Identification and functional investigation of genes involved in inherited bleeding disorders

by

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Abstract

Inherited bleeding disorders are a heterogeneous group of conditions that reflect abnormalities of the vascular endothelium, coagulation factor proteins and platelets. Clinical diagnosis is often challenging despite patients having extensive bleeding histories and 60% of patients recruited to the Genotyping and Phenotyping of Platelets (GAPP) study remain undiagnosed. A novel assay was trialled in 71 affected patients to investigate platelet spreading ability. This identified patients with abnormal platelet area and circularity who were negative for defects in aggregation and revealed that this assay could identify platelet morphological defects that might be missed by lumi-aggregometry alone. In addition, whole exome sequencing was employed in 10 unrelated families in this undiagnosed patient cohort. The definitive genetic cause of disease was identified in 1 of these families, a missense variant in *THBD*, with several plausible candidate variants remaining for the other families. In 2 of these families, and a further family from the GAPP study cohort, novel and rare genetic variants in *TTLL10* were identified. The protein tubulin tyrosine ligase-like 10 (TTLL10) shows a potential role in the post translational modification of tubulin microtubules in platelets thereby affecting normal platelet physiological processes. In conclusion, genetic variants identified in genes not previously known to cause bleeding could lead to further understanding of haemostasis and thrombosis following subsequent functional studies.

Publications arising from this work

1. KHAN, A.O., SLATER, A., MACLACHLAN, A., NICOLSON, P.L.R., PIKE, J.A., YULE, J., THOMAS, S.G., & MORGAN, N.V. 2019. Post-translational polymodification of β1 tubulin regulates motor protein localisation in platelet production and function. BioRxiv 595868 [Preprint].

2. KHAN, A.O.*, MACLACHLAN, A.*, LOWE, G.C., NICOLSON, P.L.R., AL GHAITHI, R., THOMAS, S.G., WATSON, S.P., PIKE, J.A. & MORGAN, N.V. 2019. High-throughput platelet spreading analysis: a tool for the diagnosis of platelet- based bleeding disorders. *Haematologica.* DOI: 10.3324/haematol.2019.225912. *A.O.K. & A.M. contributed equally to the work.

3. LOWE, G.C., FICKOWSKA R., AL GHAITHI, R., MACLACHLAN, A., HARRISON, P., LESTER, W., WATSON, S.P., MYERS, B., CLARK, J. & MORGAN, N.V. 2019. Investigation of the contribution of an underlying platelet defect in women with unexplained heavy menstrual bleeding. *Platelets*, 30:1, 56-65.

4. AL GHAITHI, R., MORI, J., NAGY, Z., MACLACHLAN, A., HARDY, L., PHILIPPOU, H., HETHERSHAW, E., MORGAN, N.V., SENIS, Y.A. & HARRISON, P. 2019. Evaluation of the Total Thrombus-Formation System (T-TAS): application to human and mouse blood analysis. *Platelets***,** 30:7, 893-900.

5. MACLACHLAN, A., DOLAN, G., GRIMLEY, C., WATSON, S. P., MORGAN, N.V. & ON BEHALF OF THE UK GAPP STUDY, G. 2017. Whole exome sequencing identifies a mutation in thrombomodulin as the genetic cause of a suspected platelet disorder in a family with normal platelet function. *Platelets*, 28:6, 611-613.

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List of abbreviations

ACD	Acid-citrate-dextrose
ACMG	American College of Medical Genetics and Genomics
ADP	Adenosine diphosphate
AMP	Association for Molecular Pathology
APPT	Activated partial thromboplastic time
ATP	Adenosine triphosphate
BAT	Bleeding Assessment Tool
BDP	Bleeding and platelet disorder
BSS	Bernard-Soulier syndrome
cAMP	Cyclin adenosine monophosphate
CAMT	Congenital amegakaryocytic thrombocytopenia
CLEC-2	C-type lectin receptor 2
CRISPR	Clustered regularly interspaced short palindromic repeats
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EVS	Exon Variant Server
ExAC	Exome Aggregation Consortium
Fg	Fibrinogen
GAPP	Genotyping and Phenotyping in platelets
GP	Glycoprotein
GPCR	G-protein coupled receptor
GPS	Grey platelet syndrome
GT	Glanzmann thrombasthenia
GWAS	Genome wide association studies
HGP	Human Genome Project
HLA	Human leukocyte antigen
HPA	Human Protein Atlas
HPO	Human phenotype ontology
HPS	Hermansky-Pudlak syndrome
HSC	Haematopoietic stem cell
IL	Interleukin
IMS	Invaginated membrane system
IPF	Immature platelet fraction
iPSC	Inducible pluripotent stem cell
ISTH	International Society of Thrombosis and Hemostasis
ITP	Idiopathic thrombocytopenic purpura
JAK2	Janus kinase 2
LTA	Light transmission aggregometry
MAF	Minor allele frequency
MAPK	Mitogen activated protein kinase
MEA	Multiple electrode aggregometry
MPV	Mean platelet volume
NF-E2	Nuclear factor erythroid 2
NGS	Next-generation sequencing

NO	Nitric oxide
NSAID	Non-steroidal anti-inflammatory drug
OMIM	Online Medelian Inheritance in Man
PCR	Polymerase chain reaction
PFD	Platelet function disorders
PGI_2	Prostacyclin
PI3K	Phosphoinositol-3 kinase
PLA_2	Phospholipase A 2
PLT	Platelet
PPP	Platelet poor plasma
PROVEAN	Protein Variant Effect Analyser
PRP	Platelet rich plasma
PS	Phosphatidylserine
PT	Prothrombin time
PTK	Protein tyrosine kinase
PTM	Post translational modification
RNA	Ribonucleic acid
Rpm	Revolutions per minute
RUNX1	Runt-related transcription factor 1
S1P	Sphingosine 1 phosphate
SIFT	Sorting Intolerant from Tolerant
SNP	Single nucleotide polymorphism
SNV	Single nucleotide variant
STAT5	Signal transducer and activator of transcription 5
TPM	Transcripts per million
TPO	Thrombopoietin
T-TAS	Total thrombus formation analysis system
TTL	Tubulin tyrosine ligase
TTLL	Tubulin tyrosine ligase-like
TXA_2	Thromboxane synthase
TXA_2R	Thromboxane synthase receptor
uPA	Urokinase plasminogen activator
vWD	von Willebrand disease
vWF	von Willebrand factor
WAS	Wiskott-Aldrich syndrome
WASP	Wiskott-Aldrich syndrome protein
WES	Whole exome sequencing
WGS	Whole genome sequencing
WT	Wild-type

CHAPTER 1
Introduction

1. Introduction

Platelets are small anucleate cells that circulate in the blood to provide many important biological roles. Classically, their main role is to prevent excessive bleeding upon blood vessel injury. To do this, activated platelets employ multiple signalling pathways that lead to a cascade of reactions, release of chemical mediators and recruitment of other cell types, ultimately causing the cessation of a bleed. However, when one of these steps is interrupted, it can cause detrimental bleeding in affected individuals. Platelets have, more recently, been associated with other roles such as wound healing, inflammation and cancer metastasis. Due to their wide range of functions, it is important to understand the processes that modulate platelet activity in order to investigate these when they go wrong. Furthermore, dysfunctional platelets are not the only cause of detrimental bleeding as there are many other factors to take into account. Due to the complex nature of these disorders, it is often best to investigate these other causes of bleeding using a genetic approach.

In this chapter, platelet production and physiology are introduced, as well as disorders of platelets and other non-platelet causes of bleeding. Platelet function testing will be described and the rise of genetics in general research and medicine is discussed, including the use of next-generation sequencing technologies in this field. Finally, the hypothesis and aims of this thesis will be discussed.

1.1. Haematopoiesis and megakaryopoiesis

Platelets are produced from haematopoietic stem cells (HSCs) that have differentiated through multiple precursor cell types to from megakaryocytes. These stem cells self-renew or commit to a specific cell lineage and therefore they lose their developmental potential and ultimately become a specific cell type through a number of differentiation steps

(Akashi et al., 2000). This is a complex cell differentiation process involving a great amount of regulation by cytokines and transcription factors (Schulze and Shivdasani, 2005). The whole process of haematopoiesis is summarised in Figure 1.1.

HSCs typically reside in the unique bone marrow microenvironments or niches. The osteoblastic and vascular niche allow for HSC self-renewal and differentiation (Shiozawa and Taichman, 2012). However, these cells can migrate and there have been observations of platelet production in the lungs and in early development, where they have been found in the yolk sac, foetal liver and spleen (Lefrancais et al., 2017, Machlus et al., 2014).

The first stage for the classic lineage HSC differentiation into megakaryocytes is the development of multipotent progenitors; these can commit to the myeloid or lymphoid lineage creating common myeloid progenitors or common lymphoid progenitors respectively. These progenitor cell types lack the ability to self-renew, however, remain multipotent. Common lymphoid progenitors predominantly produce T- and B-lymphocytes along with natural killer cells. Common myeloid progenitors can progress to generate erythrocytes and megakaryocytes. The common myeloid progenitors can also progress to generate monocyte derived macrophages and dendritic cell types along with granulocytes including eosinophils, basophils, and neutrophils. However, increasing evidence is challenging this idea of the hierarchical lineage progression. Recent alternative models have suggested that the progenitor cells are likely to form more fluid lineage biases in a 'continuum of low-primed undifferentiated haematopoietic stem and progenitor cells' (CLOUD-HSCs) (Velten et al., 2017, Laurenti and Gottgens, 2018).

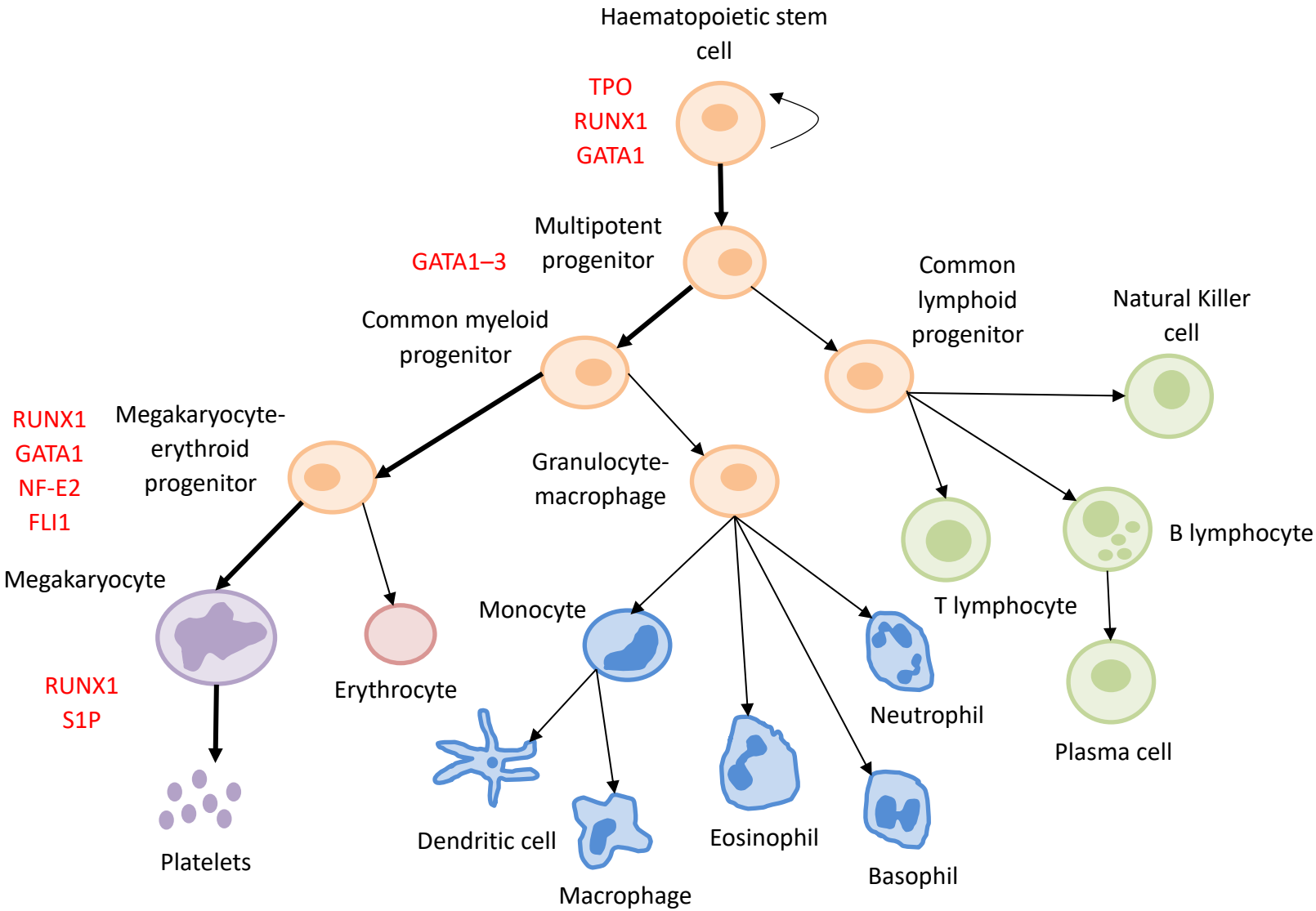


Figure 1.1. The main steps of haematopoiesis. Haematopoiesis is the formation of cellular components of the blood. Haematopoietic stem cells give rise to all the cell types that are then guided to commit to specific cell lineages by chemical messengers. The main steps of megakaryopoiesis are shown by the bold arrows. Transcription factors are shown in red.

1.1.1. The role of cytokines and transcription factors in megakaryopoiesis

Megakaryopoiesis and platelet formation is regulated at different levels by multiple cytokines. Interleukin 3 stimulates the early stages of megakaryocyte development by differentiation of multipotent HSCs to myeloid progenitor cells (Segal et al., 1988). However, the main driver of megakaryopoiesis is thrombopoietin (TPO) which is considered the most important haematopoietic cytokine involved in platelet production (Tijssen and Ghevaert, 2013, Kaushansky, 1995).

TPO, synthesised in the liver, acts on c-Mpl receptors primarily found on the surface of HSCs, megakaryocytes and platelets. TPO binds to the inactive c-Mpl receptor and creates an active receptor as it undergoes a conformational change, which allows the cytoplasmic tails of the receptor to become closer in proximity (Geddis, 2010, Kuter, 2007). c-Mpl does not have intrinsic kinase activity itself therefore, the conformational change initiates many downstream signalling events. Janus kinase 2 (JAK2) molecules associated with the receptor come together and are able to be activated by trans-autophosphorylation at multiple tyrosine residues (Varghese et al., 2017). Following the activation of JAK2, multiple signalling pathways are triggered; these include signal transducer and activator of transcription 5 (STAT5), mitogen-activated protein kinase (MAPK)/ERK and phosphoinositol-3 kinase (PI3K)/AKT pathways (Geddis, 2010, Varghese et al., 2017).

Transcription factors also play a critical role in haematopoietic cell lineage specific progression. They can bind to specific sequences of DNA neighbouring the genes they regulate to either promote or block the recruitment of RNA polymerase, thereby controlling the transcription of that exact region of DNA. Transcription factors can exert their effects alone or with other proteins in a complex. The variance in expression levels of the transcription

factors in progenitor cells forms a linear hierarchy of transcriptional regulation, therefore allowing them to selectively differentiate into their respective cell types (Tijssen et al., 2011, Shivdasani, 2001). The following transcription factors are all essential regulators of the different stages of megakaryocyte differentiation. Runt-related transcription factor 1 (RUNX1) is a key regulator of haematopoiesis and megakaryopoiesis. RUNX1 acts to maintain, proliferate and differentiate HSCs (Schlegelberger and Heller, 2017). The zinc finger protein, GATA-binding factor 1 (GATA-1) is an important regulator of early and late megakaryopoiesis (Centurione et al., 2004). There are other members of the GATA subfamily that are also transcription factors involved in haematopoiesis, GATA-2 and GATA-3 (Ferreira et al., 2005). Friend of GATA-1 (FOG1) is an essential cofactor for the GATA family members that acts via the formation of a heterodimer with the transcription factors; this is essential to activate expression of nuclear factor erythroid 2 (NF-E2) (Mancini et al., 2012). NF-E2 is a basic leucine zipper transcription factor and again is a major regulator of megakaryocyte maturation. GATA-1 and NF-E2 are increasingly expressed throughout the myelo-ethyroid lineage and most highly expressed in the megakaryocyte-erythroid progenitor cell population (Akashi et al., 2000). NF-E2 is a critical regulator for lineage specific β1 tubulin expression essential for platelet production (Lecine et al., 2000). Furthermore, the ETS gene friend of leukaemia virus integration 1 (FLI1) is critical for late megakaryopoiesis (Vo et al., 2017). Therefore, mutations in haematopoietic transcription factors can cause disruption of transcriptional control. This dysregulation can lead to thrombocytopenia due to megakaryocyte maturation failure and impaired platelet function (Johnson et al., 2016a).

1.1.2. Endomitosis and polyploidisation

In order to produce platelets, megakaryocytes undergo endomitosis whereby they become progressively larger and polyploid following multiple DNA replication cycles without cell division. Megakaryocytes follow the normal cycle of cell division passing through G1, S and G2 phases successfully followed by a restricted M phase; the cycle then repeats resulting in multiple duplicates of DNA (Zimmet and Ravid, 2000). It was revealed that within this altered form of mitosis, megakaryocytes have a cytokinesis defect which gives rise to impaired cleavage furrow formation, as well as impaired contractile ring formation (Bluteau et al., 2009, Lordier et al., 2008). This process is mediated by RUNX1 acting to downregulate non-muscle myosin IIB heavy chain (MYH10) in the contractile ring, of which filamentous actin is also part, and together they produce the contractile forces necessary for cell separation (Lordier et al., 2012). As a result of this, megakaryocytes contain multiples of normal diploid chromosome numbers with ploidy values in the range of 4N to 64N within a single multi-lobulated polyploid nucleus (Zimmet and Ravid, 2000).

The main reason for endomitosis is to generate large quantities of protein and lipids important for the formation of the invaginated membrane system (IMS) which was previously known as the demarcation membrane system (Machlus and Italiano, 2013). Therefore, following nuclear polypolidisation megakaryocytes form an IMS. A pre-IMS is initially formed which then expands (Eckly et al., 2014). The IMS is characterised as an extensive network of cisternae and tubules within the cytoplasm of the megakaryocytes continuous with the plasma membrane (Radley and Haller, 1982). The IMS is thought to be the membrane reservoir essential for the rapid increase of surface area within megakaryocytes and the formation of proplatelets (Schulze et al., 2006).

1.1.3. Platelet production

After several different models of platelet formation were hypothesised, it was shown by Italiano *et al*. that proplatelets are made at the end of extended megakaryocyte microtubules in the form of tip swellings (Italiano et al., 1999). Therefore, once megakaryocytes have fully matured, they begin to branch and form long cytoplasmic extensions termed proplatelets, which are subsequently shed into sinusoidal blood vessels of the bone marrow as shown in Figure 1.2.

Cytoskeletal rearrangements and microtubule reorganisation are of particular importance for these processes. β1 tubulin microtubules support the growing tip swellings with microtubule coils, however is it the motor protein, dynein-facilitated sliding, that is critical in driving proplatelet elongation (Machlus and Italiano, 2013). Spectrin and actin are also present in proplatelet extensions, allowing branching and bending respectively, as when these are inhibited the functions are lost (Italiano et al., 1999). Megakaryocytes capitalise on this extensive cytoskeletal network as the network also acts as a transport system enabling granules (alpha and dense granules) and organelles to be delivered to the proplatelet tip swellings. The granules are moved from the cell main body to the proplatelets in a bidirectional movement until they reach the distal proplatelet (Richardson et al., 2005). Transport along microtubules by motor kinesins as well as myosin-facilitated transport along actin filaments could both play a role in organelle movement along with other potential methods, for example, transport vesicles. (Richardson et al., 2005).

Once proplatelets have formed they are guided towards the bone marrow sinusoids by an increasing concentration gradient of sphingosine 1 phosphate (S1P) found in the blood (Zhang et al., 2012). The presence of S1P leads to downstream signalling in the megakaryocyte

by the activation of S1P receptors causing proplatelet release into the blood flow (Zhang et al., 2012). Shear stress from the flowing blood also aids in the fragmentation of the proplatelet from the tip swellings (Junt et al., 2007). The fragments that are released are larger than mature platelets therefore suggesting another step in the platelet production journey; these fragments are termed preplatelets. Preplatelets are not uniform in size, in fact, they are discoid and range from 2-10 µm which then go on to form a characteristic barbell-shape with platelet size swellings on either end (Thon et al., 2012a). This final step is likely to be instigated by the biomechanical properties of the microtubules and the later separation of the platelets caused by polar action within microtubule coils (Thon et al., 2012a). This cytoskeletal regulation of platelet production ensures that the platelets produced are a constant size although this process does have a genetic component therefore platelet size does vary within the population (Gieger et al., 2011).

While there is evidence for repression of apoptosis being required for successful proplatelet formation, once megakaryocytes have released their proplatelets, the resulting senescent cell is destroyed by apoptosis and phagocytosis (McArthur et al., 2018, Gordge, 2005).

