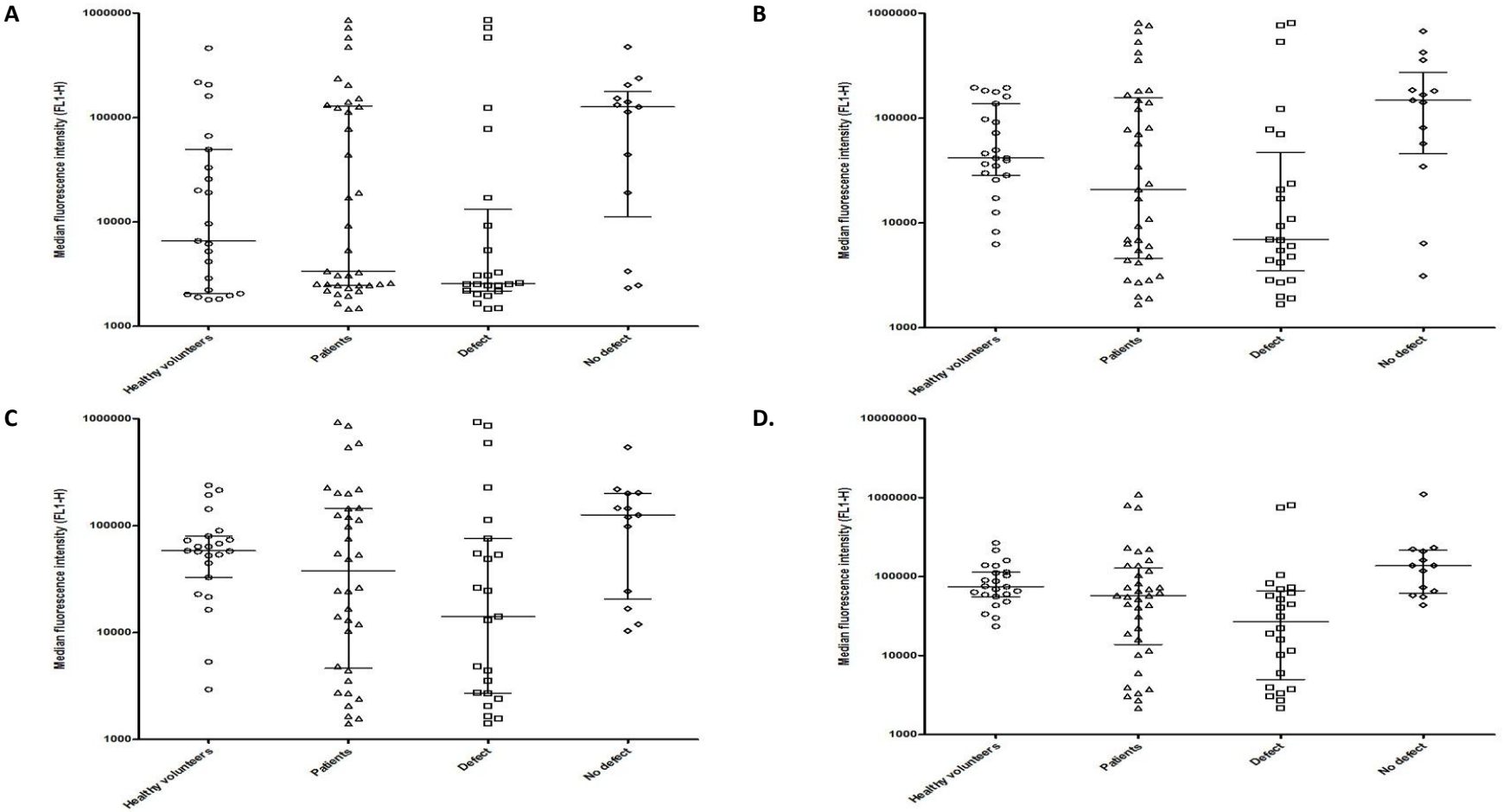

A
B
C
D.
Median fluorescence intensity (FL1-H)
1000000
100000
10000
1000
10000000
Healthy volunteers
Patients
Defect
No defect

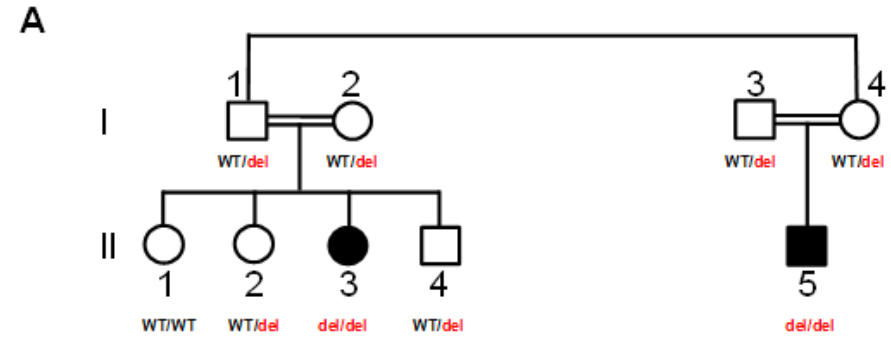
Figure 11.14 Dose response curves assess using FITC conjugated fibrinogen. CRP 0.3µg/ml (Panel A), CRP 3µg/ml (Panel B) , PAR-1 peptide 10µM (Panel C) and PAR-1 peptide 100µM (Panel D) Bars represent median and interquartile ranges. All X axes show responses for healthy volunteers, all patients, patients with a qualitative defect and patients without a qualitative defect. PRP was diluted 1 in 10 with phosphate buffered saline prior to running the assay, as outlined in methods section. For panel A $p = 0.06$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons, but $p < 0.05$ when comparing patients with and without a qualitative defect. For panel B, $p = 0.007$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons, $p < 0.05$ when comparing patients with and without a qualitative defect. For panel C, $p=0.08$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons, with non significant comparisons ($p>0.05$) between pairs of groups in all cases. For panel D, $p = 0.001$ when comparing all groups using Kruskal Wallis Test with Dunn's adjustment for multiple comparisons, $p < 0.01$ when comparing patients with and without a qualitative defect.

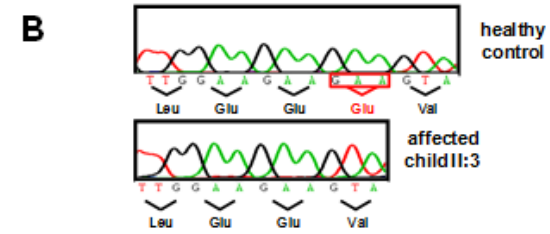
11.4.3 Genetic findings in patients with inherited thrombocytopenia

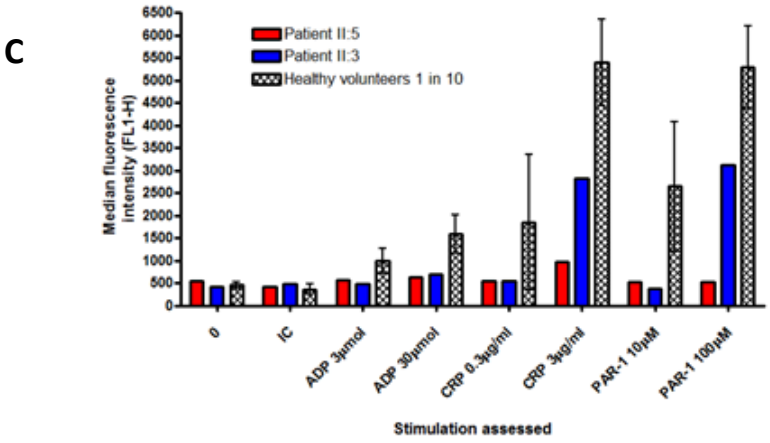
11.4.3.1 ANKRD18A (110)

A family with multiple consanguineous marriages and two affected children with severe thrombocytopenia were investigated. One of the affected children suffered from intracerebral bleeding at birth and had a platelet count of $10x10^9$/l, and the other child had a platelet count of $20x10^9$/l with several serious epistaxes requiring hospitalisation. Both children required HLA matched platelet transfusions on multiple occasions. I recruited them and assessed their platelet function using the flow cytometry assay. Sequencing the exomes of both patients was undertaken by Neil Morgan. Comparisons with dbSNP build 135, the 1000 Genomes project database and our in-house database (composed of >250 exomes) identified 77 and 83 homozygous novel variants, respectively. Of these only 2 homozygous non-synonymous variants and 1 non-frameshift deletion were found in both patients. The non-frameshift deletion was a homozygous single amino acid deletion (c.2401_2403delGAA; p.Glu801del) in exon 13 of *ANKRD18A.*

Family studies confirmed that the mutation segregated with disease status and was not detected in 312 ethnically matched control chromosomes. *ANKRD18A* encodes the ankyrin repeat domain 18A protein and the mutation maps within a coiled-coil domain of the protein. *ANKRD18A* belongs to the same family of proteins that has recently been discovered to be a major cause of a dominant form of thrombocytopenia (THC2), which is attributable to mutations in the *ANKRD26 gene* (204). The family tree, sequence traces and flow cytometry assessment of platelet function for these two patients are shown in **Figure 11.15**. Further work examining ANKRD18A mRNA expression and the function of the ANKRD18A protein by Dr Morgan and colleagues is ongoing.


A
I
II
1
2
3
4
WT/del
WT/del
WT/del
WT/del
1
2
3
4
5
WT/WT
WT/del
del/del
WT/del
del/del


B
healthy control
Leu
Glu
Glu
Glu
Val
affected child II:3
Leu
Glu
Glu
Val


C
Patient II:5
Patient II:3
Healthy volunteers 1 in 10
Median fluorescence intensity (FL1-H)
0
IC
ADP 3µmol
ADP 30µmol
CRP 0.3µg/ml
CRP 3µg/ml
PAR-1 10µM
PAR-1 100µM
Stimulation assessed

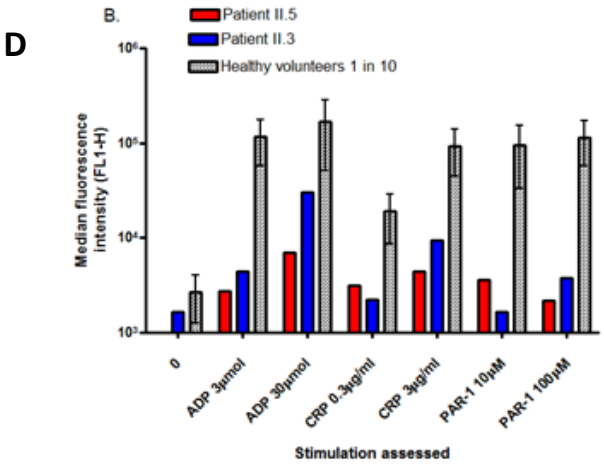

D
B.
Patient II.5
Patient II.3
Healthy volunteers 1 in 10
Median fluorescence intensity (FL1-H)
0
ADP 3µmol
ADP 30µmol
CRP 0.3µg/ml
CRP 3µg/ml
PAR-1 10µM
PAR-1 100µM
Stimulation assessed

Figure 11.15 Identification of a homozygous in frame deletion mutation in *ANKRD18A***.** (A) Pedigree from a consanguineous family with severe thrombocytopenia. Text beneath each family member represents the genotype for the c.2401_2403del (p.Glu801del) mutation in *ANKRD18A* (affected individuals are shaded). (B) Wild-type and mutant *ANKRD18A* sequence traces. The boxed sequence shows the position of the c.2401_2403 deletion sequence change in affected patient II:3. (C) Flow cytometry response using anti-CD62P antibody. (D) Flow cytometry response using fluorescent fibrinogen (NB logarithmic Y axis). Legends for (C) and (D) display PRP type. Healthy volunteers 1 in 10 = PRP from control individuals diluted 1 in 10 with PBS. Data for healthy volunteers shown as mean $\pm$ 1 s.d. (n= 9). Isotype control = IgGk1. Incubation took place at 37ºC for 2 minutes and terminated by adding a fivefold excess of ice cold PBS.

11.4.3.2 RUNX-1 and FLI-1 (205)

NGS analysis of candidate PFD genes was undertaken in 13 families who I recruited to the GAPP project and who were found to have a secretion defect. The genetic analysis was led by Neil Morgan and Jacqueline Stockley and identified a novel heterozygous alteration in either the *FLI1* or *RUNX1* gene in the index cases from 6 of the families studied (F1 to F6) (**Table 11.2**). Two nonsynonymous *FLI1* alterations, c.1009C>T and c.1028A>G, predicting p.R337W and p.Y343C substitutions in the highly conserved DNA binding domain of FLI1 were identified in affected members of F1 and F2, while a 4 bp frameshift deletion in *FLI1* (c.992-995del; p.Asn331Thrfs*4) was present in both affected members of F3. Three *RUNX1* variations predicted to cause RUNX1 haploinsufficiency were identified; donor splice site transversions, c.508+1G>T and c.351+1G>T, were detected in F4 and F5 respectively, and a nonsense mutation in codon 106 (c.317G>A; p.Trp106Stop) was detected in the index case from F6.

The presence of a *FLI1* or *RUNX1* alteration was associated with symptoms of excessive bleeding in all index cases, and their affected family members, and with mild thrombocytopenia in five of the families (**Table 11.2**). Interestingly, the missense variations in *FLI1* were also associated with alopecia, eczema or psoriasis, and recurrent viral infections

in affected individuals in F1, and with mild thrombocytopenia, alopecia and eczema in the affected individuals in F2 (**Figure 11.16A and 11.16B**).

Examination of platelet secretion and aggregation in all subjects carrying *FLI1* or *RUNX1* defects consistently revealed the predominant platelet abnormality to be a significant reduction in platelet ATP secretion in response to all agonists tested. (**Figure 11.16C, Table 11.2**, and data not shown).

Persistence of MYH10 in platelets from patients with FLI1 defects

Persistence of MYH10 in platelets was recently proposed as a biomarker for *RUNX1* alterations in patients with familial thrombocytopenia with a predisposition to AML, and for *FLI1* deletions in Paris Trousseau Syndrome (206). The detection of MYH10 in platelets from patients carrying the p.Tyr343Cys and the p.Asn331Thrfs*4 *FLI1* variations confirms the use of MYH10 detection as a biomarker for *FLI1* alterations (**Figure 11.16F**).

The identification of *RUNX1* or *FLI1* alterations in 6/13 unrelated patients with the most profound dense granule secretion disorders in this study suggests that defects in these genes will account for a significant proportion of patients with defects in platelet dense granule secretion and excessive bleeding.

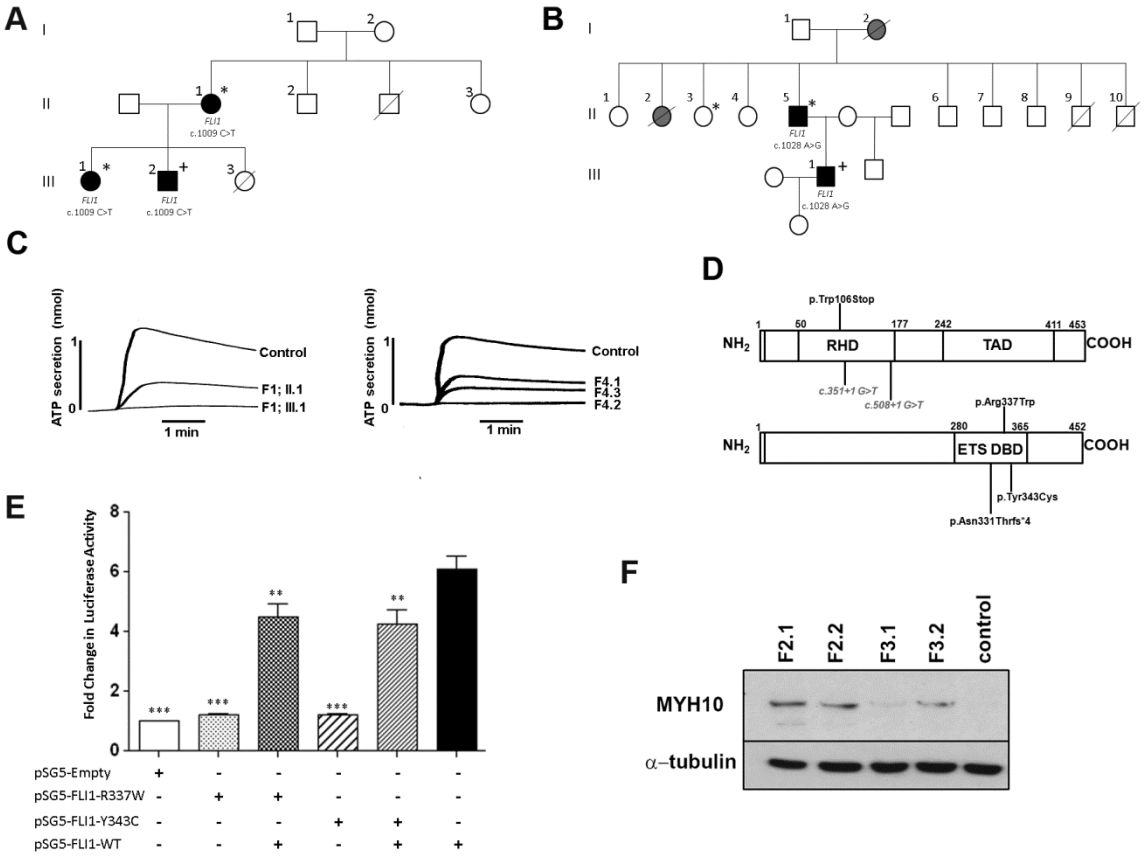


Figure 11.16 Mild bleeding symptoms and platelet dense granule secretion defects are associated with heterozygous mutations in *FLI1* and *RUNX1*. A and B: Pedigrees showing inheritance of mild bleeding, alopecia totalis and other clinical features in families 1 (A) and 2 (B). Individuals heterozygous for the c.1009C>T and c.1028A>G transitions in *FLI1* are indicated. Lines through symbols indicate deceased individuals. In A, individuals with bleeding symptoms and alopecia, and confirmed platelet dense granule secretion are indicated by black filled symbols. An asterisk indicates the presence of eczema and a history of recurrent viral infections. The presence of psoriasis is indicated by a '+' sign. In B, individuals with bleeding symptoms and alopecia are indicated by black or grey filled symbols. Black filled symbols indicate individuals with confirmed platelet dense granule secretion defect and mild thrombocytopenia. An asterisk indicates a history of infective endocarditis and the presence of eczema and colitis are indicated by a '+' sign. C. ATP secretion in response to 100μM PAR1 peptide in two members of F1 with the c.1009C>T transition in *FLI1*, and three members of F4 with the c.508+1G>T transversion in *RUNX1* alongside controls. D. Schematic diagram of RUNX1 and FLI1 showing the regions of the proteins affected by mutations identified in this study. Intronic mutations, predicted to interfere with splicing of the *RUNX1* RNA, are shown in italics. E. HEK293T cells were co-transfected with WT and variant FLI1 constructs, or combinations thereof, and pGL3.10-GP6-luciferase and pRLnull-Renilla reporters as shown, and firefly and Renilla luciferase expression assessed in cell lysates 48 hours later. Data represents the means (± SEM) of 3 independent experiments; **p<0.01, ***p<0.001. F. MYH10 protein expression in platelets from patients with *FLI1* mutations (F2.1 and F2.2 with p.Tyr343Cys FLI1 mutation; F3.1 and F3.2 with p.Asn331Thrfs*4 FLI1 mutation) and a healthy control.

Family (F)/ Patient ID	Gene and nucleotide alteration¥	Effect	Platelet count ($x10^9$/L)^	Mean MPV (fL)	ATP Secretion nmol/$1x10^8$ platelets*
F1; II.1	*FLI1;* c.1009 C>T	p.Arg337Trp	244	10.3	0.37
F1; III.1	*FLI1;* c.1009 C>T	p.Arg337Trp	150	10.5	0.07
F2; II.5	*FLI1;* c.1028 A>G	p.Tyr343Cys	92	8.8	ND **
F2; III.1	*FLI1;* c.1028 A>G	p.Tyr343Cys	100	8.6	ND **
F3.1	*FLI1;* c.992-995del	p.Asn331Thrfs*4	157	11.8	0.57
F3.2	*FLI1;* c.992-995del	p.Asn331Thrfs*4	142	11.4	0.38
F4.1	*RUNX1;* c.508+1 G>T	splicing	Unavailable	7.3	0.32
F4.2	*RUNX1;* c.508+1 G>T	splicing	70	7.5	ND
F4.3	*RUNX1;* c.508+1 G>T	splicing	130	7.1	0.62
F5	*RUNX1;* c.351+1G>T	splicing	139	8.0	0.35
F6.1	*RUNX1;* c.317 G>A	p.Trp106Stop	100	8.0	0.25

Table 11.2 Genotypic and phenotypic characteristics of subjects with platelet dense granule secretion defects. Heterozygous nucleotide changes present in FLI1 and RUNX1 and their predicted effects on the resulting RNA or protein are shown. ¥ Alterations are numbered according to positions in the NM_002017.3 and NM_001754 transcripts for *FLI1* and *RUNX1* respectively; ^Mean platelet counts are shown; *ATP secreted in response to 100μM PAR-1 receptor specific peptide SFLLRN, 5^{th} centile in healthy volunteers is 0.82 nmol / $1x10^8$ platelets; **ATP secretion in response to 1U/ml thrombin. ND= not detectable; MPV=Mean platelet volume; NR= Normal range

11.4.3.3 Whole exome sequencing of cohort of patients with inherited thrombocytopenia (117)

Analysis of the genetic findings in the patients recruited to the thrombocytopenia arm of the GAPP project (including the patients who I recruited) has been undertaken by Neil Morgan and Ben Johnson. Using the GAPP approach of combined platelet phenotyping with whole exome sequencing this work determined the possible genetic cause of disease in 73% (27/36) of index patients with thrombocytopenia. Genetic variations occurred in the 30 known genes associated with thrombocytopenia in 20 of 27 index cases (*ACTN1, ANKRD26, CYCS, FLI1, GFI1B, GP1BA, MKL1, MYH9, NBEAL2, RUNX1, TPM4* and *TUBB1*). Seven cases were of suggested novel aetiology within previously unreported genes (*ANKRD18A, MKL1, PF4* and *SLFN14).*

11.5 Discussion

The work described in this chapter is based on the premise that patients with inherited thrombocytopenia have a wide spectrum of clinically observed bleeding, and that the platelet count threshold for developing clinical bleeding in patients with thrombocytopenia is observed to vary significantly (185).

The variation in platelet count bleeding threshold may be explained by the presence of a qualitative platelet defect in addition to the numerical reduction in platelet count. In accordance with the mulitfactorial aetiology of bleeding, an individual's bleeding threshold may also be contributed to by changes in other parts of the haemostatic pathway, such as reduced von Willebrand factor levels which are not low enough to have a diagnostic label of von Willebrand disease (207). The extent of the previous bleeding history is known to predict the risk of further bleeding events (208).

My work on this patient cohort used flow cytometry in the assessment of platelet function, as this modality measures fluorescence emitted by individual platelets and is therefore independent of total platelet count. Historically, assessing platelet function in this patient group has been difficult as lumiaggregometry becomes unreliable at low platelet counts (15, 186).

My findings have demonstrated the feasibility of the flow cytometry method that I developed in assessing platelet function in thrombocytopenia. Dose response curves were demonstrated using this technique in healthy volunteers, with minimal effects observed after dilution of PRP from healthy volunteers to replicate platelet counts in PRP expected in patients with thrombocytopenia. These dilution experiments allowed construction of reference ranges for expected values at differing platelet count in PRP ranges in order to allow comparison with

results obtained from patients. Initial experiments focussed on assessing P-selectin expression after stimulation with a variety of agonists, and the testing procedure was subsequently modified to also examine fluorescent fibrinogen binding.

This newly developed technique was then applied to patients with inherited thrombocytopenia and enabled categorisation of whether patients with thrombocytopenia may also have an additional qualitative defect. The P-selectin assay was more discriminatory in this assessment, and was more reproducible in healthy volunteers. The fluorescent fibrinogen assay occasionally showed pre-activation at baseline values, and appeared to be sensitive to handling and processing, which could limit future potential utility. Future work is needed to validate these assays, which has not been possible so far due to the absence of a comparative gold standard in assessing platelet function in thrombocytopenia.

The genetic findings that have arisen from investigation of the patient cohort that I recruited are interesting, and are a good example of clinical assessment and laboratory phenotyping driving advances in genetic understanding of a rare disorder. In the cases described here, this process has led to the identification of a novel genetic defect (in *ANKRD18A*) and has identified mutations in *FLI-1* and *RUNX-1* as a relatively common and potentially significant cause in patients with inherited thrombocytopenia and secretion disorders. Interestingly, several of these patients had a platelet count of over $100x10^9$/l and a small number had a platelet count in the normal range, which is out of keeping with the commonly reported finding that associates *RUNX1* disorders with thrombocytopenia (209, 210).

Cohort analysis of genetic findings was successful in identifying underlying mutations (in known and novel genes) in almost three quarters of recruited patients with inherited thrombocytopenia.

In summary, the work described in this chapter has developed a flow cytometric based technique to allow assessment of platelet function in thrombocytopenia. This was then applied to patients with inherited thrombocytopenia to investigate whether they may have qualitative in addition to quantitative platelet defects. An underlying genetic cause for inherited thrombocytopenia was identified in a significant number of the recruited patients. Although the flow cytometric technique remains a research tool at present and requires validation, it offers promise in extending the assessment of patients with thrombocytopenia to explain their individual bleeding thresholds and to personalise clinical management plans.

CHAPTER TWELVE: GENERAL DISCUSSION

The work presented in this thesis has made the following contributions to the field of inherited platelet disorders:

- Characterisation of normal aggregation and secretion responses in healthy volunteers, with resultant compilation and development of a rationalised agonist panel for laboratory testing of suspected inherited platelet disorders.

- Confirmation that use of Chronolume® to assess secretion does not significantly impact on measured aggregation levels, suggesting that the practice of simultaneously measuring secretion and aggregation in lumiaggregometry is reliable.

- Further characterisation of inherited platelet defects in patients with a suspected inherited platelet function disorder and a normal platelet count. The majority of defects were in either dense granule secretion, G_i signalling or cycloxygenase pathway signalling, with a defect not identified in approximately 40% of recruited patients.

- Assessment of ISTH BAT scores in patients with suspected inherited platelet function defects, with confirmation that this score is raised in this patient group, but that the score value is not discriminative for the presence of an underlying platelet defect. This supports the use of this tool in assessing and recording bleeding symptoms from a clinical and research perspective in patients with suspected platelet disorders.

- Confirmation that some patients with unexplained menorrhagia are noted to have an underlying platelet disorder.

- Development of an assay to assess platelet function in thrombocytopenia, with application of this assay to patients with thrombocytopenia to ascertain whether they have a qualitative platelet defect in addition to the numerical reduction in their platelet count.

The ultimate aim of GAPP was to identify mutations in known and novel platelet and megakaryocyte genes to explain inherited platelet defects, and to further understanding of basic platelet biology. Use of phenotyping data to guide relevant genetic investigation has been vital in achieving this. The nature of the search for mutations is governed by a number of factors, including the presence of consanguineous parents, evidence of autosomal dominant inheritance patterns, and the nature of the platelet function defect. The greatest successes have been achieved in identifying mutations in patients with a clear pattern of inheritance such as thrombocytopenia (117) and conditions associated with characteristic laboratory findings (such as ADP $P2Y_{12}$ receptor defects), or other phenotypic changes (as exemplified by HPS) (68).

The use of next generation sequencing has facilitated the use of targeted arrays and whole exome approaches in identifying mutations to explain other gene defects in patients recruited to GAPP (99, 117, 155). The whole exome approach allows for rapid generation of large amounts of data and is now financially viable, but does require expert interpretation and may not detect mutations in genes outside the coding region, or if unaffected family members are not available for comparative purposes. It has the potential to identify genetic defects in large cohorts of unrelated patients and this is a very important future development (101, 116, 117).

The need to further genetic understanding of platelet disorders is recognised by major stakeholder associations, including funders such as the NHLBI working group of the NIH in the USA (211). The difficulty in unravelling genetic defects in a complex trait such as bleeding will be in linking mutations identified to the underlying bleeding disorder, and as such it is helpful to use platelet phenotyping to interpret and guide genetic investigations. In

some cases, genetic mutations may represent a risk factor (rather than a solely causative agent) in giving rise to excessive bleeding (68).

An improved ability to diagnose underlying platelet function disorders has important clinical implications, as it allows appropriate management of patients at times of high risk of bleeding (such as perioperatively, peripartum, during menstruation and after injury), prevents unnecessary treatment with immunosuppresion and allows appropriate family counselling. An awareness of the possibility of an underlying platelet function disorders in patients with excessive bleeding (such as patients with unexplained menorrhagia) is vital in directing patients towards the correct investigation and in making this diagnosis. A more detailed assessment of bleeding risk than platelet count alone has potential importance in patients with inherited thrombocytopenia as it could change frequency of clinical assessment, lifestyle advice, treatment thresholds and consideration of newer agents (such as thrombopoietin mimetics) in treatment (212), thereby personalising management plans.