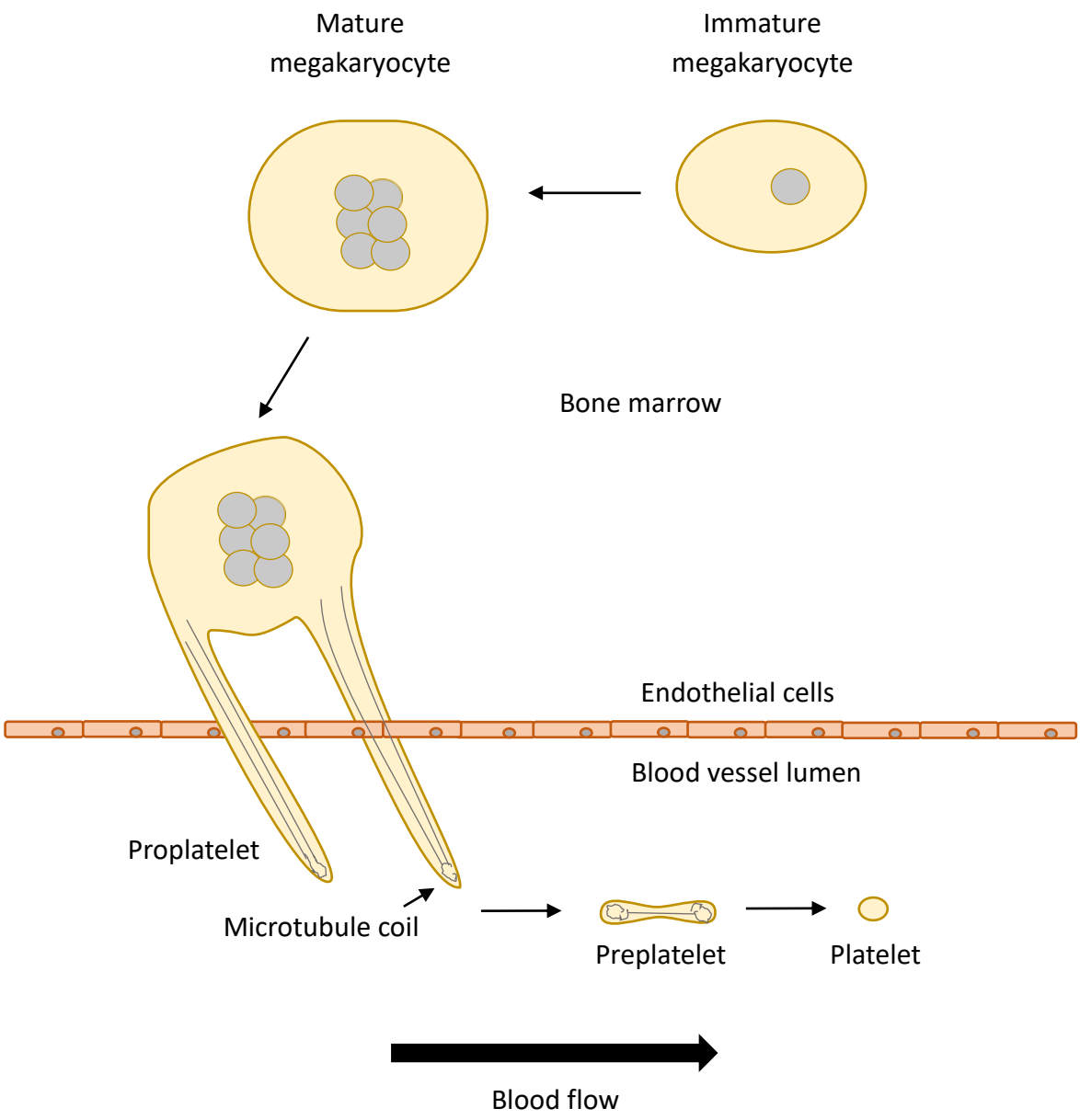


Figure 1.2. Megakaryocyte maturation and platelet release. Platelets are produced in the bone marrow from mature megakaryocytes. Megakaryocytes mature by becoming progressively larger by endomitosis and polyploidisation. The megakaryocytes then produce tip swellings, lined with microtubules, which extend through the cells lining the blood vessel. Preplatelets are released into the circulation due to the shear stress within the blood flow. The preplatelets then split further to give rise to mature platelets.

1.2. Platelet physiology

Platelets were first described by Schultze in 1865 however the association between platelets and their role as effectors of haemostasis and thrombosis was observed *in vivo* by Bizzozero in 1882 (Schultze, 1865, Bizzozero, 1882). The principal role of platelets is to prevent excessive blood loss by their activation, aggregation and the formation of a thrombus. Platelets are a highly specialised cell type and their structure allows them to carry out their functions successfully.

1.2.1. Platelet structure

Platelets are the smallest component of the blood measuring only 2-3 μm in diameter and 0.5 μm in depth. Platelets do not have a nucleus but within their unique cytoplasm they do contain other organelles including but not limited to mitochondria, Golgi apparatus, endoplasmic reticulum and lysosomes. Platelets have an extensive and specialised cytoskeleton, of which actin is the most abundant protein along with microtubules (Hartwig and DeSisto, 1991). The actin is cross-linked by other proteins such as filamin to form a strong framework to support the platelet (Hartwig and DeSisto, 1991). Filamin can initiate interactions between glycoprotein (GP) Ibα involved in initial platelet adhesion and αIIbβ3 involved in firm adhesion, therefore providing the mechanical force to stop and adhere the platelets from the blood flow (Clemetson, 2012). The actin and microtubule networks undergo rapid reorganisation upon platelet activation to form morphologically different shapes, characteristic of activated platelets.

Due to the platelets' small size and shape they are most abundant towards the peripheral blood flow within a blood vessel so they are in close contact with the vessel wall allowing quick and easy recruitment if necessary (Tangelder et al., 1985). Under normal conditions, platelets

circulate in the blood in a quiescent state. They flow around unresponsive as they are inhibited by nitric oxide (NO) and endothelial derived prostacyclin (PGI_2). The effect of NO on platelets is concentration dependant. Low levels of NO act to maintain the normal flow of blood and platelets however when platelets are activated, higher concentrations of NO are released and this acts to aid in the platelet aggregation response. Similarly, the antithrombotic effect of PGI_2 can be overridden when platelets are activated and the physiological effects of thromboxane synthase (TXA_2) predominate.

1.2.2. Platelet granules

Platelets contain granules which are vital to the normal functioning of platelets as previously mentioned in this chapter. Alpha (α-) granules are the most abundant granule type with each platelet containing approximately 50-80 α- granules, each measuring from 200-500 nm in size (Blair and Flaumenhaft, 2009). These golgi derived granules have a highly organised internal structure that contains membrane bound proteins that upon activation become expressed on the surface of the platelet. These include the cell surface receptors P-selectin, GPIb-IV-V and αIIbβ3. α- granules also contain soluble adhesive proteins which are released from the granule including: platelet factor 4, fibrinogen, von Willebrand factor (vWF) and thrombospondin (Harrison and Cramer, 1993).

Dense granules are smaller in size at approximately 250 nm, are less abundant within the platelets and characterised by an electron dense core when imaging using electron microscopy (Blair and Flaumenhaft, 2009). These granules contain serotonin, adenosine diphosphate (ADP), adenosine triphosphate (ATP) and calcium. The contents of both granule types are secreted upon platelet activation however, due to the differing contents of the granules, the release of α- granules facilitates platelet adhesion whereas dense granules aid

in the activation and release from other platelets and help to amplify the platelet response (Koseoglu and Flaumenhaft, 2013).

Lysosomes are often known as the third platelet granule as they have been shown to release acid hydrolsases *in vitro*, but their physiological role remains understudied (Heijnen and van der Sluijs, 2015). T-granules have also been described and named referring to their tubular morphology (Thon et al., 2012b).

1.2.3. Platelet activation and adhesion

Platelets undergo a series of reactions through several different mechanisms, all acting in synergy to achieve adhesion, aggregation and hence effective haemostasis. Upon vessel injury, the endothelial cell lining is damaged and matrix proteins and the subendothelial membrane are exposed to the flow of blood. This allows platelets to interact with the proteins and begin to tether, even in regions of high shear. To achieve this, platelets express cell surface proteins, including protein tyrosine kinase (PTK) receptors, for the initial activation of the platelets, integrins to initiate platelet adhesion and G-protein coupled receptors (GPCRs) for the secondary wave of platelet activation from released soluble activation proteins (Figure 1.3).

vWF is now present in the blood flow around the site of injury as circulating multimers and binds to exposed collagen (types I and III) leading to vWF elongation and exposure of an A1 domain that can bind to GPIbα on the platelet surface (Clemetson, 2012). This is essential in small arteriole blood vessels where shear rates can reach >5000 s^{-1}. Once the platelets have slowed to a roll, this allows platelet surface integrins to interact with other components of the subendothelial membrane to provide further activation signalling via PTKs. These interactions are the first critical steps for platelet adhesion by increasing platelet surface receptor

interaction with the subendothelial membrane and therefore initiating firmer adhesion reactions. The immunoglobulin-like surface receptor GPVI binds to collagen, inducing tyrosine phosphorylation dependent intracellular signalling, activating the FcRγ chain (Nieswandt et al., 2009). This leads to strong and sustained activation of the platelet and activates inside-out signalling of platelet integrins; the integrins are in a low-affinity state until the platelet is activated when they shift to a high-affinity binding state (Nieswandt et al., 2009). αIIbβ3 is the most abundant integrin on the platelet surface and mediates aggregation by binding to vWF, fibronectin and fibrinogen. α2β1 is the second most abundant integrin which binds to collagen (Watson, 2009, McGrath et al., 2010). Furthermore, integrins α5β1 and α6β1 bind to fibronectin and laminin respectively. C-type lectin receptor 2 (CLEC-2) is also important to consider in the process of platelet activation and thrombus formation. Its ligand podoplanin is a transmembrane glycoprotein widely expressed outside of the vascular system (Lorenz et al., 2015). When CLEC-2 binds to its ligand, this initiates platelet activation via the tyrosine phosphorylation cascade similarly to GPVI (Lorenz et al., 2015).

When the platelets have fully adhered, they undergo conformational changes such as cytoskeletal remodelling. The binding of αIIbβ3 to fibrinogen allows for filamin and α-actinin mediated extensions of filopodia and lamellipodia, in order for the platelets to spread along the endothelium while increasing their surface area for further platelet-platelet interactions (Durrant et al., 2017).

Secretory proteins within the platelet granules are also released following platelet activation and spreading. Dense granules secrete ADP which interacts with the P2Y12 receptor on the platelet surface to provide GPCR mediated platelet activation, it also interacts with PLA_2 which forms TXA_2 from arachidonic acid (AA), which then acts as a soluble agonist on the

thromboxane synthase receptor (TXA_2R) (Watson, 2009). A positive feedback mechanism is created which leads to increased production and release of said agonists in order to recruit more platelets to the site of injury and aid in creation of the vascular plug (Salles et al., 2008).

When platelets are activated, the exposure of platelet membrane phosphatidylserine (PS) promotes blood coagulation (Lentz, 2003). PS usually resides on the inner leaflet of the phospholipid bilayer. However, when activated, the enzyme scramblase catalyses the bidirectional movement of PS to flip to the outer leaflet providing a platform to facilitate the attachment of cofactors and clotting factors (Lentz, 2003, Bevers and Williamson, 2010). Platelets have the highest rate of scramblase activity accelerating the reactions of the coagulation cascade to generate thrombin and form fibrin (Bevers and Williamson, 2010, Schoenwaelder et al., 2009). The generation of thrombin again forms a positive feedback mechanism and further platelet activation by acting on protease activated receptors (PARs) namely PAR1 and PAR4 which signal via G-proteins (Coughlin, 2000). Finally, fibrinogen is converted to fibrin during the coagulation cascade to help form a fibrin mesh and thus further stabilises the thrombus. Once the thrombus has formed, it retracts in order to minimise obstruction of the normal blood flow in the vessel. This is due to contractile forces from non-muscle myosin II on the actin cytoskeleton and fibrinogen bound to αIIbβ3 to pull neighbouring platelets closer together and reduce the overall thrombus size (Durrant et al., 2017).

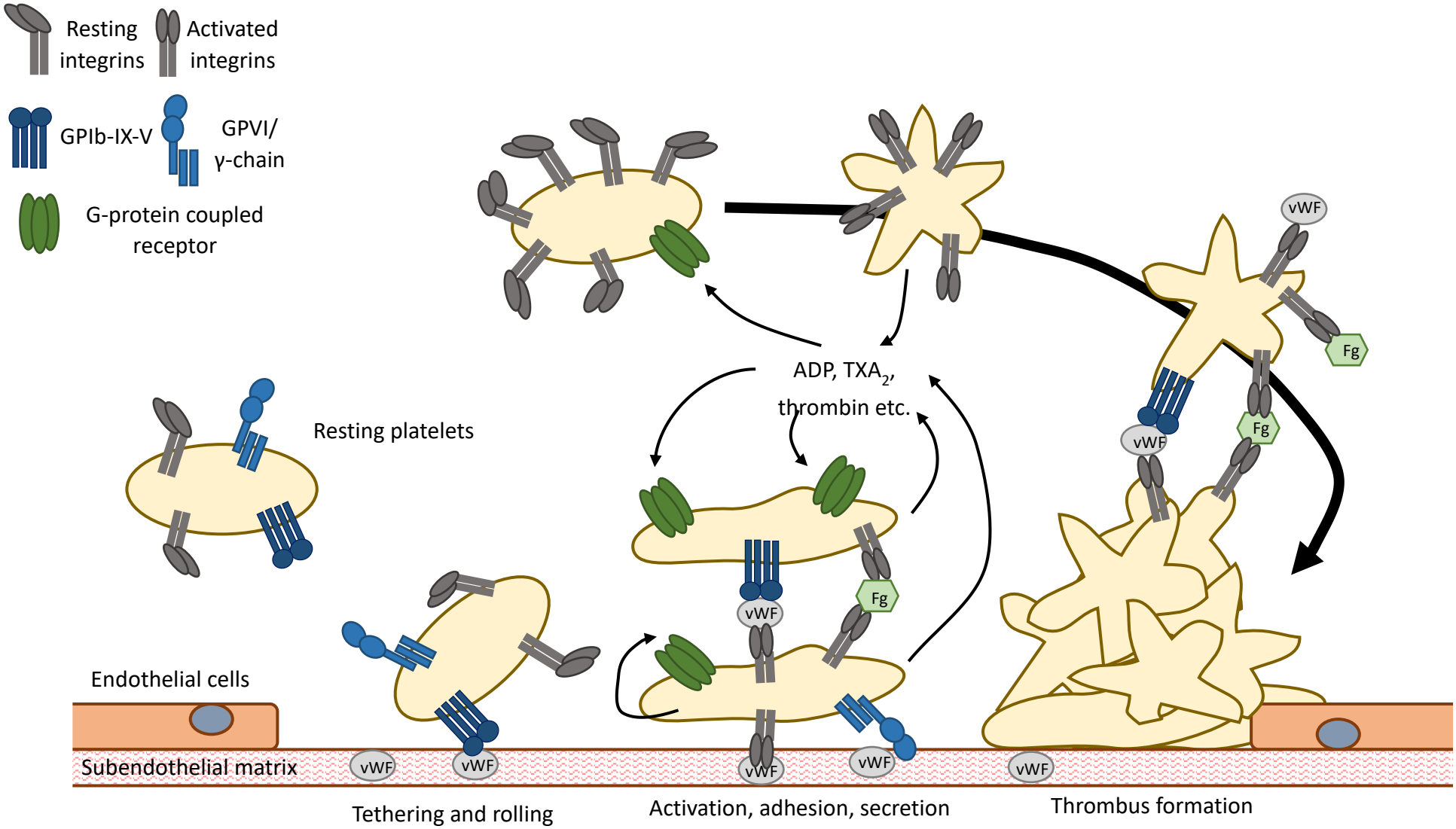


Figure 1.3. The main stages of thrombus formation. Vascular injury causes exposure of the subendothelial matrix. vWF binds to GPIb-IX-V resulting in initial tethering and platelet rolling. Other components of the matrix bind to platelet receptors causing activation, further adhesion and secretion. Contents of platelet granules amplify the activation response by recruiting cells to the site of injury. Platelets also undergo a conformation change by extending filopodia and lamellipodia. This process results in platelet aggregation and the formation of a thrombus.

1.2.4. Platelet lifespan

Non-activated platelets have a lifespan of 7-10 days and this is due to a fine balance between platelet production and clearance which varies from individual to individual. Platelet production is tightly regulated to avoid too much clearance leading to spontaneous bleeding or excessive production leading to vessel occlusion. Like megakaryocytes, platelets also express the c-Mpl receptor and regulate TPO by binding and removing it from the circulation. When platelet counts are low, less TPO is bound and therefore it is able to stimulate thrombopoiesis to produce more platelets to keep the level physiologically stable. It has also been shown that chemokine ligand 5 (CCL5) signalling may act to help increase platelet numbers during eposides of physiological stress (Machlus et al., 2016). Platelets are cleared from the circulation by phagocytosis in the liver and spleen and recognised by clearance signals, PS, P-selectin exposure and autoantibodies to platelet receptors (Mason et al., 2007). The balance between apoptotic proteins BAX and BAK and pro-survival proteins from the Bcl-2 family also govern their lifespan (Wagner et al., 2000). More recent research has shown that desialyation of platelet surface receptors leads to the exposure of galactose sugars in a time-dependant manner. The Ashwell-Morrell receptor recognises the desialylated platelets and this receptor-ligand pairing directly regulates TPO synthesis in the liver, via the recruitment of JAK2 and STAT3 signalling molecules in order to promote thrombopoiesis (Grozovsky et al., 2015). However, this regulatory mechanism requires further research in order to completely understand the maintenance of healthy platelet numbers in the circulation and how these findings could impact current therapeutics.

1.3. Platelet function testing

Platelet function testing is necessary when the ability of an individual's platelet function is suspected to be impaired. This is important for the treatment and management of the frequent bleeding episodes in these individuals that can have a negative impact on their quality of life. Individuals normally present with a history of frequent bleeding episodes including easy bruising, epistaxis, menorrhagia, excessive bleeding after injury or minor surgery and post-partum haemorrhage. There are multiple platelet function assays that can test different parameters to produce both qualitative and quantitative results regarding the platelets' function. Many of these tests are used in clinical practice however, these are constantly evolving to provide more rapid yet accurate tests to produce results for the clinical and research settings, in the most efficient manner.

The first recorded platelet function test, from 1910, was known as the Duke Procedure. This test was designed to evaluate the bleeding of a small wound made to the skin and the time taken for the wound to stop bleeding; hence, the time taken for a platelet plug to form. This test has decreased in popularity due to the invasive nature of the procedure and because it is insensitive and of poor reproducibility (Harrison et al., 2011). Platelet function testing is now more specialised and can focus on specific areas of platelet function from aggregation, adhesion and secretion under different conditions to the analysis of different cell surface markers in resting and activated platelet states.

1.3.1. Light transmission aggregometry

The Duke procedure was superseded by the gold standard platelet function assay- light transmission aggregometry (LTA), first described by Born (Rodgers and Levin, 1990, Born, 1962). LTA is still the most widely used test for the identification and diagnosis of platelet

function disorders as the responses to multiple concentrations of different agonists can be monitored. The method simply measures the light transmission in real time through samples of platelet rich plasma or washed platelets from when the agonists are added. If the platelets are functioning normally, light transmission increases as more light can pass through the sample when the platelets aggregate. Furthermore, LTA can be coupled with lumi-aggregometry to simultaneously measure the secretion of ATP from platelet granules. When the luciferin-luciferase reagent is added to the platelet mixture, the luciferase enzyme oxidises luciferin in the presence of ATP, which in turn produces a fluorescent product which can then be quantified (Holmsen et al., 1972). If a higher concentration of ATP is present in the platelet sample, a stronger fluorescent signal in the form of released light would be produced. However, the results of this assay vary between controls and so the use of standard control ranges is essential. This assay is also specialised and requires time and a fairly large volume of fresh blood which is not always possible for young children; an experienced scientist is also necessary to carry out the test and to interpret the results. This assay has given rise to alternative forms, for example, the Optimul assay; a 96-well platelet aggregation assay with high levels of sensitivity and specificity (Lordkipanidze et al., 2014, Chan et al., 2018). Multiple electrode aggregometry (MEA) is also increasingly used which measures whole blood impedance aggregation (Wurtz et al., 2014, Al Ghaithi et al., 2017). This assay allows for rapid platelet function analysis but the response is heavily influenced by platelet count (Toth et al., 2006, Wurtz et al., 2014). This assay is widely used for monitoring individuals under anti-platelet therapy.

1.3.2. Platelet adhesion assays

Platelet adhesion assays were developed to determine the ability of platelets to adhere and aggregate under the shear stresses of blood flow, therefore making these assays more physiologically relevant. Many of these assays require specialised microfluidic systems or chips that can be coated in particular proteins that cause platelet activation for example collagen or fibrinogen. The platelet function analyser (PFA-100®) tests the aggregation and adhesion of platelets under high shear. In this test, platelet function is measured as a function of the time taken to occlude an aperture within test cartridges (Harrison, 2005a, Harrison, 2005b). The Total Thrombus Formation Analysis System (T-TAS) is an automated microchip flow chamber also developed to quantitatively assess the formation of thrombi. This system uses variable flow conditions to mimic both physiological and pathological shear rates using two types of thrombogenic surfaces (Hosokawa et al., 2012). The T-TAS measures the flow pressure through the chips, requiring very small blood volumes, whilst video recording in real-time. This test is deemed a useful preliminary test (Daidone et al., 2016) (Al Ghaithi et al., 2019).

1.3.3. Flow cytometry

In order to measure the platelet secretion and the expression of cell surface markers, flow cytometry is most commonly used. The basic principle of flow cytometry is the passage of single cells through a laser to be detected, counted and sorted. The fluorescently labelled cell components are excited by the laser beam which then emit different wavelengths allowing them to be measured. Flow cytometry can also be used to measure levels of platelet activation by the addition of agonists where the level of the platelet α-granule marker, P-selectin, is the most commonly used marker. In addition, the quantity of platelet glycoproteins can be measured (Gordon et al., 1995, Michelson, 1996, Cohn et al., 1997). The very small volume of

blood needed for this assay is advantageous particularly in cases of thrombocytopenia where specialist assays have been developed (De Cuyper et al., 2013). Again, fresh blood samples are required, and the test is restricted to specialised laboratories that possess flow cytometry instruments. These downfalls are being addressed with the evolution of a test that can be performed by activating then fixing the blood remotely, at the point of collection (Platelet Solutions, UK). These blood samples are then stable for up to 9 days while they are delivered to a specialised laboratory for analysis (Dovlatova et al., 2014).

However, when a potential platelet function disorder is discovered as a result of the testing methods above, the outcomes usually stop short of giving the clinicians and patients a final and definitive diagnosis. This explains the need to involve a level of genetic analysis either to confirm the observed phenotype or to suggest candidate genetic variants when a diagnosis is not obvious.

1.4. Inherited platelet disorders – Symptoms and treatments

There are many diseases that affect the function and/or number of platelets present in the circulation. Functional disorders of platelets can be either inherited or acquired. An acquired platelet dysfunction is more common and normally due to medications, food/supplements but most often, secondary to another multisystem disease also affecting the immune system. With this cause, platelet function and/or number can decline in the disease state leading to a thrombocytopenia and clinical bleeding disorder, but this can return to normal levels during recovery. Thrombocytopenia is classed as a platelet count <150 $x10^9$/l (normal range 150-400 $x10^9$/l) which results in bleeding diathesis that can again be either inherited or acquired. Inherited bleeding disorders, as well as being rarer, present with more variable severities.

Symptoms of these disorders can range from mild to severe, but most suffer from bleeding episodes including: unexplained cutaneous bruising, petechiae, epistaxis, bleeding from mouth and gums, menorrhagia, post-partum haemorrhage and bleeding following dental extraction or surgery. In some cases, the knowledge of a bleeding disorder in affected individuals can go undiagnosed due to the lack of haemostatic challenges in their lives. This is more common in males as females undergo monthly haemostatic challenges from puberty and face other challenges such as childbirth. Many individuals do not need treatment for their bleeding disorder however, the need for regular monitoring and clinical management is necessary to avoid bleeding risks especially in the case of pregnancy or planned surgery.

1.4.1. Treatments

There are some treatments for individuals with moderate to severe bleeding particularly in paediatric cases. Local antifibrinolytic treatments are often successful such as the application of gauze soaked with tranexamic acid/ thrombin to superficial wounds or nasal packing with thrombin (Bolton-Maggs et al., 2006). These fibrinolysis inhibitors work to interfere with the formation of fibrinolytic enzyme plasmin from its precursor plasminogen to prevent the formed blood clot from breaking down. Desmopressin (DDAVP) is also a common therapy option for bleeding following trauma or for a planned surgical procedure. This synthetic analog of vasopressin induces a release of vWF from endothelial cells with subsequent release of coagulation factor VIII and tissue plasminogen activator, thereby reducing overall bleeding times (Kaufmann and Vischer, 2003). For severe bleeds or in preparation for surgery where a great bleeding risk is known, a platelet transfusion may need to be considered or the use of recombinant Factor VIIa (RVIIa) or a combination of the two (Bolton-Maggs et al., 2006). In cases of platelet transfusions either human leukocyte antigen (HLA) matched donors are

required or leukocyte depleted blood products must be used to avoid undesirable alloimmunisation. Furthermore, as advancements in medicine are being made, the use of treatments such as TPO mimetics and gene therapy have been used to improve outcomes in patients with specific bleeding disorders (Kuter, 2009, Morris et al., 2017). Gene therapy has even been used to cure individuals with haemophilia B and now haemophilia A (Nathwani et al., 2014, Rangarajan et al., 2017).

1.4.2. Disorders of platelet adhesion and aggregation

All disorders are shown in the brief guide Table 1.1. Bernard-Soulier syndrome (BSS) is a disorder of both platelet formation and platelet adhesion. The expression of glycoproteins (GP)Ibα, GPIbβ or GPIX are reduced or absent thus affecting the GPIb-IX-V receptor complex on the platelet surface membrane (Nurden et al., 2012). Most patients affected by BSS present with thrombocytopenia (platelet count $<150x10^9$/L) and atypically large platelets (Pecci and Balduini, 2014). This rare biallelic autosomal recessive disease presents clinically as a severe phenotype with severe bleeding episodes. Individuals with monoallelic mutations present with a milder phenotype and a diagnosis can easily be missed (Othman and Emsley, 2017). Platelet aggregation in an individual with BSS will show a decreased response upon the addition of ristocetin due to decreased vWF –GPIb binding. Flow cytometry is also used to diagnose this disease to confirm the absence of specific glycoproteins on the platelet surface (Beltrame et al., 2009).

von Willebrand disease (vWD) is an autosomal dominant disorder, although Type 2N and Type 3 are autosomal recessive, which are caused by a lack of, or defective von Willebrand factor. vWF binds to GP1bα through its A1 domain upon vessel injury when the protein is exposed within the subendothelial membrane. However, lack of this interaction can lead to increased

bleeding times. In Types 1 and 3 vWD, a quantitative deficiency of vWF causes the bleeding diathesis. In Type 2, individuals have qualitative defects of vWF. These are broken down into four subtypes: type 2A, Type 2B, Type 2M and Type 2N which depend on the presence and nature of the vWF multimers and chains of vWF. vWD Type 2B is caused by genetic variants affecting expression of the A1 domain; this causes a gain of function phenotype as GP1bα will more readily bind to circulating vWF leading to the presence of multimers in the blood and enhances platelet clearance (Nurden et al., 2006).

Platelet $P2Y_{12}$ deficiencies result in defects of platelet activation. This principal G-protein coupled receptor for the agonist ADP is required for normal platelet coagulation activity (Dorsam and Kunapuli, 2004). ADP acting on $P2Y_{12}$ is responsible for the amplification of the platelet response to ADP and other soluble agonists including TXA_2 and thrombin. $P2Y_{12}$ defects are inherited in an autosomal recessive manner and manifest by mild to severe prolonged bleeding times as well as other bleeding episodes. Diagnosis of this condition by platelet aggregation is followed by genetic analysis to confirm a suspected diagnosis of a $P2Y_{12}$ deficiency (Cattaneo et al., 2003).

Glanzmann thrombasthenia (GT) is a disorder of platelet aggregation caused by mutations in the genes *ITGA2B* and *ITGB3* which lead to qualitative and quantitative defects of the integrin αIIbβ3 (Salles et al., 2008, Nurden and Nurden, 2011, Nurden and Pillois, 2018). This molecule is essential for platelet aggregation and blood clotting as without it, the platelets' ability to bind fibrinogen and also form platelet-platelet interactions is severely disrupted (Nurden et al., 2012). This rare autosomal recessive disorder is normally characterised by severe bleeding episodes and affected individuals often have to undergo platelet transfusions. GT can be diagnosed after an abnormal result following platelet aggregation studies, confirmed by a

deficiency of αIIbβ3 using flow cytometry and genetic testing to identity and confirm genetic variant responsible for the disease (Nurden et al., 2011).

Scott Syndrome is known as a defect of platelet procoagulant activity since there is defective scrambling of the phospholipid membrane. Upon platelet activation PS is normally transported from the inner to the outer leaflet of the platelet membrane where coagulation factors are converted to their active forms. This autosomal recessive disorder leads to failure in production of the procoagulant surface upon platelet activation (Zwaal et al., 2004). The only treatment for this disorder is a platelet transfusion during bleeding episodes.

Table 1.1. Brief guide of the main inherited disorders that affect platelets. The disorders are classified by platelet abnormality. The disease and the gene causative of the disease is described as well as the phenotype and any additional findings associated with the disease; all disorders can be found described in the review for the United Kingdom Haemophilia Centre Doctors' Organisation by Bolton-Maggs *et al*. (Bolton-Maggs et al., 2006).

Platelet abnormality	Disease	Inheritance	Gene	Phenotype and other findings
Platelet adhesion and aggregation	Bernard Soulier syndrome	Autosomal recessive	*GPXI* *GPIbα* *GP1bβ*	Thrombocytopenia, large platelets Anomalies in components of the GPIb-IX-V complex
	Glanzmann thrombasthenia	Autosomal recessive	*ITGA2B* *ITGB3*	Normal platelet count Absent aggregation with all agonists
	$P2Y_{12}$ receptor defects	Autosomal recessive	*P2Y12*	Normal platelet count Transient aggregation
	Scott syndrome	Autosomal recessive	*TMEM16F*	Normal platelet count Factor X activation deficiency
	Thromboxane A_2	Autosomal recessive	*TBXA2R*	Normal platelet count Aggregation reduced in response to arachidonic acid and U46619
	von Willebrand disease	Autosomal dominant (Type 2B) Autosomal dominant (Platelet-type)	*vWF* *GPIbα*	Thrombocytopenia Reduced or absent vWF multimers
Platelet secretion	Chediak-Higashi syndrome	Autosomal recessive	*LYST*	Normal platelet count Skin and hair hypopigmentation Immunodeficiency Reduced α-granules
	Grey Platelet syndrome	Autosomal recessive	*NBEAL2*	Thrombocytopenia, large platelets Grey platelet colour on blood film Absent α-granules
	Hermansky-Pudlak syndrome	Autosomal recessive	*HPS1-9*	Normal platelet count Decreased/absent dense granules Oculocutaneous albinism

	Jacobsen/ Paris-Trousseau syndrome	Autosomal dominant	*FLI1*	Thrombocytopenia, large platelets Developmental delay, facial abnormalities
	Quebec platelet disorder	Autosomal dominant	*PLAU*	Thrombocytopenia Urokinase plasminogen activator storage in platelets
Platelet abnormality	**Disease**	**Inheritance**	**Gene**	**Phenotype and other findings**
Disorders of megakaryopoiesis, proplatelet formation and release	Actin disorders	Autosomal dominant	*ACTN1*	Thrombocytopenia, large platelets
	Congenital amegakaroycytic thrombocytopenia	Autosomal recessive	*MPL*	Thrombocytopenia Pancytopenia Absent megakaryocytes in bone marrow
	Tubulin disorders	Autosomal dominant	*TUBB1*	Thrombocytopenia, platelets not uniform in shape or size
	Wiskott-Aldrich syndrome	X-linked	*WAS*	Thrombocytopenia, small platelets Immunodeficiency
Disorders of platelet clearance and other disorders	Familial platelet disorder with predisposition to acute myeloid leukaemia	Autosomal dominant	*RUNX1*	Thrombocytopenia Abnormal aggregation
	Increased platelet clearance disorders	Autosomal recessive	*GNE*	Thrombocytopenia, large platelets
	Upshaw-Schulman syndrome	Autosomal recessive	*ADAMTS13*	Thrombocytopenia Ultra large vWF multimers Acute thrombotic microangiopathy

1.4.3. Disorders of platelet secretion

The disorders of platelet secretion and storage can affect platelet α- or dense granules with few patients having defects in both sets of granules (Bolton-Maggs et al., 2006). These are a heterogeneous group of disorders that can affect platelet responses upon activation; the disorders are shown in the brief guide Table 1.1.

Grey Platelet syndrome (GPS) is characterised by a complete absence of platelet α-granules caused by homozygous or compound heterozygous mutations in *NBEAL2* which was initially identified by whole exome sequencing (WES) (Albers et al., 2011, Gunay-Aygun et al., 2011, Kahr et al., 2011). This defect causes platelets to be characteristically grey in colour on a blood smear. Platelets can vary in their ability to function however most sufferers have thrombocytopenia due to decreased platelet survival times. Other disorders that affect α-granules include Jacobsen Syndrome and its associated syndrome, Paris-Trousseau thrombocytopenia, and Quebec platelet disorder in which urokinase plasminogen activator (uPA) in α-granules produces profibrinolytic platelets (Pecci and Balduini, 2014, Hayward and Rivard, 2011). For this disorder, individuals present with delayed-onset bleeding following trauma.

Dense granule disorders such as Chediak-Higashi and Hermansky-Pudlak syndrome (HPS) both lead to a dense granule deficiency (Nurden and Nurden, 2011). Chediak-Higashi is recognised as a separate disorder due to the severity of the immune defects resulting from mutations in the protein LYST, while HPS has been shown to be due to defects in ten genes in humans, although fifteen causative genes have been identified in mice (Di Pietro and Dell'Angelica, 2005, Maaloul et al., 2016). The bleeding diathesis in affected individuals is caused by overall impaired aggregation due to lack of dense granule secretion. Other disorders that affect dense

granules include Jacobsen Syndrome and its associated syndrome, Paris-Trousseau thrombocytopenia caused by deletion of a region on chromosome 11, del11q23 which contains the gene that encodes FLI1 (White, 2007, Raslova et al., 2004). Paris-Trousseau thrombocytopenia has now been described without the 11q deletion caused only by genetic variants in *FLI1* (Stockley et al., 2013, Stevenson et al., 2015).

1.4.4. Disorders of megakaryopoiesis, proplatelet formation and release

The production of platelets from megakaryocytes and primarily HCSs is a highly controlled process and there are a multitude of disorders that are associated with megakaryopoiesis defects, disruption in proplatelet formation and release. These can range from altered transcriptional control to disruption in signalling pathways associated with the differentiation process. As a result, platelet numbers and function are often reduced (Johnson et al., 2016a).

Genetic variants in the gene which encodes the thrombopoietin receptor, c-Mpl, have been described to cause congenital amegakaryocytic thrombocytopenia (CAMT) which results in a severe pancytopenia due to bone marrow failure (Stoddart et al., 2013). The only feasible treatment for this rare condition is stem cell transplant from matched related or unrelated donors (Frangoul et al., 2010). The transcription factor GATA1 is involved in the cell lineage progression of HSCs to MEPs therefore dysfunctional GATA1 can affect thrombopoiesis and erythropoiesis. Genetic variants that affect the ability of GATA1 to bind to its cofactor FOG1 cause X-linked thrombocytopenia whereas variants that affect GATA1's ability to bind to DNA cause X-linked thrombocytopenia and thalassemia (Nichols et al., 2000). These platelets are typically abnormal in number, size and shape (Nichols et al., 2000). Furthermore, variants in the transcription factor RUNX1 give rise to an autosomal dominant disorder characterised by

quantitative and qualitative disorders of platelets along with a predisposition to myelodysplasia or leukaemia (Schlegelberger and Heller, 2017).

During proplatelet formation and release, the control of the cytoskeletal rearrangement process during thrombopoiesis is also highly regulated in order to produce healthy, functioning platelets. Wiskott-Aldrich syndrome arises from mutations in the *WAS* gene which encodes the WAS protein (WASP) (Pecci and Balduini, 2014). WASP is known to affect platelets during megakaryopoiesis where it plays a key role in actin cytoskeletal regulation and typically results in microthrombocytopenia, therefore leading to the mild bleeding phenotype, in addition to other symptoms such as immunodeficiency which are often associated with this X-linked recessive disorder (Balduini and Savoia, 2012, Pecci and Balduini, 2014, Bolton-Maggs et al., 2006).

Genetic variants in *TUBB1* encode dysfunctional β1-tubulin. This leads to the expressed protein being unstable and unable to interact normally with other microtubules (Kunishima et al., 2014). The reduced number of proplatelets that are formed have a marked reduction in the number of tip swellings and these are more variable in size causing few platelets to be released into the circulation of varying sizes. Individuals with variants in the *ACTN1* gene are affected in a similar way. α-actinin is an actin binding protein also involved in cytoskeletal remodelling during proplatelet formation (Kunishima et al., 2013). Megakaryocytes in individuals with these variants possess disorganised actin cytoskeletons resulting in abnormally large proplatelet tips which are reduced in number (Kunishima et al., 2013).

There are many more known disorders of megakaryopoiesis, platelet production and platelet release and the study of these disorders has provided insights into the mechanisms of action of the now well-known transcription factors and megakaryocyte and platelet biology.

1.4.5. Disorders of platelet clearance, other and unknown disorders

There are a number of genes affected by detrimental genetic variants which cause platelets to be cleared prematurely from the circulation. This includes variants in the gene encoding metalloproteinase *ADAMTS13* resulting in Upshaw-Schulman syndrome (Levy et al., 2001). Affected individuals with this rare syndromic disorder have inactive ADAMTS13 leading to an increase and hence accumulation of vWF multimers. These large multimers, therefore begin to interact with circulating platelets causing them to aggregate. This inappropriate pre-activation of the platelets causes them to be removed from the circulation leading to continually low platelet counts and characteristic of the disease phenotype (Levy et al., 2001). vWD Tybe 2B, described previously, manifests in a similar way however platelets also fail to aggregate. In both disease states, the vWF complexes are removed by macrophage action in the liver and spleen.

Variants in the gene *GNE* cause a secondary symptom of thrombocytopenia due to excessive platelet clearance. The bifunctional enzyme the gene encodes, UDP-N-acetylglucosamine 2-epimerase (GNE), initiates the synthesis of a precursor to sialic acid (Favier and Raslova, 2015). The affected individuals have reduced GNE activity and sialic acid biosynthesis failure, therefore sialic acid residues on the platelets' surface are decreased (Futterer et al., 2018, Revel-Vilk et al., 2018). This causes increased platelet clearance from the liver hence thrombocytopenia. This disease requires further investigation as some affected individuals present with additional myopathy, but not in all cases (Futterer et al., 2018, Revel-Vilk et al., 2018).

1.5. Non-platelet bleeding disorders

There are numerous other causes of inherited bleeding that are not related to platelet dysfunction. Excluding the more common and regularly screened von Willebrand disease and haemophilia A and B, much less is known about these other bleeding disorders due to their rarity and difficulty in clinical diagnosis. For example, fibrinogen abnormalities are rare and are not as well characterised as some of the platelet function disorders (Acharya and Dimichele, 2008).

1.5.1. Inherited fibrinogen disorders

Inherited fibrinogen disorders can, like platelet function disorders, be due to quantitative defects (afibrinogenemia and hypofibrinogenemia) or qualitative defects (dysfibrinogenemia) (Acharya and Dimichele, 2008). Fibrinogen is a 340kDa glycoprotein synthesised in the liver encoded by genes: *FGA*, *FGB* and *FGG,* and as it is a precursor of fibrin, plays an essential role in the formation of the vascular plug (Tiscia and Margaglione, 2018). Variants in these genes lead to abnormal polymerisation of the protein, defective cross-linking and defective fibrinolysis. Fibrinogen is a multifaceted molecule and hence disease causing variants within the aforementioned genes can result in both bleeding and thrombotic phenotypes (Acharya et al., 2004).There are over 70 causative mutations identified in the fibrinogen genes with the majority being in FGA (Neerman-Arbez and de Moerloose, 2007, Tiscia and Margaglione, 2018). A valuable tool for investigating human fibrinogen variants can be found in the Fibrinogen Database (http://site.geht.org/base-de-donnees-fibrinogene/) (Hanss and Biot, 2001).

1.5.2. Coagulation factor deficiencies

Coagulation factor deficiencies also cause varying severities of bleeding most of which are routinely screened at Haemophilia Comprehensive Care Centres across the UK (Mannucci et al., 2004). These include prothrombin (FII) deficiency, FV deficiency, FVII deficiency, FX deficiency, FXI deficiency, FXIII deficiency and combined FV and FVIII deficiency. Most patients with these deficiencies require replacement therapy regularly, usually with fresh-frozen plasma which contains all the necessary coagulation factors (Mannucci et al., 2004). The treatment options are improving now with the availability of single coagulation factors for replacement therapy, however neither of the therapies are straightforward and haemostatic levels need to be constantly monitored (Mannucci et al., 2004).

Other proteins implicated in bleeding include protein C which is a vitamin K-dependant serine protease and when activated acts as an anticoagulant (Danese et al., 2010). Variants within the gene *PROC* that encodes protein C, could hypothetically, lead to gain of function by increasingly inactivating cofactors of the coagulation cascade and hence reduce clotting (Danese et al., 2010). Protein S, encoded by genes *PROS1* and *PROSP*, is also a vitamin K-dependent anticoagulant glycoprotein and acts as a cofactor to activated protein C (Lind-Hallden et al., 2012). Again, a genetic variant within these genes could affect haemostasis. Finally, genetic variants in *THBD*, which encodes the glycoprotein thrombomodulin, have been shown to cause a bleeding phenotype in affected individuals (Langdown et al., 2014, Dargaud et al., 2015). Thrombomodulin is expressed on the surface of endothelial cells and normally serves as a cofactor for thrombin, however, when its function is impaired the coagulation cascade is disrupted (Weiler and Isermann, 2003).

1.6. Genetics and genomics

Genomics is defined as the study of the genetic material of an organism and its associated functions (World Health Organization, 2002). Genomics therefore aims to address all genes and their interrelationships in order to study and identify their combined influence on growth and development; this is in comparison to genetics which mainly scrutinises the function of a single gene (World Health Organization, 2002). In our now 'post-genomic era', massive advancements in genomic technology have been achieved.

The Human Genome project (HGP) began in the mid-1980s and was an international, collaborative project with the goal to completely map and improve the understanding of an individual's entire nucleic material known collectively as our genome (Venter et al., 2001, Sachidanandam et al., 2001). The initial sequence data was first published in the journal *Nature* in February 2001 when it was 90% complete, and was finally proclaimed 99% complete in 2003 (Lander et al., 2001, Collins et al., 2003). This project required and will continue to require new technologies to refine the draft and carry on research with regard to the complete human genome.

Following the HGP, the 1000 Genomes Project was the first of its kind to sequence the genomes of a large group of individuals in order to provide a valuable resource to the scientific community on human genetic variation. The primary goal was to elucidate the genetic variants that have frequencies of at least 1% in the populations studied. The main drawback of the 1000 Genomes Project was that the samples were anonymous and therefore no phenotype data was available. However, the 1000 Genomes data can be combined with genome-wide association studies (GWAS) for additional variants beyond those already genotyped and allow for disease-associated genes, regions, and variants to be researched in more depth.

GWAS focus on the associations between single nucleotide polymorphisms (SNPs) and the varying phenotypes of a specific syndrome or disease which are typically seen in inherited platelet disorders (Bunimov et al., 2013). Family-based designs for GWAS are particularly beneficial as they enrich for genetic defects and have more power than unrelated individuals to detect genetic defects (Ott et al., 2011). Family-based designs often include parent-offspring trios, sibling pairs, consanguineous families and large extended families with multiple affected family members (Ott et al., 2011). The statistical principals underlying these designs such as the McNemar-type statistic are often complicated and will not be described here in detail (Ott, 1989, Terwilliger and Ott, 1992, Knapp et al., 1993).

Platelet genetics is a challenging area of research due to the absence of a nucleus. Platelets do have residual levels of megakaryocyte-derived RNA, and have been shown to make low amounts of proteins most notably interleukin 1 (Macaulay et al., 2005, Booyse and Rafelson, 1968, Loppnow et al., 1998). The platelet genome is yet to be fully defined but data enlightening on how genetic variation, at the level of gene transcription and translation, perturbs platelet function has been highly beneficial (Macaulay et al., 2005). This has led to further analysis in recent years with the birth of more specialised techniques within genomics and more manageable costs of genomic investigation.

1.6.1. Next-generation sequencing

DNA sequencing has been revolutionising medical research since the invention of Sanger sequencing by Sanger *et al*. and the polymerase chain reaction (PCR) by Mullis *et al*. (Mullis, 1986, Sanger et al., 1977). These traditional methods have been replaced with next-generation sequencing technologies and led to an exponential reduction in the costs involved in sequencing whole genomes/exomes (Rabbani et al., 2014). Whole genome sequencing

(WGS) and whole exome sequencing (WES) are used to identify genetic factors causing human diseases; some of which were previously undetectable. These high-throughput experimental approaches are now more widely available than ever and have been adopted in both research and clinical settings in relation to patients with platelet and bleeding disorders (Singleton, 2011).