LIST OF REFERENCES

1. Hartley PS. Platelet senescence and death. Clin Lab. 2007;53:157-66.
2. In: Platelets in Hematologic and Cardiovascular Disorders. Editors: Gresele P, Fuster V, Lopez H, Page C, Vermylen J Cambridge University Press; 2008
3. In: Platelets. 3rd ed. Michelson AD, London: Academic Press; 2013
4. Marie Lordkipanidzé GC Lowe, P Harrison. Tests of platelet function. In Practical Haemostasis and Thrombosis 3rd edition: Editors: Nigel Key, Mike Makris and David Lilliicrap, Wiley-Blackwell. Submitted 24th September 2013
5. Watts T, Barigou M, Nash GB. Comparative rheology of the adhesion of platelets and leukocytes from flowing blood: why are platelets so small? Am J Physiol Heart Circ Physiol. 2013 Jun 1;304(11):H1483-94.
6. Heemskerk JW, Bevers EM, Lindhout T. Platelet activation and blood coagulation. Thromb Haemost. 2002 Aug;88(2):186-93.
7. Kaplan ZS, Jackson SP. The role of platelets in atherothrombosis. Hematology Am Soc Hematol Educ Program. 2011;2011:51-61.
8. Bos CL, Richel DJ, Ritsema T, Peppelenbosch MP, Versteeg HH. Prostanoids and prostanoid receptors in signal transduction. Int J Biochem Cell Biol. 2004 Jul;36(7):1187-205.
9. Willoughby S, Holmes A, Loscalzo J. Platelets and cardiovascular disease. Eur J Cardiovasc Nurs. 2002;1(4):273-88.
10. Freedman JE. Molecular regulation of platelet-dependent thrombosis. Circulation. 2005 Oct 25;112(17):2725-34.
11. Jackson SP. The growing complexity of platelet aggregation. Blood. 2007 Jun 15;109(12):5087-95.
12. Wei AH, Schoenwaelder SM, Andrews RK, Jackson SP. New insights into the haemostatic function of platelets. Br J Haematol. 2009 Jul 28;147:415-30.
13. Lordkipanidze M, Pharand C, Palisaitis DA, Diodati JG. Aspirin resistance: truth or dare. Pharmacol Ther. 2006;112(3):733-43.
14. SP Watson and P Harrison. The Vascular Function of Platelets. In Postgraduate Haematology (5th Edition). Editors V Hoffbrand, E Tuddenham and D Cavotsky. Blackwell; 2004.
15. Dawood B. Study of patients with suspected platelet based bleeding disorders: a search for patients with a defect in the P2Y12 ADP receptor: Thesis submitted to the University of Birmingham; 2009. ID Code in eTheses Repository 1519
16. Hollopeter G, Jantzen HM, Vincent D, Li G, England L, Ramakrishnan V, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. Nature. 2001;409(6817):202-7.
17. Takasaki J, Kamohara M, Saito T, Matsumoto M, Matsumoto S, Ohishi T, et al. Molecular cloning of the platelet P2T(AC) ADP receptor: pharmacological comparison with another ADP receptor, the P2Y(1) receptor. Mol Pharmacol. 2001;60(3):432-9.
18. Bernat A, Mares AM, Defreyn G, Maffrand JP, Herbert JM. Effect of various antiplatelet agents on acute arterial thrombosis in the rat. Thromb Haemost. 1993;70(5):812-6.
19. Andre P, Delaney SM, LaRocca T, Vincent D, DeGuzman F, Jurek M, et al. P2Y12 regulates platelet adhesion/activation, thrombus growth, and thrombus stability in injured arteries. J Clin Invest. 2003;112(3):398-406.
20. Cattaneo M. Bleeding manifestations of congenital and drug-induced defects of the platelet P2Y12 receptor for adenosine diphosphate. Thromb Haemost. 2011;105(1):11-0742.
21. Cattaneo M. The platelet P2Y(1)(2) receptor for adenosine diphosphate: congenital and drug-induced defects. Blood. 2011;117(7):2102-12.
22. Lecchi A, Razzari C, Paoletta S, Dupuis A, Nakamura L, Ohlmann P, et al. Identification of a new dysfunctional platelet P2Y12 receptor variant associated with bleeding diathesis. Blood. 2015;125(6):1006-13.

23. Pozgajova M, Sachs UJ, Hein L, Nieswandt B. Reduced thrombus stability in mice lacking the alpha2A-adrenergic receptor. Blood. 2006;108(2):510-4.
24. Offermanns S. Activation of platelet function through G protein-coupled receptors. Circ Res. 2006;99(12):1293-304.
25. Offermanns S, Toombs CF, Hu YH, Simon MI. Defective platelet activation in G alpha(q)-deficient mice. Nature. 1997;389(6647):183-6.
26. Gachet C. Regulation of platelet functions by P2 receptors. Annu Rev Pharmacol Toxicol. 2006;46:277-300.
27. Leon C, Hechler B, Freund M, Eckly A, Vial C, Ohlmann P, et al. Defective platelet aggregation and increased resistance to thrombosis in purinergic P2Y(1) receptor-null mice. J Clin Invest. 1999;104(12):1731-7.
28. Bird JE, Wang X, Smith PL, Barbera F, Huang C, Schumacher WA. A platelet target for venous thrombosis? P2Y1 deletion or antagonism protects mice from vena cava thrombosis. J Thromb Thrombolysis. 2012;34(2):199-207.
29. Habib A, FitzGerald GA, Maclouf J. Phosphorylation of the thromboxane receptor alpha, the predominant isoform expressed in human platelets. J Biol Chem. 1999;274(5):2645-51.
30. Williams CD, Chan AT, Elman MR, Kristensen AH, Miser WF, Pignone MP, et al. Aspirin use among adults in the u.s.: results of a national survey. Am J Prev Med. 2015;48(5):501-8.
31. Kamae T, Kiyomizu K, Nakazawa T, Tadokoro S, Kashiwagi H, Honda S, et al. Bleeding tendency and impaired platelet function in a patient carrying a heterozygous mutation in the thromboxane A2 receptor. J Thromb Haemost. 2011;9(5):1040-8.
32. Mumford AD, Nisar S, Darnige L, Jones ML, Bachelot-Loza C, Gandrille S, et al. Platelet dysfunction associated with the novel Trp29Cys thromboxane A(2) receptor variant. J Thromb Haemost. 2013;11(3):547-54.
33. Mumford AD, Dawood BB, Daly ME, Murden SL, Williams MD, Protty MB, et al. A novel thromboxane A2 receptor D304N variant that abrogates ligand binding in a patient with a bleeding diathesis. Blood. 2010;115(2):363-9.
34. Nisar SP, Lordkipanidze M, Jones ML, Dawood B, Murden S, Cunningham MR, et al. A novel thromboxane A2 receptor N42S variant results in reduced surface expression and platelet dysfunction. Thromb Haemost. 2014;111(5):923-32.
35. Thomas DW, Mannon RB, Mannon PJ, Latour A, Oliver JA, Hoffman M, et al. Coagulation defects and altered hemodynamic responses in mice lacking receptors for thromboxane A2. J Clin Invest. 1998;102(11):1994-2001.
36. Alberelli MA, De Candia E. Functional role of protease activated receptors in vascular biology. Vascul Pharmacol. 2014;62(2):72-81.
37. Muehlschlegel JD, Perry TE, Liu KY, Fox AA, Smith SA, Lichtner P, et al. Polymorphism in the protease-activated receptor-4 gene region associates with platelet activation and perioperative myocardial injury. Am J Hematol. 2012;87(2):161-6.
38. Smith SM, Judge HM, Peters G, Armstrong M, Dupont A, Gaussem P, et al. PAR-1 genotype influences platelet aggregation and procoagulant responses in patients with coronary artery disease prior to and during clopidogrel therapy. Platelets. 2005;16(6):340-5.
39. Dupont A, Fontana P, Bachelot-Loza C, Reny JL, Bieche I, Desvard F, et al. An intronic polymorphism in the PAR-1 gene is associated with platelet receptor density and the response to SFLLRN. Blood. 2003;101(5):1833-40.
40. Patel YM, Lordkipanidze M, Lowe GC, Nisar SP, Garner K, Stockley J, et al. A novel mutation in the P2Y12 receptor and a function-reducing polymorphism in protease-activated receptor 1 in a patient with chronic bleeding. J Thromb Haemost. 2014;12(5):716-25.
41. Li Z, Delaney MK, O'Brien KA, Du X. Signaling during platelet adhesion and activation. Arterioscler Thromb Vasc Biol. 2010;30(12):2341-9.

42. Gibbins J, Asselin J, Farndale R, Barnes M, Law CL, Watson SP. Tyrosine phosphorylation of the Fc receptor gamma-chain in collagen-stimulated platelets. J Biol Chem. 1996;271(30):18095-9.
43. Moroi M, Jung SM, Okuma M, Shinmyozu K. A patient with platelets deficient in glycoprotein VI that lack both collagen-induced aggregation and adhesion. J Clin Invest. 1989;84(5):1440-5.
44. Gibbins JM, Briddon S, Shutes A, van Vugt MJ, van de Winkel JG, Saito T, et al. The p85 subunit of phosphatidylinositol 3-kinase associates with the Fc receptor gamma-chain and linker for activitor of T cells (LAT) in platelets stimulated by collagen and convulxin. J Biol Chem. 1998;273(51):34437-43.
45. Clemetson JM, Polgar J, Magnenat E, Wells TN, Clemetson KJ. The platelet collagen receptor glycoprotein VI is a member of the immunoglobulin superfamily closely related to FcalphaR and the natural killer receptors. J Biol Chem. 1999;274(41):29019-24.
46. Chen H, Locke D, Liu Y, Liu C, Kahn ML. The platelet receptor GPVI mediates both adhesion and signaling responses to collagen in a receptor density-dependent fashion. J Biol Chem. 2002;277(4):3011-9.
47. Nieswandt B, Watson SP. Platelet-collagen interaction: is GPVI the central receptor? Blood. 2003;102(2):449-61.
48. Jarvis GE, Atkinson BT, Snell DC, Watson SP. Distinct roles of GPVI and integrin alpha(2)beta(1) in platelet shape change and aggregation induced by different collagens. Br J Pharmacol. 2002;137(1):107-17.
49. Kato K, Kanaji T, Russell S, Kunicki TJ, Furihata K, Kanaji S, et al. The contribution of glycoprotein VI to stable platelet adhesion and thrombus formation illustrated by targeted gene deletion. Blood. 2003;102(5):1701-7.
50. Kalia N, Auger JM, Atkinson B, Watson SP. Critical role of FcR gamma-chain, LAT, PLCgamma2 and thrombin in arteriolar thrombus formation upon mild, laser-induced endothelial injury in vivo. Microcirculation. 2008;15(4):325-35.
51. Nieswandt B, Schulte V, Bergmeier W, Mokhtari-Nejad R, Rackebrandt K, Cazenave JP, et al. Long-term antithrombotic protection by in vivo depletion of platelet glycoprotein VI in mice. J Exp Med. 2001;193(4):459-69.
52. Dumont B, Lasne D, Rothschild C, Bouabdelli M, Ollivier V, Oudin C, et al. Absence of collagen-induced platelet activation caused by compound heterozygous GPVI mutations. Blood. 2009;114(9):1900-3.
53. Hermans C, Wittevrongel C, Thys C, Smethurst PA, Van Geet C, Freson K. A compound heterozygous mutation in glycoprotein VI in a patient with a bleeding disorder. J Thromb Haemost. 2009;7(8):1356-63.
54. Matus V, Valenzuela G, Saez CG, Hidalgo P, Lagos M, Aranda E, et al. An adenine insertion in exon 6 of human GP6 generates a truncated protein associated with a bleeding disorder in four Chilean families. J Thromb Haemost. 2013;11(9):1751-9.
55. Savoia A, Kunishima S, De Rocco D, Zieger B, Rand ML, Pujol-Moix N, et al. Spectrum of the mutations in Bernard-Soulier syndrome. Hum Mutat. 2014;35(9):1033-45.
56. Clemetson KJ, McGregor JL, James E, Dechavanne M, Luscher EF. Characterization of the platelet membrane glycoprotein abnormalities in Bernard-Soulier syndrome and comparison with normal by surface-labeling techniques and high-resolution two-dimensional gel electrophoresis. J Clin Invest. 1982;70(2):304-11.
57. Nurden AT, Caen JP. An abnormal platelet glycoprotein pattern in three cases of Glanzmann's thrombasthenia. Br J Haematol. 1974;28(2):253-60.
58. Nurden AT, Pillois X, Fiore M, Alessi MC, Bonduel M, Dreyfus M, et al. Expanding the Mutation Spectrum Affecting alphaIIbbeta3 Integrin in Glanzmann Thrombasthenia: Screening of the ITGA2B and ITGB3 Genes in a Large International Cohort. Hum Mutat. 2015;36(5):548-61.

59. Ihara K, Ishii E, Eguchi M, Takada H, Suminoe A, Good RA, et al. Identification of mutations in the c-mpl gene in congenital amegakaryocytic thrombocytopenia. Proc Natl Acad Sci U S A. 1999;96(6):3132-6.
60. Baxter EJ, Scott LM, Campbell PJ, East C, Fourouclas N, Swanton S, et al. Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet. 2005;365(9464):1054-61.
61. Tefferi A, Barbui T. Polycythemia vera and essential thrombocythemia: 2015 update on diagnosis, risk-stratification and management. Am J Hematol. 2015;90(2):162-73.
62. Phillips DR, Agin PP. Platelet membrane defects in Glanzmann's thrombasthenia. Evidence for decreased amounts of two major glycoproteins. J Clin Invest. 1977;60(3):535-45.
63. Nurden AT, Caen JP. Specific roles for platelet surface glycoproteins in platelet function. Nature. 1975;255(5511):720-2.
64. Berndt MC, Gregory C, Chong BH, Zola H, Castaldi PA. Additional glycoprotein defects in Bernard-Soulier's syndrome: confirmation of genetic basis by parental analysis. Blood. 1983;62(4):800-7.
65. Sugiyama T, Okuma M, Ushikubi F, Sensaki S, Kanaji K, Uchino H. A novel platelet aggregating factor found in a patient with defective collagen-induced platelet aggregation and autoimmune thrombocytopenia. Blood. 1987;69(6):1712-20.
66. Jurk K, Schulz AS, Kehrel BE, Rapple D, Schulze H, Mobest D, et al. Novel integrin-dependent platelet malfunction in siblings with leukocyte adhesion deficiency-III (LAD-III) caused by a point mutation in FERMT3. Thromb Haemost. 2010;103(5):1053-64.
67. Cattaneo M, Zighetti ML, Lombardi R, Martinez C, Lecchi A, Conley PB, et al. Molecular bases of defective signal transduction in the platelet P2Y12 receptor of a patient with congenital bleeding. Proc Natl Acad Sci U S A. 2003;100(4):1978-83.
68. Watson SP, Lowe GC, Lordkipanidze M, Morgan NV. Genotyping and phenotyping of platelet function disorders. J Thromb Haemost. 2013;1:351-63.
69. Eikelboom JW, Hirsh J, Spencer FA, Baglin TP, Weitz JI. Antiplatelet drugs: Antithrombotic Therapy and Prevention of Thrombosis, 9th ed: American College of Chest Physicians Evidence-Based Clinical Practice Guidelines. Chest. 2012;141(2 Suppl):11-2293.
70. Daly ME, Dawood BB, Lester WA, Peake IR, Rodeghiero F, Goodeve AC, et al. 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;113(17):4110-3.
71. Bolton-Maggs PH, Chalmers EA, Collins PW, Harrison P, Kitchen S, Liesner RJ, et al. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. Br J Haematol. 2006;135(5):603-33.
72. Harrison P, Mackie I, Mumford A, Briggs C, Liesner R, Winter M, et al. Guidelines for the laboratory investigation of heritable disorders of platelet function. Br J Haematol. 2011;155(1):30-44.
73. Gresele P. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. J Thromb Haemost. 2015;13(2):314-22.
74. Lehman CM, Blaylock RC, Alexander DP, Rodgers GM. Discontinuation of the bleeding time test without detectable adverse clinical impact. Clin Chem. 2001;47(7):1204-11.
75. Born GV. Aggregation of blood platelets by adenosine diphosphate and its reversal. Nature. 1962;194:927-9.
76. Harrison P. Assessment of platelet function in the laboratory. Hamostaseologie. 2009;29(1):25-31.
77. 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;18(5):329-45.
78. Miller JL. Platelet function testing: an improved approach utilizing lumi-aggregation and an interactive computer system. Am J Clin Pathol. 1984;81(4):471-6.

79. Nieuwenhuis HK, Akkerman JW, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on one hundred six patients. Blood. 1987;70(3):620-3.
80. Hayward CP, Harrison P, Cattaneo M, Ortel TL, Rao AK. Platelet function analyzer (PFA)-100 closure time in the evaluation of platelet disorders and platelet function. J Thromb Haemost. 2006;4(2):312-9.
81. Carcao MD, Blanchette VS, Stephens D, He L, Wakefield CD, Butchart S, et al. Assessment of thrombocytopenic disorders using the Platelet Function Analyzer (PFA-100). Br J Haematol. 2002 Jun;117(4):961-4.
82. Christie D, Avari T, Carrington L, Cohen E, DeBiase B, Harrison P, et al. Platelet Function Testing by Aggregometry: Approved guideline. Clinical and Laboratory Standards Institute. 2008;28:1-45.
83. Naik S, Teruya J, Dietrich JE, Jariwala P, Soundar E, Venkateswaran L. Utility of platelet function analyzer as a screening tool for the diagnosis of von Willebrand disease in adolescents with menorrhagia. Pediatr Blood Cancer. 2013 Jul;60(7):1184-7.
84. Gadisseur A, Hermans C, Berneman Z, Schroyens W, Deckmyn H, Michiels JJ. Laboratory diagnosis and molecular classification of von Willebrand disease. Acta Haematol. 2009;121(2-3):71-84.
85. Harrison P, Lordkipanidzé M. Testing platelet function. Hematol Oncol Clin North Am 2013;27:411-41.
86. Schimmer C, Hamouda K, Sommer SP, Ozkur M, Hain J, Leyh R. The Predictive Value of Multiple Electrode Platelet Aggregometry (Multiplate) in Adult Cardiac Surgery. The Thoracic and cardiovascular surgeon. 2013 Feb 18.
87. Siller-Matula JM, Francesconi M, Dechant C, Jilma B, Maurer G, Delle-Karth G, et al. Personalized antiplatelet treatment after percutaneous coronary intervention: The MADONNA study. Int J Cardiol. 2013 May 30;167:2018-23.
88. Sibbing D, Steinhubl SR, Schulz S, Schomig A, Kastrati A. Platelet aggregation and its association with stent thrombosis and bleeding in clopidogrel-treated patients: initial evidence of a therapeutic window. J Am Coll Cardiol. 2010 Jul 20;56(4):317-8.
89. Bonello L, Tantry US, Marcucci R, Blindt R, Angiolillo DJ, Becker R, et al. Consensus and future directions on the definition of high on-treatment platelet reactivity to adenosine diphosphate. J Am Coll Cardiol. 2010 Sep 14;56(12):919-33.
90. Wijns W, Kolh P, Danchin N, Di Mario C, Falk V, Folliguet T, et al. Guidelines on myocardial revascularization: The Task Force on Myocardial Revascularization of the European Society of Cardiology (ESC) and the European Association for Cardio-Thoracic Surgery (EACTS). Eur Heart J. 2010 Aug 29;31:2501-55.
91. Levine GN, Bates ER, Blankenship JC, Bailey SR, Bittl JA, Cercek B, et al. 2011 ACCF/AHA/SCAI Guideline for Percutaneous Coronary Intervention: A Report of the American College of Cardiology Foundation/American Heart Association Task Force on Practice Guidelines and the Society for Cardiovascular Angiography and Interventions. Circulation. 2011 Nov 7;124:e574-651.
92. van Werkum JW, Harmsze AM, Elsenberg EH, Bouman HJ, ten Berg JM, Hackeng CM. The use of the VerifyNow system to monitor antiplatelet therapy: a review of the current evidence. Platelets. 2008 Nov;19(7):479-88.
93. Campbell J, Ridgway H, Carville D. Plateletworks®: a novel point of care platelet function screen. Mol Diagn Ther. 2008;12(4):253-8.
94. Chitlur M, Lusher J. Standardization of thromboelastography: values and challenges. Semin Thromb Hemost. 2010 Oct;36(7):707-11.
95. Scharbert G, Auer A, Kozek-Langenecker S. Evaluation of the Platelet Mapping Assay on rotational thromboelastometry ROTEM. Platelets. 2009 Mar;20(2):125-30.

96. Lordkipanidze M, Lowe GC, Kirkby NS, Chan MV, Lundberg MH, Morgan NV, et al. Characterization of multiple platelet activation pathways in patients with bleeding as a high-throughput screening option: use of 96-well Optimul assay. Blood. 2014;123(8):2013-08.
97. Dovlatova N, Lordkipanidze M, Lowe GC, Dawood B, May J, Heptinstall S, et al. Evaluation of a whole blood remote platelet function test for the diagnosis of mild bleeding disorders. J Thromb Haemost. 2014;12(5):660-5.
98. Watson S, Daly M, Dawood B, Gissen P, Makris M, Mundell S, et al. Phenotypic approaches to gene mapping in platelet function disorders - identification of new variant of P2Y12, TxA2 and GPVI receptors. Hamostaseologie. 2010;30(1):29-38.
99. Leo VC, Morgan NV, Bem D, Jones ML, Lowe GC, Lordkipanidze M, et al. Use of next-generation sequencing and candidate gene analysis to identify underlying defects in patients with inherited platelet function disorders. J Thromb Haemost. 2015 13(4):643-50.
100. Jones ML, Norman JE, Morgan NV, Mundell SJ, Lordkipanidze M, Lowe GC, et al. Diversity and impact of rare variants in genes encoding the platelet G protein-coupled receptors. Thromb Haemost. 2015;113(4):826-37
101. Sarah Fletcher Ben Johnson, Gill Lowe, Danai Bem, Sian Drake, Marie Lordkipanidzé, Isabel Sánchez Guiú,, Ban Dawood JR, Michael Simpson, Martina Daly, Jayashree Motwani, Peter Collins, Steve Watson and Neil Morgan on behalf of the GAPP collaborative. Consecutive SLFN14 mutations in 3 unrelated families with an inherited bleeding disorder, thrombocytopenia and secretion defects. Presented abstract (oral presentation) at International Society on Thrombosis and Haemostasis 2015 Congress; Toronto, Canada. Abstract reference AS011
102. http://www.birmingham.ac.uk/research/activity/mds/domains/cardio-resp-neuro/vita/platelet-gapp/index.aspx.
103. Dawood BB, Lowe GC, Lordkipanidze M, Bem D, Daly ME, Makris M, et al. Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. Blood. 2012;120(25):5041-9.
104. Henson J, Tischler G, Ning Z. Next-generation sequencing and large genome assemblies. Pharmacogenomics. 2012;13(8):901-15.
105. Desai AN, Jere A. Next-generation sequencing: ready for the clinics? Clin Genet. 2012;81(6):503-10.
106. Pareek CS, Smoczynski R, Tretyn A. Sequencing technologies and genome sequencing. J Appl Genet. 2011;52(4):413-35.
107. Tennessen JA, Bigham AW, O'Connor TD, Fu W, Kenny EE, Gravel S, et al. Evolution and functional impact of rare coding variation from deep sequencing of human exomes. Science. 2012;337(6090):64-9.
108. Morgan NV, Pasha S, Johnson CA, Ainsworth JR, Eady RA, Dawood B, et al. A germline mutation in BLOC1S3/reduced pigmentation causes a novel variant of Hermansky-Pudlak syndrome (HPS8). Am J Hum Genet. 2006;78(1):160-6.
109. Lowe GC, Sanchez Guiu I, Chapman O, Rivera J, Lordkipanidze M, Dovlatova N, et al. Microsatellite markers as a rapid approach for autozygosity mapping in Hermansky-Pudlak syndrome: identification of the second HPS7 mutation in a patient presenting late in life: Thromb Haemost. 2013 Apr;109(4):766-8
110. Lowe G Motwani J, Williams M, Simpson M, Watson S and Morgan N. A mutation in ANKRD18A, ankyrin repeat domain 18A, causes a severe form of congenital thrombocytopenia. Presented at 58th Scientific and Standardization Committee Meeting of the International Society on Thrombosis and Haemostasis; 2012; Liverpool, United Kingdom. Abstract reference ref SSC12-PC01.
111. Albers CA, Cvejic A, Favier R, Bouwmans EE, Alessi MC, Bertone P, et al. Exome sequencing identifies NBEAL2 as the causative gene for gray platelet syndrome. Nat Genet. 2011;43(8):735-7.

112. Gunay-Aygun M, Falik-Zaccai TC, Vilboux T, Zivony-Elboum Y, Gumruk F, Cetin M, et al. NBEAL2 is mutated in gray platelet syndrome and is required for biogenesis of platelet alpha-granules. Nat Genet. 2011;43(8):732-4.
113. Kahr WH, Hinckley J, Li L, Schwertz H, Christensen H, Rowley JW, et al. Mutations in NBEAL2, encoding a BEACH protein, cause gray platelet syndrome. Nat Genet. 2011;43(8):738-40.
114. Klopocki E, Schulze H, Strauss G, Ott CE, Hall J, Trotier F, et al. Complex inheritance pattern resembling autosomal recessive inheritance involving a microdeletion in thrombocytopenia-absent radius syndrome. Am J Hum Genet. 2007;80(2):232-40.
115. Albers CA, Paul DS, Schulze H, Freson K, Stephens JC, Smethurst PA, et al. Compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit RBM8A causes TAR syndrome. Nat Genet. 2012;44(4):435-9.
116. Westbury SK, Turro E, Greene D, Lentaigne C, Kelly AM, Bariana TK, et al. Human phenotype ontology annotation and cluster analysis to unravel genetic defects in 707 cases with unexplained bleeding and platelet disorders. Genome Med. 2015;7(1):015-0151.
117. Ben Johnson, Gillian C Lowe, Jane Futterer, Marie Lordkipanidzé, David MacDonald, Michael A Simpson, Isabel Sánchez Guiú, Sian Drake, Danai Bem, Vincenzo Leo, Sarah J Fletcher, Ban Dawood, David Allsup, Tina Biss, Paula Bolton-Maggs, Peter Collins, Nicola Curry, Charlotte Grimley, Beki James, Mike Makris, Jayashree Motwani, Sue Pavord, Katherine Talks, Jecko Thachil, Jonathan Wilde, Mike Williams, Paul Harrison, Paul Gissen, Stuart Mundell, Andrew Mumford, Martina Daly, Steve P Watson and Neil V Morgan on behalf of the UK GAPP Study Group. Whole exome sequencing in patients with inherited thrombocytopenia and excessive bleeding is an efficient way to identify genetic variants in known and novel genes. Submitted to Blood. 2015.
118. Dickinson RE, Griffin H, Bigley V, Reynard LN, Hussain R, Haniffa M, et al. Exome sequencing identifies GATA-2 mutation as the cause of dendritic cell, monocyte, B and NK lymphoid deficiency. Blood. 2011;118(10):2656-8.
119. Jones ML, Murden SL, Bem D, Mundell SJ, Gissen P, Daly ME, et al. Rapid genetic diagnosis of heritable platelet function disorders with next-generation sequencing: proof-of-principle with Hermansky-Pudlak syndrome: J Thromb Haemost. 2012 Feb;10(2):306-9
120. Collins PW, Baglin TP, Dang R, Evans G, Greaves M, Laffan M, et al. Non-malignant haematology research in the UK: looking forward to new opportunities. Br J Haematol. 2010;150(6):732-6.
121. National Institute of Health Research. Specialty Group News. October 2012; Available from: http://www.birmingham.ac.uk/Documents/college-mds/research/plategappubs/SGN-October-2012.pdf.
122. Blais N, Pharand C, Lordkipanidze M, Sia YK, Merhi Y, Diodati JG. Response to aspirin in healthy individuals. Cross-comparison of light transmission aggregometry, VerifyNow system, platelet count drop, thromboelastography (TEG) and urinary 11-dehydrothromboxane B(2). Thromb Haemost. 2009;102(2):404-11.
123. Castilloux JF, Moffat KA, Liu Y, Seecharan J, Pai M, Hayward CP. A prospective cohort study of light transmission platelet aggregometry for bleeding disorders: is testing native platelet-rich plasma non-inferior to testing platelet count adjusted samples? Thromb Haemost. 2011;106(4):675-82.
124. Linnemann B, Schwonberg J, Mani H, Prochnow S, Lindhoff-Last E. Standardization of light transmittance aggregometry for monitoring antiplatelet therapy: an adjustment for platelet count is not necessary. J Thromb Haemost. 2008;6(4):677-83.
125. Lowe GC Lordkipanidzé M, Watson S. Defining normal ranges for human platelet responses by lumiaggregometry using a rationalised agonist panel. Presented at United Kingdom Platelet Group Meeting; 2013; Birmingham, United Kingdom.
126. Cariappa R, Wilhite TR, Parvin CA, Luchtman-Jones L. Comparison of PFA-100 and bleeding time testing in pediatric patients with suspected hemorrhagic problems. J Pediatr Hematol Oncol. 2003;25(6):474-9.

127. Gresele P et al. Validation of the ISTH/SSC Bleeding Assessment Tool for Inherited Platelet Disorders. [accessed 02/04/2015]; Protocol available from: http://c.ymcdn.com/sites/www.isth.org/resource/group/1801fd48-7b38-4094-a922-c424340ee0b3/projects/protocol_final.pdf.
128. Hurst BS, Lange SS, Kullstam SM, Usadi RS, Matthews ML, Marshburn PB, et al. Obstetric and gynecologic challenges in women with Ehlers-Danlos syndrome. Obstet Gynecol. 2014;123(3):506-13.
129. Lordkipanidze M, Lowe GC, Watson SP. Simultaneous measurement of ATP release and LTA does not potentiate platelet aggregation to epinephrine: Thromb Haemost. 2013;110(1):199-201.
130. Hayward CP, Moffat KA, Castilloux JF, Liu Y, Seecharan J, Tasneem S, et al. Simultaneous measurement of adenosine triphosphate release and aggregation potentiates human platelet aggregation responses for some subjects, including persons with Quebec platelet disorder. Thromb Haemost. 2012;107(4):726-34.
131. Callan MB, Shofer FS, Wojenski C, Giger U. Chrono-lume and magnesium potentiate aggregation of canine but not human platelets in citrated platelet-rich plasma. Thromb Haemost. 1998 Jul;80(1):176-80.
132. Mehta P, Mehta J, Ostrowski N, Aguila E. Potentiation of platelet aggregation by Chronolume. Thromb Res. 1983 Dec 1;32(5):509-12.
133. Zwierzina WD, Kunz F. A method of testing platelet aggregation in native whole blood. Thromb Res. 1985 Apr 1;38(1):91-100.
134. Pfueller SL, Broadway M. Inhibition of platelet aggregation and [14C] serotonin release by luciferin: luciferase preparations. Thromb Res. 1986 Nov 15;44(4):539-42.
135. Thompson NT, Scrutton MC. Inhibition by luciferin-luciferase reagents of aggregatory responses to excitatory agonists in washed platelet suspensions. Thromb Res. 1985 Apr 15;38(2):109-19.
136. Dawood BB, Lowe GC, Lordkipanidzé M, Bem D, Daly ME, Makris M, et al. Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. Blood. 2012 Sep 21;120:5041-9.
137. Rydz N, James PD. The evolution and value of bleeding assessment tools. J Thromb Haemost. 2012;10(11):2223-9.
138. Rodeghiero F, Castaman G. Congenital von Willebrand disease type I: definition, phenotypes, clinical and laboratory assessment. Best Pract Res Clin Haematol. 2001;14(2):321-35.
139. Rodeghiero F, Castaman G, Tosetto A, Batlle J, Baudo F, Cappelletti A, et al. The discriminant power of bleeding history for the diagnosis of type 1 von Willebrand disease: an international, multicenter study. J Thromb Haemost. 2005;3(12):2619-26.
140. Tosetto A, Rodeghiero F, Castaman G, Goodeve A, Federici AB, Batlle J, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). J Thromb Haemost. 2006;4(4):766-73.
141. Katsanis E, Luke KH, Hsu E, Li M, Lillicrap D. Prevalence and significance of mild bleeding disorders in children with recurrent epistaxis. J Pediatr. 1988;113(1 Pt 1):73-6.
142. Dean JA, Blanchette VS, Carcao MD, Stain AM, Sparling CR, Siekmann J, et al. von Willebrand disease in a pediatric-based population--comparison of type 1 diagnostic criteria and use of the PFA-100 and a von Willebrand factor/collagen-binding assay. Thromb Haemost. 2000;84(3):401-9.
143. Bowman M, Riddel J, Rand ML, Tosetto A, Silva M, James PD. Evaluation of the diagnostic utility for von Willebrand disease of a pediatric bleeding questionnaire: J Thromb Haemost. 2009; 7(8):1418-21.
144. Higham JM, O'Brien PM, Shaw RW. Assessment of menstrual blood loss using a pictorial chart. Br J Obstet Gynaecol. 1990;97(8):734-9.