It is well known that approximately 1.5% of the human genome contains protein-coding sequences (exons), within which 85% of alleles that underlie Mendelian disorders, that disrupt protein-coding sequences, reside (Rabbani et al., 2014). Therefore, exome sequencing alone is sufficint to harbour enough data to uncover most rare and potential genetic disorders and predisposing genetic variants (Majewski et al., 2011, Rabbani et al., 2014). The first report of selectively sequencing all exomes successfully was published by Ng *et al*. (Ng et al., 2009). There are several slightly different methods which all follow the same principle for the rapid identification of protein-coding variants; mutations include: missense and nonsense to small insertion/deletion mutations and splice site mutations (Teer and Mullikin, 2010, Singleton, 2011). WES can also be used to identify the genetic causes of multifactorial disorders that can be more common in bleeding disorders (Leinoe et al., 2017).

1.6.2. Limitations and challenges of NGS

There are some fundamental limitations of WES, the most obvious being that WES does not assess the impact of non-coding regions, since it is only limited to coding and splice site variants (Rabbani et al., 2014). A further limitation is that the method is still time consuming and the coverage of regions of interest is not always complete. This aspect of WES has improved since the first experiments where 8% of regions of interest were not captured by the enrichment strategy, but it is not expected that 100% coverage will ever be reached

(Singleton, 2011, Ng et al., 2009). WES is often less cost effective than a large targeted panel and still has difficulty in identifying repeat mutations and copy number variants, often leading to false positives (Singleton, 2011). Confirmation and validation of possible disease causing variants by Sanger sequencing is nearly always required following WES as it has a very high level of coverage and the possibility of false-positive calls is little to none.

Challenges also arise with the lack of development of bioinformatic analysis tools that are already lagging behind the advances in the substantial amount of data generated by NGS. This often leads to the analysis of bioinformatic data post NGS being the rate limiting step in the process of gene identification. Therefore, the second challenge faced within platelet genomics is the identification of the causal gene and variant among multiple possible variants and their link to disease and function (Majewski et al., 2011). This is often equivocal, and several candidates remain. Presently, sequence variant analysis is imperfect and a tremendous amount of work is needed to introduce a large-scale statistical automated framework for the calling of variants (Richards et al., 2015, MacArthur et al., 2014). There have been standards and guidelines published for the interpretation of sequence variants, as recommended by the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology which will be discussed in Methods section 2.14.2 (Richards et al., 2015).

Another potential challenge arises with the inevitable discovery of genetic variants not related to the disease state in question, so called incidental or secondary findings (Wolf et al., 2008). This is a complex issue with many confounding factors involving ethics. The issue faced by clinicians is, should they disclose secondary findings with potential clinical relevance to their patients and if so, when and how? The interpretation of the information delivered would require adequate education of the patient and family, and with some inherited bleeding

disorders, possible genetic counselling. The ACMG have published a policy statement concerning NGS with emphasis on incidental findings in clinical pretesting, genetic testing and the reporting of results (ACMG Board of Directors, 2012). The recommendations of the ACMG Working Group on Incidental Findings in Clinical Exome and Genome Sequencing have also been published and outline a list of conditions, genes and variants they recommend for return of incidental findings in clinical sequencing (Green et al., 2013). This is deemed by many to go beyond targeted sequencing and true incidental findings to suggest automatic screening for genetic conditions in all patients undergoing WES/WGS (Allyse and Michie, 2013). It is a controversial area of interest that requires further social and professional debate both of which should proceed with ethical caution and concern (Allyse and Michie, 2013).

1.6.3. Advancements of genomic technologies in discovering genetic variants in platelet disorders

One of the main applications and challenges of NGS is proving that a certain genetic variant or variants are responsible for the clinical presentation of patients. In the case of bleeding and inherited platelet disorders, the phenotypes can vary greatly from one individual to another even with the same genotype. This makes it very difficult to generate genotype-phenotype correlations and elucidate a disease causing mechanism within these individuals.

The first use of NGS in a platelet function defect identified *NBEAL2* as the causative gene for grey platelet syndrome (GPS) (Albers et al., 2011). Many causative mutations within the gene which encodes a BEACH domain protein cause differential bleeding severities in the individuals with GPS (Gunay-Aygun et al., 2011). In the past few years however, several research groups and their collaborators have employed NGS to discover the causes of unexplained bleeding. We now have knowledge of platelet genes that are known or predicted

to have a role in regulation of platelets along with platelet physiological processes and we are therefore able to diagnose patients with specific platelet defects as a result (Leo et al., 2015). It is important to emphasise the need for cell biology to explore novel unclassified variants in order to investigate expression analysis and protein function to ensure a variant is well characterised and proven to be functionally disrupting (Watson et al., 2013). As more data is accumulated about the genetics of bleeding and platelet disorders, the list of candidate variants will decrease. Therefore, the likelihood of finding a disease causing mutation will increase. A table of genes involved in bleeding disorders discovered by NGS can be seen in Table 1.2.

Table 1.2. Novel genomic variants reported in genes discovered in patients with an inherited bleeding disorder following next-generation sequencing. Gene and phenotypes associated with variants are shown. Heterozygous nucleotide changes (unless indicated) present in patients with inherited bleeding and their predicted effects on the resulting RNA or protein are also shown. Genomic variations are numbered according to positions in the publication of the reference indicated. dbSNP ID is given for each variant if known or is a novel variant not reported in the available databases (Bhagwat, 2010).

Gene Associated phenotypes	**Genomic variation**	**Protein effect**	**Variation type**	**dbSNP ID**	**Reference**
NBEAL2 Thrombocytopenia, large platelets, lack of α-granules.	c.256 A>G c.1163T>C c.1928 A>T c.2044 >T c.6299 C>T c.6802-1_6805dup c.6806 C>T c.7658 G>A	p.Ile86Val p.Leu388Pro p.Glu643Val p.Ile682Phe p.Pro2100Leu p.Ser2269Leu p.Gly2553Glu	Missense Missense Missense Missense Missense Splice site Missense Missense	rs754407148 rs387907113 rs387907114 rs773164015 rs387907115 rs749896920 rs144664865	(Albers et al., 2011) (Albers et al., 2011) (Albers et al., 2011) (Albers et al., 2011) (Albers et al., 2011) (Albers et al., 2011) (Bolton-Maggs et al., 2006) (Albers et al., 2011)
ACTN1 Thrombocytopenia, large platelets.	c.64 G>A c.94 C>A c.136 C>T c.137 G>A c.313 G>A c.673 G>A c.751 G>C c.2210 C>A c.2212 C>T c.2255 G>A c.2289 G>A c.2305 G>A	p.Asp22Asn p.Gln32Lys p.Arg46Trp p.Arg46Gln p.Val105Ile p.Glu225Lys p.Gly251Arg p.Thr737Asn p.Arg738Trp p.Arg752Gln p.Gly764Ser p.Glu769Lys	Missense Missense Missense Missense Missense Missense Missense Missense Missense Missense Missense Missense	rs387907346 rs747559032 rs387907348 rs387907345 rs387907350 rs387907349 rs387907347	(Bottega et al., 2015) (Kunishima et al., 2013) (Bottega et al., 2015) (Bottega et al., 2015, Kunishima et al., 2013) (Kunishima et al., 2013) (Bottega et al., 2015, Kunishima et al., 2013) (Bottega et al., 2015) (Bottega et al., 2015) (Bottega et al., 2015, Kunishima et al., 2013) (Bottega et al., 2015, Kunishima et al., 2013) (Bottega et al., 2015) (Bottega et al., 2015)

Gene Associated phenotypes	**Genomic variation**	**Protein effect**	**Variation type**	**dbSNP ID**	**Reference**
SRC Thrombocytopenia, sometimes large platelets, lack of α-granules.	c.1579 G>A	p.Glu527Lys	Missense		(Turro et al., 2016)
SLFN14 Thrombocytopenia, reduced δ-granules.	c.652 A>G c.657 T>A c.659 T>A c.667 C>T	p.Lys218Glu p.Lys219Asn p.Val220Asp p.Arg223Trp	Missense Missense Missense Missense	 rs766076920	(Fletcher et al., 2015) (Fletcher et al., 2015) (Fletcher et al., 2015) (Marconi et al., 2016)
ETV6 Thrombocytopenia, haematologic malignancy.	c.641 C>T c.1106 G>A c.1195 C>T	p.Pro214Leu p.Arg369Gln p.Arg399Cys	Missense Missense Missense	rs724159947 rs724159946 rs724159945	(Zhang et al., 2015) (Zhang et al., 2015) (Zhang et al., 2015)
FYB Thrombocytopenia, small platelets.	c.393 G>A^{Hom}	p.Trp131Stop^{Hom}	Nonsense		(Levin et al., 2015)
PRKACG Thrombocytopenia, giant platelets.	c.222 C>G^{Hom}	p.Ile74Met^{Hom}	Missense	rs724159972	(Manchev et al., 2014)
RBM8A Thrombocytopenia, absence of radius bones.	c.-21 G>A c.67+32G>C		Regulatory SNP Regulatory SNP	rs139428292 rs201779890	(Albers et al., 2012) (Albers et al., 2012)
GNE Thrombocytopenia, giant platelets	c.1246 G>A^{Hom} c.1516_1517delinsTT c. 1457 T>V^{Hom}	p.Gly416Arg^{Hom} p.Gly475Phe p.Leu486Pro^{Hom}	Missense Missense Missense		(Futterer et al., 2018) (Revel-Vilk et al., 2018) (Revel-Vilk et al., 2018)

1.7. Genetic studies of bleeding and platelet disorders

There are a number of genetic studies for the investigation of potential bleeding and platelet disorders. These all use different approaches with the aim to find a causative genetic variant, determine the effect(s) of the variant and then link it to the disease phenotype. Research groups in the U.K., Italy, Spain, Denmark as well as the USA are progressing in the field by incorporating NGS to their investigation of unknown bleeding diatheses (Leinoe et al., 2017, Sanchez-Guiu et al., 2014, Bastida et al., 2018, Johnson et al., 2018).

1.7.1. BRIDGE-Bleeding and Platelet Disorders

The BRIDGE consortium was introduced as the NIHR BioResource funded body for projects involving NGS related to rare disease causing variants. The BRIDGE-Bleeding and Platelet Disorders (BDPs) study (B20) takes an alternative approach to the discovery of novel and known variants by high throughput sequencing (HTS) of a large patient cohort (> 1000 donors) and annotation with adapted Human Phenotype Ontology (HPO) terms (https://bridgestudy.medschl.cam.ac.uk/bpd.shtml) (Westbury et al., 2015). Their aim is to characterise cases with similar HPO terms and variants in the same gene to aid in gene discovery (Westbury et al., 2015).

The BRIDGE-BPD study used their novel approach to gene discovery using HPO-encoded phenotype data for novel variants in *ACTN1* along with other genes that have been implicated with bleeding including *MYH9* (Kunishima et al., 2013, Westbury et al., 2015). A gain-of-function mutation in *SRC* was also found to cause bleeding and other bone pathologies following WGS and HPO patient coding within the BRIDGE-BDP study as well as a gain-of-function variant in *DIAPH1* (Turro et al., 2016, Stritt et al., 2016). In order to ensure accurate annotation of the particular phenotypes, the study added 80 terms and associated symptoms

to HPO in parallel with the development of the software hpoPlot that summarises the HPO codes of the cases (Kohler et al., 2014). It was hypothesised that individuals with rare coding variants in the disease gene would cluster on the basis of the HPO-encoded phenotypes they were given upon recruitment to the study (Westbury et al., 2015). The data that was generated suggested that despite minor anomalies, bleeding disorders caused by common genetic backgrounds would tend to cluster on the basis of their HPO terms (Westbury et al., 2015). This approach to gene discovery was pioneered in bleeding and platelet disorders but is applicable to other rare diseases. The HPO data can be linked to large online databases such as Online Mendelian Inheritance in Man (OMIM) which can be used to prioritise disease gene candidates based on comparison to other ontological phenotype terms from scientific literature (Westbury et al., 2015).

1.7.2. ThromboGenomics Platform

The ThromboGenomics platform has also been implementing NGS with regard to bleeding, thrombotic and platelet disorders (http://www.thrombogenomics.org.uk). They have developed a targeted gene capture platform which encompasses approximately 70 candidate genes with the aim to provide a multi-gene high-throughput sequencing platform to obtain diagnoses for patients with suspected bleeding or platelet disorders (Simeoni et al., 2016). Again the use of HPO term based coding of patient phenotypes is being utilised in order to elucidate genotype-phenotype correlations (Simeoni et al., 2016). The ThromboGenomics platform has used WGS data and is critically dependent on the recruitment of patients with strong, clinically well characterised phenotypes. WES has been carried out on the genes *ITGA2B* and *ITGB3* with input from ThromboGenomics in order to identify novel variants within these genes and predict whether the said variants are likely to cause Glanzmann

Thrombasthenia (Buitrago et al., 2015). ThromboGenomics has multiple national and international collaborators that are involved at various levels to continue the development of the platform.

1.7.3. The Genotyping and Phenotyping of Platelets study

The Genotyping and Phenotyping of Platelets (GAPP) study run from the University of Birmingham has recruited approximately 1000 participants with bleeding disorders of unknown cause from over twenty five collaborating Haemophilia Comprehensive Care Centres across the UK and involves collaborations with the Universities of Bristol, Nottingham and Sheffield (Figure 1.4) (http://www.birmingham.ac.uk/research/activity/cardiovascular-sciences/research/platelet-group/platelet-gapp/index.aspx) (Watson et al., 2013).

The GAPP study team use a combination of platelet phenotyping by LTA and WES in a different approach to variant discovery within platelet genomics with the aim to identify the likely causative gene(s) within each recruited individual (Watson et al., 2013, Jones et al., 2012). Blood from individuals suspected to have a platelet function disorder is subjected to LTA which is extensively used as the gold standard for platelet function testing with a streamlined panel of agonists (Dawood et al., 2007, Dawood et al., 2012). This has shown that most platelet function disorders can be reliably diagnosed using key platelet agonists at specified concentrations (Dawood et al., 2012). In some cases, additional phenotyping is performed including flow cytometry and the measurement of cyclic adenosine monophosphate (cAMP) to gain further information. The study emphasises the importance of the genotype-phenotype correlation to improve the classification and diagnosis of patients with unclassifiable, novel or unknown platelet and bleeding disorders (Watson et al., 2013). The GAPP study workflow can be seen in Figure 1.5.

Figure 1.4. Map of the GAPP study network. Orange pins show all Haemophilia Comprehensive Care Centres from which patients are referred to the main laboratory at the University of Birmingham shown by the blue circle.

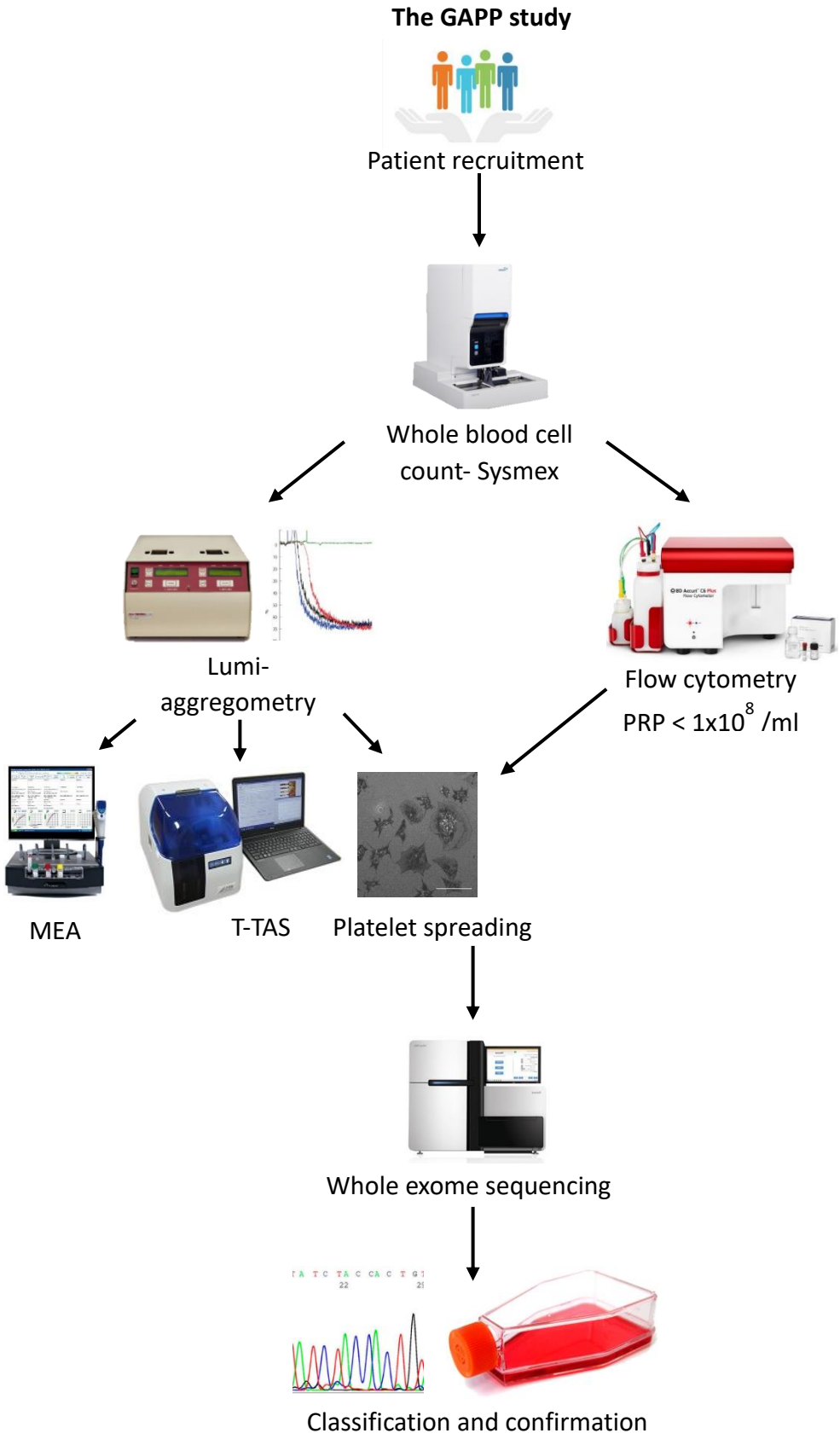


Figure 1.5. The GAPP study normal workflow. Patients suitable for the study are recruited. Blood is then taken and analysed using the Sysmex**.** Depending on the platelet count lumi-aggregometry or flow cytometry is carried out. If diagnosis is still not possible, DNA is selected to go forward for WES. Genetic variants are then confirmed by Sanger sequencing and functional work may need to be carried out.

The GAPP study workflow has allowed for the diagnosis of hundreds of patients since its inception through either phenotyping, genotyping or both (Jones et al., 2012, Johnson et al., 2018). Patients with novel *SLFN14* variants as well as those with *RUNX1* and *FLI1* variants implicated in bleeding were discovered through the GAPP study methodology of employing NGS, showing effective proof of principle for this approach (Fletcher et al., 2015, Stockley et al., 2013). The *SLFN14* variants were narrowed down from eight potential disease causing single nucleotide variants (SNVs) shared by the three patients of the same family in the initial investigation. The affected individuals presented with thrombocytopenia and excessive bleeding disproportionate to their platelet counts (Fletcher et al., 2015). The platelets also have a reduced number of dense granules and therefore ADP secretion is also reduced (Fletcher et al., 2015).

This study is not exempt from challenges and over the years, the GAPP study team have identified a large group of individuals in which no platelet defect was identified despite there being extensive bleeding histories (Figure 1.6). It is this cohort of patients that require further investigation by WES in order to elucidate their causes of disease. Recently, a novel variant in *GNE* was found by WES to be the causative of a family's thrombocytopenia and bleeding (Futterer et al., 2018). The GAPP study continues to investigate this 'no defect' group in patients with normal platelet counts however, interpreting genetic variants remains difficult.

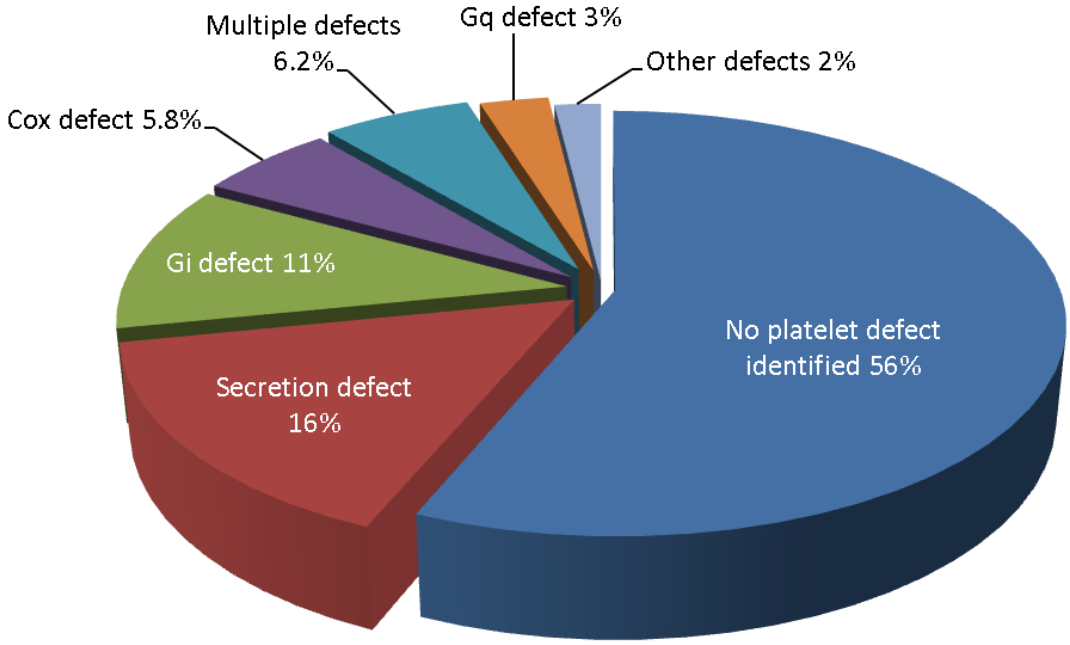


Figure 1.6. The main findings and classification of defects from the GAPP study after Lumi-aggregometry. Following lumi-aggregometry, the platelet defects were classified. The most common platelet defect was found to be secretion defects followed by Gi signalling pathway defects. In the largest group however, there were found to be no identifiable platelet defects. This illustrates the limit of particular assays to detect platelet function disorders and how they fail to address other possible causes of patients' bleeding.

1.8. Thesis hypothesis and aims

We propose that a significant proportion of patients (≈60%) with no obvious platelet defect despite having extensive bleeding history, have defective genes/mutations in other genes involved in haemostasis. We believe that some of these give rise to impaired platelet function but are not readily picked up in available tests. We will investigate this hypothesis using next generation sequencing approaches to identify the underlying gene mutations in a cohort of patients with no apparent laboratory platelet defect despite having an extensive bleeding history. Furthermore, we will explore the use of a platelet spreading assay in detecting platelet defects that may not otherwise be detected.

The overarching aim is to elucidate disease mechanisms in novel inherited bleeding disorders. The objectives are as follows:

1. To use a forward genetic approach to identify genes and pathways in patients with inherited bleeding disorders that are characteristic of a defect in platelet function but where laboratory testing has not identified a defect in platelet function.
2. To determine whether an assay to test the ability of activated platelets to spread is a useful to investigate suspected platelet function defects.
3. To determine how a proportion of these novel disease-causing mutations impair platelet, endothelial cell or coagulation using *in vitro* models.

CHAPTER 2
Materials and Methods

2. Materials and Methods

2.1. Materials

All agonists, antibodies, software, and databases used in this thesis are listed in Tables 2.1, 2.2, 2.3 and 2.4 respectively. If unstated, reagents used in this thesis are from Sigma-Aldrich™, UK and all plastics were purchased from StarLab, UK.

2.2. Study approval

The Genotyping and Phenotyping of Platelets (GAPP) study was approved by the UK National Research Ethics Service by the Research Ethics Committee of West-England (REC reference: 06/MRE07/36) and participants gave full, written, informed consent in accordance with the Declaration of Helsinki. The GAPP study is included in the National Institute of Health Research Non-Malignant Haematology study portfolio (ID9858). This study is also registered at ISRCTN (https://www.isrctn.com/) as ISRCTN77951167.

Table 2.1. Platelet agonists for light transmission aggregometry

Agonist	Concentrations used	Source
ADP	3, 10, 30 and 100 µM	Sigma-Aldrich™, UK, #A2754
Adrenaline	3, 10 and 30 µM	Sigma-Aldrich™, UK, #E4375
Arachidonic acid	0.5, 1 and 1.5 mM	Cayman Chemical Company, USA, #10006607
Collagen	1, 3 and 10 µg/ml	Takeda, Japan, #1130630
Collagen-related peptide, cross-linked	1 and 3 µg/ml	Provided by Dr Richard Farndale, University of Cambridge, #CRP-XL
PAR-1 peptide	30 and 100 µM	Severn Biotech, UK, custom product
Ristocetin	1.5 and 2 mg/ml	Sigma-Aldrich™, UK, #R7752
U46619	1 and 3 µM	Cayman Chemical Company, USA, #16450

Table 2.2. Antibodies

Antibodies	Host Species	Source	Working concentration/dilution
Primary antibodies			
Anti-GAPDH	Rabbit polyclonal	Abcam, UK, #ab9485	WB: 1/5,000
Anti-PolyG	Rabbit	Gifted from Koji Ikegami, Hamamatsu School of Medicine, Japan	WB: 1/2000 IF: 1/200
Anti-TTLL10 1	Guinea Pig	Gifted from Koji Ikegami, Hamamatsu School of Medicine, Japan	WB:1/5,000-10,000
Anti-TTLL10 2	Rabbit polyclonal	Proteintech, USA, #26014-1-AP	WB: 1/1000
Anti-TTLL10 3	Rabbit polyclonal	Thermo Fisher Scientific, USA, #PA5-31631	WB: 1/1000
Anti-TTLL10 4	Rabbit polyclonal	OriGene Technologies, USA, #TA332190	WB: 1/1000
Anti-α-Tubulin	Mouse monoclonal	Sigma-Aldrich®, UK, #T6199	WB: 1/1,000 IF: 1/200
Phalloidin (F-actin)	Alexa Fluor®488-conjugated Phalloidin	Thermo Fisher Scientific, USA, #A12379	IF: 1/300
Secondary antibodies			
Donkey anti-Rabbit IgG	Donkey, HRP-conjugated	GE Healthcare, UK, #NA934	WB 1:5,000-10,000
Sheep anti-Mouse IgG	Sheep, HRP-conjugated	GE Healthcare, UK, #NA931	WB 1:10,000
Goat anti-Guinea Pig IgG	Goat, HRP-conjugated	Bethyl Laboratories, USA, #A60-210P	WB 1:5,000
Goat anti-Rabbit IgG	Goat, Alexa Fluor® 488-conjugated	Thermo Fisher Scientific, USA, #A11008	IF 1:250
Goat anti-Rabbit IgG	Goat, Alexa Fluor® 647-conjugated	Thermo Fisher Scientific, USA, #A21244	IF 1:250
Goat anti-Mouse IgG	Goat, Alexa Fluor® 647-conjugated	Thermo Fisher Scientific, USA, #A21235	IF 1:250

Table 2.3. Software used throughout this project

Software	Supplier
AGGRO/LINK® Interface	Chrono-Log, USA
Chromas	Technelysium, Australia
ExonPrimer	Helmholtz Center Munich, Germany
GeneSnap	Syngene, USA
Ilastik	Heidelberg Collaboratory for Image Processing- University of Heidelberg, Germany
ImageJ	Optical and Computational Instrumentation- University of Wisconsin-Madison, USA
KNIME	KNIME, Switzerland
Mutation Surveyor®	Softgenetics®, USA
Mutation Taster	Charité, Germany
Novoalign	Novocraft Technologies, Malaysia
Polymorphism Phenotyping v2	BWH- Harvard Medical School, USA
Primer3	Whitehead Institute for Biomedical Research, USA
Protein Variation effect analyser	J. Craig Venter Institute, USA
SAMtools	Wellcome Trust Sanger Institute, Cambridge
SoftMax® Pro	Molecular Devices, USA
Sorting Intolerant from Tolerant	J. Craig Venter Institute, USA

Table 2.4. Databases used throughout this project

Database	URL	Date last accessed
1000 Genomes Project	http://www.internationalgenome.org/1000-genomes-browsers/	21 Sep. 19
BDGP	http://www.fruitfly.org/	21 Sep. 19
Blueprint Progenitors	https://blueprint.haem.cam.ac.uk/admin/query_genes	21 Sep. 19
ClinVar	https://www.ncbi.nlm.nih.gov/clinvar/	21 Sep. 19
dbSNP135	https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi	21 Sep. 19
Ensembl	https://www.ensembl.org/index.html	21 Sep. 19
EVS	http://evs.gs.washington.edu/EVS/	21 Sep. 19
ExAC	http://exac.broadinstitute.org/	21 Sep. 19
GAPP Database	https://collaborate.bham.ac.uk/mds/gapp/SitePages/Home.aspx	21 Sep. 19
HGMD	http://www.hgmd.cf.ac.uk/ac/index.php	21 Sep. 19
Haem Atlas	https://haemgen.haem.cam.ac.uk/haematlas/	21 Sep. 19
The Human Protein Atlas	https://www.proteinatlas.org/	21 Sep. 19
UCSC Genome Browser	https://genome.ucsc.edu/	21 Sep. 19

2.3. Patient recruitment

Patients have been recruited to the GAPP study since the study first opened to patients, in 2006. Since then, nearly 1000 patients have been successfully recruited from multiple collaborating Haemophilia Centres across the UK and Ireland. Recruitment of patients to the study is based on a diagnosis of bleeding or thrombocytopenia of unknown cause as defined by the International Society of Thrombosis and Haemostasis (ISTH) Bleeding Assessment Tool (BAT) by a specialist Haematologist. Patients were deemed thrombocytopenic if their platelet count was < 150 $x10^9$/l in whole blood. Owing to the likely inheritance patterns of such bleeding disorders and thrombocytopenias, if the recruits had potentially affected family members, the relatives were also recruited and where appropriate, genetic pedigrees were taken.

Selection for recruitment to the GAPP study required the patients to meet certain inclusion criteria. Patients who fulfilled all criteria and who were consented in writing by signing the consent form (Appendix, Figure B), were invited to participate. Any patients who fulfilled one or more of the exclusion criteria were not invited to participate in the study.

Inclusion criteria:

a. Aged 0-85 years
b. Bleeding episodes in line with a platelet function disorder
c. Normal coagulation factor screen results
d. Absence of evidence suggesting acquired platelet dysfunction

Exclusion criteria:

a. Recently undergone major surgery
b. Suffer from chronic renal failure requiring dialysis
c. Severely anaemic (Hgb <8 g/dl)
d. Recently received any blood products
e. Receiving any known platelet function affecting drugs

Patients were also excluded if they had a known cause of their bleeding or thrombocytopenia. Routine clinical screening tests from each referring centre differed; however, usually consisted of full blood cell count, peripheral blood smear, prothrombin time (PT) and activated partial thromboplastic time (APPT). From these tests, the most common causes of bleeding were excluded. Peripheral blood smears allowed platelets to be visualised. Large platelets with leukocyte inclusions (indicative of MYH9-related disorder) or grey platelets (Gray Platelet Syndrome) often lead to definitive diagnoses that were followed up with genetic tests if offered. Non-platelet bleeding based disorders including coagulation factor deficiencies and von Willebrand's Disease were also diagnosed and these patients were not invited to participate in the study. Furthermore, patients were excluded if they were suspected to have idiopathic thrombocytopenic purpura (ITP) due to an autoimmune cause of disease.

2.4. Healthy controls

Healthy donor volunteers were required to be over the age of 18 years and physically well in the run up to and time of donation. The healthy donor volunteers should not take any known platelet disrupting drugs e.g. aspirin or other non-steroidal anti-inflammatory drugs (NSAIDs) in the two weeks before donation. Donors were unable to give blood to the NHSBT Blood Donation service up to two weeks before taking part in the study.

2.5. Sample collection

Peripheral blood was collected simultaneously from patient and healthy donor volunteers where possible. If this was not possible, control blood from a healthy donor volunteer was collected at the site of blood analysis within similar time points as the patient blood collection. The blood was transported by courier at room temperature from the referring centre to the site of analysis in Birmingham. Previous investigations into the blood transport by members of the GAPP Study found that results from blood samples transported in this way were highly comparable to blood samples collected locally (Dawood et al., 2007).

For adult patients, approximately 40 ml blood was collected in 3.2% trisodium citrate by venepuncture. For paediatric patients (<18 years) up to 20 ml blood was taken, depending on the age and weight of the patient, into 3.2% trisodium citrate by venepuncture. Blood was usually collected into BD Vacutainer® 2.7 ml citrate blood collection tubes (BD, UK, #363083) but collection varied occasionally due to different protocols within the referring centres. Control samples taken at the site of analysis were collected by venepuncture into 4% citrate concentrated solution (Sigma-Aldrich™, UK, #S5770) at a ratio of 1:9 (v:v), citrate to blood volume. A further 4 ml of ethylenediaminetetraacetic acid (EDTA) anticoagulated blood was taken from both patients and healthy donor volunteers for whole blood cell counts. Blood was collected into BD Vacutainer® blood collection tubes, coated in spray dried K2EDTA (BD, UK, #367835), by venepuncture.

2.6. Platelet counts in EDTA-anticoagulated blood

Whole blood cell counts were obtained from analysing EDTA anticoagulated blood on the Sysmex XN-1000™ Haematology analyser (Sysmex, UK). The XN-Series offers high diagnostic capabilities by using fluorescent flow cytometry to determine highly accurate whole blood cell

counts. The instrument has five core channels that provide readings of almost all components of the blood including white blood cells, basophils, nucleated red blood cells and haemoglobin. The most important channel for this project was the PLT channel that solely measured different platelet parameters. Within this channel there are three methods of obtaining platelet counts: platelet fluorescence (PLT-F), platelet impedance (PLT-I) and platelet optical (PLT-O). The PLT-F method required the fluorescent RNA staining dye, oxazine, which improved current methods due to its increased ability to differentiate between platelets and apoptotic white cells that other analysers were unable to do previously. Platelet counts were checked against the PLT-I and the PLT-O results and the data was collated to provide the overall platelet count. Mean platelet volume (MPV) and immature platelet fraction (IPF), a measure of platelet production, were also recorded within the PLT channel. A normal range record was determined by a study of analysis of blood from 40 healthy control volunteers in collaboration with Sysmex. Blood from both patients and controls recruited to the GAPP study was analysed in this way. Daily quality control was carried out with control blood (XN-CHECK [Sysmex, UK]) to ensure instrument performance throughout the study. An example of the Sysmex analysis and full blood count can be seen in Figure 2.1.

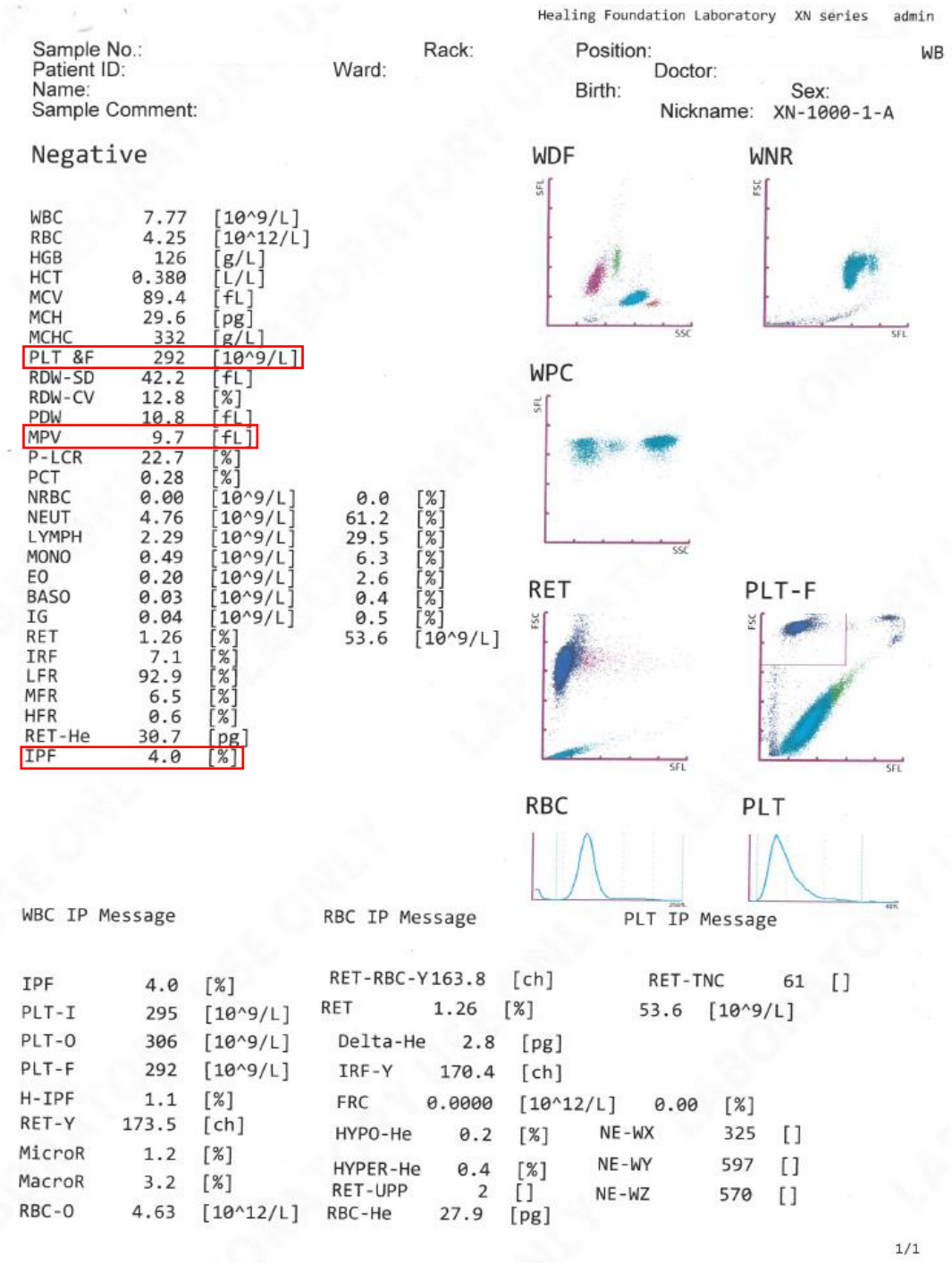


Figure 2.1. Full blood count using the Sysmex XN-1000™ Haematology analyser. This analysis shows that all components are within the normal ranges however; this read is for research purposes only. The parameters highlighted in red are of most interest when receiving new patient GAPP samples in order to process and filter the samples correctly.

PLT, platelet count; MPV, mean platelet volume; IPF, immature platelet fraction.

2.7. Preparation of whole citrated blood

Platelet rich plasma (PRP) was prepared for platelet function testing by LTA and lumi-aggregometry. PRP was obtained by slow centrifugation of whole blood at 200 g for 20 minutes at room temperature using the Heraeus™ Megafuge™ 16R centrifuge (Thermo Fisher Scientific™, USA). If a patient was suspected to have macrothrombocytopenia (MPV > 13 fl), PRP was prepared by centrifugation at 100 g for 30 minutes to retain the large platelets within the PRP layer. The PRP was removed and added to labelled 15 ml Falcon™ tubes. Platelets were then counted; 5 µl of PRP was added to 10 ml COULTER® ISOTON® II Diluent (VWR, USA, #8448011) and the platelets counted three times using the Z™2 Series COULTER COUNTER® (Beckman Coulter®, USA) and the counts averaged. The remaining blood was centrifuged again at 1000 g for 10 minutes to obtain platelet poor plasma (PPP) for use as a reference and to set the aggregation scale during LTA and lumi-aggregometry. The PPP was removed and added to labelled 15 ml Falcon™ tubes.

2.8. Preparation of washed platelets

Washed platelets were prepared for platelet spreading assays in this thesis. 50 ml Tyrode's buffer (134 mM NaCl, 2.9 mM KCl, 10 mM HEPES, 12 mM NaHCO3, 0.34 mM NA2HPO4, 1 mM $MgCl_2$, [all reagents available from Sigma-Aldrich™, UK]) was warmed in a 37 °C water bath along with 10 ml acid-citrate-dextrose (ACD) (39mM citric acid, 75 mM sodium citrate, 135 mM dextrose [all reagents available from Sigma-Aldrich™, UK]). 45 mg of glucose was added to make modified Tyrode's buffer and the pH was adjusted to 7.3. Blood was taken into sterile 4% citrate concentrated solution (Sigma-Aldrich™, UK, #S5770) as an anticoagulant at 10% of the desired blood volume. 10% of the blood volume in ACD was added to the blood-anticoagulant mixture and transferred to polypropylene tubes. The anticoagulated

blood was then centrifuged at 200 g for 20 minutes at room temperature using the Heraeus™ Megafuge™ 16R centrifuge (Thermo Fisher Scientific™, USA). Platelet rich plasma (PRP) was pipetted off and added to a 50 ml Falcon™ tube. 10 µl prostaglandin I2 (PGI_2) (10 µl of stock solution at 1 mg/ml [Cayman Chemical Company, USA, #18220]) per 10ml PRP was added and the tube gently inverted to mix before centrifuging at 1000 g for 10 minutes. The supernatant was removed and discarded and the pellet was resuspended in 1 ml warmed modified Tyrode's buffer. Another 24 ml of modified Tyrode's buffer and PGI_2 were added and the tube was centrifuged at 1000g for 10 minutes to wash and pellet the platelets. This process was repeated once more. The supernatant was then removed, discarded and the platelet pellet gently resuspended in 1 ml modified Tyrode's buffer before a further 4 ml was added.

In order to count the platelets, 5 µl of washed platelets was added to 10 ml COULTER® ISOTON® II Diluent (VWR, USA, #8448011) and the platelets counted three times using the Z™2 Series COULTER COUNTER® (Beckman Coulter®, USA) and an average value calculated. The platelet solution was diluted to a final concentration of $2x10^7$ /ml with modified Tyrode's buffer and allowed to rest for 20-30 minutes before spreading.

2.9. Platelet function testing- LTA with lumi-aggregometry

LTA with lumi-aggregometry was carried out on all patients and controls with a platelet count of > 1 $x10^8$/ml in PRP as described and published previously by members of the GAPP study (Dawood et al., 2012, Dawood et al., 2007). LTA for the GAPP study was firstly performed by Dr Gill Lowe and Miss Sian Drake but was later performed by Dr Rashid Al Ghaithi.

Patient and control PRP were processed using the same method and agonist conditions; LTA was carried out within 6 hours of the PRP preparation. All aggregations were preformed using a dual channel Chrono-log 460 VS Lumi-Aggregometer (Chrono-Log, USA). Briefly, this

technique, first described by Born, measures light transmission through the PRP platelet suspension before and after the addition of different agonists (Born, 1962). These agonists cause platelet aggregation therefore permitting greater light transmission through the solution that is detected by a photocell and recorded as a function of time. LTA now has the capacity to simultaneously measure ATP secretion from platelet dense granules using the luciferase assay (Holmsen et al., 1972).

400 µl of the PRP samples was added to siliconized mini glass cuvettes and incubated at 37 °C for 2 minutes. This was followed by a further 1 minute under stirring conditions (1200 rpm) to set the baseline; the PPP was also used to set the aggregation scale. The platelets were then stimulated with a range of concentrations of different agonists (

Table 2.1. Platelet agonists for light transmission aggregometry). Platelet aggregation was measured for 5 minutes after the addition of the agonists and the maximal percentage of aggregation was recorded using the AGGRO/LINK® Interface (Chrono-Log, USA). Utilisation of the full panel of agonists depended on the individual patient responses. If the aggregation traces were abnormal, stimulation of PRP to that concentration of agonist was repeated. Furthermore, if responses to ADP or arachidonic acid were abnormal, further aggregation experiments were carried out in response to varying concentrations of U46619, the synthetic thromboxane A_2 receptor agonist.

Dense granule release was measured by detecting ATP secretion by lumi-aggregometry. 30 µl of Chronolume® (Chrono-Log, USA, #395) containing D-luciferin was added to PRP samples during the 37 °C incubation stage for 1 minute. Platelets were then stimulated with the agonists and aggregation measured for 5 minutes, as previously mentioned. At the start of the experiment, an internal calibration was carried out to allow quantification of ATP secretion by

the addition of a 2 nM ATP standard (Chrono-Log, USA) for each channel. The AGGRO/LINK® Interface (Chrono-Log, USA) calculated total ATP released automatically.