145. Philipp CS, Faiz A, Heit JA, Kouides PA, Lukes A, Stein SF, et al. Evaluation of a screening tool for bleeding disorders in a US multisite cohort of women with menorrhagia. Am J Obstet Gynecol. 2011;204(3):17.
146. Rodeghiero F, Tosetto A, Abshire T, Arnold DM, Coller B, James P, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. J Thromb Haemost. 2010;8(9):2063-5.
147. Bidlingmaier C, Grote V, Budde U, Olivieri M, Kurnik K. Prospective evaluation of a pediatric bleeding questionnaire and the ISTH bleeding assessment tool in children and parents in routine clinical practice. J Thromb Haemost. 2012;10(7):1335-41.
148. Lowe GC, Lordkipanidze M, Watson SP. Utility of the ISTH bleeding assessment tool in predicting platelet defects in participants with suspected inherited platelet function disorders. J Thromb Haemost. 2013;11(9):1663-8.
149. Meade TW, Vickers MV, Thompson SG, Stirling Y, Haines AP, Miller GJ. Epidemiological characteristics of platelet aggregability. Br Med J. 1985;290(6466):428-32.
150. Watson SP, Lowe GC, Lordkipanidze M, Morgan NV. Genotyping and phenotyping of platelet function disorders. J Thromb Haemost. 2013;1:351-63
151. Rodeghiero F, Tosetto A, Castaman G. How to estimate bleeding risk in mild bleeding disorders. J Thromb Haemost. 2007 Jul;5 Suppl 1:157-66.
152. Rodeghiero F, Tosetto A, Abshire T, Arnold DM, Coller B, James P, et al. ISTH/SSC Bleeding Assessment Tool: A Standardized Questionnaire and a Proposal for a New Bleeding Score for Inherited Bleeding Disorders. J Thromb Haemost. 2010;8:2063-5.
153. Tosetto A, Castaman G, Plug I, Rodeghiero F, Eikenboom J. Prospective evaluation of the clinical utility of quantitative bleeding severity assessment in patients referred for hemostatic evaluation. J Thromb Haemost. 2011 Jun;9(6):1143-8.
154. Zhang X. Exome sequencing greatly expedites the progressive research of Mendelian diseases. Front Med. 2014;8(1):42-57.
155. Daly ME, Leo VC, Lowe GC, Watson SP, Morgan NV. What is the role of genetic testing in the investigation of patients with suspected platelet function disorders? Br J Haematol. 2014;165(2):193-203.
156. Hallberg L, Nilsson L. Determination of Menstrual Blood Loss. Scand J Clin Lab Invest. 1964;16:244-8.
157. Fonseca-Moutinho JA, Barbosa LS, Torres DG, Nunes SM. Abnormal uterine bleeding as a presenting symptom is related to multiple uterine leiomyoma: an ultrasound-based study. Int J Womens Health. 2013;5:689-94.
158. In: Christine A. Lee, Rezan A Kadir, Peter A Kouides. Inherited Bleeding Disorders in Women: Wiley-Blackwell; 2009
159. James AH, Ragni MV, Picozzi VJ. Bleeding disorders in premenopausal women: (another) public health crisis for hematology? Hematology Am Soc Hematol Educ Program. 2006:474-85.
160. In: Assessment of menorrhagia BMJ Best Practice 2014: Available from: http://bestpractice.bmj.com/best-practice/monograph/171.html. Last accessed 03/07/2015
161. Peuranpaa P, Heliovaara-Peippo S, Fraser I, Paavonen J, Hurskainen R. Effects of anemia and iron deficiency on quality of life in women with heavy menstrual bleeding. Acta Obstet Gynecol Scand. 2014;93(7):654-60.
162. Wasiak R, Filonenko A, Vanness DJ, Law A, Jeddi M, Wittrup-Jensen KU, et al. Impact of estradiol valerate/dienogest on work productivity and activities of daily living in women with heavy menstrual bleeding. J Womens Health. 2013;22(4):378-84.
163. Ahuja SP, Hertweck SP. Overview of bleeding disorders in adolescent females with menorrhagia. J Pediatr Adolesc Gynecol. 2010;23(6 Suppl):006.
164. Von Mackensen S. Quality of life in women with bleeding disorders. Haemophilia. 2011;1:33-7.

165. Chi C, Pollard D, Tuddenham EG, Kadir RA. Menorrhagia in adolescents with inherited bleeding disorders. J Pediatr Adolesc Gynecol. 2010;23(4):215-22.
166. Kouides PA, Kadir RA. Menorrhagia associated with laboratory abnormalities of hemostasis: epidemiological, diagnostic and therapeutic aspects. J Thromb Haemost. 2007;1:175-82.
167. Diaz R, Dietrich JE, Mahoney D, Jr., Yee DL, Srivaths LV. Hemostatic abnormalities in young females with heavy menstrual bleeding. J Pediatr Adolesc Gynecol. 2014;27(6):324-9.
168. Nilsson IM. Commentary to Erik von Willebrand's original paper from 1926 'Hereditar pseudohemofili'. Haemophilia. 1999;5(3):220-1.
169. Saxena R, Gupta M, Gupta PK, Kashyap R, Choudhry VP, Bhargava M. Inherited bleeding disorders in Indian women with menorrhagia. Haemophilia. 2003;9(2):193-6.
170. Miller CH, Philipp CS, Stein SF, Kouides PA, Lukes AS, Heit JA, et al. The spectrum of haemostatic characteristics of women with unexplained menorrhagia. Haemophilia. 2011;17(1):1365-2516.
171. Lohse J, Gehrisch S, Tauer JT, Knofler R. Therapy refractory menorrhagia as first manifestation of Hermansky-Pudlak syndrome. Hamostaseologie. 2011;31(1):S61-3.
172. Santos XM, Bercaw-Pratt JL, Yee DL, Dietrich JE. Recurrent menorrhagia in an adolescent with a platelet secretion defect. J Pediatr Adolesc Gynecol. 2011;24(2):28.
173. White JG, Keel S, Reyes M, Burris SM. Alpha-delta platelet storage pool deficiency in three generations. Platelets. 2007;18(1):1-10.
174. Mills HL, Abdel-Baki MS, Teruya J, Dietrich JE, Shah MD, Mahoney D, Jr., et al. Platelet function defects in adolescents with heavy menstrual bleeding. Haemophilia. 2014;20(2):249-54.
175. Seravalli V, Linari S, Peruzzi E, Dei M, Paladino E, Bruni V. Prevalence of hemostatic disorders in adolescents with abnormal uterine bleeding. J Pediatr Adolesc Gynecol. 2013;26(5):285-9.
176. Quiroga T, Mezzano D. Is my patient a bleeder? A diagnostic framework for mild bleeding disorders. Hematology Am Soc Hematol Educ Program. 2012:466.
177. Matthews DC. Inherited disorders of platelet function. Pediatr Clin North Am. 2013;60(6):1475-88.
178. NICE (National Institute of Clinical Excellence) guidelines . Heavy menstrual bleeding [CG44]. 2007; Available from: http://www.nice.org.uk/guidance/cg44. Last accessed 03/07/2015.
179. NHS Choices - Hysterectomy. Available from: http://www.nhs.uk/conditions/Hysterectomy/Pages/Introduction.aspx. Last accessed 03/07/2015.
180. Albright BB, Witte T, Tofte AN, Chou J, Black JD, Desai VB, et al. Robotic Versus Laparoscopic Hysterectomy for Benign Disease: A Systematic Review and Meta-Analysis of Randomized Trials. J Minim Invasive Gynecol. 2015;10(15):003.
181. Lonnee-Hoffmann R, Pinas I. Effects of Hysterectomy on Sexual Function: Curr Sex Health Rep. 2014;6(4):244-251.
182. Ray S, Ray A. Non-surgical interventions for treating heavy menstrual bleeding (menorrhagia) in women with bleeding disorders. Cochrane Database Syst Rev. 2014;26(11).
183. United Kingdom Haemophilia Centres Doctors Organisation.. Bleeding disorder statistics for April 2013 to March 2014. Available from http://www.ukhcdo.org/docs/AnnualReports/2014/Bleeding_Disorder_Statistics_For_Website_2013-2014.pdf. Last accessed 03/07/2015
184. Balduini CL, Pecci A, Savoia A. Recent advances in the understanding and management of MYH9-related inherited thrombocytopenias. Br J Haematol. 2011;154(2):161-74.
185. Friedmann AM, Sengul H, Lehmann H, Schwartz C, Goodman S. Do basic laboratory tests or clinical observations predict bleeding in thrombocytopenic oncology patients? A reevaluation of prophylactic platelet transfusions. Transfus Med Rev. 2002;16(1):34-45.
186. Hayward CP. Diagnostic evaluation of platelet function disorders. Blood Rev. 2011;25(4):169-73.

187. Michelson AD. Flow cytometry: a clinical test of platelet function. Blood. 1996 Jun 15;87(12):4925-36.
188. Schmitz G, Rothe G, Ruf A, Barlage S, Tschope D, Clemetson KJ, et al. European Working Group on Clinical Cell Analysis: Consensus protocol for the flow cytometric characterisation of platelet function. Thromb Haemost. 1998 May;79(5):885-96.
189. Ault KA. The clinical utility of flow cytometry in the study of platelets. Semin Hematol. 2001 Apr;38(2):160-8.
190. Michelson AD. Evaluation of platelet function by flow cytometry. Pathophysiol Haemost Thromb. 2006;35(1-2):67-82.
191. Frelinger AL, 3rd, Li Y, Linden MD, Barnard MR, Fox ML, Christie DJ, et al. Association of Cyclooxygenase-1-Dependent and -Independent Platelet Function Assays With Adverse Clinical Outcomes in Aspirin-Treated Patients Presenting for Cardiac Catheterization. Circulation. 2009 Dec 7;120:2586-96.
192. Fox SC, May JA, Shah A, Neubert U, Heptinstall S. Measurement of platelet P-selectin for remote testing of platelet function during treatment with clopidogrel and/or aspirin. Platelets. 2009 Jun;20(4):250-9.
193. Psaila B, Bussel JB, Frelinger AL, Babula B, Linden MD, Li Y, et al. Differences in Platelet Function In Patients with Acute Myeloid Leukaemia and Myelodysplasia Compared to Equally Thrombocytopenic Patients with Immune Thrombocytopenia: J Thromb Haemost. 2011; 9(11): 2302-10.
194. Connor DE, Ma DD, Joseph JE. Flow cytometry demonstrates differences in platelet reactivity and microparticle formation in subjects with thrombocytopenia or thrombocytosis due to primary haematological disorders. Thromb Res. 2013;132(5):572-7.
195. SHOT (Serious Hazards of Transfusion) Annual Report 2013. Available from: http://www.shotuk.org/shot-reports/report-summary-supplement-2013. Last accessed 03/07/2015.
196. Bradburn R. Blood and DTS Pricing Proposals for 2014/15: NHS Blood and Transplant Available from http://www.nhsbt.nhs.uk/download/board_papers/sept13/Blood_and_DTS_Pricing_Proposals_for_2014_15.pdf Last accessed 03/07/2015.
197. Slichter SJ. Relationship between platelet count and bleeding risk in thrombocytopenic patients. Transfus Med Rev. 2004;18(3):153-67.
198. Gaydos LA, Freireich EJ, Mantel N. The quantitative relation between platelet count and hemorrhage in patients with acute leukemia. N Engl J Med. 1962;266:905-9.
199. Stanworth SJ, Estcourt LJ, Powter G, Kahan BC, Dyer C, Choo L, et al. A no-prophylaxis platelet-transfusion strategy for hematologic cancers. N Engl J Med. 2013;368(19):1771-80.
200. Wandt H Frank M, Schaefer-Eckart K, and Wilhelm M Routine Prophylactic Platelet Transfusions Are Not Necessary in Patients with Acute Myeloid Leukemia - A Therapeutic Transfusion Strategy Is Safe and Cost Effective. Blood (ASH Annual Meeting Abstracts) 2005;106:428.
201. Wandt H, Schaefer-Eckart K, Frank M, Birkmann J, Wilhelm M. A therapeutic platelet transfusion strategy is safe and feasible in patients after autologous peripheral blood stem cell transplantation. Bone Marrow Transplant. 2006;37(4):387-92.
202. Provan D, Stasi R, Newland AC, Blanchette VS, Bolton-Maggs P, Bussel JB, et al. International consensus report on the investigation and management of primary immune thrombocytopenia. Blood. 2010;115(2):168-86.
203. Neunert C, Lim W, Crowther M, Cohen A, Solberg L, Jr., Crowther MA. The American Society of Hematology 2011 evidence-based practice guideline for immune thrombocytopenia. Blood. 2011;117(16):4190-207.

204. Noris P, Perrotta S, Seri M, Pecci A, Gnan C, Loffredo G, et al. Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: analysis of 78 patients from 21 families. Blood. 2011;117(24):6673-80.
205. Stockley J, Morgan NV, Bem D, Lowe GC, Lordkipanidze M, Dawood B, et al. Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects. Blood. 2013;122(25):4090-3.
206. Antony-Debre I, Bluteau D, Itzykson R, Baccini V, Renneville A, Boehlen F, et al. MYH10 protein expression in platelets as a biomarker of RUNX1 and FLI1 alterations. Blood. 2012;120(13):2719-22.
207. Laffan MA, Lester W, O'Donnell JS, Will A, Tait RC, Goodeve A, et al. The diagnosis and management of von Willebrand disease: a United Kingdom Haemophilia Centre Doctors Organization guideline approved by the British Committee for Standards in Haematology. Br J Haematol. 2014;167(4):453-65.
208. Civaschi E, Klersy C, Melazzini F, Pujol-Moix N, Santoro C, Cattaneo M, et al. Analysis of 65 pregnancies in 34 women with five different forms of inherited platelet function disorders. Br J Haematol. 2015;170(4):559-63.
209. Jongmans MC, Kuiper RP, Carmichael CL, Wilkins EJ, Dors N, Carmagnac A, et al. Novel RUNX1 mutations in familial platelet disorder with enhanced risk for acute myeloid leukemia: clues for improved identification of the FPD/AML syndrome: Leukemia. 2010; 24(1): 242-6.
210. Bejar R, Stevenson K, Abdel-Wahab O, Galili N, Nilsson B, Garcia-Manero G, et al. Clinical effect of point mutations in myelodysplastic syndromes. N Engl J Med. 2011;364(26):2496-506.
211. Fuster V, Bhatt DL, Califf RM, Michelson AD, Sabatine MS, Angiolillo DJ, et al. Guided antithrombotic therapy: current status and future research direction: report on a National Heart, Lung and Blood Institute working group: Circulation. 2012 Sep 25;126(13):1645-62.
212. Palandri F, Zoli M, Polverelli N, Noris P, Sollazzo D, Catani L, et al. MYH9-related thrombocytopenia and intracranial bleedings: a complex clinical/surgical management and review of the literature: Br J Haematol. 2015; 170(5): 729-31

Appendix 1 – Example GAPP Patient Information Sheet (v8, 12/11/2012) and Consent Form (v5, 12/11/2012)

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

UNIVERSITY OF BIRMINGHAM

Birmingham Platelet Group

Participant Information Sheet on 'Mild Platelet Disorders'

PART 1

1. Study title: Mild bleeding disorders caused by platelet defects

2. Invitation paragraph:

You are being invited to take part in a study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives, your GP and your Consultant if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part.

The UK Clinical Research Collaboration (UK CRC) publishes a leaflet entitled "Understanding Clinical Trials". This leaflet gives more information about medical research and looks at some questions you may wish to ask. A copy may be obtained from the internet at http://www.ukcrc.org/publications/informationbooklets. If you do not have access to the Internet, please let us know and we will print a copy off for you.

3. What is the purpose of the study?

This study aims to understand why some patients bleed excessively. We are going to look at the following groups of patients:

1. Patients with mild bleeding who have been seen by a blood specialist who feels their symptoms could be due to a problem in the platelets ("clinically diagnosed platelet defects")
2. Patients who have unexpectedly heavy periods with no obvious cause
3. Patients with lifelong low platelet counts in the blood with no known cause

In addition, we will also include some "control" individuals who do not suffer from the above problems and are important to allow us to compare responses between those who bleed excessively and those who do not. If we only examined affected patients it would be difficult to tell how their response compared to that expected in a normal individual.

You will be included in the highlighted group above, if you choose to participate in this study.

For all populations except the control group, we suspect that you have a problem in small cells in the blood, known as platelets, but we are not certain that this is the case. This study will use new and more specialist tests in an attempt to confirm that there is a problem in your platelets and to use this information to help identify the gene or genes that cause this problem. More information on platelets and what the study aims to achieve is given below:

What are platelets?

Platelets are small cells within the blood that are involved in the clotting mechanism. Following injury, platelets 'stick' to each other and block (or plug) the site of injury. Patients with too few platelets or with platelets that are less sticky may bleed more than we would expect, and in combination with other factors such as medications or injury may cause the loss of a great deal of blood. By carrying out certain tests on platelets, it is possible to find out whether somebody has a problem with their platelets and whether this is likely to explain their bleeding problem.

The testing of platelet function

Platelet tests are routinely performed in the Haematology Clinic to investigate whether a patient has a platelet problem. In many cases, however, the tests do not give a clear answer on whether the patient has a platelet problem or on the cause of the problem. The present study will use a range of more specialist platelet tests to establish whether the patient has a platelet problem and to gain further information on the nature of the defect. This will require analysis of platelet function in freshly donated blood. Work will be carried out by the Birmingham Platelet Group who have a large amount of experience in this field.

The search for defects in genes that could cause the platelet problem

The most likely explanation for a platelet problem is a defect (or problem) in one or more of the genes that you have been passed down (or inherited) from your mother and/or father. Genes can be described as the set of "instructions" that tell the body how to develop and work. A problem in one or more genes could cause the bleeding problem.

The results from the study of platelet function will have provided important clues on genes that may have caused the bleeding problem. The present study will look for errors in these genes using techniques that have been developed to identify gene defects. These studies require a small amount of blood that can be stored frozen for many years.

Is it possible that the gene defect will not be identified?

Yes. There is no guarantee that we will detect a change in either the platelet tests or in your gene. If we are unable to find a problem, we will tell you. If we fail to identify a problem, it does not mean you do not have a platelet or gene disorder – it is just that we have not been able to identify a problem with the techniques that we have used.

Are my family members likely to have the same gene defects and will they participate in the study?

Your mother, father and other family members may have a bleeding problem caused by a defect in the same gene or genes. Alternatively, your mother, father and other family members may have (or 'carry') one or more of the genes that is responsible for the bleeding problem, but may not have a bleeding problem.

If we are able to identify one or more gene defects that cause your platelet problem, we may wish to investigate, with their consent, other members of your family to see if they have a problem with their platelets and whether they have a defect in the same gene or genes. A separate information sheet is available for members of your family explaining these studies.

Will my blood be analysed for gene defects other than those which could cause the bleeding problem?

No. We are only interested in identifying defects that could cause your bleeding problem and we will target our search for abnormal genes to those involved in the way that platelets work under normal circumstances.

In patients with low platelet counts, we will need your consent to also look for mutations in the RUNX1 gene. Mutations in this gene have been shown to cause low platelet numbers and may also predispose to several types of leukemia. You are free to decline your permission for this testing without compromising your participation in this study. This testing will not be carried out in the other participants in this study.

4. Why have I been chosen?

You have been chosen due to one of the following factors:

1. Your hospital doctor suspects that you have a problem with your platelets, but does not know the cause of the problem.
2. You have been identified as a suitable "control" or healthy volunteer.

5. Do I have to take part?

It is up to you to decide whether or not to take part. If you decide to take part you will be given this information sheet to keep and be asked to sign an agreement (or consent) form. By signing the consent form you are saying that you are happy to take part in the study and that you understand what has been explained to you. If you choose not to take part, the clinical care you receive will not be affected in any way.

6. What will happen to me if I take part?

If you agree to take part you will be asked some questions about your medical history including questions to complete a bleeding questionnaire.

You will also be asked to give a sample of blood (between 10–50 ml, which is about the same volume as that of 2–10 teaspoonfuls). The blood will usually be taken at a local Haematology Clinic at a time that is suitable for your yourself and the Haematology Clinic, ideally early in the day so that the analyses can be carried out on the same day.

Depending on the results of the tests, we may ask you to give blood on up to three further occasions. This is to enable us to carry out different types of tests on your platelets and therefore find out more information on the problem. We will again ask for between 10–50 ml of blood, and this again can be taken either during a 'regular' visit to the Haematology Clinic / surgery or through a specific appointment. There will be a minimum of one month between the dates that the blood is taken and no upper time limit on the gap between the donations.

What will happen to my blood?

Your blood samples will be sent to research laboratories in Birmingham, Bristol and/or Sheffield for analysis of your platelets. We will also keep a frozen sample for a maximum of 10 years, which may be used to look for gene defects that could cause the platelet problem or changes in blood proteins that may increase your chance of bleeding. The sample will be destroyed at this time, or earlier if you request.

We may wish to keep the sample for this length of time in the event that we are unable to find a problem with your genes in our first set of investigations. It is possible that, as we gain more knowledge, that we will wish to investigate the sample at later times to see if we can find a gene defect that may be the cause of the bleeding problem.

Please note that a frozen sample will not be stored for "control" individuals.

7. What are the possible disadvantages and risks of taking part?

You may experience a little pain and bruising around the vein where the blood sample is taken from. The needle used to take the blood will be identical to what has been used on you previously to take blood samples.

8. What are the possible benefits of taking part?

The study will generate important information on possible defects in your platelets and may also identify the genes that are responsible for the defects. The identification of a defect in your platelets may influence your clinical treatment.

If a gene defect is found, it will be possible to investigate whether one or more members of your family have the same gene defect, should they wish. You will not receive any money for taking part in this study.

9. When will the study be completed?

Recruitment of patients will end in February 2015. Because we anticipate receiving many samples from throughout the UK, it may be many months before we can let you know if we have found a gene defect in your blood.

The nature of the gene defect will not be identified in all patients. However, developments may take place over the next few years that will provide important, new information on the likely cause of platelet bleeding disorders. We will therefore continue to follow developments in the field and, if appropriate, use this information to direct our efforts to identify the defective gene(s) in your frozen blood sample, which will be kept frozen for up to ten years, unless you ask for it to be destroyed. We will contact you as soon as we have identified a genetic defect in the sample.

10. Will my taking part in this study be kept confidential?

Yes. All information which is collected about you during the course of the research will be kept strictly confidential.

11. What will happen if I don't want to carry on with the study?

If you decide that you do not want to be in the study at any time after you have had your blood sample taken, you can ask for us to destroy your stored sample.

12. Further information on genetic testing

If we find a genetic defect related to bleeding, we will let you know of this. You will have the right to choose not to access this information. We may refer you for retesting by genetic services outside of this study for confirmation of the gene analysis.

We will discuss the full implications of the genetic studies with you with regard to your own children, having children in the future, insurance status and available counselling, as appropriate. Stored samples will not be used for other genetic studies without additional consent and presentation to an ethics committee for consideration.

13. Further tests that may be undertaken on your blood sample

In some circumstances, we will pursue more detailed testing of your blood samples. The types of detailed tests include, for example, using the stem cells from your blood sample to look at platelet production or examining the proteins in your platelets. Only tests for which permission has been formally granted to the Birmingham Platelet Group from the Research Ethics Committee (REC) will be performed. The purpose of the REC is to protect the rights, safety, dignity and well-being of research participants, and to facilitate and promote ethical research that is of potential benefit to participants, science and society.

14. Contact for further information

Further information about this study can be obtained by contacting one of our group on telephone number [redacted] You will be able to leave a message if there is no-one in the office and we will get back to you. The members of the group are Mrs Gayle Halford (general enquires), Dr Marie Lordkipanidzé, Dr Gillian Lowe and Ms Rachael Taylor.

The study co-ordinator is a British Heart Foundation Professor, Stephen Watson (Centre for Cardiovascular Sciences, School of Clinical and Experimental Medicine, Institute of Biomedical Research, College of Medical and Dental Sciences, Wolfson Drive, University of Birmingham, Edgbaston, Birmingham, B15 2TT).

This completes Part 1 of the information sheet. If the information in Part 1 has interested you and you are considering participation, please continue to read the additional information in Part 2 before making any decisions.

PART 2

15. Who is funding this study?

The work is supported by the British Heart Foundation Research Charity.

16. Will I get paid for taking part?

You will not be paid for taking part.

17. Who has reviewed this study?

This study has been reviewed and approved by the West Midlands Multi-Centre Research Ethics Committee and by the Local Research Ethics Committee for your hospital.

18. What if something goes wrong?

If you are hurt by taking part in this research project, there are no special compensation arrangements.

If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. Regardless of this, if you wish to complain, or have any concerns about any aspect of the way you have been approached or treated during the course of this study, the normal National Health Service complaints mechanisms should be available to you. At present this would be via PALS (Patient Advice and Liaison Service) at your local hospital, or www.pals.nhs.uk

19. What will happen to the results of this study?

Your results will be passed on to your haematology doctor who will be asked to give them to you and to answer any questions you may have.

The collective results from all of the patients in the study may be presented in a scientific report to help other doctors who are treating patients with these bleeding disorders. No patient names will be used and it will not be possible to identify you from any report of the results of the study.

20. What if I wish to complain about the way in which the study has been conducted?

If you have any cause to complain about any aspect of the way in which you have been approached or treated during the course of the study, the normal National Health Service complaints mechanisms are available to you and are not compromised in any way because you have taken part in a research study. Complaints should be dealt with by an independent body such as PALS. To use the normal hospital complaints procedure, you should contact the PALS officer at your hospital or get more information at www.pals.nhs.uk

Thank you very much for reading through this information sheet.

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

UNIVERSITY OF BIRMINGHAM

Birmingham Platelet Group

INFORMED CONSENT FORM

PATIENT / FAMILY MEMBER

Thank you for reading the information about our research project. If you would like to take part, please read and sign this form.

Patient Identification Code for this study:

Title of Project: Mild bleeding disorders caused by platelet defects

Contact details for research team:

Your referring doctor / research nurse ……..……..

or

The study team: Mrs Gayle Halford (general enquiries), Dr Marie Lordkipanidzé and Dr Gillian Lowe

Birmingham Platelet Group, Institute for Biomedical Research, School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, Birmingham B15 2TT, ☎ [redacted] Please leave a message if no-one is in the office and we will get back to you.

Please initial all boxes

1. I have read the attached information sheet on this project, and have been given a copy to keep. I have been able to ask questions about the project and I understand why the research is being done and any risks involved. ☐

2. I agree to give a sample of blood for research in this project. I understand how the sample will be collected, that giving a sample for this research is voluntary and that I am free to withdraw my approval for the use of the sample at any time without giving a reason and without my medical treatment or legal rights being affected. ☐

3. I give permission for someone from the local research team to look at my medical records to get information on my bleeding history. I understand that the information will be kept confidential. ☐

4. I agree to answering some questions about my medical history including those needed to complete a bleeding assessment by questionnaire. ☐

5. I understand that my referring doctor and I will be informed if any of the results of the medical tests done as a part of the research are important for my health. ☐

6. I understand that I will not benefit financially if this research leads to the development of a new treatment or medical test. ☐

7. I know how to contact the research team if I need to, and how to get information about the results of the research. ☐

8. Consent for storage of sample

I agree that the sample I have given and the information gathered about me can be stored in the Medical Schools of the Universities of Birmingham, Bristol and Sheffield for the purpose of analysing platelets, bleeding tendency and detection of the gene change responsible for the platelet disorder in myself or a family member, for a maximum of 10 years, after which it will be destroyed.

If I wish I may request the return of the sample after analysis has been completed. ☐

9. Consent for genetic research.

I give permission for genetic analysis to be carried out on the sample I give, as part of this project. I have received written information about this test and I understand what the result could mean to me and/or members of my family. ☐

10. Consent for stem cell studies

I give permission for the stem cells in my blood sample to be used to understand how my platelets are produced.

YES ☐

NO ☐

11. Consent for RUNX1 mutation testing **(ONLY FOR PARTICIPANTS WITH LOW PLATELET COUNTS).**
I give permission for genetic analysis to be carried out on the sample I give to specifically look at the RUNX1 gene. I understand that this mutation is associated with low platelet counts and can in some cases predispose to certain types of leukemia.

YES ☐

NO ☐

12. I want to be told the results of the tests undertaken in this study. I understand I can change my mind about this later.