2.10. Sample storage- protein and buffy coat

Any patient PRP remaining after lumi-aggregometry was saved and processed for storage of platelet protein. Depending on the platelet count of the patient and control samples, the volumes of PRP containing 3 $\times 10^8$ platelets were removed from the samples. These were then added to separate, labelled 1.5 ml Eppendorf tubes. 1 µl of PGI_2 (1 mg/ml [Cayman Chemical Company, USA, #18220]) was added to the PRP samples and inverted gently to mix. The samples were immediately centrifuged at 1000g for 10 minutes in the Heraeus™ Pico™ 21 centrifuge (Thermo Fisher Scientific™, USA). The supernatants were carefully removed by aspiration to avoid disruption of the platelet pellets. The pellets were then resuspended in 1 ml of phosphate buffered saline (PBS, [Sigma-Aldrich™, UK, #P4417]). 1 µl of PGI_2 was added to the tubes, inverted gently to mix, and centrifuged at 13000 rpm for 2 minutes. The supernatant was removed and the remaining cell pellets were resuspended in 600 ml of 1 x sample buffer (60 mM Tris-HCl pH 6.8 [Sigma-Aldrich™, UK, #T6455] , 2% sodium dodecyl sulphate [SDS, Sigma-Aldrich™, UK, #L3771], 10% glycerol [Sigma-Aldrich™, UK, #G5516], 0.002% Bromophenol Blue, 5% β-mercaptoethanol [Sigma-Aldrich™, UK, #M6250]). Samples were stored at -80 °C until needed.

After the PPP was removed from the blood, the visible white layer separating the red cell layer, known as the buffy coat, was also removed by pipetting and transferred to 1.5 ml, labelled Eppendorf tubes. The buffy coat contains white blood cells that can be used in subsequent experiments involving DNA extraction. Only the patient buffy coats were stored at -80 °C until needed.

2.11. Preparation of platelet lysates from washed platelets

Platelet lysates were made from particular samples for platelet protein analysis. 10 ml of extraction buffer was prepared from 2 x lysis buffer (300 mM NaCl, 20 mM Tris, 2 mM EDTA, 2 mM EGTA and 1% Nonidet p-40 [NP-40, Sigma-Aldrich™, UK, #I8896]). Protease inhibitors, all on ice, were then added (250 µl sodium orthovanadate [Sigma-Aldrich™, UK, #450243], 100 µl leupeptin [Enzo Life Sciences, USA, #260-009-M100], 10 µl aprotinin [Sigma-Aldrich™, UK, #A1153], 4 µl pepstatin [Sigma-Aldrich™, UK, #P5318] and 100 µl AEBSF [Calbiochem, UK, #101500]). An equal volume of extraction buffer was added to platelets and was incubated on ice for 40 minutes, vortexing every 10 minutes to aid lysis. Tubes containing buffer and lysed platelets were then centrifuged at 13,000 rpm for 20 minutes at 4 °C using the Refrigerated Microcentrifuge Model 5417R (Eppendorf®, USA). The supernatant containing the protein was removed and transferred to new, labelled tubes.

2.12. DNA extraction from buffy coat and whole blood

DNA was extracted from buffy coats or whole blood using the Gentra Puregene Blood DNA Extraction Kit (Qiagen, UK, #158389) according to the standard protocol. Blood was added 1:3 (v:v) to RBC Lysis Solution and incubated for 10 minutes, inverted once during incubation. This was then centrifuged at 2000g for 10 minutes using the Heraeus™ Megafuge™ 16R centrifuge (Thermo Fisher Scientific™, USA). The supernatant was removed and discarded and tube vortexed at high speed to resuspend pellet in residual liquid. 3 ml Cell Lysis Solution was added to the resuspended pellet and tube inverted to aid lysis. The solution was incubated overnight at 37 °C if cell clumps were still visible. When the solution was homogenous, 15 µl RNase A (Sigma-Aldrich™, UK, #R6148) was added, tube inverted to mix and incubated at 37 °C for 30 minutes.

The sample was cooled to room temperature and 3 ml Protein Precipitation Solution was added. This was vortexed at high speed for 20 seconds and centrifuged at 2000 g for 10 minutes to pellet the precipitated protein.

The supernatant containing the DNA was transferred into a clean, labelled 15 ml Falcon™ tube. 3 ml 100% isopropanol (Fisher Scientific, USA, #389710025) was added and sample mixed by inverting then centrifuged at 2000 g for 3 minutes. The supernatant was removed and discarded and 3 ml 70% ethanol (EtOH) (VWR, USA, #20821.33) added to wash DNA pellet. The tube was inverted and centrifuged at 2000 g for 1 minute to re-pellet the DNA. Again, the EtOH was poured off and pellet left to air dry with tube inverted on paper towel. Once dry, 100–250 µl DNA Hydration Solution was added depending on the size of the DNA pellet and hence quantity of DNA present. The mixture was vortexed for 10 seconds and solution incubated overnight at 55 °C to dissolve the DNA pellet.

DNA was stored at -80 °C until needed. To avoid excess freeze thawing, DNA samples used regularly were aliquoted.

2.13. DNA quantification

The concentration of DNA for whole exome sequencing was determined using the Qubit® 3.0 Fluorometer (Life Technologies™, USA) following the standard protocols and using the Qubit® reagents. The Qubit® dsDNA Broad Range (BR) Assay kit (Thermo Fisher Scientific, UK, #Q32850) was used to make up the Qubit® working solution. Either the standards (10 µl) or samples (3 µl) were added to the working solution to give a total volume of 200 µl in 500 µl thin-walled polypropylene Qubit® Assay Tubes (Invitrogen, UK, #Q32856). The mixes were briefly vortexed and incubated at room temperature for 2 minutes before being centrifuged

to collect the contents. The standards for the dsDNA BR Assay were run before samples were read and the DNA concentrations recorded.

DNA for any other use was quantified using the NanoPhotometer® P360 (IMPLEN, Germany). The NanoPhotometer® P-Class Submicrolitre Cell was loaded into the cell holder. The well was blanked with 1 µl of water before samples were measured. Samples were vortexed and 1 µl was loaded into the well to be tested; the well was cleaned, and this was repeated three times. The average DNA concentration was calculated, and 260/280 ratio was recorded as an indicator of the sample quality.

2.14. Whole exome sequencing

Whole exome sequencing is a commonly used technique and classed as next generation sequencing technology. The exome (the protein coding regions of the genome) makes up approximately 1.5% of the human genome but contains around 85% of known disease-related variants. WES employed by the GAPP study is therefore a more cost-effective and rapid alternative to whole genome sequencing. Confirmatory Sanger sequencing is usually carried out post-WES.

The whole exome sequencing for the GAPP study was carried out externally by a collaborating group headed by Professor Michael Simpson from King's College, London, until 2016. After this time, the whole exome sequencing for the GAPP study was carried out by collaboration with Dr Yvonne Wallis from the West Midlands Regional Genetics service at Birmingham Women's Hospital.

A minimum of 1µg of DNA was required per patient for successful WES. The DNA was transported to the sequencing centre in sealed 1.5 ml Eppendorf tubes at room temperature. Enrichment of the coding regions and intron/exon boundaries was then completed with the

SureSelect Human All Exon kit (Agilent Technologies, USA) according to the manufacturer's instructions. Following this, WES was carried out using the HiSeq® 2500 Sequencing System (illumina®, USA) with 100 bp paired-end reads. Sequencing reads were then aligned to the human reference genome (hg19) by applying the Needleman-Wunsch algorithm using NovoAlign (Novocraft Technologies, Malaysia). Duplicate reads and reads that mapped to multiple regions of the exome were excluded from the bioinformatic analysis. Small insertions, deletions, and single nucleotide variants were identified and filtered for quality by the SAMtools software (Wellcome Trust Sanger Institute, Cambridge) (Li et al., 2009). Furthermore, any calls with a read coverage of < 4 were again excluded from downstream bioinformatic analysis.

The remaining variants were then filtered for novelty by comparison to known variants present in a number of databases including: dbSNP139 that now contains over 40 million validated human reference single nucleotide polymorphism clusters, the 1000 Genomes Project, Exon Variant Server (EVS) and the variants from the GAPP database of over 800 whole exome sequences.

Post alignment and variant called sequencing bioinformatics was approached in a manner unique to the GAPP study and will be discussed in Chapter 4.

2.14.1. Pathogenicity prediction and conservation

The potential pathogenicity of each variant was analysed using five main pathogenicity prediction tools that use different methods to assess whether a variant will affect the protein's functionality. The prediction tools are as follows:

1. MutationTaster2 assesses the results of WES by a Naive Bayes classifier to predict the disease potential of a variant by deciding whether the effect might be deleterious.

MutationTaster2 can predict the effects of frameshift and non-frameshift insertions and deletions, SNPs and splice-site changes. Data of common polymorphisms and known disease causing mutations are included and if these variants are present, they are described automatically (Schwarz et al., 2014).

2. Polymorphism Phenotyping version 2 (PolyPhen-2) calculates the probability of an amino acid substitution being damaging to the protein structure therefore affecting its function. This prediction is dependent on the sequence, the phylogenetic and structural information of the substitution. PolyPhen-2 will extract the above features of the substitution location and compute them to a probabilistic classifier (Adzhubei et al., 2013). For this study, the HumVar model was used, which consists of all human disease causing mutations from UniProtKB along with common SNPs not involved in disease.
3. Sorting Intolerant From Tolerant (SIFT) is based on the degree of conservation within the amino acid residues under question in sequence alignments derived from closely-related sequences. SIFT assumes that important positions are highly conserved in a protein sequence throughout evolution and therefore substitutions within these regions may affect protein function (Kumar et al., 2009). This allows a prediction of whether an amino acid substitution will be tolerated or whether it will be deleterious to the protein function based on the sequence homology.
4. Protein Variant Effect Analyser (PROVEAN) predicts the functional effect of non-synonymous amino acid substitutions as well as insertions and deletions by using an alignment-based score. The change between the variant sequence and the reference sequence homology is measured and those variants scoring below the neutral

threshold (-2.5) are deemed deleterious, any scoring above the threshold are deemed neutral therefore, have no effect (Choi et al., 2012).

5. Splice site variants were analysed using the Berkeley Drosophila Genome Project that works to predict whether donor or acceptor splice sites are altered by such variants.

The levels of mRNA expression of each of the candidate genes in the different haematopoietic cells was also noted using the Blueprint Progenitors database along with RNA-seq data from the human and mouse platelet transcriptomes study (Rowley et al., 2011).

Conservation at the site of the variant in query is determined by PhastCons and PhyloP- both available from MutationTaster (Pollard et al., 2010). MutationTaster uses precomputed values produced by UCSC. PhastCons values range between 0 and 1 to reflect the probability of a nucleotide belonging to a conserved region, based on the multiply aligned sequences of over 46 species. The closer the value is to 1, the more likely the variant is conserved. PhyloP in contrast to phastCons, measures conservation without considering the effects of neighbouring nucleotides. PhyloP values range between -14 and 6, positive scores were assigned to sites predicted to be conserved while negative scores were assigned to sites predicted to be fast evolving.

2.14.2. Variant classification

Recently, there have been standards and guidelines published for the interpretation of sequence variants as recommended from the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) (Richards et al., 2015). The genetic variants from WES of the GAPP study patients were classified in this way. Dominant inheritance was assumed for all patients unless the family histories obtained from the patients suggested otherwise. The report recommends the use of specific consensus

terminology – ‘pathogenic’, ‘likely pathogenic’, ‘uncertain significance’, ‘likely benign’ and ‘benign’ to describe variants. Candidate variants identified in possible disease causing genes were classified based on evidence for a set of criteria and the strength of the criteria (Richards et al., 2015). The classification of each variant was applied when predicting the pathogenicity of each variant.

2.15. Polymerase chain reaction

All PCRs were performed according to a standardised laboratory protocol. Each reaction consisted of a total 25 µl containing: 12.5 µl REDTaq® ReadyMix™ PCR Reaction Mix (DNA polymerase) (Sigma-Aldrich®, UK, #R2523), 0.5 µl of forward primer, 0.5 µl of reverse primer both at a concentration of 10 µM in ddH_2O, 6.5 µl ddH_2O (Sigma-Aldrich®, UK, #R2523) and 5 µl DNA at 20 ng/µl. All the primer pairs were designed using ExonPrimer and purchased from Sigma-Aldrich®, UK.

The universal cycling conditions of denaturation, annealing and elongation were conducted under the following conditions in the DNA Engine Tetrad® 2, Peltier Thermal Cycler (Bio-Rad, USA):

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

  x 30 cycles
- 72 °C for 5 minutes

Results from the PCR were visualised using agarose gel electrophoresis. The gel was 0.0001% ethidium bromide (Sigma-Aldrich®, UK, #E8751-1G) stained in 1.5% Ultrapure™ Agarose

(Invitrogen™, UK, #16500-500) and TAE (40 mM Tris, 20mM acetic acid and 1 mM EDTA). All gels were loaded with 10 µl of 1 Kb DNA ladder (Invitrogen, UK, #1078-018) and run at 120 V using a suitable power pack for 20-60 minutes. The gel was imaged with an ultraviolet transilluminator (Syngene, Gene Genius Bio Imaging System) and GeneSnap software version 6.03.00 (Synoptics, UK).

2.16. Sanger sequencing

DNA sequencing has been revolutionising medical research since the invention of Sanger sequencing by Sanger *et al*. in 1977 and was the most used sequencing method for 40 years (Sanger et al., 1977). Sanger sequencing was used to verify WES candidate variants identified.

The PCR products were cleaned before Sanger sequencing was carried out. 2.4 µl of PCR product, both for forward and reverse reads, were transferred to a clean plate. 2.4 µl of microCLEAN solution (Microzone, UK, #2MCL) was added to each well using the Multipette® Stream multi-pipette (Eppendorf, Germany). The plate was centrifuged at 4,000 rpm for 40 minutes in the Universal 320R centrifuge (Hettich®, Germany). The plate was then turned upside down and centrifuged at 500 rpm for 30 seconds.

Sequence amplification occurred on the dry pellet present in each well following the microclean step. Each reaction consisted of a total of 10 µl containing: 0.5 µl BigDye™ Ready Reaction Mix, 2 µl BigDye® Terminator 5x Sequencing Buffer (BigDye™ Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems™, USA, #4337455), 0.4 µl forward or reverse primer both at 10 µM and 7.1 µl ddH_2O. The plate was vortexed and briefly centrifuged. The universal cycling sequencing conditions of denaturation, annealing and elongation were conducted under the following conditions in the DNA Engine Tetrad® 2, Peltier Thermal Cycler (Bio-Rad, USA):

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

    } x 30 cycles
- 4 °C for ∞

The sequencing reactions were cleaned-up using 2x EtOH washes. Firstly, 2 µl 0.125 M EDTA (Sigma-Aldrich™, UK, #E7889) and 30 µl 100% EtOH (VWR, USA, #208121.33) were added to each reaction well. The plate was covered and centrifuged at 2,000 rpm for 20 minutes then turned upside down and centrifuged at 500 rpm for 30 seconds. 90 µl of freshly made 70% EtOH diluted in dH_2O were added to each reaction well. The plate was then centrifuged at 2000 rpm for 10 minutes and again turned upside down and centrifuged at 500 rpm for 30 seconds. The pellets were left to air dry until no EtOH was visible in the wells.

10 µl of Hi-Di™ Formamide (Thermo Fisher Scientific™, USA, #4311320) was added to each well and pipette mixed to ensure the DNA pellet was resuspended. A new adhesive film sealed the plate and the plate was placed in DNA Engine Tetrad® 2, Peltier Thermal Cycler (Bio-Rad, USA) at 94 °C for 2 minutes to denature the samples. Immediately after finishing, the plate was placed on ice to snap chill in order to stop single stranded DNA from re-annealing.

The plate was then sequenced by fragment separation by capillary electrophoresis using the ABI 3730XL Automated Sequencer (Applied Biosystems™, USA) at the Core Sequencing Facility, Functional Genomics at the School of Biosciences, University of Birmingham. The sequences were analysed by Mutation Surveyor® (Softgenetics®, USA) that aligned the sequences against reference sequences. Chromas Version 2.4 (Technelysium, Australia) sequence chromatogram view was also used. Sequencing traces with high levels of

background signal required repeat sequencing. Furthermore, unsuccessful Sanger sequencing usually required repeat sequencing, repeat amplification and sequencing or complete redesign of sequence primers and repetition of the whole process.

2.17. Preparation of lysate from cell culture and lysates from tissues

Cell lysates were prepared from cell culture for western blot analysis to detect specific proteins in the samples. Adherent cells were washed with sterile PBS (Gibco™, UK, #12549079). The PBS was then aspirated and ice cold lysis buffer (300 mM NaCl, 20 mM Tris, 2 mM EDTA, 2 mM EGTA and 1% Nonidet p-40 [NP-40, Sigma-Aldrich™, UK, #I8896]) with protease inhibitors (250 µl sodium orthovanadate [Sigma-Aldrich™, UK, #450243], 100 µl leupeptin [Enzo Life Sciences, USA, #260-009-M100], 10 µl aprotinin [Sigma-Aldrich™, UK, #A1153], 4 µl pepstatin [Sigma-Aldrich™, UK, #P5318] and 100 µl AEBSF [Calbiochem, UK, #101500]) was added to the cell flask. The cells were then scraped using a plastic cell scraper. The cell suspension was transferred to a 15 ml Falcon™ tube and incubated on ice for 30 minutes vortexing every 10 minutes to increase cell lysis. The cell suspension was then centrifuged at 4 °C using the Heraeus™ Megafuge™ 16R centrifuge (Thermo Fisher Scientific™, USA) at 12000 rpm for 20 minutes. The supernatant was removed and placed in a new 15 ml Falcon™ tube and kept on ice.

Preparation of lysates from tissues was carried out in largely the same manner. The tissue of interest was dissected from wild-type mice by Dr Sian Lax and placed on ice to avoid tissue degradation by proteases. For a 5 mg piece of tissue 300 µl of ice-cold lysis buffer was added to the 1.5 ml Eppendorf tube containing the tissue. The tissue was homogenised either by an electric homogeniser or by sonication using the Ultrasonic processor, Vibra-Cell™ (Sonics and Materials, USA). For sonication, the probe was first rinsed in lysis buffer and the power was

set to 180 W. Sonication was carried out in rounds of 10 seconds with 10 second breaks to avoid sample warming and continued until the tissue was no longer visible. The sample was then incubated on ice for 30 minutes vortexing every 10 minutes to increase cell lysis. The tissue lysate was then centrifuged at 13,000 rpm for 20 minutes at 4 °C using the Refrigerated Microcentrifuge Model 5417R (Eppendorf®, USA). The supernatant containing the protein was removed and transferred to new, labelled tubes kept on ice.

A Bradford assay was carried out on all cell and tissue lysates before western blotting; this is a colourimetric assay used to measure the concentration of protein in the solution. The DC Protein Assay kit (Bio-Rad, USA, #5000111) was used for all Bradford assays and carried out according to the manufacturer's instructions. This assay was carried out in 96 well, flat-bottom plate and absorbances were read at 750 nm using the VersaMax Microplate Reader (Molecular Devices, USA). The results were collected by SoftMax® Pro software, version 6.3 (Molecular Devices, USA). The concentration of the protein was determined by the generation of a standard curve using Microsoft® Excel. The proteins were adjusted and the appropriate amount of sample buffer (60 mM Tris-HCl pH 6.8 [Sigma-Aldrich™, UK, #T6455] , 2% sodium dodecyl sulphate [SDS, Sigma-Aldrich™, UK, #L3771], 10% glycerol [Sigma-Aldrich™, UK, #G5516], 0.002% Bromophenol Blue , 5% β-mercaptoethanol [Sigma-Aldrich™, UK, #M6250]) was added to the sample. Samples were stored at -80 °C until needed.

2.18. Western blot analysis

Western blot is a widely used technique that aims to resolve and detect specific proteins in a sample of interest. The samples used for western blots in this thesis include cell lysates, tissue lysates, and stored platelet protein or platelet lysates depending on availability.

Before use, samples are boiled at 105 °C for five minutes to be denatured. Precast polyacrylamide 10 and 12 well Bolt™ Bis-Tris Plus gels (Invitrogen, UK, #NW04120BOX and #NW04122BOX) were loaded into Bolt™ Mini Gel Tanks (Invitrogen, UK, no longer in production). 20X Bolt™ MES SDS Running Buffer (Thermo Fisher Scientific, USA, #B000202) diluted to 1x in dH_2O was added to the gel tank up to the fill line. 5 µl of Colour Prestained Protein Standard, Broad Range (11-245 KDa) (New England BioLabs, USA, #P7712) was added to the first well of the Bolt™ Bis-Tris Plus gel in order to observe protein separation, verify transfer and approximate protein size. 20 µl of denatured sample were then added to the remaining wells. The Bolt™ Mini Gel Tanks were attached to a suitable power pack and run at 60 V for 10 minutes followed by 120 V for 1–2 hours depending on the size of the protein of interest.

The protein bands were transferred from the polyacrylamide gels to low fluorescence polyvinylidene difluoride (LV PVDF) membranes using the Trans-Blot® Turbo™ Transfer System (Bio-Rad, UK). PVDF membranes from the Trans-Blot® Turbo™ RTA Transfer Kit (BioRad, UK, #1704274) were activated by immersion in methanol (VWR, USA, #20847.307) for 2 minutes. Blot absorbent filter paper was pre-soaked in 1 x Trans-Blot® Turbo™ Transfer Buffer (BioRad, UK, #10026938) prior to transfer. Filter paper, PVDF membrane, and polyacrylamide gel were stacked and rolled to expel air bubbles and topped with a final layer of filter paper. The stack was loaded into the Trans-Blot® Turbo™ Transfer System Cassette (Bio-Rad, UK) and run at 1.5 V for 10 minutes.

Membranes were blocked with 4% bovine serum albumin (BSA) (First Link UK Ltd., UK, #41-00-450) and 0.1% sodium azide (Sigma-Aldrich®, UK, #S2002) in tris-buffered saline with

Tween® (TBS-T, [200 nM Trizma base, 1.37 M NaCl, 0.1% Tween-20]) for 1 hour at room temperature.

Membranes were then incubated overnight in primary antibody solution diluted in 4% BSA and 0.1% sodium azide in TBS-T solution at 4 °C on a tube roller. Western blots were probed with anti-GAPDH (Abcam, UK, #ab9485) or anti-α-tubulin (Sigma-Aldrich®, UK, #T6199) antibodies for loading control validation. After primary antibody incubation, membranes were washed 3 x for 5 minutes in TBS-T.

Horseradish peroxidase-conjugated secondary antibodies were added to TBS-T. Membranes were then incubated with secondary antibodies diluted in TBS-T for one hour at room temperature on a tube roller.

After secondary antibody incubation, membranes were washed 5 x in TBS-T before being developed. Proteins on the membranes were stained using an enhanced chemiluminescence system with the Pierce™ ECL Western Blotting Substrate Kit (Fisher Scientific, UK, #32106). 1 ml Detection Reagent 1 Peroxide Solution was added to 1 ml Detection Reagent 2 Luminol Enhancer Solution and mixed. Membranes were placed protein-side-down in the solution for 5 minutes. Membranes were blotted dry and mounted into a film cassette. The chemiluminescence was visualised by exposure of the membrane to Amersham Hyperfilm ECL (Fisher Scientific, UK, #GZ28906837) and developed in the dark room using the XOGRAPH Compact X4 Film Processor (Xograph, UK,).

Bound antibodies were stripped from the membrane by incubating in stripping buffer (TBS-T containing 2% SDS) and 1% β-mercaptoethanol (Sigma-Aldrich™, UK, #M6250) for 20 minutes at 80 °C. Membranes were stripped again in stripping buffer without β-mercaptoethanol for 20 minutes at 80 °C. Membranes were then blocked again in 4% BSA (First Link UK Ltd., UK,

#41-00-450) and 0.1% sodium azide (Sigma-Aldrich®, UK, #S2002) in TBS-T for 1 hour at room temperature and primary antibodies were added as previously mentioned.

2.19. Platelet spreading

Coverslips (VWR, USA, #631-0150) were prepared in advance of the experiment. Coverslips were washed in 100% EtOH (VWR, USA, #208121.33) and rinsed with 1 x PBS, (Sigma-Aldrich™, UK, #P4417) prior to coating in fibrinogen. 400 µl of fibrinogen (Enzyme Research Laboratories, UK, #FIB3) at 100 µg/ml was added to wells and incubated overnight at 4 °C. The fibrinogen solution was removed and replaced with 300 µl 5 mg/ml denatured BSA (First Link UK Ltd., UK, #41-00-450) in PBS and incubated for 1 hour at room temperature. Coverslips were washed 3 x in PBS.

The washed platelet solution was diluted to 2 $\times 10^7$ /ml with modified Tyrode's buffer and left to rest for 20–30 minutes before spreading. 300 µl of washed platelets at 2 $\times 10^7$ /ml were added to the coverslips and allowed to spread for 45 minutes in a 37 °C incubator. The platelet solution was then removed, and coverslips washed 1 x in PBS. The platelets were fixed in 500 µl 10% Formalin (Sigma-Aldrich®, UK, #HT5012-1CS) for 10 minutes then washed 3 x in PBS.

2.19.1. Immunofluorescence

The coverslips were incubated for 5 minutes with 0.1% triton x 100 (Sigma-Aldrich®, UK, #9002-93-1) and washed 3 x with PBS. Coverslips were then incubated for 20–30 minutes in blocking buffer (1% BSA [First Link UK Ltd., UK, #41-00-450], 5% goat serum [Gibco™, USA, #11530526] in PBS). α-tubulin fibres were stained with primary antibody, Monoclonal Anti-α-Tubulin DM1A (Sigma-Aldrich®, UK, #T6199), which was added (1:200) to blocking buffer and coverslips placed platelet-side down on para-film in 50 µl of mix for 1 hour; other antibodies

were also used. Coverslips were washed 3 x in PBS and secondary antibodies Alexa Fluor® 647 goat anti-mouse IgG (1:250) (Thermo Fisher Scientific™, USA, #A21235) and Alexa Fluor® 488-conjugated Phalloidin (1:300) (Thermo Fisher Scientific™, USA, #A12379) to stain actin fibres, were added to blocking buffer and coverslips were incubated in 50 µl in the dark, on para-film for 1 hour. Coverslips were then washed with PBS, dipped in H_2O, blotted dry and mounted on glass microscope slides (Fisher Scientific, USA, #7101) with Hydromount (Scientific Laboratory Supplies Ltd., UK, #NAT1324). Before imaging, the slides were left to dry overnight in the dark at 4 °C.

2.19.2. Confocal microscopy

All images were taken using a DM IRE2 Leica Inverted microscope, SP2 confocal system running Leica Confocal Software Version 2.61 Build 1537. Spread platelet confocal imaging was performed using the 488 nm line of an Argon-Ion laser 457-514 nM (to image Alexa Fluor® 488) and the 633 nm line of an Helium-Neon-Ion laser (to image Alexa Fluor® 647) with an HCX Plan Apo lbd.BL 63x NA 1.4, Leica objective. Z-stacks were taken and the average intensity projection of 10 fields of view per control or patient were analysed. The detection parameters (gain and offset settings) were kept the same each time to enable comparisons from multiple patients who attended clinic on different days. Images were analysed and quantified using ImageJ and other image analysis sotfware and will be discussed in Chapter 3.

2.20. Transfection of Human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVECs) were plated on 0.1% gelatine coated, 6 cm plates at a density of 360,000 cells per plate. 1M HCl acid washed coverslips (VWR, USA, #631-0150) were added to plates in which the cells were being used for immunofluorescence. The cells were then kept in a 37 °C incubator for 24 hours prior to transfection. M199 media

with the following supplements was used for HUVEC culture: 2.2 g/l $NaHCO_3$, 0.1 g/l L-Glutamine, 90 mg/l heparin, 10% (v:v) Foetal Calf Serum (FCS) (Sigma-Aldrich®, UK, C8056) and 0.1% (v:v) Bovine Brain Extract as per Maciag *et al.* (Sigma-Aldrich®, UK, B3635-1G) (Maciag et al., 1979). As the HUVECs were being used for siRNA-mediated knockdown of genes, they were cultured in media without antibiotics. All cell culture work was carried out in a laminar flow cell culture hood.

Silencer® Select siRNAs (Thermo Fisher Scientific™, USA, #4427037) were made up to 20 µM using H_2O. The Gibco™ Opti-MEM™ reduced serum media (Thermo Fisher Scientific™, USA, #31985070) was filtered before use using a sterile 50 ml syringe and Ministart filter (Sigma-Aldrich®, UK, 16534K). 3.6 µl of each siRNA was mixed with 241.4 µl Opti-MEM™ and incubated at room temperature for 10 minutes. The Lipofectamine™ mix was then made with 4.3 µl Lipofectamine™ RNAiMAX Transfection Reagent (Thermo Fisher Scientific™, USA, #13778075) and 38.7 µl Opti-MEM™ and incubated at room temperature for 10 minutes. 43 µl of the Lipofectamine™ mix was then added to the siRNA mix and incubated at room temperature for 20 minutes. Before the transfection, the cells were removed from the incubator and the media was aspirated. The cells were washed 2x with 5 ml sterile PBS (Sigma-Aldrich™, UK, 806552-500ML). 1152 µl Opti-MEM™ was added to each plate being transfected along with 288 µl of the siRNA mix added dropwise around the plate. The plates were gently rocked and incubated at 37 °C for 4 hours to allow transfection. After 4 hours, the plates were removed from the incubator and the Opti-MEM™ and siRNA mix was aspirated from the plates and replaced with 4 ml pre-warmed M199 media. The cells were then left in 37 °C incubator for 48 hours before harvesting.

To harvest the cells, plates were removed from the incubator and washed 2x in 5 ml sterile PBS. A further 1 ml PBS was then added to the plate to aid the scraping. Cells were forcefully scraped from the plate surface using a cell scraper and removed to a 1.5 ml Eppendorf. The cells were then centrifuged at 5,000 rpm for 3 minutes. If the cells were being used for RNA extraction, the pellets were frozen at -80 °C; if the cells were being used for protein assays, 40 µl of ice-cold lysis buffer was added and a Bradford assay was carried out as previously described.

2.21. RNA extraction from cultured cells

RNA was extracted from harvested HUVECs according to the Macherey-Nagel NucleoSpin® RNA kit and protocol (Macherey-Nagel, Germany, 740955.50). After cells were collected by centrifugation, they were lysed by the addition of 350 µl Buffer RA1 and 3.5 µl β-mercaptoethanol (Sigma-Aldrich™, UK, #M6250) and vortexed vigorously. The lysate was then filtered through the NucleoSpin® Filter in a collection tube and centrifuged for 1 minute at 11,000 g. The NucleoSpin® Filters were discarded and 350 µl of 70% ethanol was added to the lysate and mixed by pipetting up and down. The lysate was then added to a NucleoSpin® RNA Column in a collection tube and centrifuged for 30 seconds at 11,000 g. The Column was then placed into a new collection tube. 350 µl Membrane Desalting Buffer was added to the column and centrifuged for 1 minute at 11,000 g to dry the membrane. The DNA was then digested by addition of 95 µl rDNase reaction mixture to the centre of the membrane and incubated at RT for 15 minutes. The DNase reaction mixture was made from 10 µl reconstituted rDNase to 90 µl Reaction Buffer for rDNase for each isolation, the mixture was mixed by flicking before use.

3x washes were then carried out on the column membrane. 200 µl Buffer RAW2 was added to the column and centrifuged for 3 seconds at 11,000 g; the column was then added to a new collection tube. 600 µl Buffer RA3 was then added to the column and centrifuged for 30 seconds at 11,000 g. The flow-through was discarded and the column was placed back into the collection tube. 250 µl Buffer RA3 was added to the Column and centrifuged for 2 minutes at 11,000 g to dry the membrane. The column was then placed into a 1.5 ml nuclease-free collection tube. The RNA was eluted by the addition of 60 µl RNase-free H_2O and centrifuged for 1 minute at 11,000 g.

To check the RNA had not degraded, 5 µl RNA was mixed with 1 µl Gel Loading Dye (New England BioLabs, USA, B7024S). This was run on a 1% Ultrapure™ Agarose gel (Invitrogen™, UK, #16500-500) in TAE (40 mM Tris, 20mM acetic acid and 1 mM EDTA) for 20 minutes at 120 V. Intact RNA showed 2 bands representing the 28S and 18S ribosomal subunits.

RNA Quantification was carried out using the Qubit® 3.0 Fluorometer (Life Technologies™, USA) following the standard protocols and using the Qubit® reagents using the same method as outlined in Section 2.13. The Qubit® RNA (HS) Assay kit (Thermo Fisher Scientific, UK, #Q32852) was used to make up the Qubit® working solution along with the RNA standards.

CHAPTER 3

Patient platelet phenotyping using a platelet spreading assay

3. Patient platelet phenotyping using a platelet spreading assay

3.1. Summary of background to this results chapter

Understanding a patient's phenotype is the first crucial step to the correct diagnosis of any disorder including inherited bleeding disorders.

The GAPP study started recruiting patients in 2008, aiming to completely categorise a patient's disease with extensive phenotyping by platelet function testing and other functional assays along with genotyping by using next-generation sequencing technologies. Recruitment to the study is based on a diagnosis of clinical bleeding using the International Society of Thrombosis and Haemostasis (ISTH) bleeding assessment tool (BAT) (not used in assessing children) by a consultant haematologist following normal coagulation factor tests and the absence of any evidence that suggests an acquired platelet disorder. The study has recruited over 1000 patients in 10 years from multiple collaborating haematology referral centres across the UK and Ireland. As a multicentre study, this incorporates 28 centres across the countries accounting for over two-thirds of the total number of Haemophilia Comprehensive care centres within the UK and Ireland. Given the likely genetic component of such disorders, the recruited patients have potentially affected family members, so relatives are also recruited where possible.

There is still a lack of a single and robust platelet function test, therefore diagnosis can be difficult in this patient group. To add to the difficulty, the phenotypes of patients with inherited bleeding disorders can be very heterogeneous despite similar genetic aetiologies. This variable phenotypic presentation suggests that patients should be individually considered instead of carrying out one particular method of diagnosis for all.

Over 10 years of research by the GAPP study has identified that a large proportion of patients (≈ 60%) who are still classified as having 'no identifiable platelet defect' despite the extensive phenotyping carried out and the patients' lifelong bleeding histories. This illustrates the limit of particular assays to detect some platelet function disorders and fails to address those individuals who may have other causes for their unexplained bleeding. Therefore, in this thesis, an alternative platelet phenotyping assay was selected and tested on a selection of patients to address those patients who required further investigation into their bleeding disorder. The platelet spreading assay is a well-known assay carried out in a research setting, however its clinical application and full utility for a complete diagnosis is currently unknown.

3.2. Aims of this chapter

This chapter aims to build upon previous work carried out in the GAPP study to completely categorise the patients' bleeding phenotype. The overarching aim is to combine platelet spreading with other methods of platelet function testing to try and elucidate platelet function disorders that are not identified by other methods of testing.

3.3. GAPP Study workflow and platelet spreading assay

The GAPP study workflow for patients is followed as outlined in Chapter 1, Figure 1.5. Patients were successfully recruited and blood was sent to the GAPP study laboratory team in the Birmingham Platelet Group. A full blood count was initially carried out followed by platelet function analysis. For patients with a platelet count in PRP above $1x10^8$ /ml, lumi-aggregometry was carried out as the standard platelet function test. The GAPP study has previously trialled different methods for phenotyping including Multiplate® multiple electrode aggregometry and the Total Thrombus-Formation Analysis System. In addition, platelet spreading was recently introduced (for the work of this thesis) as a method of phenotyping.

These further assays were carried out alongside the gold-standard method of platelet function testing- lumi-aggregometry. Following phenotyping, in selected cases, genotyping was carried out by WES. All genetic variants were then classified and confirmed by PCR amplification followed by Sanger sequencing.

Over the last 15 years, investigation into platelets' adhesion and activation mechanisms has been carried out by many research groups. However, it was over 40 years ago that the reports of activated platelet conformational change was recorded cinematographically (Allen et al., 1979). Allen *et al*. recorded the conformational change of platelets from disks to spheres with the extension and retraction of 'pseudopodia' using a sensitive differential interference contrast (DIC) microscope (Allen et al., 1979). Furthermore, Savage *et al*. showed that the mechanisms of platelet adhesion with agonists fibrinogen and vWF are complementary to achieve stable adhesion (Savage et al., 1996). Later on, it was shown that there are a multitude of molecules all acting in synergy to facilitate platelet adhesion spreading upon activation (Obergfell et al., 2002, Wonerow et al., 2003, Inoue et al., 2006). Along with these investigations it was also shown that deficiencies in platelet membrane glycoproteins can cause defects in platelet spreading (Nieuwenhuis et al., 1986). Platelet spreading assays have the ability to highlight platelet structural defects that may not be picked up by other assays. This could include defects of the platelet cytoskeleton which may be detrimental to the function of platelets, reducing their ability to spread and hence cause prolonged bleeding times in affected individuals.

Since work on this thesis began, patients from the GAPP study cohort have been included in further phenotyping in the form of a platelet spreading assay. The normal GAPP study workflow was followed along with the standard laboratory procedures for these patients. The

platelet spreading assay works to activate platelets with variable activators e.g. fibrinogen, which are coated on glass coverslips. When washed platelets are added to the coverslips, they transform from their resting state to an activated state, stimulating the formation of extended filopodia and lamellipodia resulting in an increase in the platelets' surface area (Aslan et al., 2012). The morphology of the platelets can then be studied using fluorescence microscopy to identify possible platelet abnormalities (the full method is outlined in Chapter 2, Section 2.19.).

3.4. Patient cohort demographics studied in this thesis

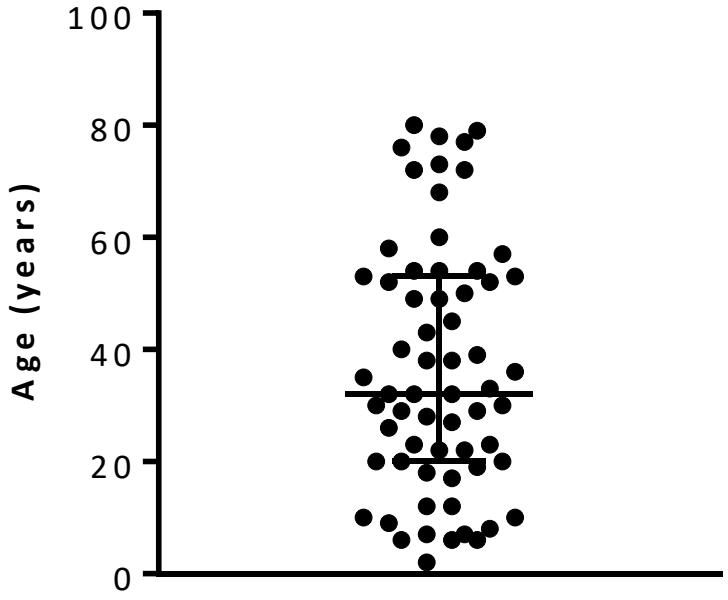
To date, 71 patients from the GAPP study cohort have been included for phenotyping using the platelet spreading assay. This number includes patients from paediatric and adult referral centres, therefore there is a considerable age range between the patients in the cohort. Patient ages were available for 64 individuals in this study. The average age of the 64 individuals was 32 years, ranging from the youngest at 2 years to the oldest at 80 years (Figure 3.1.). 32 healthy volunteer controls were also included in the platelet spreading assay however, ages were not available for this anonymised control group.

The gender of the patients was known for all 71 individuals. The cohort consisted of 77% female (n = 55) and 23% male (n = 16) participants (Figure 3.1.). Again, the genders of the control group of healthy volunteers was not made available.

The ethnicities of the patients in this cohort were not provided by the referring clinicians.

The patient group included 7 families with more than one affected family member (n = 22) and the remainder of the patients were isolated cases (n = 49).

A



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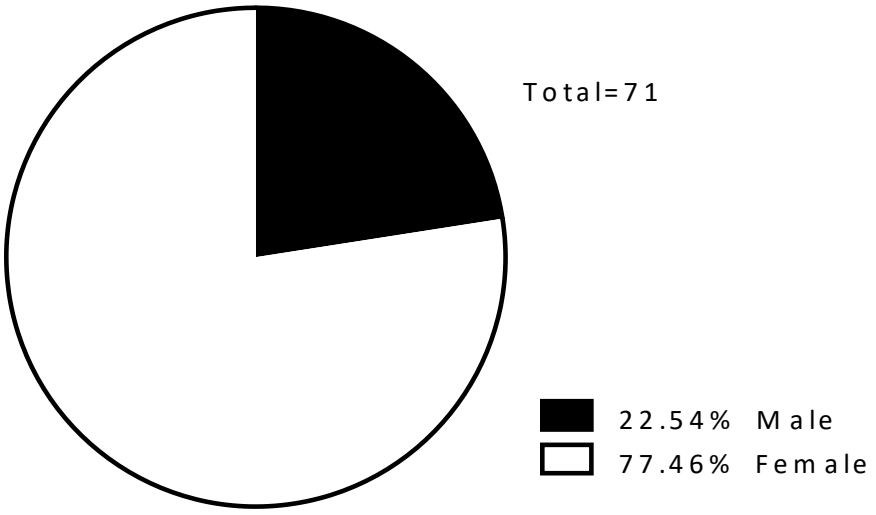


Figure 3.1. Basic patient demographics. (A) Age distribution showing the median and interquartile range of 64 individuals for which this data was available. (B) Gender distribution for 71 patients (16 males and 55 females).

3.5. Patient bleeding symptoms

Patient bleeding histories were taken by the referring clinician during the initial examination and the BAT score questionnaire was carried out in most cases (The ISTH-BAT questionnaire can be found in the Appendix, Figure A). BAT score data was available for 48 of 71 patients (40 female and 8 male). The BAT scores differed between female and male patients which can be seen in Figure 3.2. The median BAT score for the group was 10; the median for female participants was 11 whereas the median for male participants was 5. This reflects the difference in haemostatic challenges faced between females and males. The BAT score uses questions about the different patients' bleeding symptoms. The patients mostly suffered from minor bleeding symptoms including but not limited to epistaxis, cutaneous bleeding and bleeding following surgery. Some patients also suffered from more severe bleeding symptoms including post-partum haemorrhage and gastrointestinal bleeding. Bleeding symptoms and clinical findings of the participants can be seen in Table 3.1.

Bleeding symptom data was only available for 60 patients. The most common bleeding symptoms were related to cutaneous bleeding with 65% (39 of 60) stating that they had previously suffered from the symptom. Bleeding from minor wounds and oral cavity bleeding were the next most common symptoms, present in 58% (35 of 60) and 51% (31 of 60) of patients respectively. Of the 48 female patients with known symptoms, 75% (36 of 48) had suffered from menorrhagia. Several female patients had also suffered from post-partum haemorrhage following childbirth, 27% (13 of 48), however this number does not reflect the number of women who have undergone pregnancy. In most cases this bleeding was resolved by uterine packing, but some women required the use of antifibrinolytic drugs to stem the bleeding.

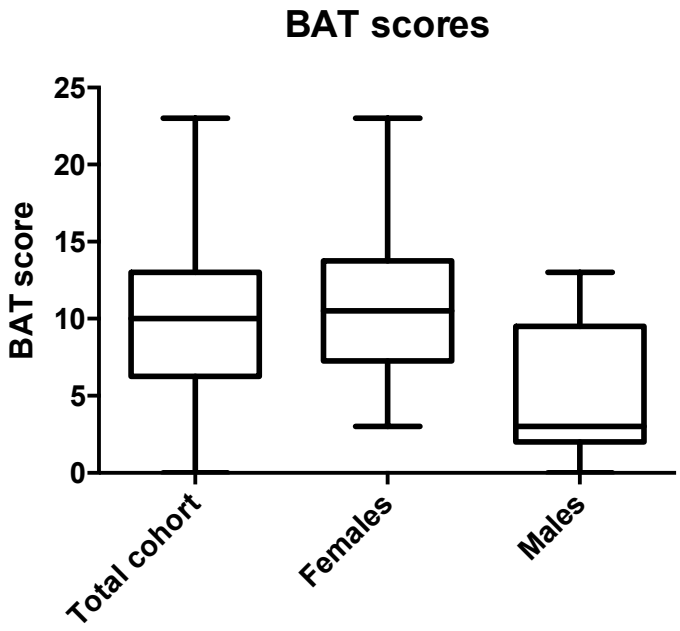


Figure 3.2: BAT score values in patients who were included in the platelet spreading assay. The values for the total cohort (48 patients) as well as separate plots for females (n = 40) and males (n = 8) is included. The boxes show the median, 25% percentile and 75% percentile. The whiskers show the minimum and maximum values.