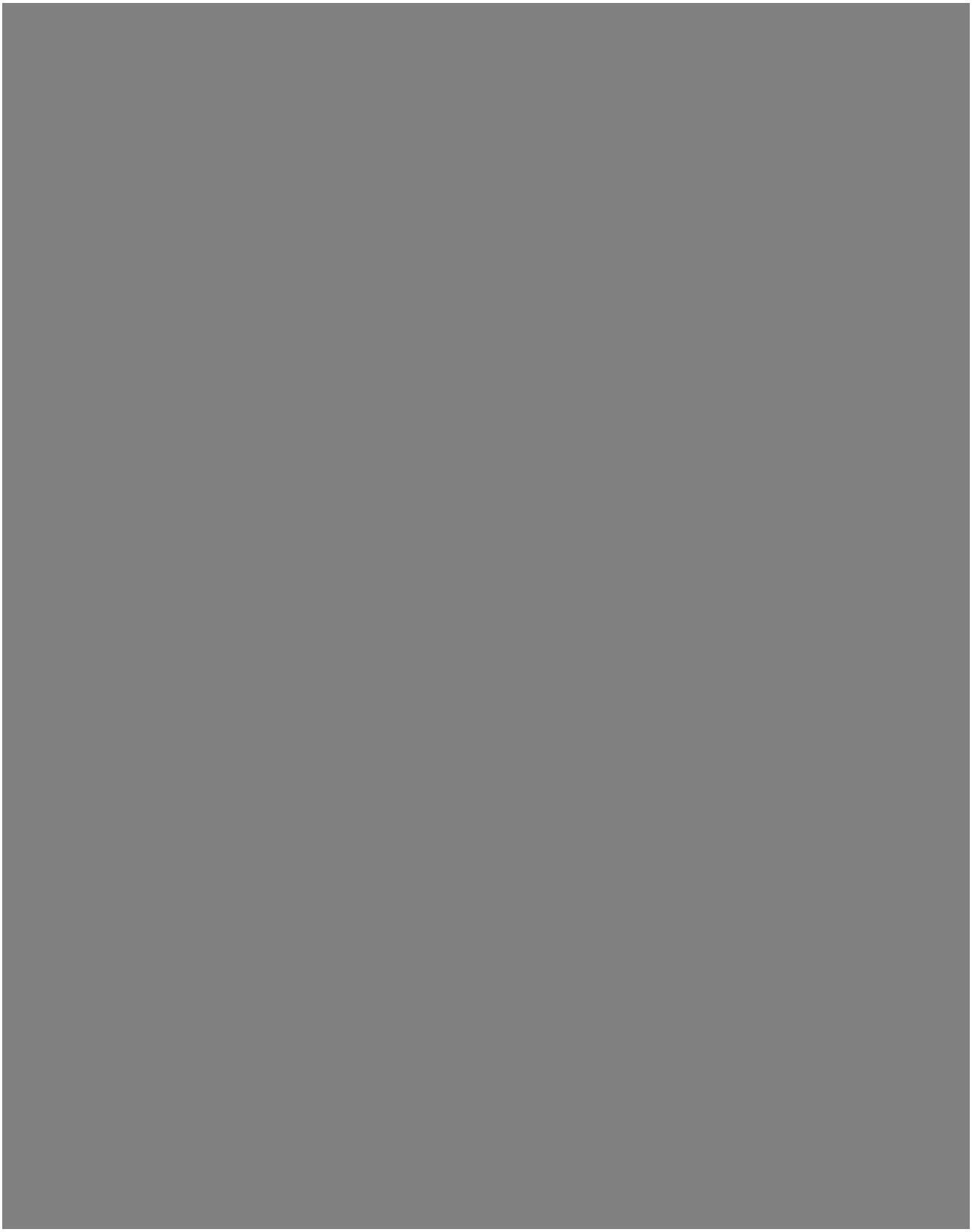
YES ☐

NO ☐

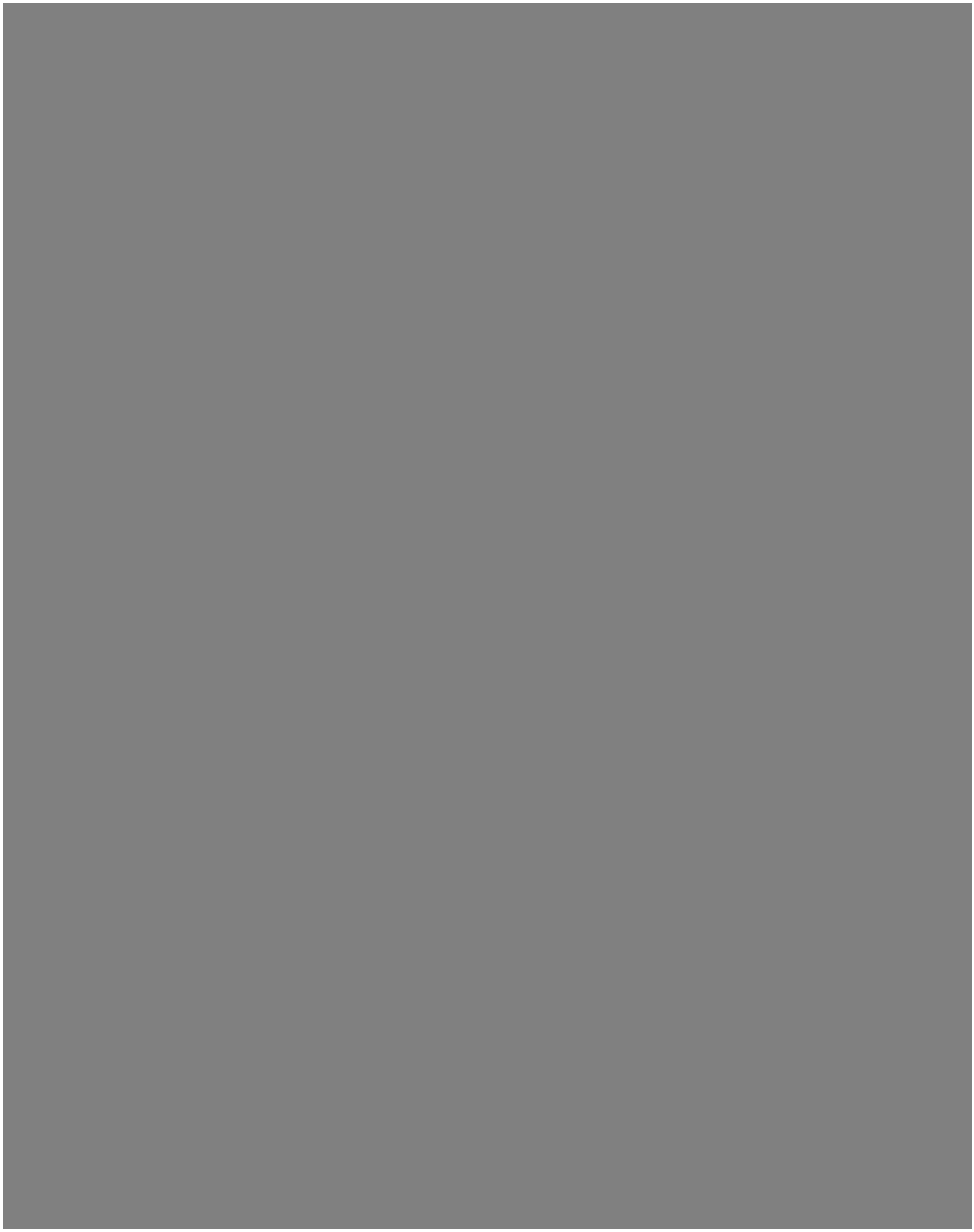
……	……	……...
Name of patient (BLOCK CAPITALS)	Date	Signature
……..	……	……...
Name of person taking consent (if different from researcher)	Date	Signature
……..	……	……...
Name of researcher	Date	Signature

Thank you for agreeing to participate in this research
1 copy for patient, 1 for hospital notes, 1 for researcher

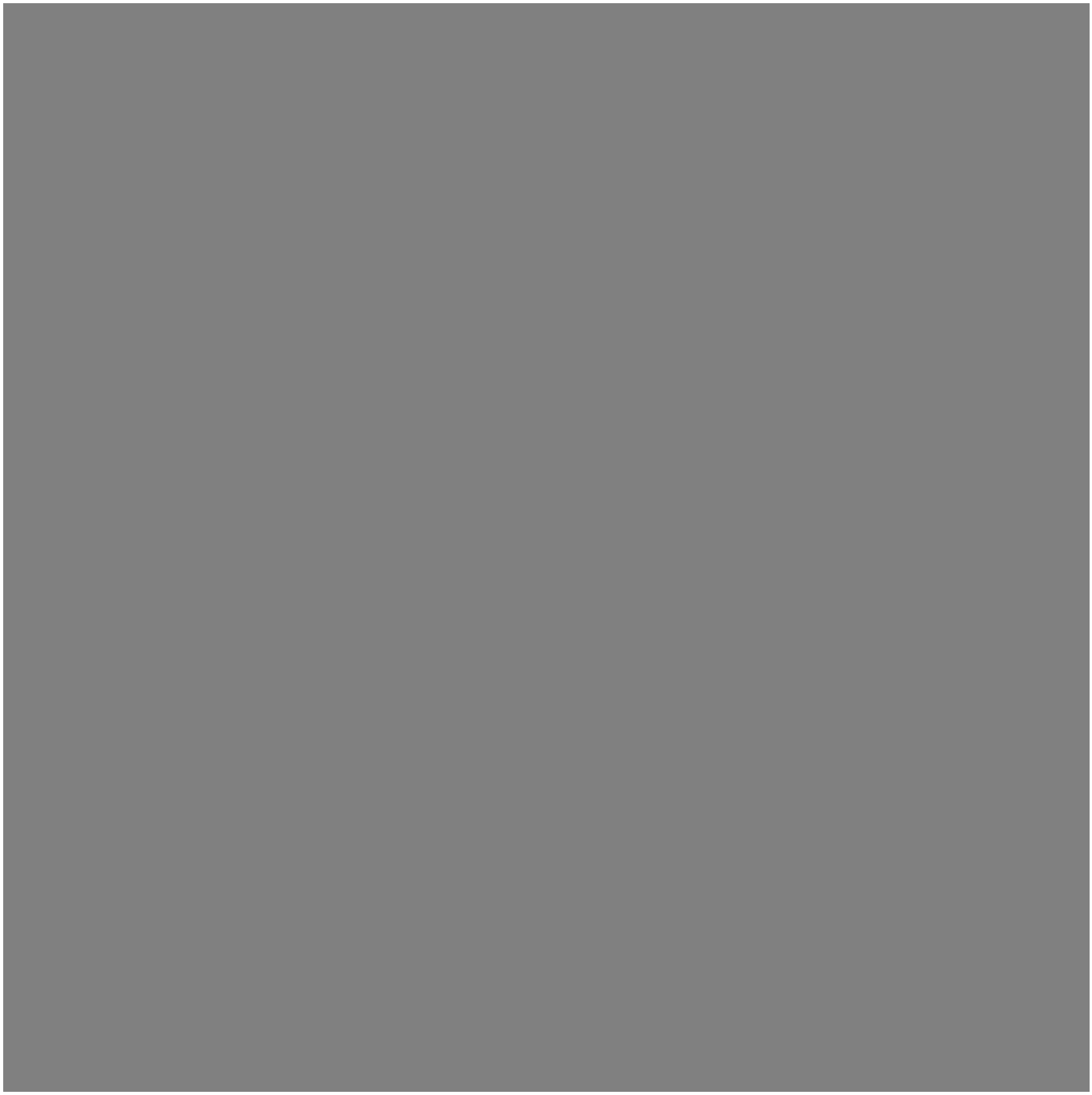
Appendix 2 – ISTH Bleeding Assessment Tool



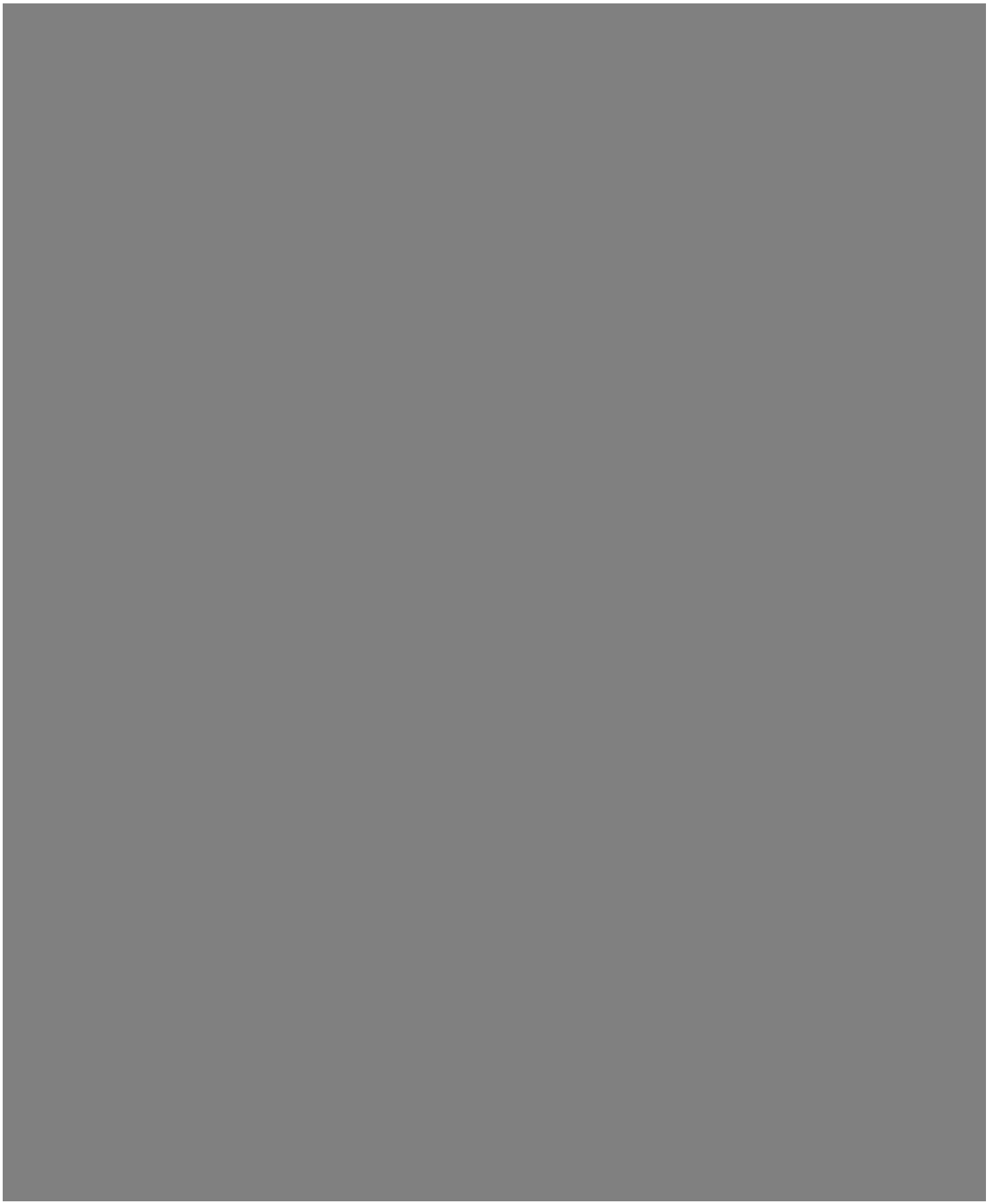
Appendix 2 – ISTH Bleeding Assessment Tool



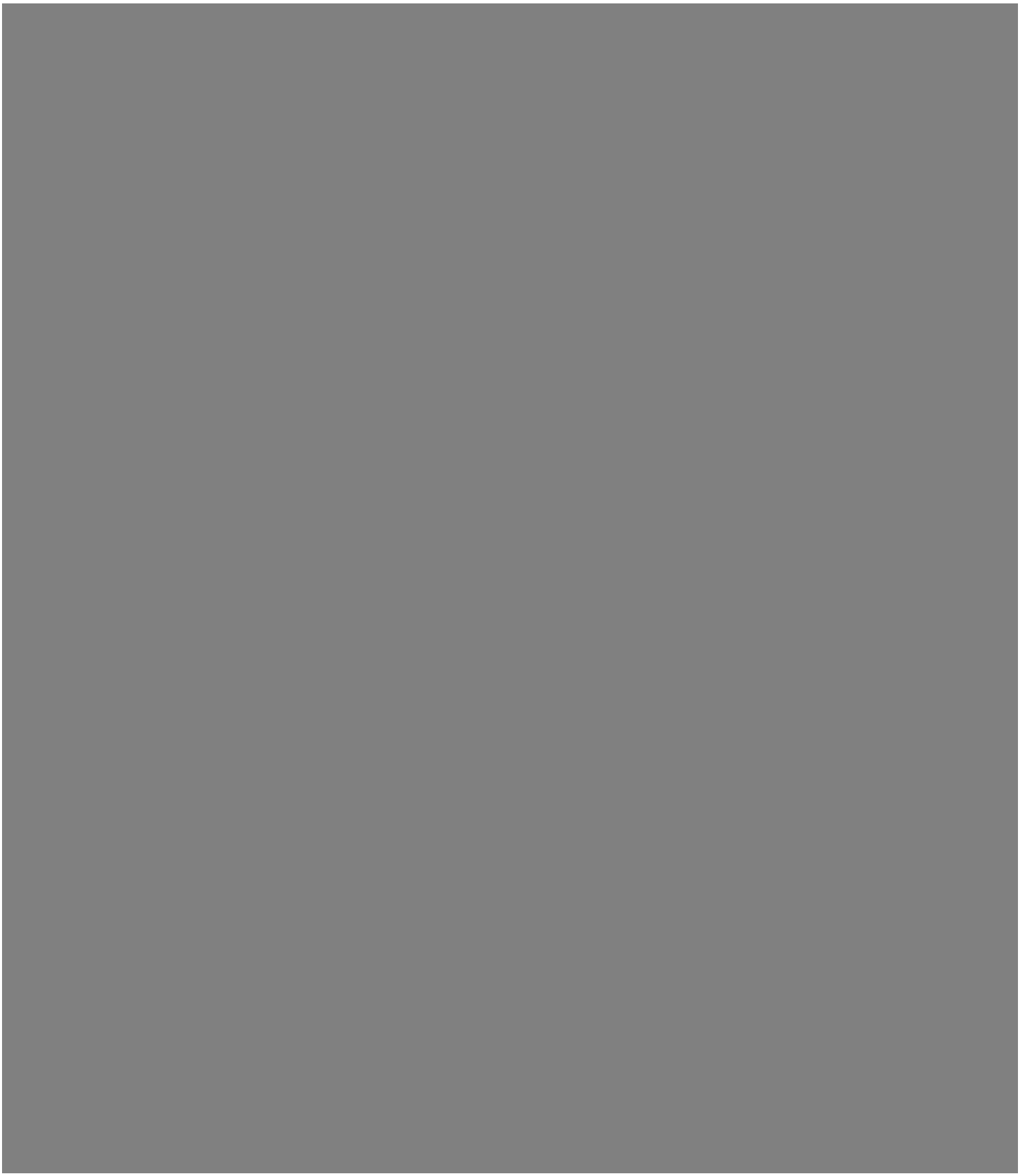
Appendix 2 – ISTH Bleeding Assessment Tool



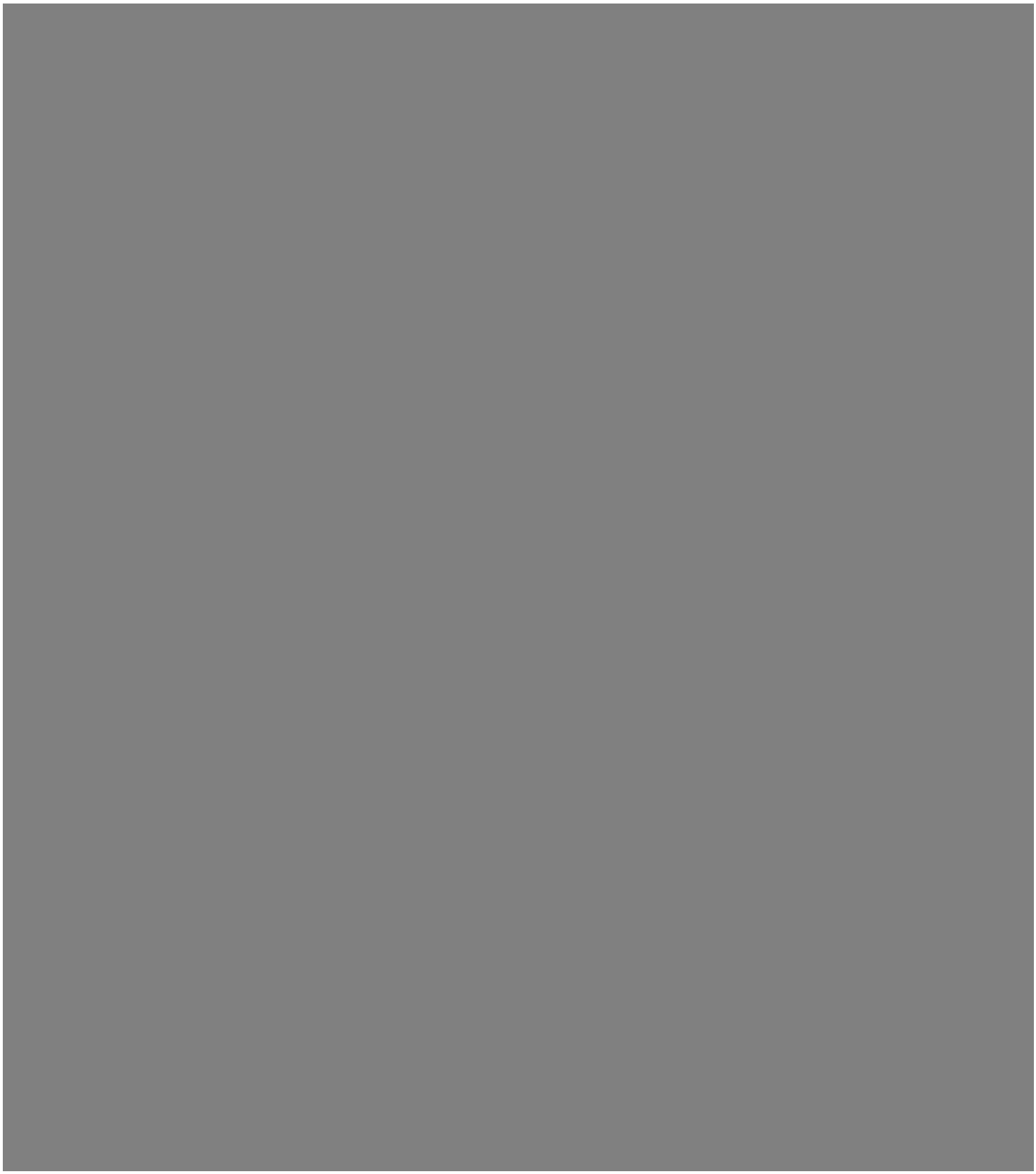
Appendix 2 – ISTH Bleeding Assessment Tool



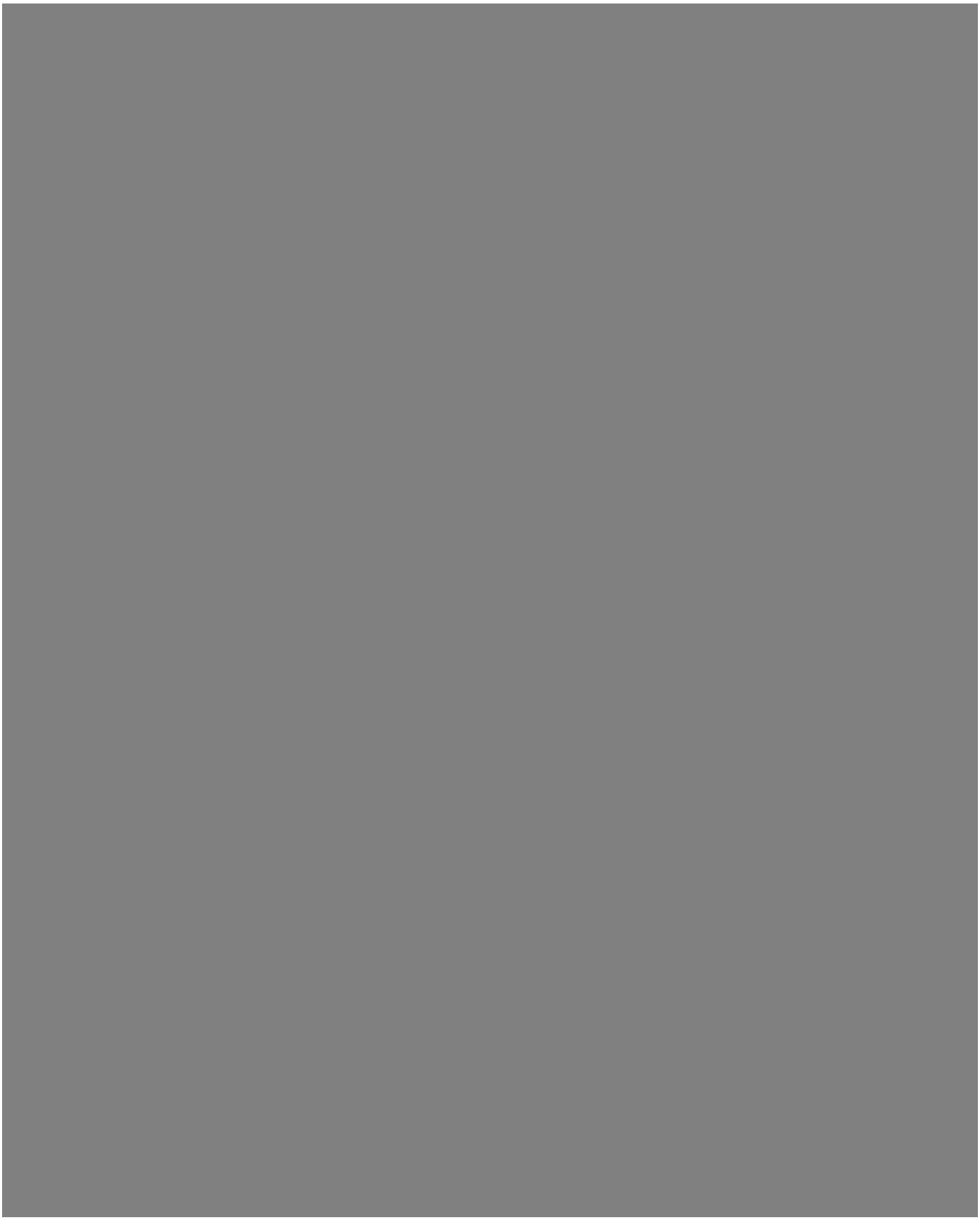
Appendix 2 – ISTH Bleeding Assessment Tool



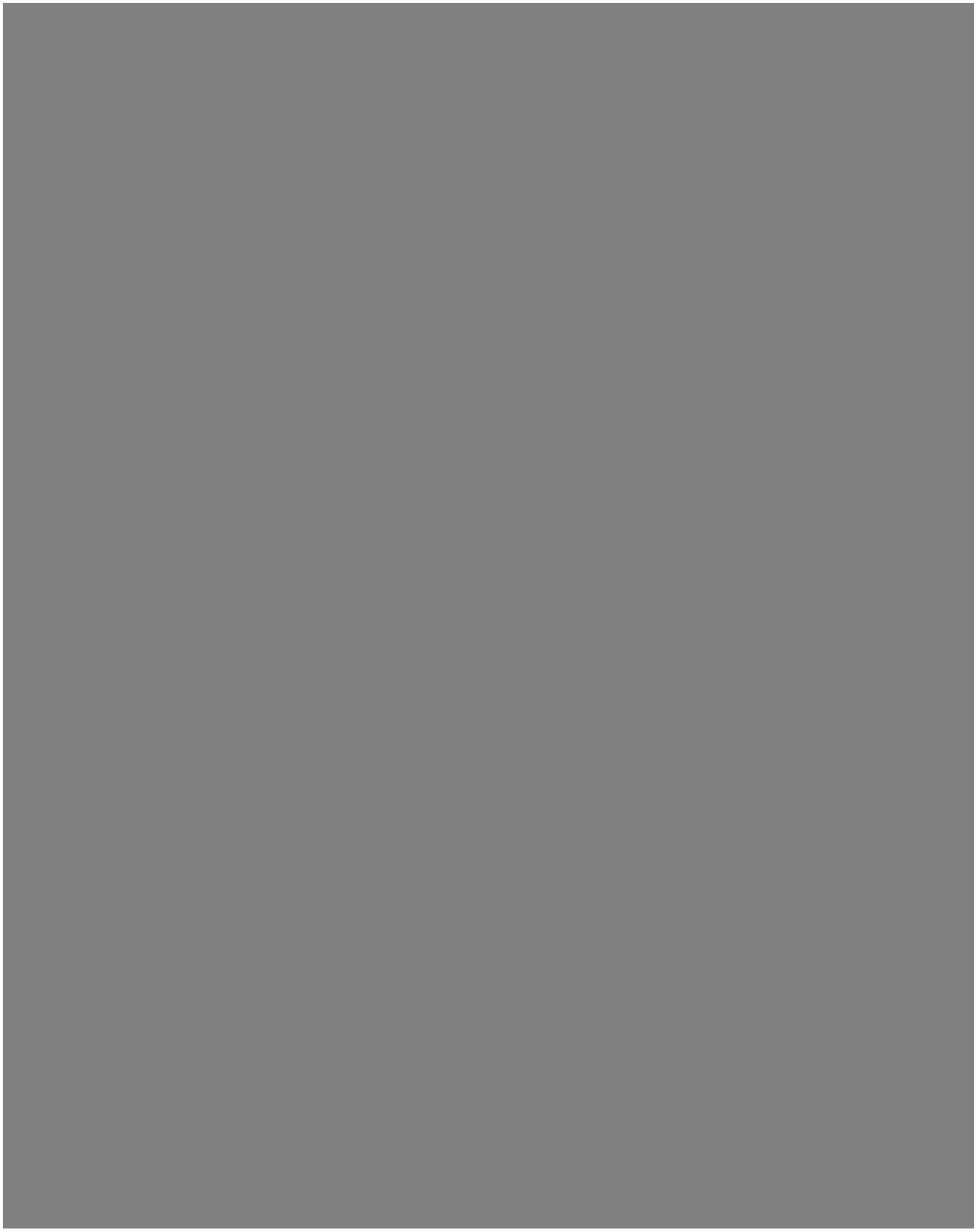
Appendix 2 – ISTH Bleeding Assessment Tool



Appendix 2 – ISTH Bleeding Assessment Tool

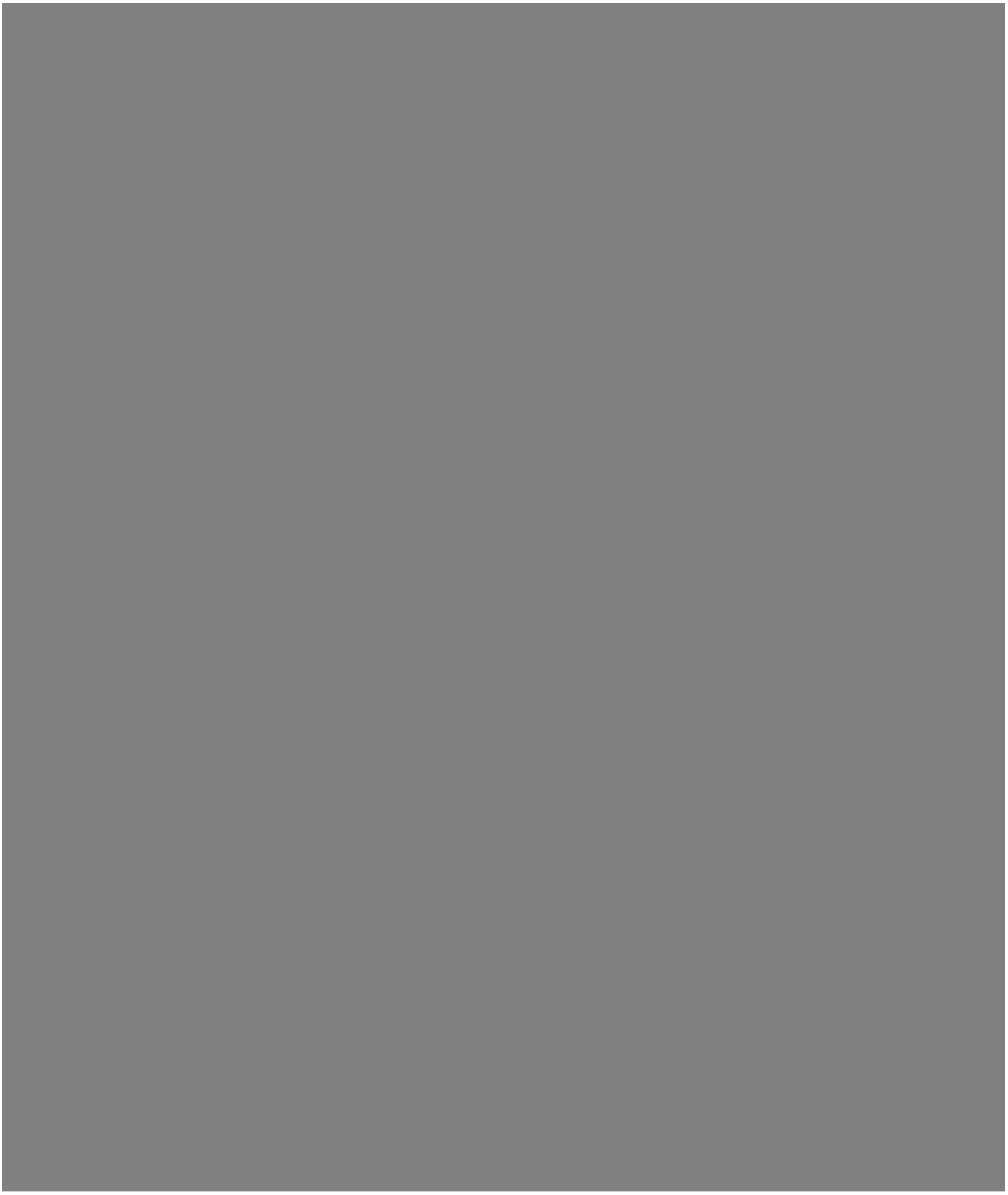


Appendix 2 – ISTH Bleeding Assessment Tool

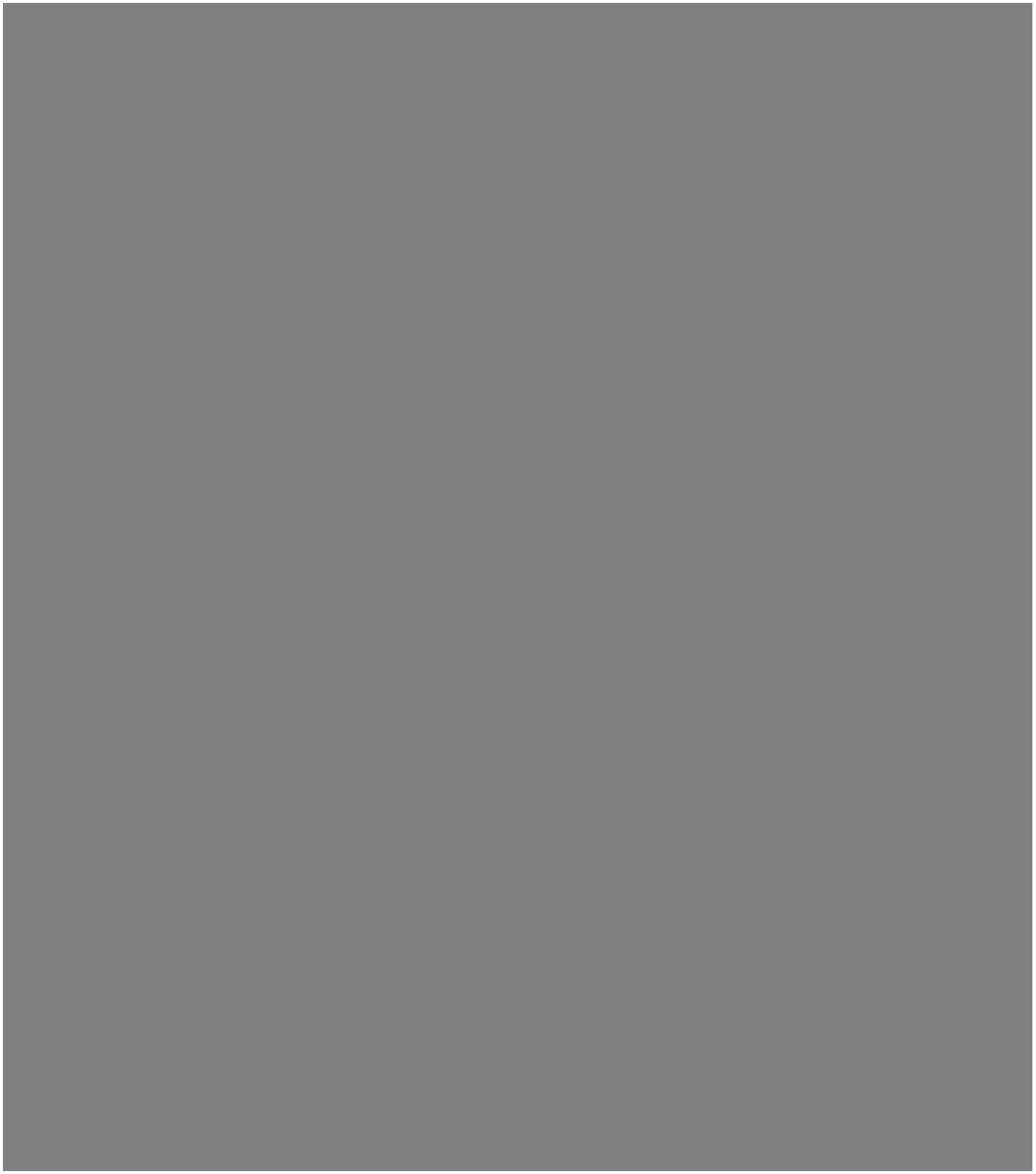


XXX

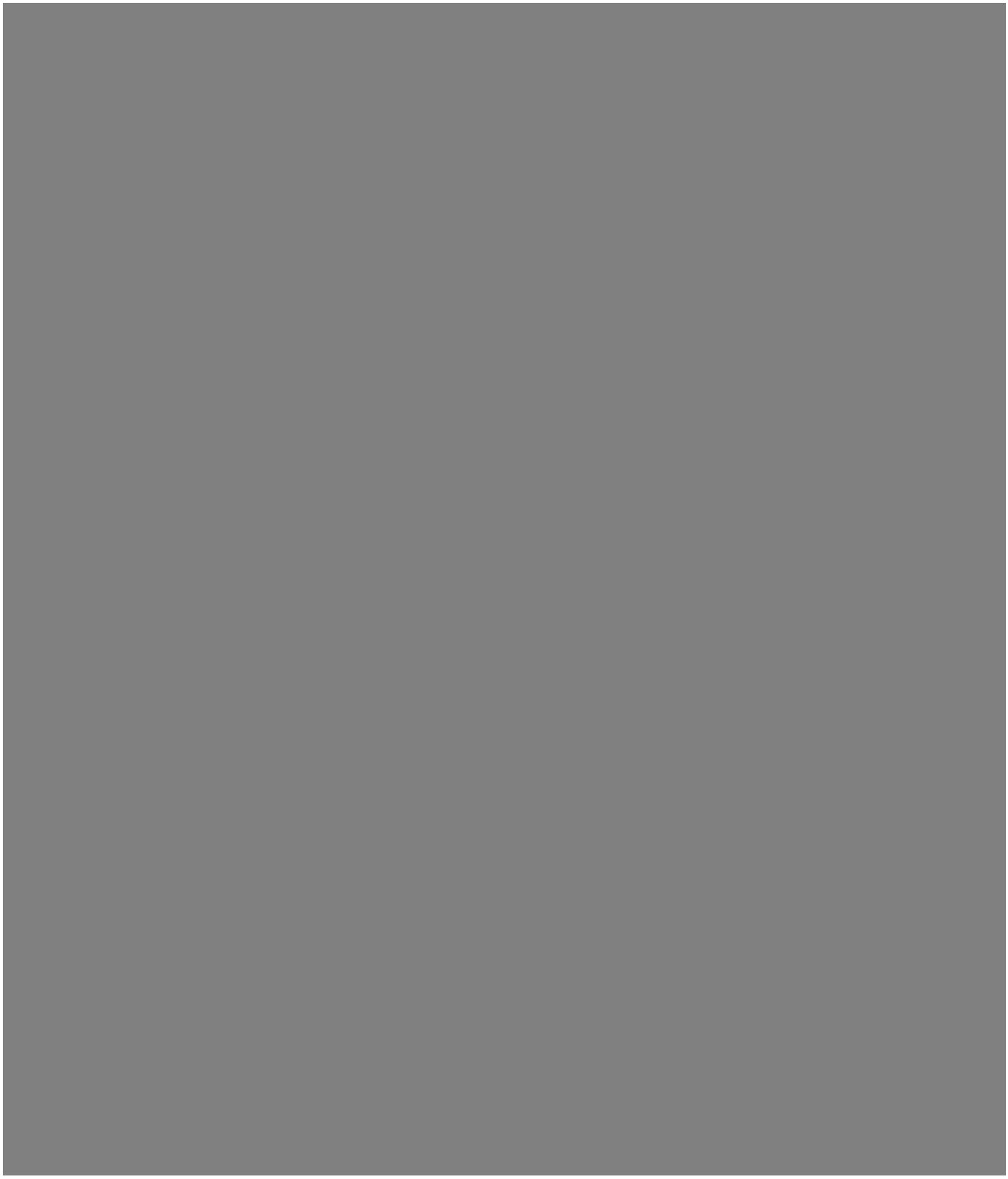
Appendix 2 – ISTH Bleeding Assessment Tool



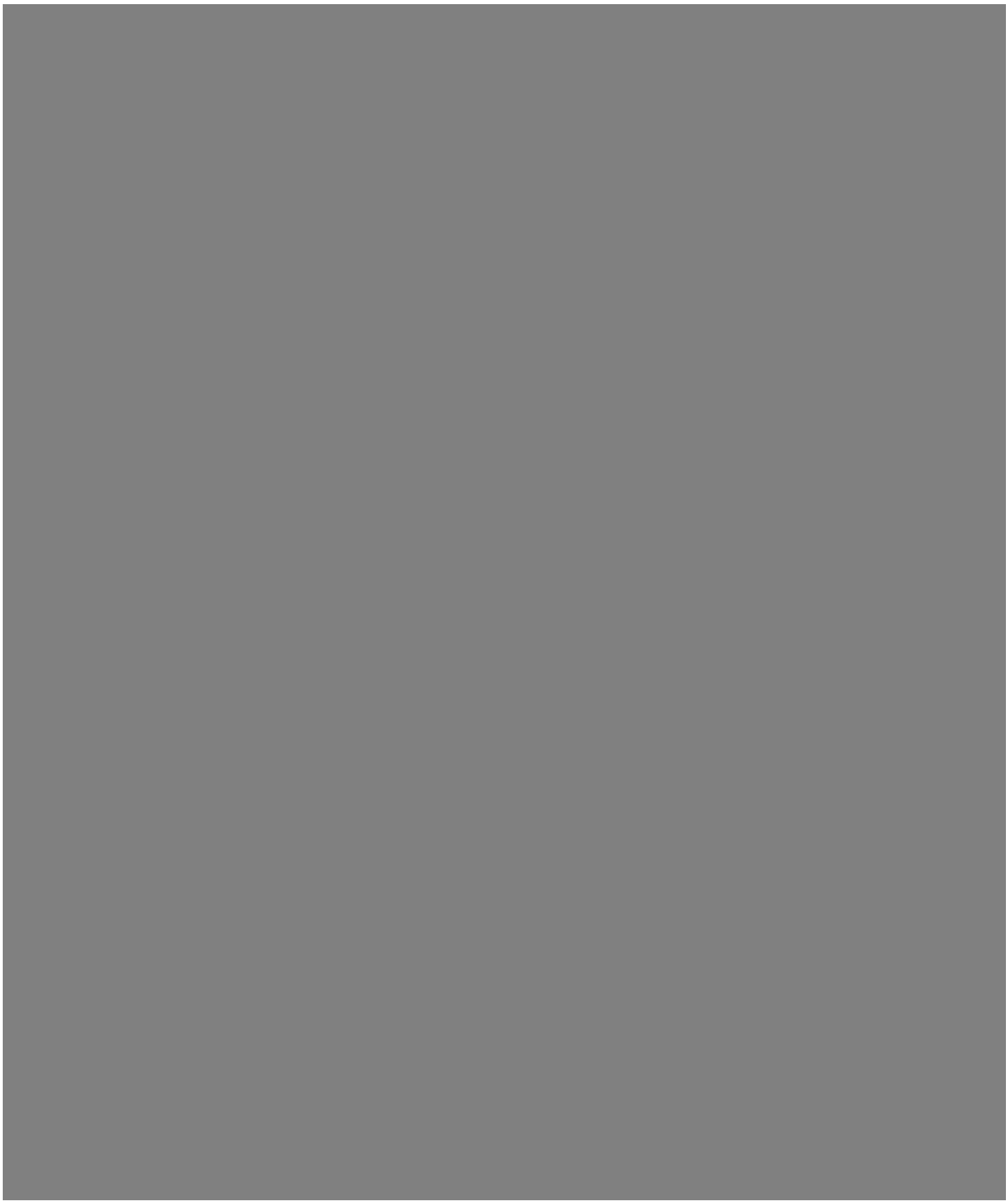
Appendix 2 – ISTH Bleeding Assessment Tool



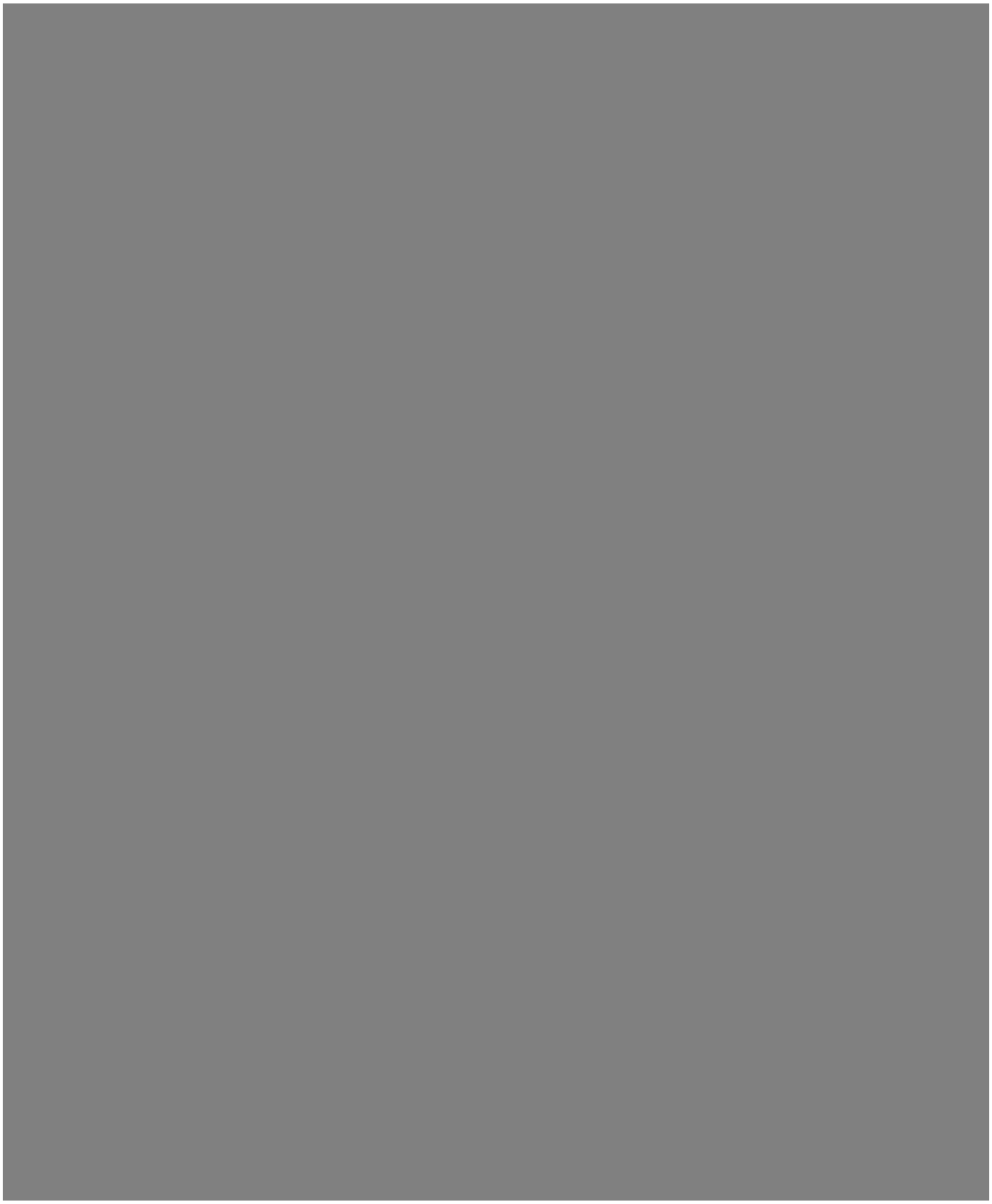
Appendix 2 – ISTH Bleeding Assessment Tool



Appendix 2 – ISTH Bleeding Assessment Tool

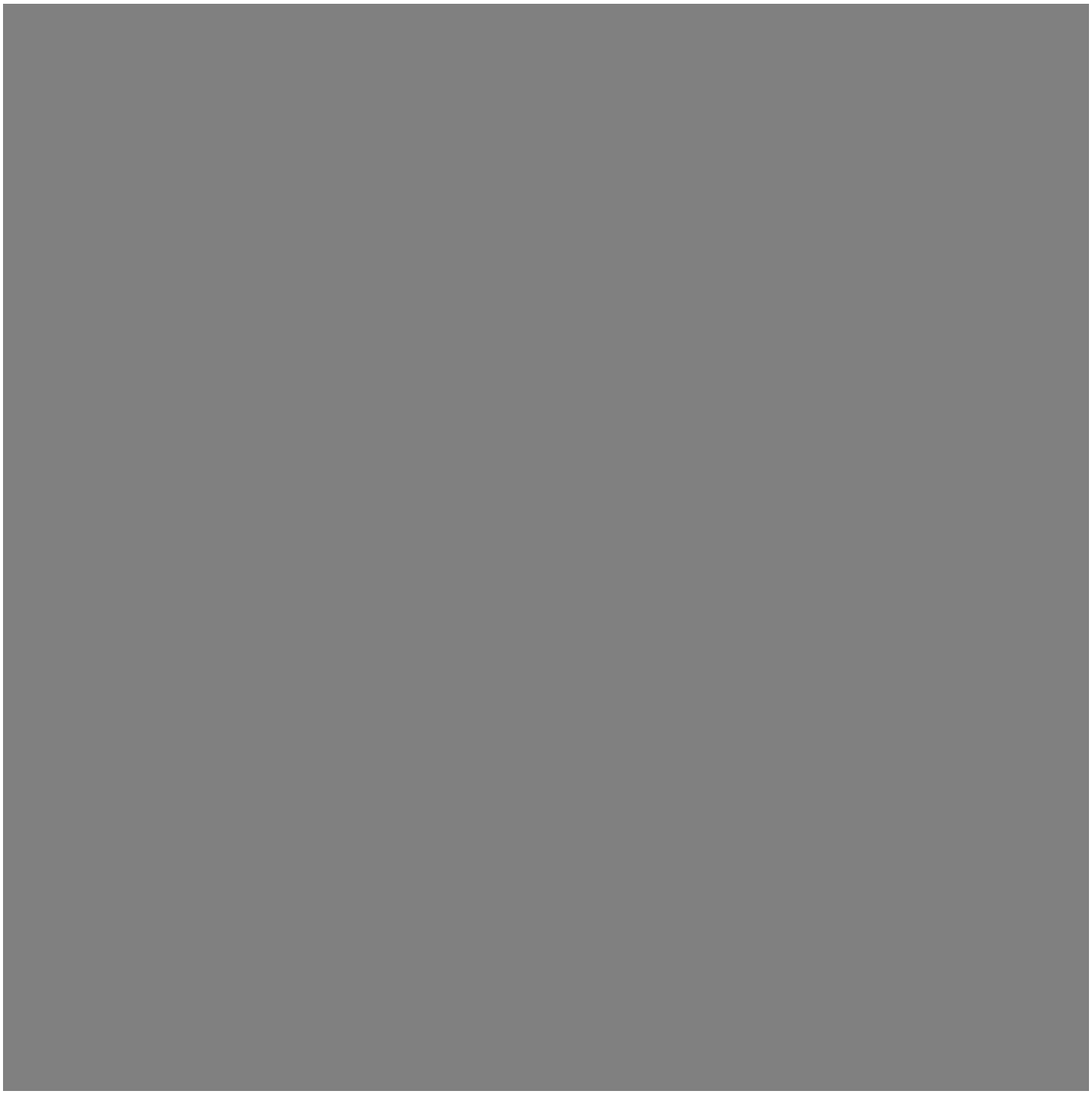


Appendix 2 – ISTH Bleeding Assessment Tool



Appendix 2 – ISTH Bleeding Assessment Tool

Appendix 2 – ISTH Bleeding Assessment Tool



Appendix 2 – ISTH Bleeding Assessment Tool

Appendix 2 – ISTH Bleeding Assessment Tool

Appendix 2 – ISTH Bleeding Assessment Tool

Appendix 2 – ISTH Bleeding Assessment Tool

Appendix 2 – ISTH Bleeding Assessment Tool

Appendix 3 – Assessment of receptor expression changes as a result of the R122C mutation (results) – Stuart Mundell and colleagues (40)

Surface expression of this receptor was found to be reduced in cell lines (**Figure A2.1**), with a significant intracellular pool of the R122C variant (**Figure A2.2**). Minimal agonist-dependent internalization was seen with the mutant receptor (**Figure A2.2**). In addition, wild type receptor co-localized with the early and endosomal recycling marker transferrin whilst the R122C variant was predominantly found to colocalize with the lysosomal marker lysotracker blue (**Figure A2.2**). Therefore unlike the wild type receptor which could recycle back to the cell surface, the R122C variant would be targeted for degradation.

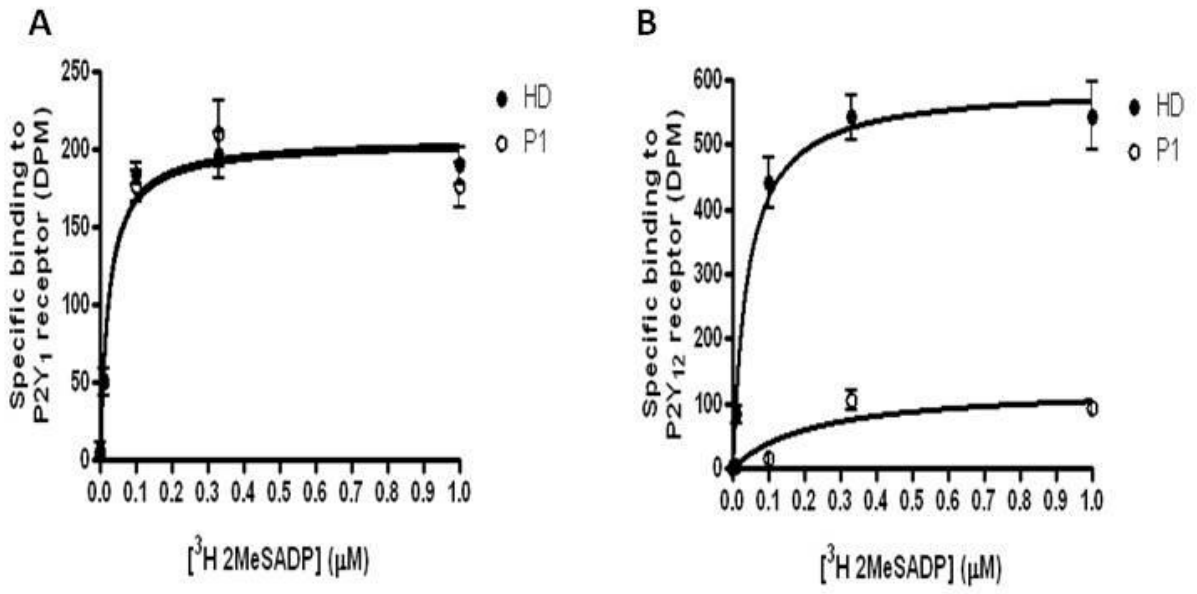


Figure A2.1 P2Y receptor expression in index case and family members. $P2Y_1$ (A) and $P2Y_{12}$ (B) surface receptor levels were measured in fixed platelets in index case (P1) and healthy volunteer (HD) by displacement of [^{3}H]2MeS-ADP (1nM – 1 μM) by receptor antagonists for $P2Y_1$ (A3P5P; 1 mM) and $P2Y_{12}$ (AR-C69931MX; 1 μM) respectively. Data are expressed as [3H]2MeS-ADP binding (DPM) and represent means ± S.E.M. of three independent experiments. Work performed by Stuart Mundell and colleagues (40).

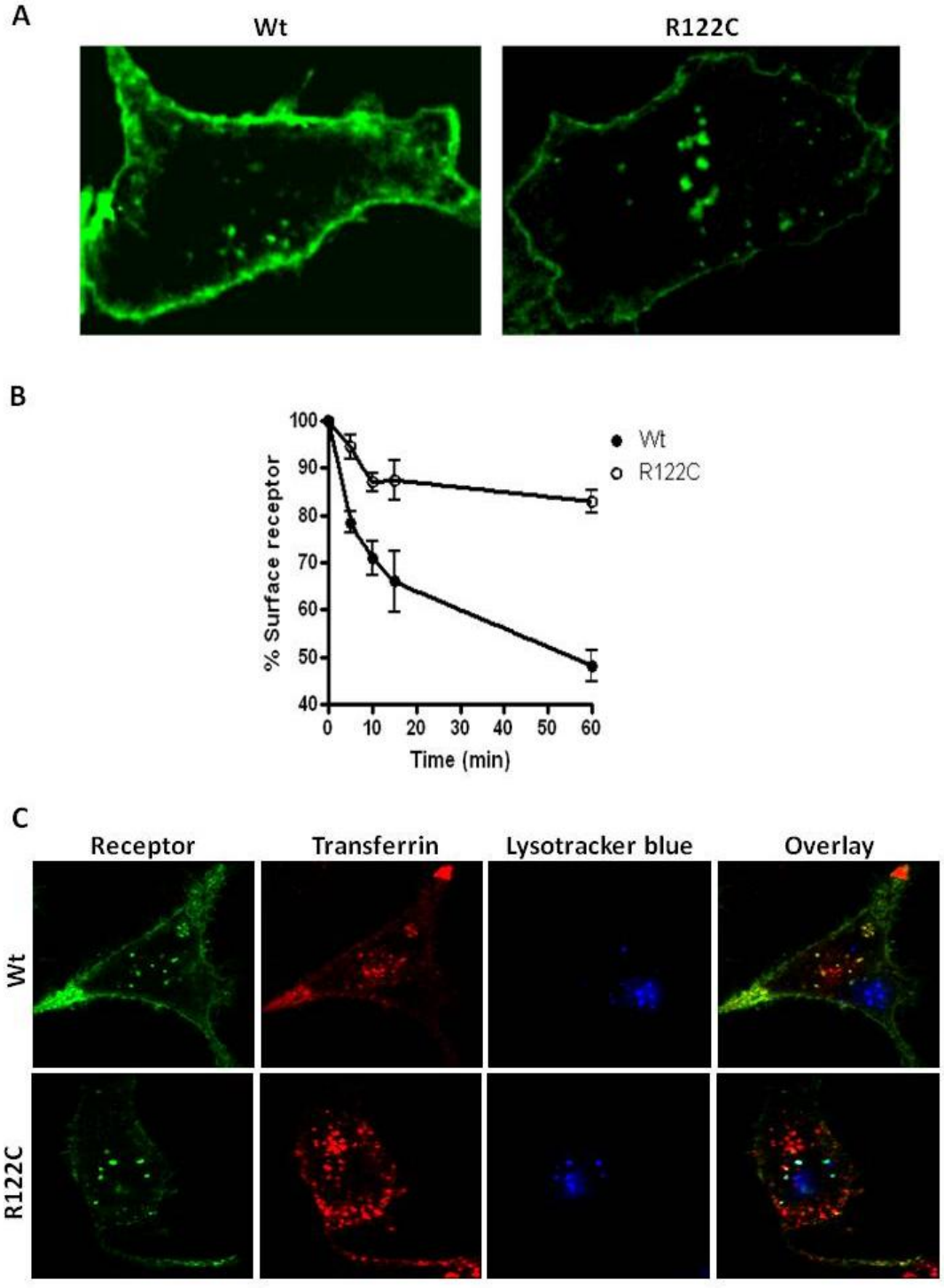


Figure A2.2 The R122C variant is poorly expressed at the cell surface as a result of rapid agonist-independent internalization followed by subsequent intracellular retention and sorting to lysosomes (A) Membrane and cellular localization of wild type and R122C variant in 1321N1 cells. 1321N1 cells stably expressing HA-tagged wild type or R122C variant receptor constructs were fixed, permeabilized and incubated with a monoclonal anti-HA antibody at 37^{o} C for 1h. Receptor localization was determined by immunofluorescence using a monoclonal fluorescein-conjugated

(green) secondary antibody. There was a clear reduction of the R122C variant expression at the cell surface and significant intracellular accumulation (B) Agonist-dependent internalization is significantly impaired in the R122C variant receptor. 1321N1 cells stably expressing HA-tagged wild type or R122C variant receptor constructs were challenged with ADP (10 μM; 0-60 min) to induce receptor internalization and surface receptor loss was subsequently assessed by ELISA. The data represent means ± S.E.M. of five independent experiments. (C) The R122C variant receptor is preferentially targeted to lysosomes following agonist-independent internalization. 1321N1 cells stably expressing HA-tagged receptor constructs were preincubated with a monoclonal anti-HA at 4^{o} C for 1h. Subsequently cells were incubated at 37^{o} C for 60 min in the absence of ADP. Receptor localization was determined by immunofluorescence in fixed cells and visualised using a monoclonal fluorescein-conjugated (green). Receptor colocalization with either rhodamine-conjugated transferrin (red) or lysotracker blue (blue) which label early and recycling endosomes or lysosomes respectively is shown. Data shown are representative of three independent experiments. Work performed by Stuart Mundell and colleagues (40).

Appendix 4 – Selected publications

All manuscripts in this section have been reproduced with permission from the relevant publishers.

PLATELETS AND THROMBOPOIESIS

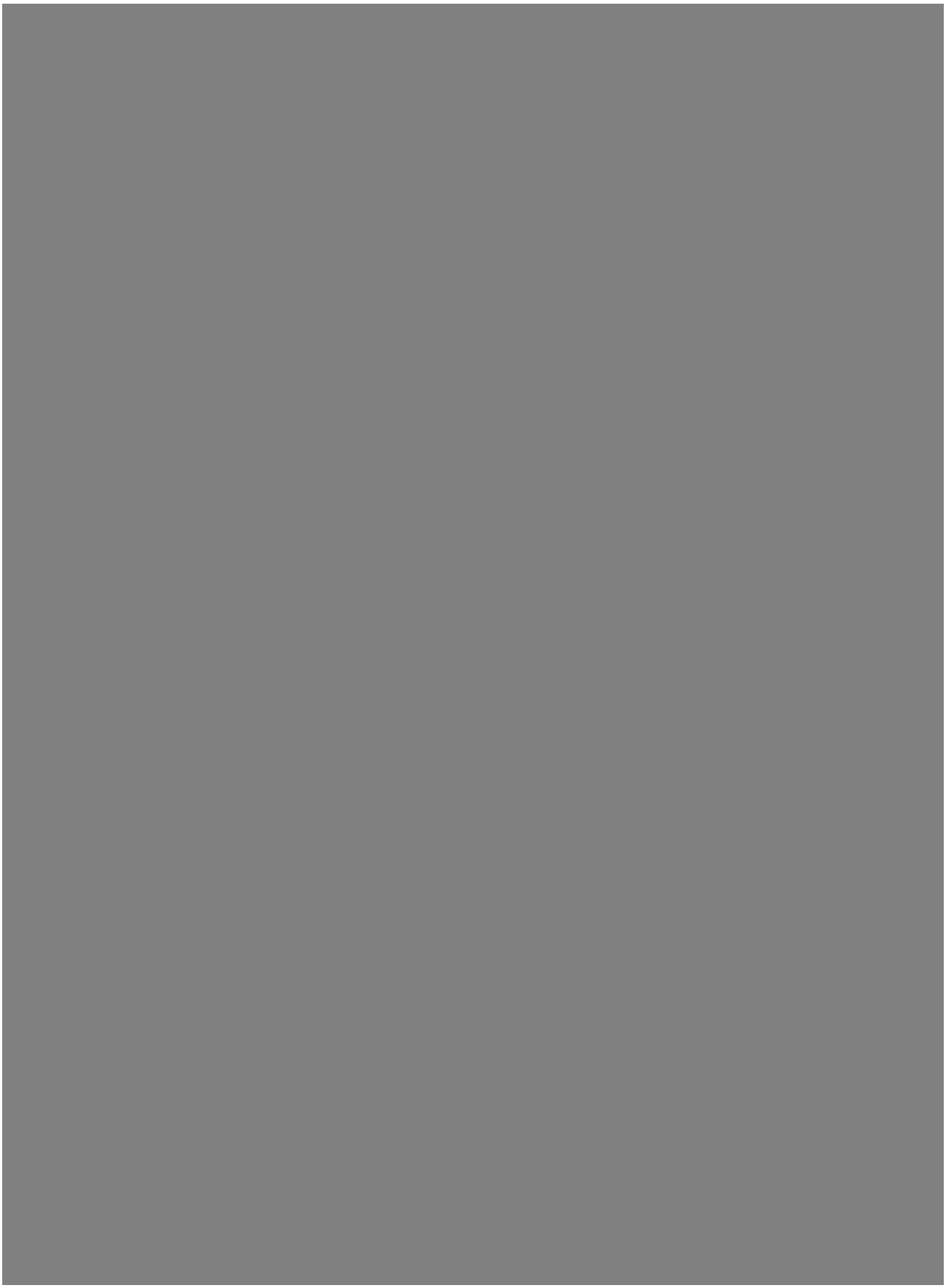
Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel

Ban B. Dawood,[1] Gillian C. Lowe,[1] Marie Lordkipanidzé,[1] Danai Bem,[1] Martina E. Daly,[2] Mike Makris,[2] Andrew Mumford,[3] Jonathan T. Wilde,[4] and Steve P. Watson[1]

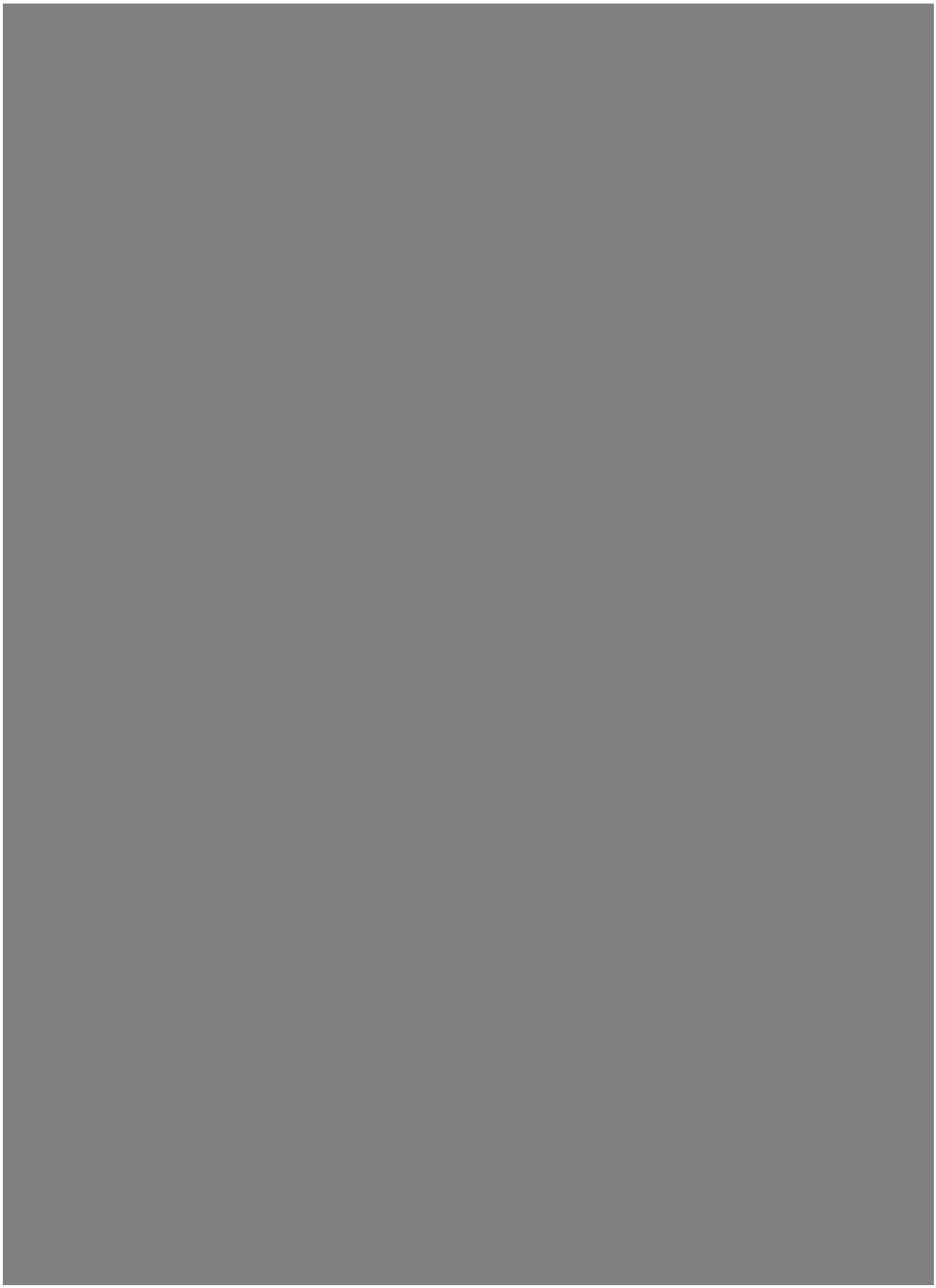
[1]Department of Cardiovascular Sciences, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom; [2]Department of Cardiovascular Science, University of Sheffield, Sheffield, United Kingdom; [3]Bristol Heart Institute, University of Bristol, Bristol Royal Infirmary, Bristol, United Kingdom; and [4]Adult Haemophilia Centre, Queen Elizabeth Hospital, Birmingham, United Kingdom

Light transmission aggregometry (LTA) is used worldwide for the investigation of heritable platelet function disorders (PFDs), but interpretation of results is complicated by the feedback effects of ADP and thromboxane A_2 (TxA_2) and by the overlap with the response of healthy volunteers. Over 5 years, we have performed lumi-aggregometry on 9 platelet agonists in 111 unrelated research participants with suspected PFDs and in 70 healthy volunteers. Abnormal LTA or ATP secretion test results were identified in 58% of participants. In 84% of these, the patterns of response were consistent with defects in Gi receptor signaling, the TxA_2 pathway, and dense granule secretion. Participants with defects in signaling to Gq-coupled receptor agonists and to collagen were also identified. Targeted genotyping identified 3 participants with function-disrupting mutations in the $P2Y_{12}$ ADP and TxA_2 receptors. The results of the present study illustrate that detailed phenotypic analysis using LTA and ATP secretion is a powerful tool for the diagnosis of PFDs. Our data also enable subdivision at the level of platelet-signaling pathways and in some cases to individual receptors. We further demonstrate that most PFDs can be reliably diagnosed using a streamlined panel of key platelet agonists and specified concentrations suitable for testing in most clinical diagnostic laboratories. (*Blood*. 2012;120(25): 5041-5049)

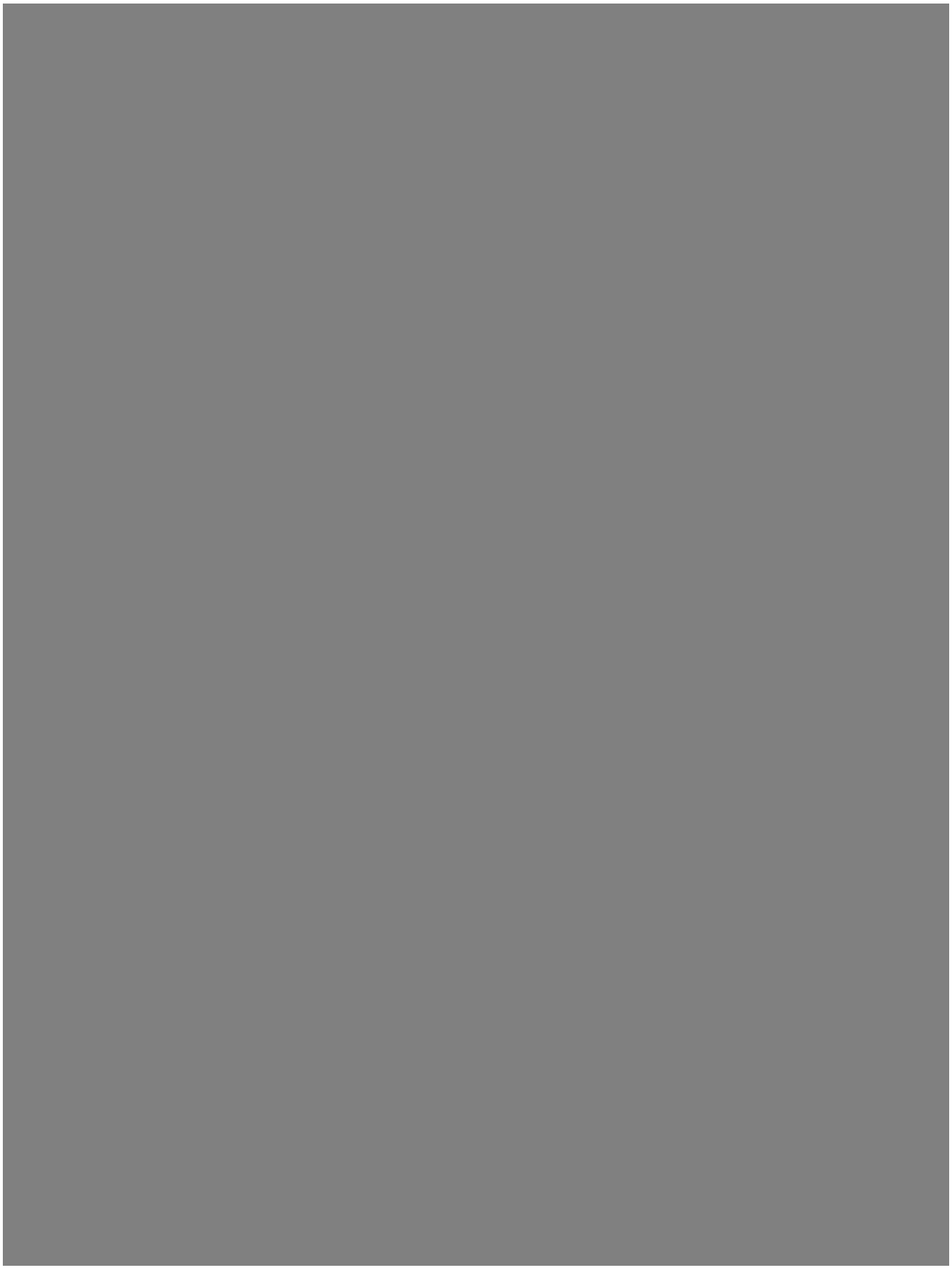
Appendix 4 – Selected publications



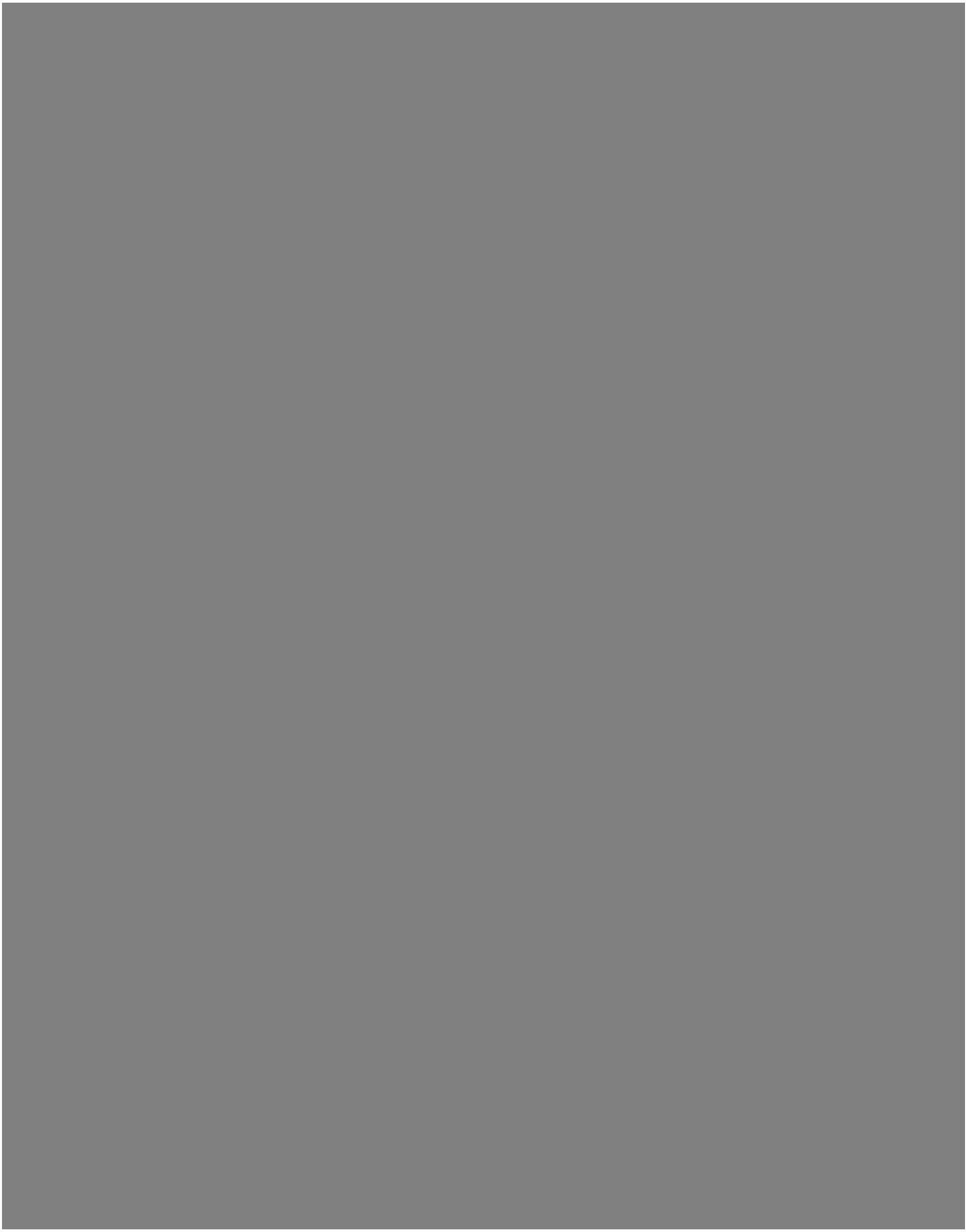
Appendix 4 – Selected publications



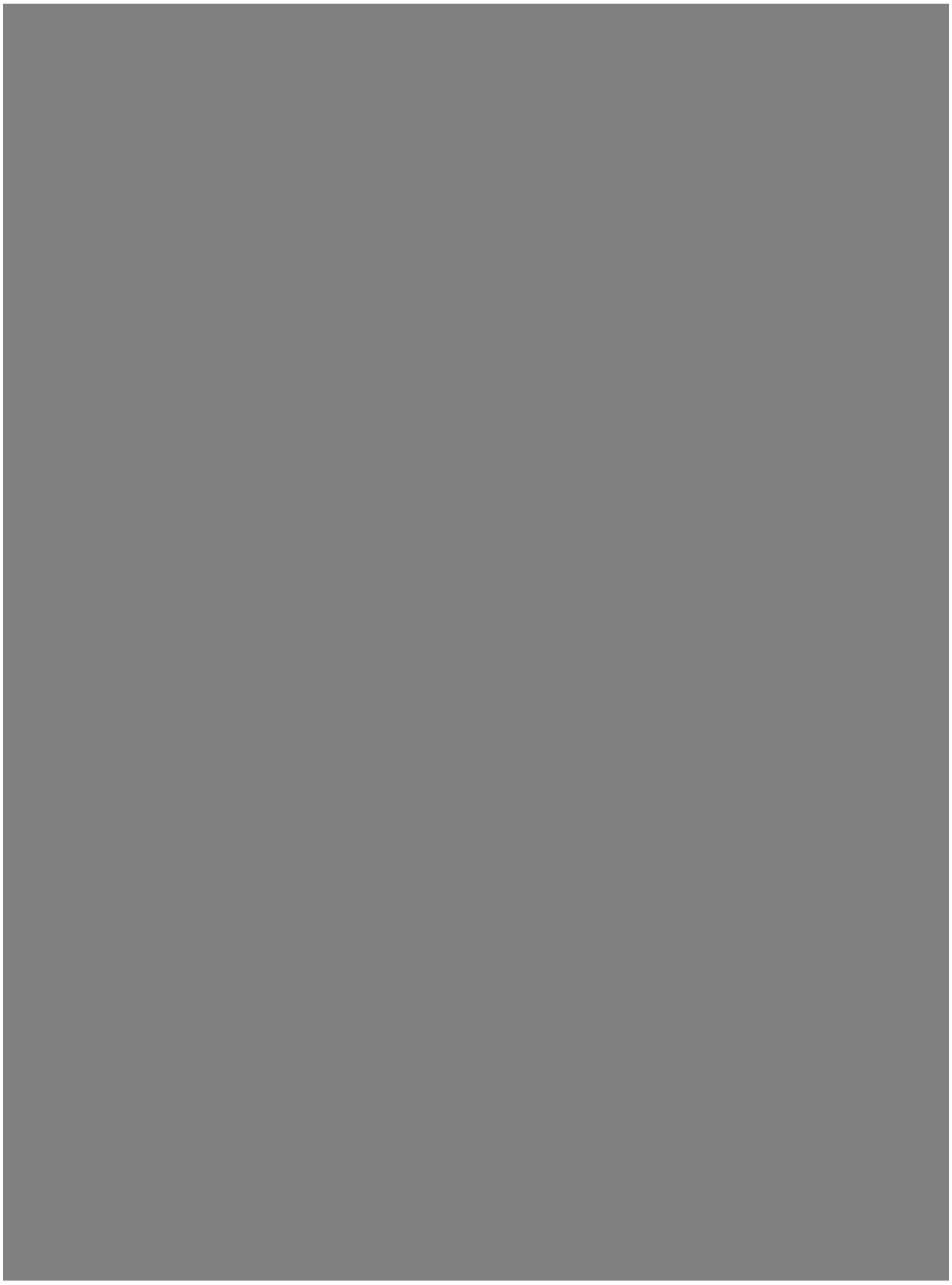
Appendix 4 – Selected publications



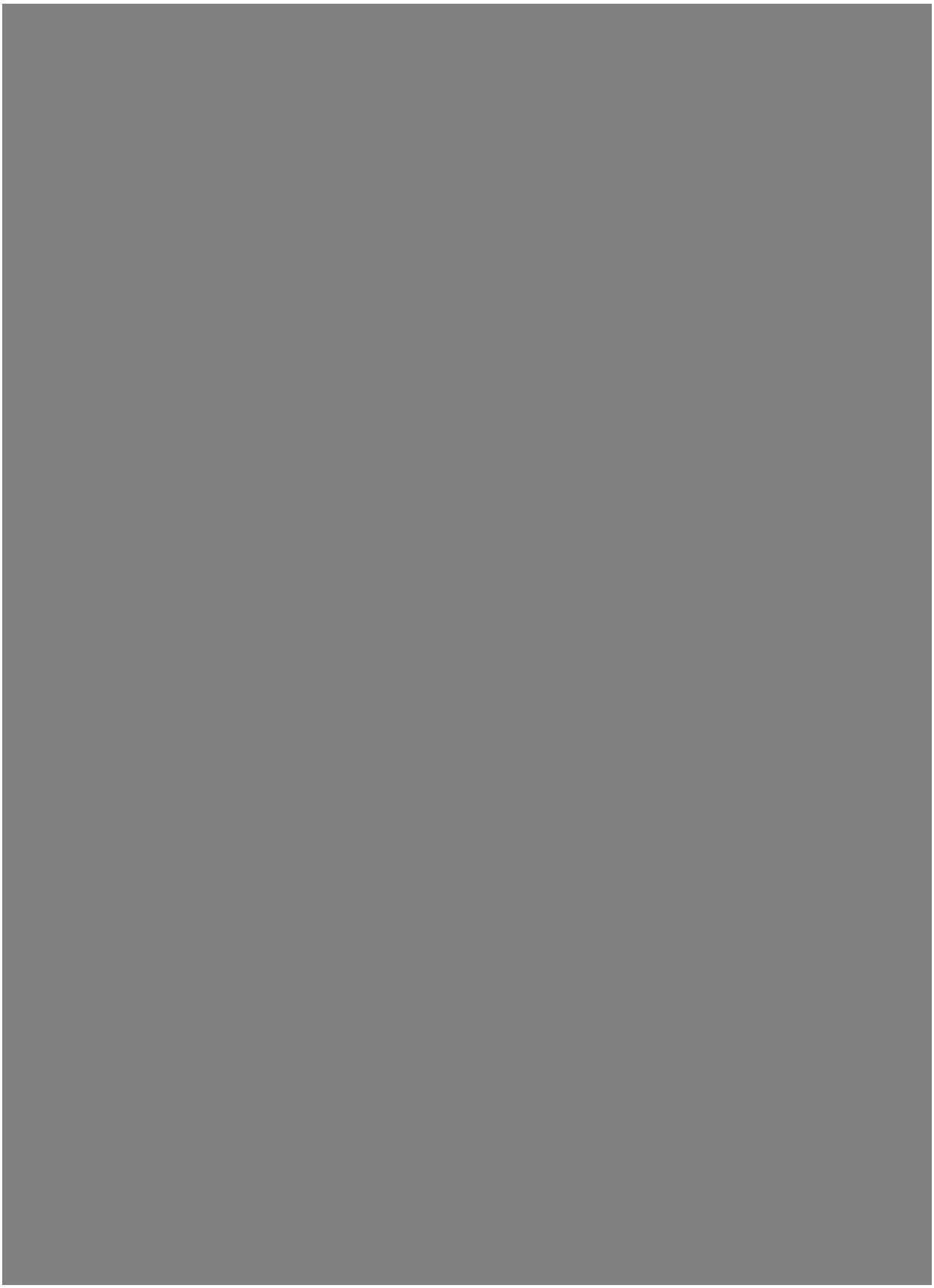
Appendix 4 – Selected publications



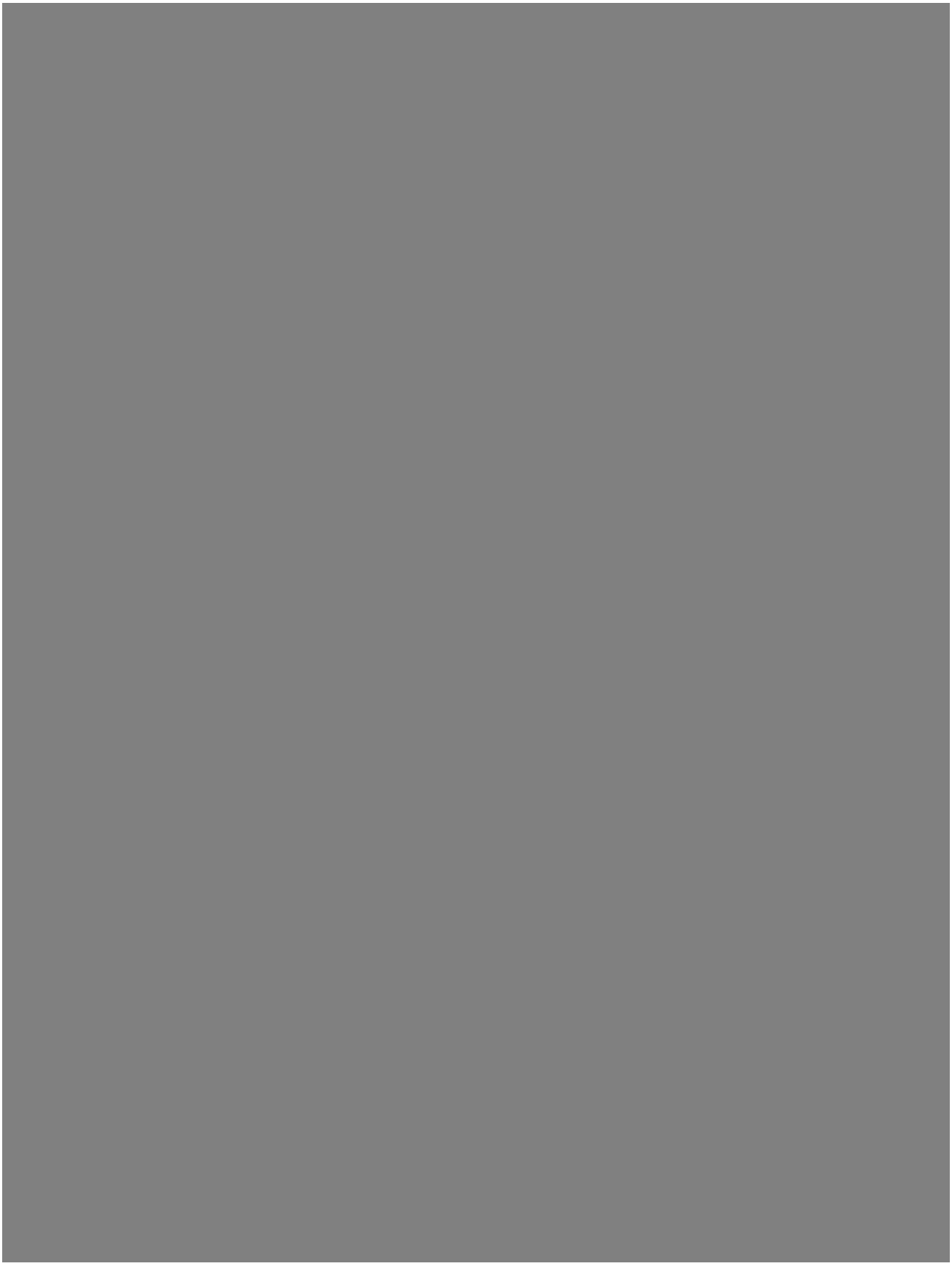
Appendix 4 – Selected publications



Appendix 4 – Selected publications



Appendix 4 – Selected publications



Appendix 4 – Selected publications

MICROSATELLITE MARKERS AS A RAPID APPROACH FOR AUTOZYGOSITY MAPPING IN HERMANSKY-PUDLAK SYNDROME: IDENTIFICATION OF THE SECOND HPS7 MUTATION IN A PATIENT PRESENTING LATE IN LIFE

Gillian C. Lowe[1]*; Isabel Sánchez Guiu[1,2]*; Oliver Chapman[3]; José Rivera[2]; Marie Lordkipanidzé[1]; Natalia Dovlatova[1,4]; Jonathan Wilde[5]; Steve P. Watson[1]; Neil V. Morgan[1]; on behalf of the UK GAPP collaborative

[1]Birmingham Platelet Group, College of Medical and Dental Sciences, University of Birmingham, Edgbaston,
Birmingham, UK; [2]Regional Blood Donation Centre and University of Murcia, Spain; [3]Haemophilia Centre, University Hospitals Coventry and Warwickshire NHS Trust, Clifford Bridge Road, Coventry, UK; [4]Thrombosis and Haemostasis Research Group, Queen's Medical Centre, University of Nottingham, Nottingham, UK; [5]West Midlands Adult Haemophilia Comprehensive Care Centre, University Hospital Birmingham, Edgbaston, Birmingham, UK

Correspondence to:

Dr. Neil Morgan
Centre for Cardiovascular Sciences
Institute for Biomedical Research
College of Medical and Dental Sciences
University of Birmingham
Birmingham, B15 2TT, UK
E-mail: n.v.morgan@bham.ac.uk

Financial support:

This work was funded by the Wellcome Trust (093994), the British Heart Foundation (RG/09/007/27917) and
Instituto de Salud Carlos III (ISG, FI10/00535 and JRP, PI10/02594).
Received: November 28, 2012

Accepted after major revision: January 17, 2013
Prepublished online: January 31, 2013
doi:10.1160/TH12-11-0876

Thromb Haemost 2013; 109: 766–768

* Joint first authors.

Dear Sirs,

Hermansky-Pudlak syndrome (HPS) is an autosomal recessive disorder characterised by oculocutaneous hypopigmentation and a bleeding diathesis caused by a lack of dense granules in platelets. HPS is genetically heterogeneous with variable skin, hair, and iris hypopigmentation, and visual impairment. In addition, some forms of HPS give rise to granulomatous colitis and pulmonary fibrosis (1). HPS has been associated with mutations in nine human genes: HPS1 (mouse model pale ear), AP3B1/HPS2 (pearl), HPS3 (cocoa), HPS4 (light ear), HPS5 (ruby-eye 2), HPS6 (ruby-eye), HPS7/dysbindin (sandy), HPS8/reduced pigmentation and HPS9/pallid (2,3). In addition, genes have been identified for a further six

mouse models of HPS (mocha, gunmetal, ashen, muted, buff, and subtle gray) (2). In general, HPS is a rare disorder, but HPS1 has a high prevalence in northwest Puerto Rico (1/1,800) due to a founder mutation (4).

The large numbers of potential HPS culprit genes (>118 coding exons), together with minimal guidance offered by the genotype-phenotype correlations between HPS genes, provides a significant challenge for the molecular diagnosis of these disorders. In cases with suspected consanguinity, autozygosity mapping can be used as a high speed tool to prioritise mutation screening for a specific HPS gene (5). Further, this can be facilitated using microsatellite markers flanking the HPS genes to exclude genes in the case of heterozygous markers/haplotypes and prioritise direct sequencing of specific genes where autozygosity is noted. We illustrate the proof of principle of this approach in the identification of the second case of a mutation in the HPS7 (encoding Dysbindin) gene in a patient with a bleeding history and hypopigmentation.

A 77-year-old Caucasian female was referred to the bleeding disorder clinic with a lifelong bleeding tendency (▸Figure 1 A). There was no history of a bleeding disorder in this participant's half siblings, parents or children. Her parents were first cousins. She had pale skin and hair and had reduced visual acuity and nystagmus throughout life. She also had spontaneous epistaxes as a teenager which lasted for several hours and required medical attention, and several episodes of prolonged bleeding from minor injuries that required surgical haemostasis. She bled for several days after dental extractions in her twenties and thirties and required packing of the tooth sockets to control the bleeding. She had menorrhagia from menarche which eventually necessitated a hysterectomy at the age of 37. She experienced heavy post partum bleeding after all of her three vaginal deliveries. Surgical procedures in this patient, including surgery for an ectopic pregnancy in the 1960s, abdominal hysterectomy and salpingo-oophrectomy in 1971 and excision of a lipoma from her back in 2002, were followed by prolonged bleeding requiring blood product transfusion. She had two episodes of severe per-rectal bleeding. The first one occurred in 1979 and was attributed to a rectal polyp which was surgically removed. She bled significantly after this procedure and required a platelet transfusion. A further episode occurred in 2009 which was attributed to Crohn's disease. During this episode the patient required red cell and platelet transfusions and tranexamic acid and was being considered for a right hemicolectomy at the time of her haematology referral. Colonic biopsies showed florid granulomatous inflammation with no co-existing infection (▸Figure 1 B). Acid fast bacilli were absent. She was reviewed by a respiratory physician in 2010 and had no evidence of any respiratory disease, with normal lung function tests (FEV1 100% predicted, FVC 95% predicted, TLC 80% predicted and KCO 105% predicted, all within normal ranges). A chest radiograph showed no active lung disease, and a computed tomography scan of the thorax with contrast showed no convincing features of fibrosis.

The patient gave informed consent and was recruited to the GAPP (Genotyping and Phenotyping of Platelets, NIHR ID 9858, Regional Ethics Committee reference 06/MRE07/36) study. Platelet function testing was performed on platelet-rich plasma (PRP) by lumiaggregometry using Chronolume® to assess secretion. The response to intermediate concentrations of PAR-1 peptide (30 μM) and collagen (1 μg/ml) was reduced relative to the control on the day and also a panel of over 70 healthy volunteers. Normal aggregation was observed at higher concentrations of these agonists (not shown). A lack of dense granule secretion with the entire panel of agonists tested (6) was noted, as illustrated for PAR-1 and PAR-4 peptides in ▸Figure 1C and D. The lack of platelet ATP secretion was consistent with an absence of platelet dense granules, and in combination with the patient's clinical features

were diagnostic of HPS. Such platelet function testing has previously been shown to be successful in diagnosing other HPS patients with complete lack of secretion from platelet dense granules (5, 6).

DNA was extracted from peripheral blood, and genetic studies were undertaken. As the patient's parents were related by blood, we were able to apply autozygosity linkage mapping by genotyping several microsatellite markers flanking all of the known human HPS genes (see Suppl. Table 1, available online at www.thrombosis-online.com). This was carried out to prioritise a limited number of HPS genes for direct sequencing. Strikingly the only HPS locus that displayed autozygosity for both flanking markers and over an extended region of genetic distance was the HPS7 locus. Therefore the most likely candidate HPS gene was *HPS7/Dysbindin* on chromosome 6p22.3. The 10 coding exons of *DTNBP1* (encoding dysbindin) including exon-intron boundaries were PCR-amplified and sequenced. Sequencing of *DTNBP1* revealed a homozygous nonsense mutation in exon 4 (c.177 G>A; p.Trp59Stop) confirming a diagnosis of HPS type 7 (Fig 1E).

Dysbindin is important for normal platelet-dense granule and melanosome biogenesis and is mutated in the sandy (sdy) mouse (7). Dysbindin is a component of the biogenesis of lysosome-related organelles complex 1 (BLOC-1) which regulates trafficking to lysosome-related organelles. The BLOC-1 complex is ubiquitously expressed and along with Dysbindin contains seven predicted coiled-coil-forming proteins (pallidin, muted, cappuccino, snapin, BLOC1S1, BLOC1S2 and BLOC1S3) (8), all of which are associated with Hermansky-Pudlak syndrome in mice.

The only previously reported HPS7 mutation in humans was a homozygous nonsense mutation (p.Q103X) found in a 48-year-old Portuguese woman with oculocutaneous hypopigmentation, ease of bruising and a bleeding tendency. She had mild shortness of breath on exertion and reduced lung compliance but otherwise normal pulmonary function (8). Although the patient described here has been diagnosed with Crohn's disease, it is likely that this represents granulomatous colitis given the histological findings reported on colonic biopsy. She has no evidence of pulmonary fibrosis. Colitis has been reported in HPS1, HPS3 and HPS4, and pulmonary fibrosis in HPS1, HPS4 and HPS2 (9, 10, 11).

In conclusion, we report a novel mutation in the HPS7 (Dysbindin) gene causing a premature stop codon which was rapidly identified following autozygosity mapping using microsatellite markers. This high-speed technique provides a rapid approach to identify candidate HPS genes for Sanger sequencing in order to identify a disease causing mutation. An alternative method to identify the genetic defect in this patient would have been to utilise second generation sequencing strategies such as whole exome sequencing or custom built arrays; however, this would have been far more costly and time consuming given the consanguinity in this family which we exploited to narrow down the culprit gene. Despite having had numerous hospital visits and lifelong excessive bleeding, the cause of this patient's bleeding was not elucidated until she was in her eighth decade. Mild inherited platelet disorders should therefore be considered in patients presenting with excessive bleeding later in life.

Acknowledgements

We would like to thank the patient for her permission to allow us to publish her case history.

Conflicts of interest

None declared.

References

Davies BH, Tuddenham EG. Familial pulmonary fibrosis associated with oculocutaneous albinism and platelet function defect. A new syndrome. Q J Med 1976; 45: 219-232.
Dell'Angelica EC. The building BLOC(k)s of lysosomes and related organelles. Curr Opin Cell Biol 2004; 16: 458-464.
Wei ML. Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. Pigment Cell Res 2006; 19: 19-42.
Witkop CJ, Almadovar C, Pineiro B, et al. Hermansky-Pudlak syndrome (HPS). An epidemiologic study. Ophthalmic Paediatr Genet 1990; 11: 245-250.
Morgan NV, Pasha S, Johnson CA, et al. A germline mutation in BLOC1S3/reduced pigmentation causes a novel variant of Hermansky-Pudlak syndrome (HPS8). Am J Hum Genet 2006; 78: 160-166.
Dawood BB, Lowe GC, Lordkipanidzé M, et al. Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. Blood 2012; 120: 5041-5049.
Swank RT, Sweet HO, Davisson MT, et al. Sandy: a new mouse model for platelet storage pool deficiency. Genet Res 1991; 58: 51-62.
Li W, Zhang Q, Oiso N, et al. Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). Nat Genet 2003; 35: 84-89.
Hermos CR, Huizing M, Kaiser-Kupfer MI, et al. Hermansky-Pudlak syndrome type 1: gene organization, novel mutations, and clinical-molecular review of non-Puerto Rican cases. Hum Mutat 2002; 20: 482.
Anderson PD, Huizing M, Claassen DA, et al. Hermansky-Pudlak syndrome type 4 (HPS-4): clinical and molecular characteristics. Hum Genet 2003; 113: 10-17.
Gochuico BR, Huizing M, Golas GA, et al. Interstitial lung disease and pulmonary fibrosis in Hermansky-Pudlak syndrome type 2, an adaptor protein-3 complex disease. Mol Med 2012; 18: 56-64.

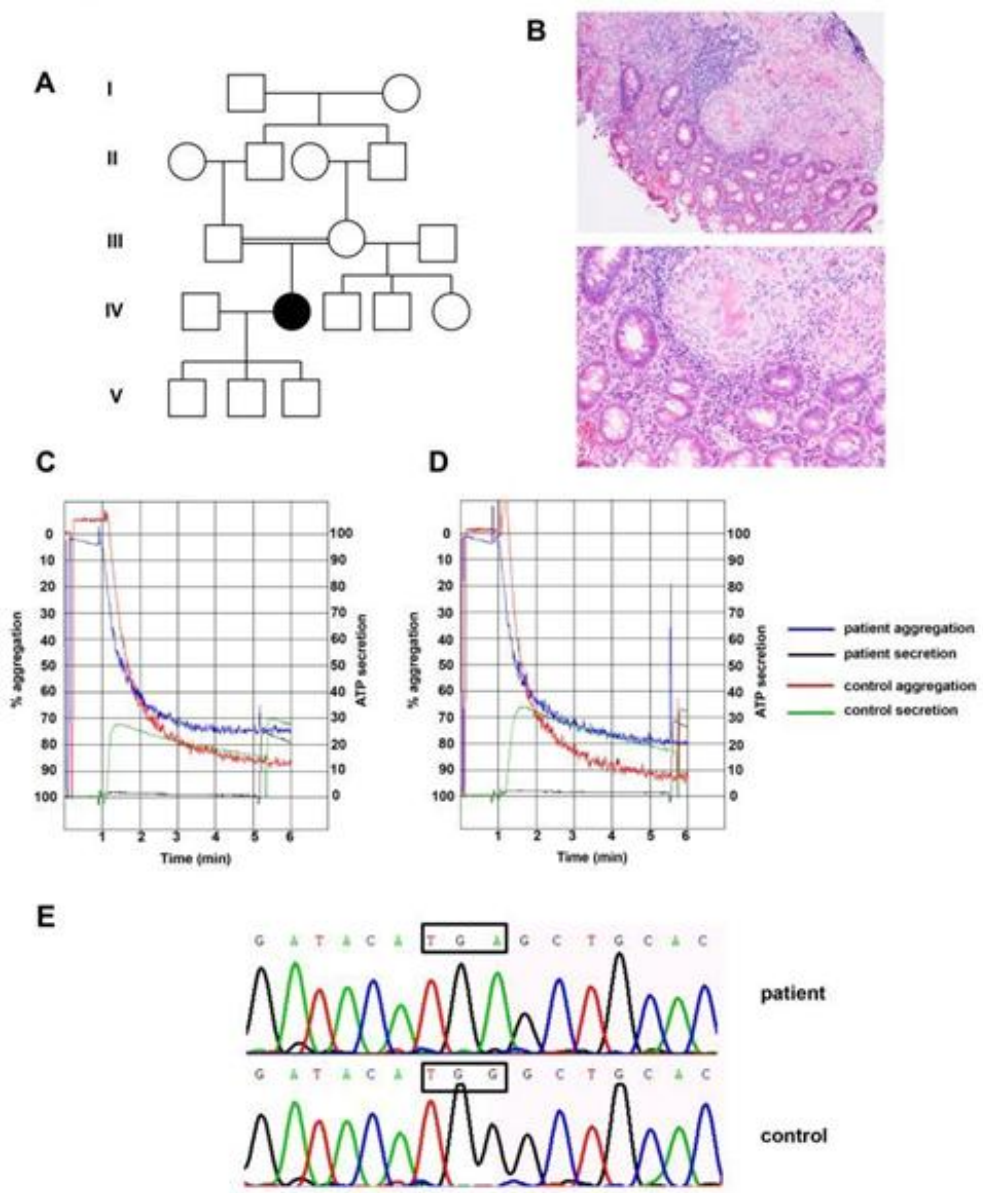


Figure 1: Identification of the second HPS7 mutation in a patient with Hermansky-Pudlak syndrome presenting late in life. A) Pedigree of a consanguineous family with Hermansky-Pudlak syndrome. The affected individual is represented by a solid symbol. B) Images of a colonic biopsy from the patient in low power (upper panel) and high power (lower panel). Both images show inflammatory infiltrates and granulomata with numerous giant cells, alongside normal bowel mucinous glands. No caseous necrosis is seen, and special stains showed no evidence of micro-organisms (including mycobacteria). C and D) Absence of secretion (black trace) to high doses of PAR-1 peptide 100 µM (C) and PAR-4 peptide 500 µM (D) in this patient. Left-sided Y axis depicts percentage aggregation, and right-sided Y axis represents platelet ATP secretion assessed using Chronolume®. 1.6 nmol of ATP standard were added to each cuvette in order to calculate absolute secretion and secretion normalised to platelet count in PRP for both patient and control. E) Identification of a homozygous single base substitution (c.177 G>A) in Dysbindin leading to a premature stop codon (p.Trp59Stop). Sanger sequencing showing wild-type and mutant *DTNBP1* sequence traces. The position of the mutation is indicated by the boxed regions.

A NOVEL THROMBOXANE A2 RECEPTOR N42S VARIANT RESULTS IN REDUCED SURFACE EXPRESSION AND PLATELET DYSFUNCTION

Shaista P. Nisar[1]; Marie Lordkipanidzé[2]; Matthew L. Jones[3]; Ban B. Dawood[2]; Sherina Murden[3]; Margaret R. Cunningham[1];
Andrew D. Mumford[3]; Jonathan T. Wilde[4]; Steve P. Watson[2]; Stuart J. Mundell[1]; Gillian C. Lowe[2]; on behalf of the UK GAPP Study Group

[1]School of Physiology and Pharmacology, Medical Sciences Building, University of Bristol, Bristol, UK; [2]Centre for Cardiovascular Sciences, Institute of Biomedical Research, College of Medical and Dental Sciences, University of Birmingham, Edgbaston, Birmingham, UK; [3]Bristol Heart Institute & School of Cellular and Molecular Medicine, University of Bristol, Bristol, Bristol, UK; 4West Midlands Adult Haemophilia Comprehensive Care Centre, University Hospital Brimingham, Edgbaston, Birmingham, UK

Summary

A small number of thromboxane receptor variants have been described in patients with a bleeding history that result in platelet dysfunction. We have identified a patient with a history of significant bleeding, who expresses a novel heterozygous thromboxane receptor variant that predicts an asparagine to serine substitution (N42S). This asparagine is conserved across all class A GPCRs, suggesting a vital role for receptor structure and function. We investigated the functional consequences of the TP receptor heterozygous N42S substitution by performing platelet function studies on platelet-rich plasma taken from the patient and healthy controls. We investigated the N42S mutation by expressing the wild-type (WT) and mutant receptor in human embryonic kidney (HEK) cells. Aggregation studies showed an ablation of arachidonic acid responses in the patient, whilst there was right-ward shift of the U46619 concentration response curve (CRC). Thromboxane generation was unaffected. Calcium mobilisation studies in cells lines showed a rightward shift of the U46619 CRC in N42S-expressing cells compared to WT. Radioligand binding studies revealed a reduction in BMax in platelets taken from the patient and in N42S-expressing cells, whilst cell studies confirmed poor surface expression. We have identified a novel thromboxane receptor variant, N42S, which results in platelet dysfunction due to reduced surface expression. It is associated with a significant bleeding history in the patient in whom it was identified. This is the first description of a naturally occurring variant that results in the substitution of this highly conserved residue and confirms the importance of this residue for correct GPCR function.

Keywords

Inherited / acquired platelet disorders, gene mutations, receptors, platelet pharmacology, platelet pathology / inherited, acquired

Correspondence to:
S. J. Mundell
School of Physiology and Pharmacology
Medical Sciences Building, University of Bristol
Bristol BS8 1TD, UK
Tel: +44 (0) 117 3311440, E-mail: s.j.mundell@bristol.ac.uk

Financial support:

This work was supported by the British Heart Foundation and the Wellcome Trust (BHF; PG/06/038 and RG/09/007/27917, Wellcome Trust ref 093994). SJM is a BHF Senior Research Fellow and SPW holds a BHF Chair. ML is supported by the Canadian Institute of Health Research (MFE-107592) and the British Heart Foundation (PG/11/31/28835). GL holds a Wellcome Trust fellowship.

Received: August 16, 2013
Accepted after major revision: December 19, 2013
Prepublished online: January 23, 2014
doi:10.1160/TH13-08-0672
Thromb Haemost 2014; 111: 923–932

Introduction

Platelet activation is a tightly regulated process that is required for haemostasis, dysregulation of which can result in thrombosis and vessel occlusion. Platelet activity is regulated by a number of cell surface receptors, including several G-protein coupled receptors (GPCRs). The thromboxane receptor (TP-α isoform) is a GPCR expressed on platelets in addition to a variety of other cell types including macrophages, vascular endothelial cells and smooth muscle cells (1).

Thromboxane A_2 (TXA_2) generated by platelets acts in an autocrine manner on TP-receptors which signal via phospholipase A2 and Rho to cause platelet activation. This pathway is currently targeted by aspirin, which exerts its antiplatelet effects by inhibition of cyclooxygenase enzymes (COX-1); however, there is considerable interest in developing direct TP receptor antagonists in order to preserve the beneficial effects of prostaglandins (such as gastric mucosal protection) that are lost upon global COX inhibition (2, 3).

As with most GPCRs, a large number of mutagenesis studies have been carried out with the TP receptor in order to further understand structure-function relationships. However, studying the impact of such mutations on platelet function is not possible; therefore an alternative approach is to study patients with naturally occurring mutations within platelet receptors, such as TP-α. Thromboxane receptor deficiency (MIM #614009) is inherited in an autosomal dominant manner and is associated with mild mucocutaneous bleeding (4). To date one quantitative defect causing reduced TP receptor expression (4) and three qualitative defects caused by TP receptor amino acid substitutions have been reported (5-7). Importantly, the naturally occurring variants that resulted in amino acid substitutions all provided important insights into structure-function of the endogenous TP receptor. For example the R60L variant resulted in reduced G protein coupling (5), whereas the D304N substitution caused an impairment in ligand binding (6). More recently, characterisation of the W29C variant showed that this tryptophan residue in TMD1 is critical for efficient receptor expression at the cell surface (7).

As part of the UK Genotyping and Phenotyping of Platelets (GAPP) consortium we have identified a number of mutations in receptor genes in patients with a bleeding tendency (8, 9). Here we used this approach to identify a novel thromboxane receptor defect in a patient with a bleeding history. The patient was heterozygous for an asparagine to serine substitution at position 42 (N42S) of the TP receptor that resulted in impaired receptor surface expression and platelet dysfunction. This is the first description of a naturally occurring variant that causes substitution of this highly conserved asparagine residue and validates findings from crystallography studies with other GPCRs (10, 11).

Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), Lipofectamine 2000 and fetal bovine serum (FBS), anti-HA-monoclonal antibody (HA-11), goat anti-mouse rhodamine, FITC and rhodamine-conjugated secondary antibodies and Fura-2 AM were purchased from Invitrogen (Carlsbad, CA, USA). Complete protease inhibitor tablets were from Roche. Arachidonic acid (AA) and rabbit polyclonal TP receptor antibody was purchased from Cayman Chemical (Ann Arbor, MI, USA). U46619 was purchased from Sigma Aldrich (St. Louis, MO, USA). PAR-1 receptor specific peptide (SFLLRN) was from Alta Biosciences (Birmingham, UK), collagen was from Nycomed (Zurich, Switzerland). Ristocetin was from Helena Biosciences (Tyne and Wear, UK). Radiochemicals were from Perkin Elmer Life Sciences (Waltham, MA, USA). The ELISA alkaline phosphatase substrate kit was from BioRad (Hercules, CA, USA). All other reagents were from Sigma.

Ethics

This study was approved by the National Research Ethics Service Committee West Midlands–Edgbaston (REC reference: 06/MRE07/36, ISRCTN 77951167, UKCRN ID 9858), and participants and controls gave written informed consent in accordance with the Declaration of Helsinki. Relevant NHS permissions were obtained in all participating institutions.

TBXA2R sequencing

Genomic DNA was purified from EDTA-anticoagulated blood using the Maxwell 16 DNA purification system (Promega, Madison, WI, USA). The *TBXA2R* gene was PCR amplified from genomic DNA. PCR reactions were performed in 15µl volumes using OneTaq PCR master mix (New England Biolabs, Ipswich, MA, USA). PCR products were treated with ExoSAP-IT (Affymetrx, Santa Clara, CA, USA) and sent to DBS Genomics (University of Durham, UK) for Sanger Sequencing.

Platelet function studies

Blood was drawn by venipuncture through a 21-gauge needle into evacuated tubes containing 3.8% sodium citrate. Platelet function was assessed using light transmission aggregometry as described previously (17). Platelet-rich plasma (PRP) was prepared by centrifugation of whole blood at 200g for 10 minutes (min) and platelet-poor plasma (PPP) by further centrifugation at 1,000g for 10 min. Platelet aggregation was measured in PRP using a dual Chronolog lumiaggregometer in response to ADP, adrenaline, arachidonic acid, PAR-1 receptor specific peptide (SFLLRN), collagen, U46619 and ristocetin. ATP secretion was assessed using the Luciferin-Luciferase reagent (Chrono-Lume®, Chrono-log Corp., Columbia, MD, USA) and an ATP standard solution.

96-well plate aggregometry – *Optimul* assay

Platelet aggregation in response to AA (0.03 – 1 mM), ADP (0.005 – 40 µM), and U46619 (0.005 – 40 µM) was assessed by 96-well plate aggregometry. The method is based on light transmission through a 96-well plate pre-coated with increasing concentrations of the agonists and assesses platelet aggregation in PRP.

Plasma thromboxane (Tx) B2 assay

After aggregation, indomethacin (30 µM) was added in relevant wells to stop COX activity. The whole plate was then centrifuged (1,300 g for 10 min) at room temperature and the supernatants were frozen at -80ºC. TxB2 levels were determined by a competitive immunoassay (Cisbio Bioassays, Codolet, France).

Radioligand binding

TP and $P2Y_{12}$ receptor ligand binding surface expression was determined by ligand binding in fixed platelets and human embryonic kidney (HEK) cells as previously described (6, 12). After incubation with [^{3}H] SQ29548 (3 Ci mM^{-1}, 0.01–2 µM) for 20 min at room temperature, either in the presence or in the absence of unlabelled ligand (10 µM), reactions were terminated with 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. Control experiments were performed with the non-specific P2Y purinergic receptor ligand [^{3}H] MeSADP (3 Ci [111 GBq mM]) in the absence or presence of the $P2Y_{12}$ receptor antagonist AR C69931MX (10 µM), to determine $P2Y_{12}$ receptor-specific radioligand binding.

Generation of receptor constructs and mutagenesis

N-terminally FLAG-tagged TP receptor constructs were generated by PCR. The N42S mutation was introduced using the primer-extension overlap PCR mutagenesis method and subcloned into the BamH1 and EcoR1 sites in pcDNA3.1+.

Cell culture and transfection

HEK293 cells were cultured in DMEM, 10% fetal calf serum, 100 units ml^{-1} penicillin, and 100 µg ml−1streptomycin and maintained at 37°C, 5% CO_2. Cells were transiently transfected with receptor DNA or pcDNA3 using LIPOFECTAMINE 2000 (Life Technologies, Paisley, UK) according to manufacturer's instructions. All assays were performed 48 hours (h) post-transfection.

Measurement of cytosolic Ca^{2+} concentration ([Ca2+]i)

HEK293 cells were grown in clear bottom, black 96-well plates (Costar). Cells were washed with HEPES-buffered saline buffer (HBBS; 130 mM NaCl, 3 mM KCl, 10 mM HEPES, 1 mM MgCl2, 2 mM $CaCl_2$, and 30 mM D-glucose, pH 7.3) and loaded with the 3 μM Fura-2 calcium sensitive dye for 1 h at 37°C. Cells were washed with HBBS and maintained at room temperature. Changes in intracellular calcium upon agonist stimulation were measured using a Flexstation III microplate reader (Molecular Devices, Sunnyvale, CA, USA).

ELISA

Surface receptor loss was assessed by immunosorbent assay (ELISA) as described previously (13, 14). Cells were split into 24-well tissue culture dishes coated with 0.1 mg/ml poly-L-lysine. Twenty-four hours later, cells were fixed with paraformaldehyde (4%). Surface receptor assessed using a monoclonal anti-HA (HA-11 1:1,000) antibody or monoclonal anti-FLAG (1:500) antibody. Receptor levels were subsequently determined by ELISA and expressed as % surface receptor with the background signal from pcDNA3-transfected controls subtracted from all receptor-transfected values.

Immunofluorescence microscopy

Cellular distribution of tagged receptors in HEK cells was assessed by immunofluorescence microscopy (13). Briefly cells were grown on poly-L-lysine coated coverslips. Twenty-four hours later, receptor distribution was assessed using a primary anti-FLAG antibody or anti-HA- polyclonal antibody (1:200) and the appropriate fluorescent-conjugated secondary antibody (1:200). For experiments where surface and total where visualised in the same cells, anti-FLAG antibody and FITC-conjugated secondary antibody were first added to non-permeabilsed cells to label surface receptor, and total receptor was stained with anti-FLAG antibody and Rhodamine-conjugated secondary antibody following permeabilisation. Confocal microscopy was carried out on a Leica SP5-AOBS confocal laser scanning microscope attached to a Leica DM I6000 inverted epifluorescence microscope with phase-contrast and a Plan-Apo 63 x 1.40 NA oil immersion objective. All images were collected on Leica TCS-NT software for 2D and 3D image analysis and processed using Adobe Photoshop CS3.

Immunoblotting

Washed platelets were lysed and subjected to SDS-PAGE, as previously described (15). Immunoblotting was carried out as using a TP-receptor antibody (1:1,000).

Clinical history

The patient was a 64-year-old female who had a history of menorrhagia from menarche that ultimately necessitated hysterectomy, excessive post-operative and post dental surgery bleeding, prolonged bleeding from minor cuts and a tendency to bruise easily. She had a raised ISTH bleeding assessment score of 13 (95^{th} centile of score in previously tested healthy volunteers in the GAPP study = score of 4) (16). She had not experienced any life-threatening bleeding episodes and had no co-morbidities. The patient had a daughter with menorrhagia and a sister with a history of easy bruising; however, both these family members were unfortunately unavailable for further study. There was no obvious history of excessive bleeding in either of the patient's parents. This history was indicative of a platelet function disorder as initial coagulation screening laboratory investigations were normal and the patient was recruited to the GAPP study, in which detailed platelet function testing was performed as previously described (17).

Results

Platelet phenotyping

Platelet aggregation and secretion in PRP was assessed by lumi-aggregometry and a luciferase assay, respectively, at three distinct time points over three years. The platelet count in PRP was within the normal range. Platelet aggregation and secretion to 1 mM and 1.5 mM arachidonic acid was consistently absent in the patient whereas these concentrations caused full aggregation of platelets collected from a healthy volunteer on the same day, and in previously tested healthy volunteers (▸Figure 1A, B and D). Aggregation in response to 3 µM U46619 (synthetic thromboxane analogue) was also markedly reduced and reversible in the patient, whereas it elicited full aggregation in the control on the same day as well as in a bank of local controls (▸Figure 1C). Secretion in response to these agonists was also abrogated in the patient (▸Figure 1A-C). Aggregation in response to a range of other agonists was also tested (▸Figure 1D). There was also a reduction in platelet responsiveness to ADP, adrenaline and intermediate concentrations of collagen and PAR-1 peptide, consistent with the amplification role of TxA_2 in platelet activation.

These findings were consistent with 96-well plate aggregometry data (*Optimul* assay) which also showed absent aggregation in the patient at a range of arachidonic acid concentrations (▸Figure 1E) and a significantly right-shifted U46619 CRC (▸Figure 1G; 10-fold shift, EC_{50} 0.22 µM vs 2.95 µM in healthy volunteers and the patient, respectively). Interestingly, the *Optimul* assay showed no difference in ADP responsiveness (▸Figure 1F), whereas there the patient showed reduced responsiveness to adrenaline and collagen (data not shown).

To rule out the possibility of a defect in arachidonic acid metabolism, thromboxane generation was measured. The plasma TxB_2 levels in the patient were 40.7 ng / 1 x 10^8 platelets, which is within the normal range and not significantly different from mean data previously collected from healthy controls (▸Figure 2). The normal COX activity in the patient, combined with reduced responsiveness to the direct-acting agonist, U46619 suggested a defect at the level of the thromboxane receptor. The reduced responses to other

thromboxane-dependent agonists, such as adrenaline and intermediate dose collagen concentrations can also be explained by incomplete activation of the thromboxane receptor resulting in reduced amplification and secondary mediator generation.

Identification of the *TBXA2R* N42S mutation

In light of the platelet phenotyping data, we sequenced the TP receptor gene *TBXA2R*. All three exons of TBXA2R were PCR amplified and sequenced, covering the entire 343 amino acid coding region. The patient was found to harbour a c.125 A>G transversion in exon 2 of *TBXA2R* that predicted a novel heterozygous missense mutation resulting in an asparagine to serine substitution at position 42 in TMD1 of the receptor. Interestingly, this asparagine residue (1.50 Ballesteros-Weinstein numbering) is the most highly conserved residue across class A GPCRs and crystallographic structure of rhodopsin suggests it is involved in a series of stabilising interactions (10). Furthermore, an asparagine to serine change in the thromboxane receptor at this position was predicted to be damaging using the PolyPhen-2 tool (18) (using both HumDiv and HumVar training datasets) and deleterious using the PROVEAN tool (19).

Heterologous expression of variant N42S TxA_2R

In order to test the functional impact of the N42S mutation, FLAG-tagged constructs of wild-type (WT) or N42S TP receptor were generated and transiently transfected into HEK293 cells. The ability of the WT and N42S receptor to signal was examined by measuring changes in cytosolic Ca^{2+} concentration in response to U46619. The CRC for N42S-expressing cells was right-shifted compared to WT (EC_{50} WT 29 nM vs N42S 0.1 μM) without any decrease in the maximal response reached (▸Figure 3A). These findings were consistent with the reduced U46619-induced aggregation seen in the patient (▸Figure 1).

To further investigate why there was a reduction in the ability of the N42S variant to function both in platelets and in cell lines, we measured binding of the TP receptor antagonist [3H]- SQ29548 to HEK293 cells expressing the WT or variant N42S receptor. Binding of [3H]-SQ29548 to transfected HEK293 cells was saturable and maximal at a ligand concentration of 0.6 μM (▸Figure 3B). Maximum binding of [3H]-SQ29548 to cells expressing N42S TP receptor was reduced by more than 60% compared with those expressing WT receptor (Bmax N42S 235 ± 12 dpm/mg protein; WT 628 ± 27 dpm/mg protein; ▸Figure 3B). Furthermore, there was almost a three-fold shift in the binding affinity of [3H]-SQ29548 to HEK293 cells expressing N42S compared to WT (Kd WT 74 ± 16 nM; N42S 200 ± 40 nM).

To investigate whether there were changes in maximal ligand binding in the patient harbouring the N42S variant, we carried out radioligand binding studies on washed platelets taken from the patient and four healthy controls. Interestingly, there was a 63% *(B*max control 355 ± 18 dpm/mg protein; patient 130 ± 15 dpm/mg protein) loss of TP receptor surface binding sites in the patient, as compared to healthy controls (▸Figure 3C), higher than might be predicted given that the patient is heterozygous for the N42S change. There was no change in total receptor expression (▸Figure 3E). There was no difference in $P2Y_{12}$ receptor surface binding sites between the patient and the control (▸Figure 3D). These data suggest surface expression of the N42S variant receptor is significantly reduced compared to WT.

N42S results in reduced receptor surface expression in cells lines

To further confirm whether the reduction in N42S TP receptor function was due to a loss of surface expression, a cell surface ELISA approach was used to quantify receptor levels. These data showed a dramatic reduction in cell surface expression of the N42S variant in HEK293 cells (3.6 ± 7.3% of WT controls; ▸Figure 4A). These data were reproducible in Chinese hamster ovary (CHO) cells (data not shown) suggesting the lack of surface expression was due to the mutation and not to a cell line-specific effect. Immunofluoresence experiments were carried out to visualise localisation of the WT and N42S TP receptors. Sequential staining with an anti-FLAG antibody was carried out in non-permeabilised and permeabilised cells to label surface and total receptor, respectively. WT receptor was localised to the cell surface whereas, in agreement with the ELISA data, there was neglible surface expression of the N42S variant (green staining, ▸Figure 4B). Total receptor expression was not affected.

Co-expression of WT receptor does not rescue surface expression of Asn42Ser

Since the patient is heterozygous for the N42S variant receptor, it is predicted that she will express both the WT and N42S receptor in equal proportions. In an attempt to replicate this in cell lines, tagged WT and N42S constructs were co-transfected into the same cells. Interestingly, in HEK293 cells, co-expression of HA-tagged WT receptor with the FLAG-tagged N42S TP receptor did not rescue expression of the N42S variant (▸Figure 5A). Furthermore, surface expression of the WT receptor was significantly reduced in presence of N42S (▸Figure 5B). Imaging of both the WT and N42S receptors in co-transfected cells revealed that the receptors were co-localised intracellularly (▸Figure 5C). Taken together, these data suggest that in addition to poorly expressing at the cell surface, the N42S receptor also causes intracellular retention of the WT receptor. These observations may explain why there was a larger than expected reduction in TP receptor surface expression in the heterozygous patient.

Discussion

Using the GAPP approach, we have identified a number of rare function-disrupting mutations in GPCRs, including two TP receptor variants (6, 17). We now report a further novel heterozygous TP receptor variant, N42S which results in reduced surface expression and thus reduced receptor function. Platelet phenotyping data showed loss of platelet responsiveness to arachidonic acid and U46619 with normal COX activity, which was consistent with a TP receptor defect. This report takes the number of TP receptor variants identified in patients with abnormal bleeding to a total of five, indicating that these remain rare contributors to bleeding risk even in selected populations. It is possible that this patient had other unidentified factors which also contributed to her bleeding phenotype, consistent with a multifactorial model for individual bleeding risk.

The patient described here had a history of significant post-operative and mucocutaneous bleeding. Three of the previously identified TP variants (c.167dupG, R60L & D304N) were also identified in patients with mucocutaneous bleeding (4-6), whereas the W29C patient had abnormal post-operative bleeding without a history of mucocutaneous bleeding (20).

However, similarly to reported *P2Y12R* mutations, these patients had family members who harboured the same heterozygous change in the *TBXA2R* gene, but did not have any bleeding symptoms (6, 20). In the absence of a family study, which would be recommended wherever possible in all patients with excessive bleeding and a potential causative genetic lesion, it is impossible to confirm whether the N42S mutation is causing the significant bleeding seen in the patient. Interestingly, however, the patient did have family members with menorrhagia and a history of easy bruising, although unfortunately these were unavailable for further study. It is possible that there are other unidentified factors that are contributing to bleeding symptoms. In the platelet function studies we were unable to detect any responses to the endogenous agonist, arachidonic acid in either the LTA or in the *Optimul* assay, which is in line with a "all-or-none" type of response seen with this agonist. Specific assessment of the TP receptor with the synthetic agonist, U46619, showed that platelet responses were strongly impaired in response to low and intermediate concentrations, whereas very high concentrations were able to elicit full aggregation in the patient despite a significant reduction in surface expression, a finding which is likely explained by receptor reserve. As thromboxane is involved in the amplification of other platelet responses (21), it is possible that loss of TP receptor function could have a more profound effect *in vivo.* Reduced platelet activation could also result in reduced thrombin generation, which may also contribute to impaired global haemostasis.

This study also provides important insights into endogenous TP receptor structure and function. Asparagine 42 (1.50 Ballesteros-Weinstein numbering) is the most highly conserved residue in class A GPCRs and has been shown to be involved in hydrogen bonding with Gly51, Ala299, Asp83 in bovine rhodopsin (10, 11), all of which are conserved in human TP-α. Crystallographic studies suggest that this network of hydrogen-bonding stabilizes the packing of TMD1, 2 and 7. Furthermore, mutagenesis studies with the thyrotropin-releasing hormone receptor showed that substitution of this asparagine residue results in reductions in maximal ligand binding and affinity, in addition to an increased EC_{50} (22), observations which are consistent with the findings reported here. This is the first report of a naturally occurring mutation which results in the substitution of this highly conserved asparagine in TMD1 and demonstrates the importance of this residue to structure and function of an endogenously expressed GPCR.

Cell line studies show that the N42S mutant receptor was retained intracellularly, likely in a Trans Golgi Network (TGN)/ER compartment (data not shown). Furthermore, the thromboxane receptor sequence also contains a potential arginine based-ER retention motif (RxR) within intracellular loop 3 (ICL3) of the receptor. These motifs have been shown to regulate anterograde traffic of proteins including GPCRs (23, 24). The notable intracellular localisation of this N42S variant and the ability of this site to interact with other residues through hydrogen bonding mean that this substitution has the potential to impact conformational rearrangement of the receptor. Such rearrangement may give rise to exposure to motifs such as the RxR motif, which may otherwise be masked in native WT form. This may result in impaired ER-to-plasma membrane export, although this remains to be explored in further detail.

We recently described the characterisation of another TP receptor variant, W29C, which also resulted in reduced surface expression in platelets taken from a heterozygous patient and in cell lines expressing the mutant. Like the asparagine 42 residue, tryptophan 29 is also located in TMD1. Recent mutagenesis studies have also demonstrated the importance of TMD1 in the formation of TP receptor dimer formation at the cell surface (25). Interestingly, studies with the $5HT_{2C}$ receptor also demonstrated that residues within TMD1 (N55 and W56)

played an important role in dimer formation (26). Therefore the reduction of N42S cell surface expression maybe, in part, due to disruption of interactions required for homodimer assembly at the cell surface.

Here we show that in addition to reduced surface expression, N42S also causes intracellular retention of WT receptor. Efficient traffic of any protein through intracellular compartments such as the ER/Golgi apparatus can be directed through interaction with ER chaperone proteins such as calnexin (27). Calnexin targets N-linked glycoproteins and can direct the ER export of proteins or target misfolded proteins for intracellular retention through a glycan-independent mechanism. Whilst the TP mutant described here results in a loss of an asparagine residue, the transmembrane positioning of this mutation makes it unlikely to be a residue for N-glycan addition due to its inaccessibillty within the ER lumen. The chaperone ANKRD13C has been implicated in biogenesis and ER exit of GPCRs, including TP-α (28). A possible explanation for the retention of the WT receptor may be the inability of WT-N42S heterodimers to interact efficiently with molecular chaperones in the ER. Taken together with previous studies, the description of the N42S variant provides further evidence for the role of TMD1 in regulating TP receptor surface expression.

Acknowledgements

We would like to thank Dr Sophie Minaee for assistance with TP receptor mutagenesis, Dr Timothy D. Warner for providing *Optimul* plates and Dr Nicholas Kirkby for TxB2 analysis.

Conflict of interests

None declared.

What is known about this topic?

Rare thromboxane (TP) receptor mutations associated with platelet dysfunction have previously been described in four index cases with bleeding symptoms.

Mutagenesis studies together with the recently reported W29C variant suggest that transmembrane domain 1 (TMD1) of the TP receptor is important for cell surface expression.

Asparagine 42 in TMD1 is a highly conserved residue across class A GPCRs and crystallography studies suggest it interacts with a number of other residues.

What does this paper add?

This is the first report of a naturally occurring receptor variant that results in substitution of this highly conserved asparagine and confirms its importance for function of an endogenously expressed GPCR.

This study confirms the importance of the TMD1 for TP surface expression.

The intracellular retention of WT receptors with the N42S variant may explain larger than expected platelet dysfunction in a heterozygous patient with a significant bleeding history.

References

Woodward DF, Jones RL, Narumiya S. International Union of Basic and Clinical Pharmacology. LXXXIII: classification of prostanoid receptors, updating 15 years of progress. Pharmacol Rev 2011; 63: 471-538.

Ting HJ, Murad JP, Espinosa EV, et al. Thromboxane A2 receptor: biology and function of a peculiar receptor that remains resistant for therapeutic targeting. J Cardiovasc Pharmacol Ther 2012; 17: 248-259.

Bousser MG, Amarenco P, Chamorro A, et al. Terutroban versus aspirin in patients with cerebral ischaemic events (PERFORM): a randomised, double-blind, parallel-group trial. Lancet 2011; 377: 2013-2022.

Kamae T, Kiyomizu K, Nakazawa T, et al. Bleeding tendency and impaired platelet function in a patient carrying a heterozygous mutation in the thromboxane A2 receptor. J Thromb Haemost 2011; 9: 1040-1048.

Hirata T, Kakizuka A, Ushikubi F, et al. Arg60 to Leu mutation of the human thromboxane A2 receptor in a dominantly inherited bleeding disorder. J Clin Invest 1994; 94: 1662-1667.

Mumford AD, Dawood BB, Daly ME, et al., A novel thromboxane A2 receptor D304N variant that abrogates ligand binding in a patient with a bleeding diathesis. Blood 2010; 115: 363-369.

Mumford AD, Nisar S, Darnige L, et al. Platelet dysfunction associated with the novel Trp29Cys thromboxane A(2) receptor variant. J Thromb Haemost 2013; 11: 547-554.

Watson S, Daly M, Dawood B, et al. Phenotypic approaches to gene mapping in platelet function disorders - identification of new variant of P2Y12, TxA2 and GPVI receptors. Hamostaseologie 2010; 30: 29-38.

Watson SP, Lowe GC, Lordkipanidze M, et al. Genotyping and phenotyping of platelet function disorders. J Thromb Haemost 2013; 11 (Suppl 1): 351-363.

Smith SO. Structure and activation of the visual pigment rhodopsin. Annu Rev Biophys 2010; 39: 309-328.

Venkatakrishnan AJ, Deupi X, Lebon G, et al. Molecular signatures of G-protein-coupled receptors. Nature 2013; 494: 185-194.

Mundell SJ, Jones ML, Hardy AR, et al. Distinct roles for protein kinase C isoforms in regulating platelet purinergic receptor function. Mol Pharmacol 2006; 70: 1132-1142.

Mundell SJ, Luo J, Benovic JL, et al. Distinct clathrin-coated pits sort different G protein-coupled receptor cargo. Traffic 2006; 7: 1420-1431.

Pula G, Mundell SJ, Roberts PJ, et al. Agonist-independent internalisation of metabotropic glutamate receptor 1a is arrestin- and clathrin-dependent and is suppressed by receptor inverse agonists. J Neurochem 2004; 89: 1009-1020.

Nisar SP, Cunningham M, Saxena K, et al. Arrestin scaffolds NHERF1 to the P2Y12 receptor to regulate receptor internalisation. J Biol Chem 2012; 287: 24505-24515.

Lowe GC, Lordkipanidze M, Watson SP, et al. Utility of the ISTH bleeding assessment tool in predicting platelet defects in participants with suspected inherited platelet function disorders. J Thromb Haemost 2013; 11: 1663-1668.

Dawood BB, Lowe GC, Lordkipanidze M, et al. Evaluation of participants with suspected heritable platelet function disorders including recommendation and validation of a streamlined agonist panel. Blood 2012; 120: 5041-5049.

Adzhubei IA, Schmidt S, Peshkin L, et al. A method and server for predicting damaging missense mutations. Nat Methods 2010; 7: 248-249.

Choi Y, Sims GE, Murphy S, et al. Predicting the functional effect of amino acid substitutions and indels. PLoS One 2012; 7: e46688.

Mumford A, Nisar S, Darnige L, et al. Platelet dysfunction associated with the novel Trp29Cys thromboxane A(2) receptor variant. J Thromb Haemost 2013; 11: 547-554.

FitzGerald GA. Mechanisms of platelet activation: thromboxane A2 as an amplifying signal for other agonists. Am J Cardiol 1991; 68: 11B-15B.

Appendix 4 – Selected publications

Perlman JH, Colson AO, Wang W, et al. Interactions between conserved residues in transmembrane helices 1, 2, and 7 of the thyrotropin-releasing hormone receptor. J Biol Chem 1997; 272: 11937-11942.

Michelsen K, Yuan H, Schwappach B. Hide and run. Arginine-based endoplasmic-reticulum-sorting motifs in the assembly of heteromultimeric membrane proteins. EMBO Rep 2005; 6: 717-722.

Cunningham MR, McIntosh KA, Pediani JD, et al. Novel role for proteinase-activated receptor 2 (PAR2) in membrane trafficking of proteinase-activated receptor 4 (PAR4). J Biol Chem 2012; 287: 16656-16669.

Fanelli F, Mauri M, Capra V, et al. Light on the structure of thromboxane A(2)receptor heterodimers. Cell Mol Life Sci 2011; 68: 3109-3120.

Mancia F, Assur Z, Herman AG, et al. Ligand sensitivity in dimeric associations of the serotonin 5HT2c receptor. EMBO Rep 2008; 9: 363-369.

Vassilakos A, Michalak M, Lehrman MA, et al. Oligosaccharide binding characteristics of the molecular chaperones calnexin and calreticulin. Biochemistry 1998; 37: 3480-3490.

Parent A, Roy SJ, Iorio-Morin C, et al. ANKRD13C acts as a molecular chaperone for G protein-coupled receptors. J Biol Chem 2010; 285: 40838-40851.

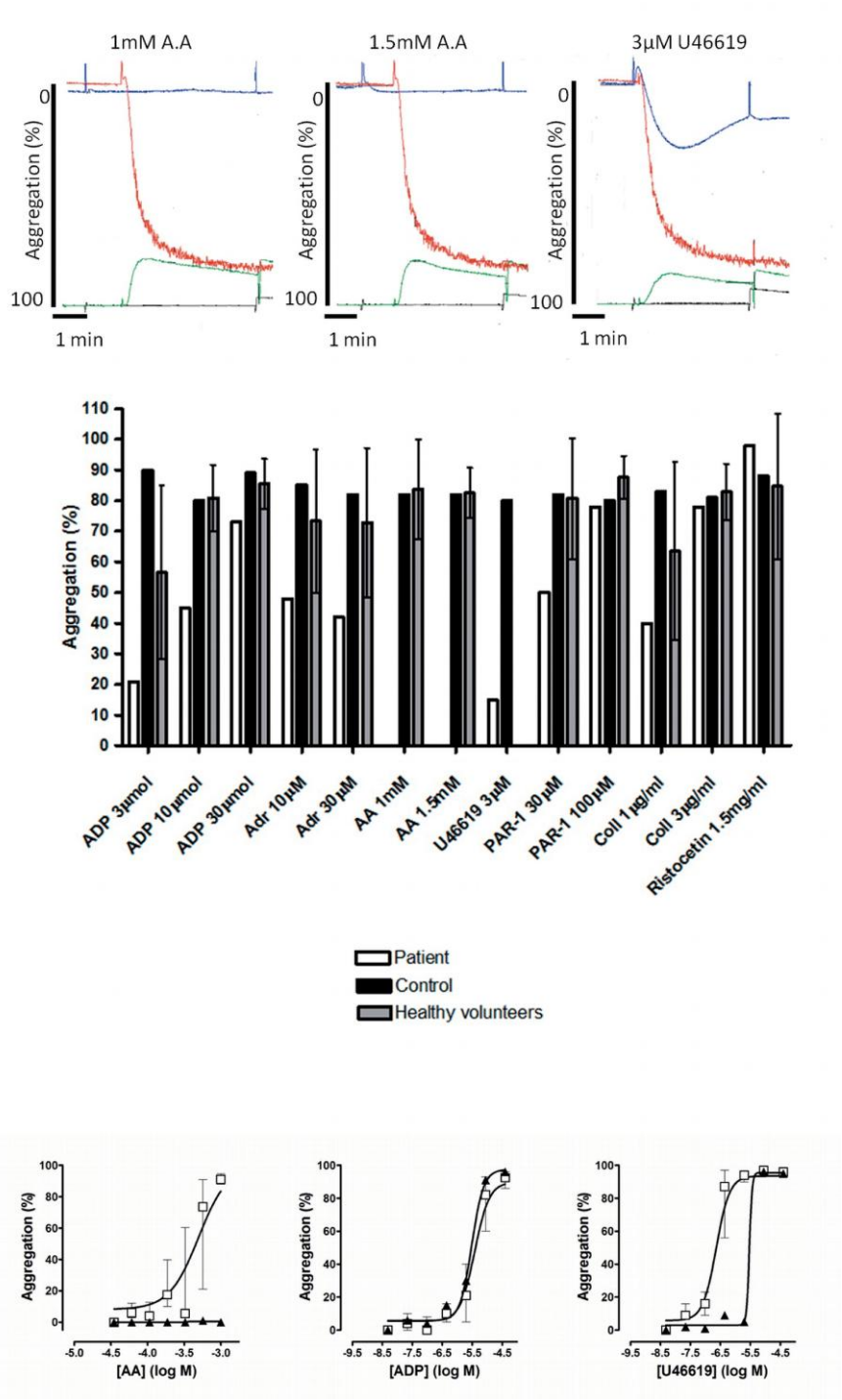


Figure 1: Agonist-induced platelet activation in the patient. Light transmission aggregation and secretion of platelets in PRP from a healthy control or patient in response to A) A.A (1 mM), B) A.A (1.5 mM) or C) U46619 (3 µM). Key: Control aggregation (black), patient aggregation (blue), control secretion (green), patient secretion (black). (D) Quantification of all aggregation responses for patient, control and healthy volunteers. X axis shows agonist used (ADP = adenosine diphosphate, Adr = adrenaline, A.A.= arachidonic acid, U46619 is a direct TP receptor agonist, PAR-1=PAR-1 peptide (thrombin receptor agonist), Coll=collagen. Y axis shows aggregation. For all agonists other than adrenaline, this was measured at 5 min after agonist addition. Adrenaline-dependent aggregation was measured at 10 min post agonist addition. Ristocetin responses represent agglutination rather than aggregation. Data from healthy volunteers collected from responses of 68 individuals. E-G) 96-well *Optimul a*ggregometry. Aggregation dose-response curves to E) A.A, F) ADP and G) U46619 presented as median and interquartile range. Open squares represent healthy volunteers (n=50), black triangles represent the patient.

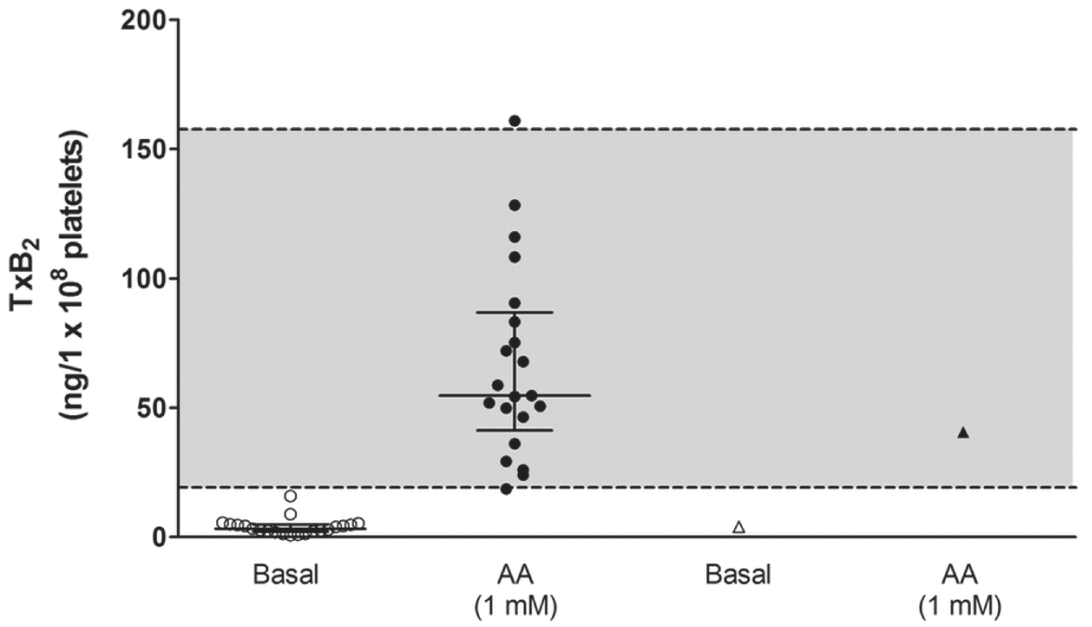


Figure 2: Thromboxane generation in the patient. TxB_2 concentration in plasma supernatants from healthy volunteers and the participant with a thromboxane receptor mutation. The circles represent healthy volunteers, the triangles represent the participant with a thromboxane receptor mutation. Line and whiskers represent median and interquartile range; the grey band represents the normal range derived from a bank of healthy volunteers.

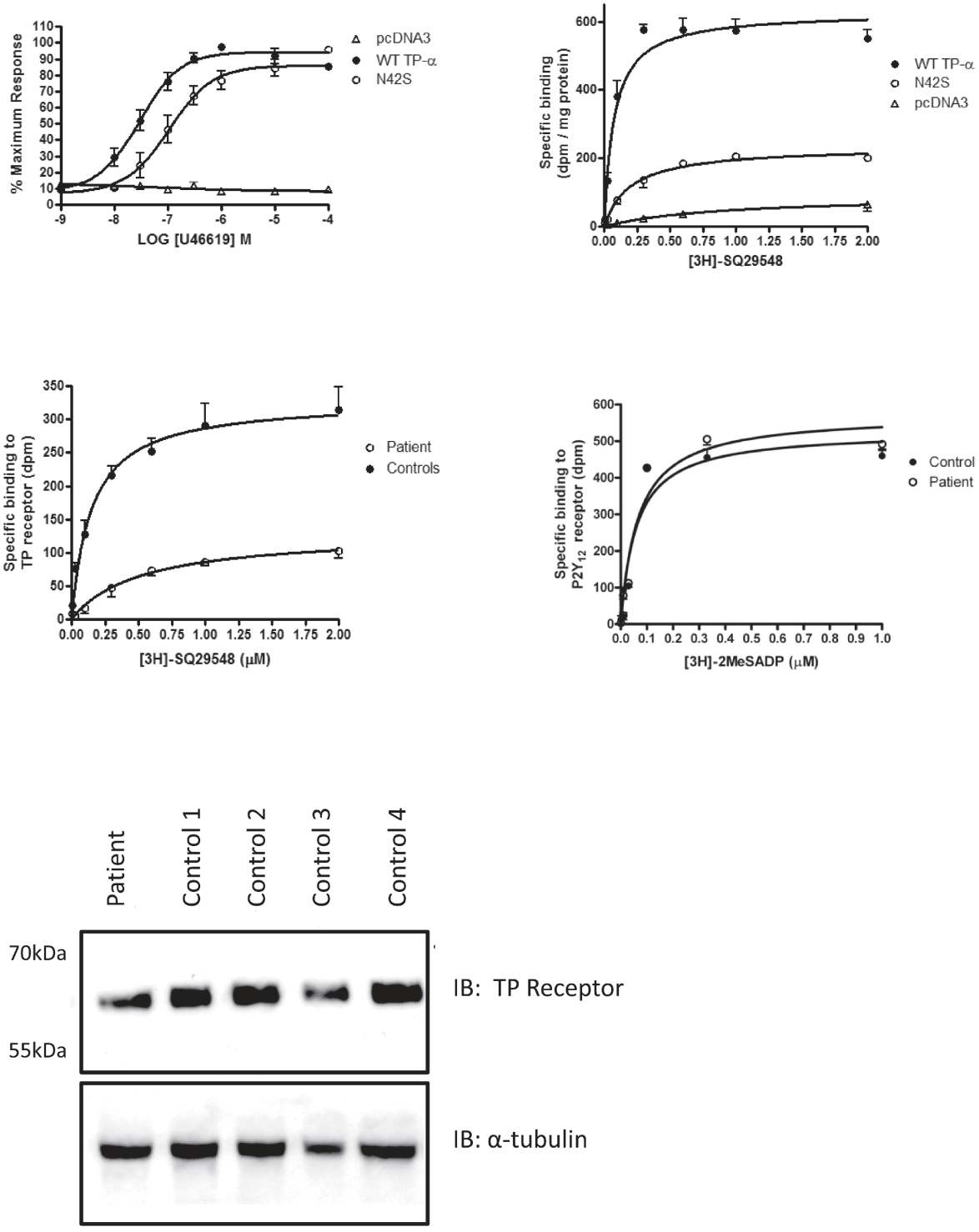


Figure 3: N42S mutant displays reduced ligand binding and signalling. A-B) HEK293 cells were transiently transfected with FLAG-tagged WT TP-α (black circles) or N42S (open circles). A) Changes in cytoplasmic calcium concentration $[Ca^{2+}]i$ were measured in fura-2/AM loaded cells before addition of U46619. (Data are mean ± SEM, n=3). B) TP receptor levels were measured with the use of TP receptor antagonist [3H]-SQ29548 (3 Ci/mmol, 0.01–2 ìM) in the presence of unlabelled ligand (10 ìM) to determine specific binding. Data are expressed as [3H]-SQ29548 binding (dpm) and represent means ± SEM of three independent experiments. C-D) Surface TP receptor (C) and P2Y12 purinergic receptor expression (D) was studied by measuring binding of the specific receptor ligands [3H]SQ29548 and [3H]MeSADP, respectively, in washed fixed platelets in the absence and the presence of unlabelled ligand to assess receptor-specific radioligand binding. Patient (open circles); healthy control (black circles). E) Total receptor expression was assessed in platelet lysates by SDS-PAGE and immunoblotting with a TP-receptor antibody (Cayman).

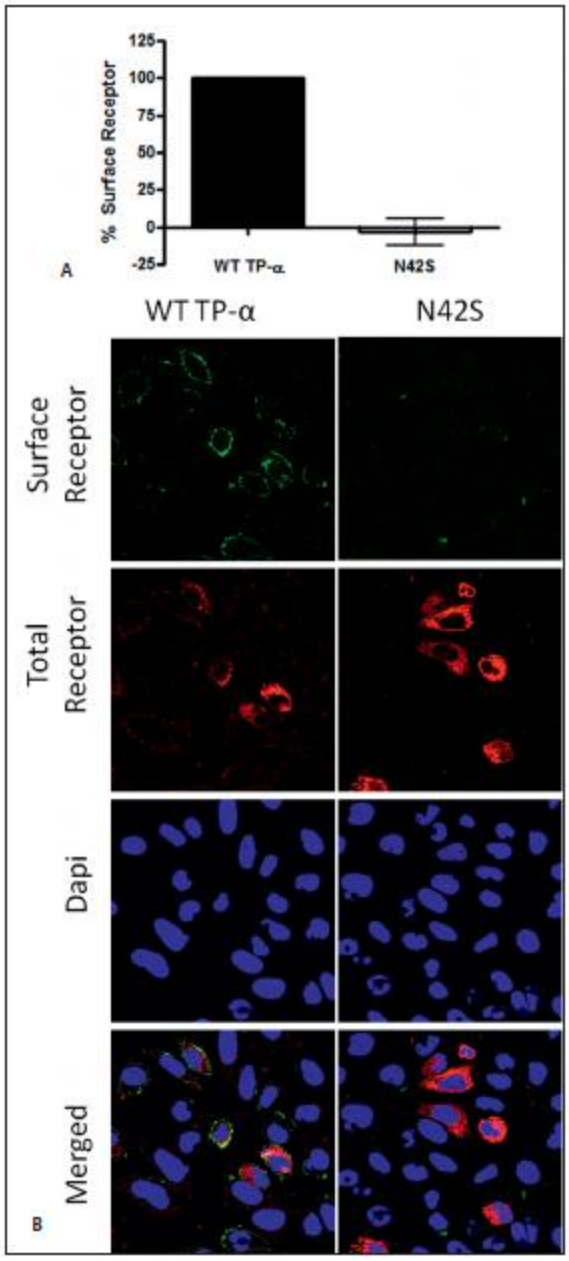


Figure 4: Reduction in expression of N42S variant at the cell surface. Surface receptor levels were assessed in HEK293 cells were transiently transfected with FLAG-tagged WT TP-α or N42S. A) FLAG tagged surface receptor was measured in fixed cells using an ELISA-based assay. Data are expressed as percentage of WT surface expression and represent mean ± SEM (n=3). B) WT and N42S expressing cells were stained for surface (green) and total receptor (red) using an anti FLAG antibody under non-permeabilising and permeabilising conditions, respectively. Cells were imaged by confocal microscopy and are representative of three independent experiments.

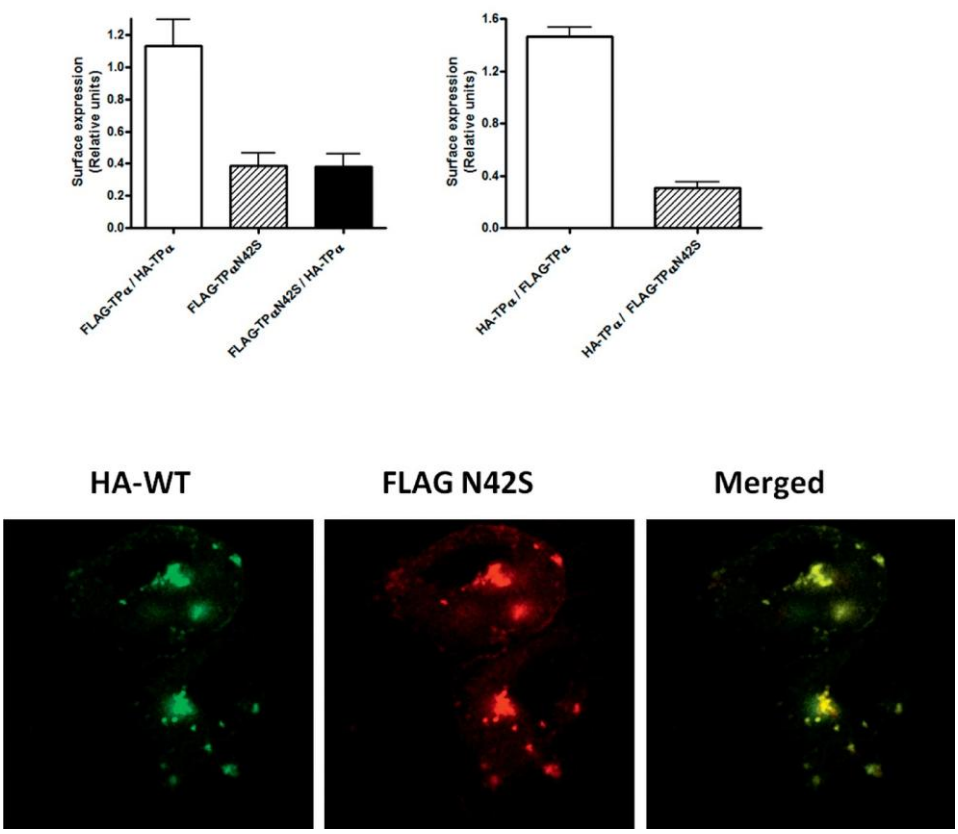


Figure 5: Co-expression of N42S with the WT receptor causes intracellular retention of WT TP receptor. HEK cells were transiently co-transfected with HA-WT TP-α and FLAG-N42S. Surface expression of N42S (A) and WT (B) was assessed using anti-FLAG and anti-HA antibodies, respectively, by cell surface ELISA (mean ± SEM) (n=3). C) WT (green) and TP-α (red) were visualised in the same cells by staining with anti-HA and anti-FLAG, respectively. Colocalisation in the merged images is shown in yellow. Cells were imaged by confocal microscopy and are representative of three independent experiments.