Table 3.1. Phenotypes of all patients recruited to the GAPP study that were included in platelet spreading investigations. F, Female; M, Male; MPV, Mean Platelet Volume; BAT Score, Bleeding Assessment Tool; n/a, not applicable e.g. for children.
Code of bleeding:
1 – Epistaxis, 2 – Cutaneous bleeding, 3 – Petechiae, 4 – Oral cavity bleeding, 5 – Bleeding from minor wounds, 6 – bleeding after tooth extraction, 7- Bleeding after surgery, 8 – Hematuria, 9 – GI bleeding, 10 – Ovulation bleeding, 11 – Menorrhagia, 12 – Post-partum haemorrhage, 13 – Muscle haematomas.

Individual/ Family	Age	Gender	Platelet count (x10^9/l)	MPV (fL)	secretion (nmol ATP/1x108 platelets)	Platelet defect	BAT score	Bleeding phenotype
1	72	F	270	10.1	0.57	Mild secretion defect	13	1, 2, 6, 11
2	32	F	74	14.3	0.89	Thrombocytopenia/delayed secretion	17	1, 2, 4, 5, 6, 10, 11, 12
3	80	F	204	11.6	0.74	No defect	9	1, 2, 4, 5
4	—	F	281	10.5	1.06	No defect	10	2, 4, 5, 6, 11
5	—	F	265	10.7	1.25	No defect	13	1, 2, 4, 6, 8, 11, 12
6	—	F	371	10.5	0.86	No defect	5	4, 5, 10, 11
7	—	F	281	11.3	1.04	No defect	12	2, 4, 5, 6, 8, 11
8	38	F	273	9.8	0.99	No defect	13	1, 2, 4, 5, 11, 12
9	58	F	286	10.4	2.46	No defect	9	2, 4, 5, 11, 12
10	54	F	238	10.9	0.92	No defect	7	2, 4, 5, 11, 12
11	—	F	270	12	1.11	Medication - Clopidogrel	7	2, 4, 8, 9, 11
12	—	F	352	10.5	1.27	No defect	15	4, 5, 7, 9, 11
13	38	F	101	12.1	—	Thrombocytopenia	—	1, 2
14	32	F	292	9.7	0.61	COX defect	—	1, 2, 3, 7, 8, 12
15	40	F	232	10.2	0.77	No defect	6	2, 7, 11

16 I	39	F	221	13.9	—	ITP/Thrombocytopenia/ no defect	—	—
16 II	54	M	107	—	—	ITP/very weak evidence of secondary defect	—	—
16 III	28	M	85	—	—	Thrombocytopenia/ secondary defect	—	—
17	54	F	239	11.9	0.74	No defect	16	2, 7, 8, 10, 11
18	49	F	289	11.5	1.3	No defect	6	4, 5, 11
19	77	F	417	10.1	1.72	No defect	23	1, 2, 4, 6, 8, 11, 12
20	45	F	375	10	1.22	Gi defect	8	4, 5, 6, 7
21	20	F	182	12.5	2.35	Gi defect	5	4, 5, 11
22	72	M	113	10.2	1.05	Thrombocytopenia/signalling defect	13	1, 7, 8, 9
23	32	F	122	—	1.47	Thrombocytopenia/Gi defect	10	2, 4, 11, 12
24	49	F	195	10.4	0.63	No defect	14	2, 4, 5, 7, 11, 12
25	52	F	—	—	1.49	No defect	16	1, 4, 5, 11, 12
26	20	F	—	—	—	No defect	—	2, 5, 9
27 I	79	F	105	14.5	0.73	Thrombocytopenia/mild qualitative defect	9	1, 2, 7
27 II	53	F	172	13.9	1.03	Thrombocytopenia	7	11, 12
27 III	35	F	107	15.1	0.26	Thrombocytopenia/Gq signalling defect	4	1, 9,
28	36	F	221	11.7	1.13	No defect	13	1, 2, 4, 11, 12
29	57	F	261	9.3	0.82	COX defect	12	1, 5, 6, 11
30	22	F	291	11	1.03	No defect	13	2, 4, 8, 11
31 I	30	M	59	13	—	Thrombocytopenia/ secondary defect	8	1, 4, 5, 7
31 II	52	M	183	12	0.66	No defect	2	5, 7

32	6	M	140	10.1	0.53	Thrombocytopenia/mild secretion defect	n/a	2, 3
33	12	F	355	8.3	0.75	No defect	n/a	—
34	68	F	43	—	0.34	Congenital thrombocytopenia	—	—
35	—	F	241	9	0.11	Secretion defect	14	1, 4, 5, 6, 11
36	43	F	331	10	0.56	Gi Defect	14	1, 2, 4, 5, 11, 12
37	29	F	225	12.1	0.55	No Defect	10	2, 4, 5, 11, 12
38	76	M	120	14.6	0.97	Thrombocytopenia with COX defect	3	—
39 I	23	M	428	9.1	0.39	Secretion Defect	10	1, 4, 5, 7, 9
39 II	20	F	345	10.2	0.59	COX Defect	12	2, 4, 5, 6, 11
39 III	17	M	329	10.9	0.25	Secretion defect	3	2, 5
40	6	F	230	10.3	0.39	Secretion defect	n/a	1, 2
41	18	F	235	11	0.97	No defect	11	1, 2, 4, 5, 10, 11
42	53	F	298	11.1	0.72	No defect	13	2, 4, 7, 9, 11
43	78	M	91	—	0.45	Thrombocytopenia	2	2, 4
44 I	7	F	287	8.8	1.01	No defect	n/a	—
44 II	73	F	150	10.5	0.5	Secretion defect	—	—
45	6	M	104	8.9	0.87	Thrombocytopenia	n/a	2, 3
46	7	F	170	10.5	0.55	Secretion defect	n/a	—
47	27	F	256	11.1	0.75	No defect	9	1, 2, 5, 6, 7, 11
48	60	F	184	12.2	0.46	COX defect	21	1, 2, 4, 5, 7, 9, 10, 11, 13
49	23	F	204	12.4	0.59	Secretion defect	8	1, 2, 5, 10, 11
50	22	F	323	11.5	0.66	No defect	3	1, 5, 6
51	19	F	269	11.9	0.73	No defect	16	1, 2, 4, 5, 7, 9, 11
52	26	F	295	10.8	0.89	No defect	3	1, 5
53	29	F	225	12.1	0.55	No defect	10	2, 4, 5, 10, 11

54	50	M	270	11.4	1.39	Gi Defect	—	1
55 I	9	F	329	12.1	—	No defect	n/a	1, 2, 5, 7
55 II	10	F	246	11.3	—	No defect	n/a	2, 5
55 III	30	F	259	11.7	—	No defect	—	11
55 IV	33	M	196	12.6	—	No defect	0	n/a
56 I	—	F	93	12.8	2.14	No defect	0	n/a
56 II	8	M	126	11	1.68	No defect	n/a	1
56 III	2	M	217	8.8	0.91	No defect	n/a	1
56 IV	10	M	196	9.4	1.81	No defect	n/a	1
56 V	12	F	185	10.8	1.38	No defect	n/a	1, 2, 5, 10, 11

3.6. Platelet counts and morphology

Platelet counts and mean platelet volumes were measured using the Sysmex XN-1000™ as outlined in Chapter 2, Section 2.6. The platelet counts were available for 69 of the 71 patients and are shown in Table 3.1. Platelet counts were also determined for the control group of 32 individuals and shown in Table 3.2.

The platelet counts for both groups were compared and analysed (Figure 3.3.). The overall platelet counts in the patient group ranged from 43–428 $\times 10^9$ /l. The average platelet count in this group of individuals was 225 $\times 10^9$/l (n = 67). 17 patients had a platelet count of <150 $\times 10^9$/l which is below the normal range and therefore labelled as thrombocytopenia. The overall platelet counts in the control group ranged from 67–420 $\times 10^9$/l. The average platelet count in this group was 217 $\times 10^9$/l (n = 32). After carrying out an unpaired t test to compare both groups, the data was not found to be significantly different.

The mean platelet volume (MPV) was measured for 64 patients (Figure 3.3.). The normal range is between 9.2–12.9 fL. The MPV in the patient group ranged from 8.3–15.1 fL. The average MPV for the patient group was 11.17 fL. The MPV for the control group was measured in 31 individuals and it ranged from 8.9–13.4 fL. The average measurement was 11.54 fL. Again, an unpaired t test was carried out to compare the two groups. The data was not found to be significantly different.

Table 3.2. Platelet counts, MPVs and platelet secretion values for health volunteer control group.

Individual	Platelet count (x10^9 /l)	MPV (fL)	Secretion (nmol ATP /1x10^8 platelets)
1	258	11.7	1.47
2	67	12.1	0.6
3	288	12.2	1.29
4	285	12.4	1.24
5	234	12.3	1.06
6	281	12.2	1.77
7	175	12.5	—
8	187	10.1	1.04
9	72	10.1	—
10	235	12.5	1.24
11	246	11.9	1.68
12	218	12.7	1.6
13	233	12.3	1.29
14	—	—	1.31
15	260	11.5	1.64
16	259	12	1.76
17	227	12.6	1.37
18	165	12.1	1.03
19	194	12.5	1.11
20	204	10.6	0.46
21	205	10.7	0.57
22	202	13.4	0.62
23	247	11.9	0.64
24	277	10.5	0.55
25	126	11.1	1.45
26	322	8.9	0.99
27	185	10.7	0.93
28	187	11.9	0.76
29	297	10.3	0.74
30	248	10.7	1.21
31	420	9.6	—
32	138	11.6	2.03

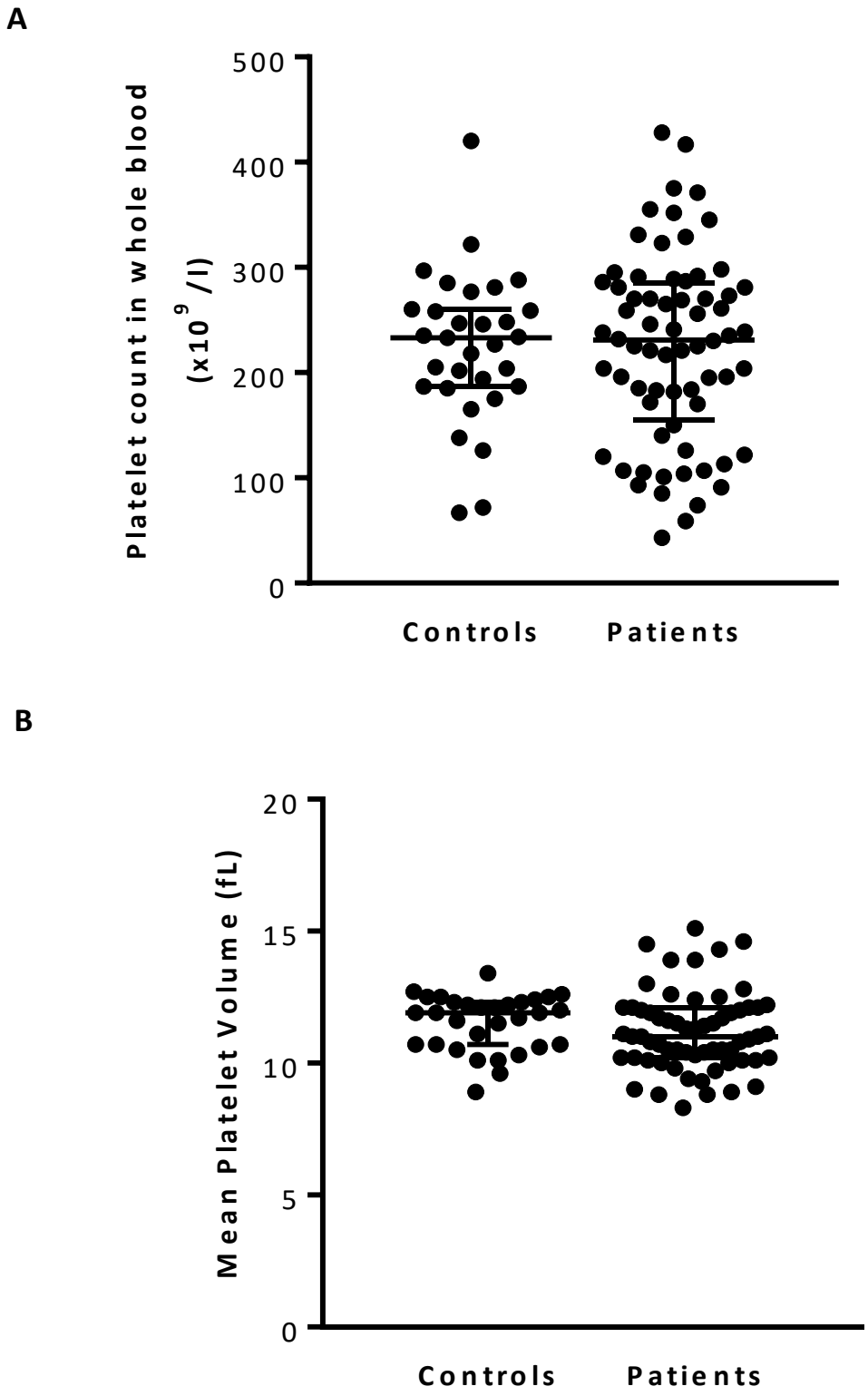


Figure 3.3. Platelet parameters for Patient and Control groups. (A) Platelet counts in whole blood showing the median and interquartile range for 31 control individuals and 67 patients for which this data was available. (B) MPV measurements are also shown as the median and interquartile range for 31 control individuals and 64 patients.

3.7. Platelet function testing

Platelet function testing by lumi-aggregometry was carried out on all 71 patients as per the standard GAPP study workflow.

3.7.1. Light transmission aggregometry

LTA remains the reference method for the investigation of platelet function. This has been the main method for over 50 years as the core principles of the original method have not changed (Born, 1962). Platelets' aggregation responses to agonists which target different receptors and signalling pathways are measured. By interpreting the aggregation traces, certain disorders of specific pathways can be identified.

The main findings of the 71 patients' LTA can be seen in Figure 3.4. The largest group with 54% (38/71) of the patients had no platelet defect identified by LTA. This further reiterated the need for other, perhaps more specialised, platelet function tests and this is where the platelet spreading assay comes into play. The second largest group, with 16% (11 of 71) of patients, was the group where more than one platelet defect was identified in the patients. This mostly included patients with thrombocytopenia as well as a secondary defect for example, secretion or signalling defects. Platelet secretion defects were the third largest group with 10% (7 of 71) of patients affected. These are patients with secretion values below the cut-off value of 0.65 nmol ATP/1x10^8 platelets. Secretion data for the control group was also measured. The patients' median secretion value was 0.87 nmol ATP/1x10^8 platelets. For the controls, the median secretion was 1.2 nmol ATP/1x10^8 platelets. An unpaired t test suggested that the values between the control group and the patient group were in fact, statistically significant ($p = 0.049$). This reflects the difference between the patient and control

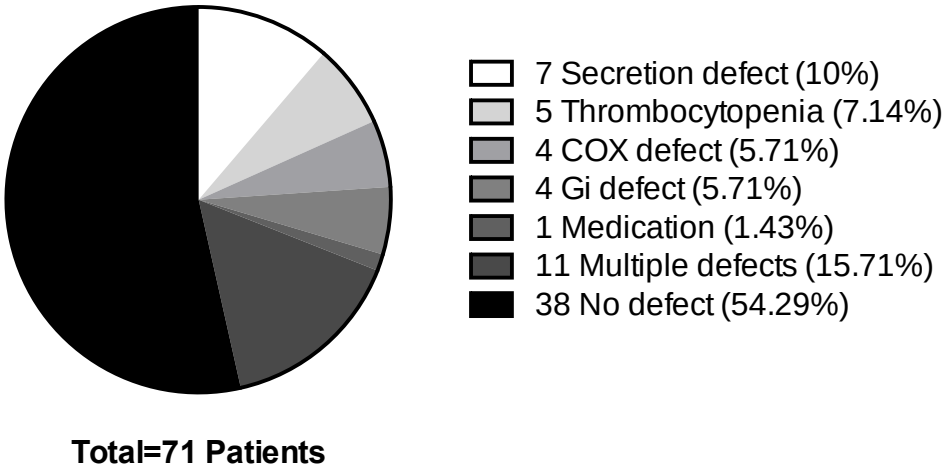


Figure 3.4. Main findings of 71 patients recruited to the GAPP study following phenotyping by Lumi-aggregometry. The largest group of patients were classified as 'no defect' due to normal platelet aggregation and secretion.

groups as all the patients have a history of bleeding symptoms. Furthermore, 10% of the patient group were diagnosed with secretion disorders alone. A graph showing the patient and control secretion values can be seen in Figure 3.5.

A few examples of representative patient aggregation traces are displayed in Figure 3.6. Patient 39.1 was diagnosed with a secretion defect due to abnormal aggregation in response to all doses of ADP and low doses of collagen and PAR-1 peptide along with a secretion value that was markedly reduced (0.39 nmol ATP/ 1×10^9). Patient 39.2 was diagnosed with a COX pathway defect due to abnormal aggregation responses to all concentrations of arachidonic acid and collagen but normal responses to U46619 and collagen-related peptide. However, aspirin intake can replicate the same defective responses observed. Therefore, it is necessary to proceed with caution and perhaps repeat this patient's aggregometry and explain the important of refraining from taking any NSAIDs up to 10 days prior to the blood test appointment.

In addition, it was established after the blood was taken that Patient 11 was taking clopidogrel at the time of testing which therefore meant that the results of her LTA phenotyping could not be analysed. Clopidogrel usually mimics a platelet function defect (P2Y12 receptor defect) when the patient's blood is tested by LTA. In order to repeat the test, she would need to stop taking the medication if possible and return to clinic for a repeat blood test.

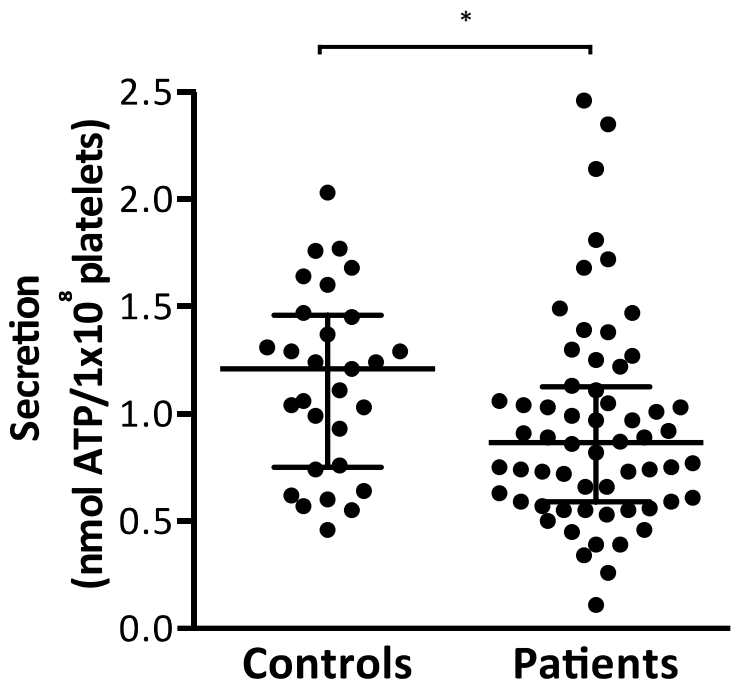
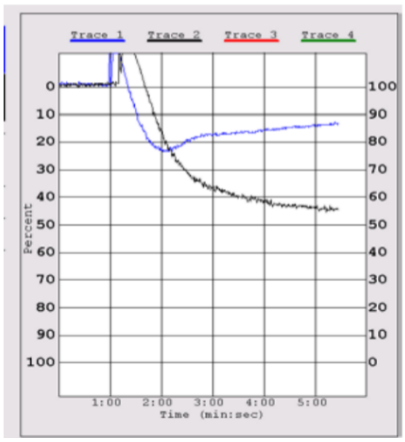
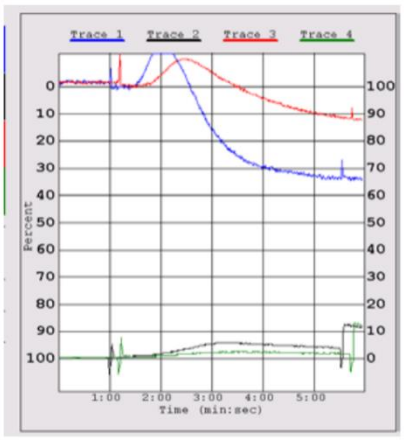


Figure 3.5. Platelet secretion for Patient and Control groups. Platelet secretion was measured in both groups in PRP during lumi-aggregometry with PRP. The median and interquartile ranges for 29 control individuals and 61 patients are shown for which this data was available. A value of p = 0.049 after an unpaired t test indicated that the difference in secretion values between the two groups is statistically significant.

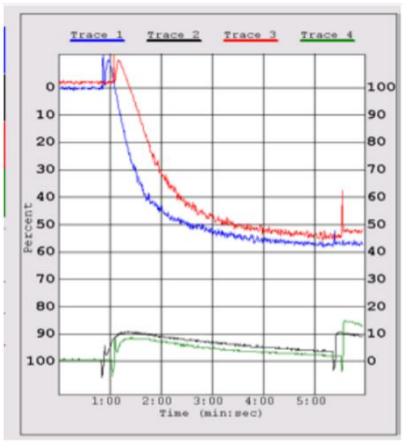
A



ADP 10 µM

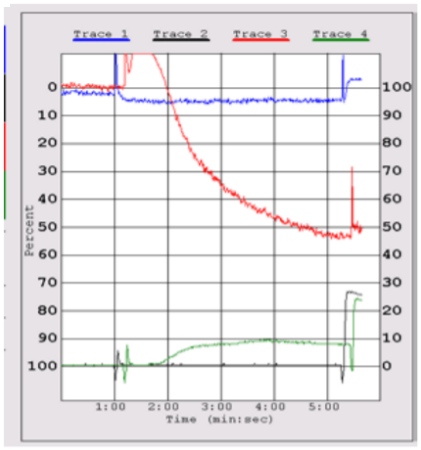


Collagen 3 µg/ml

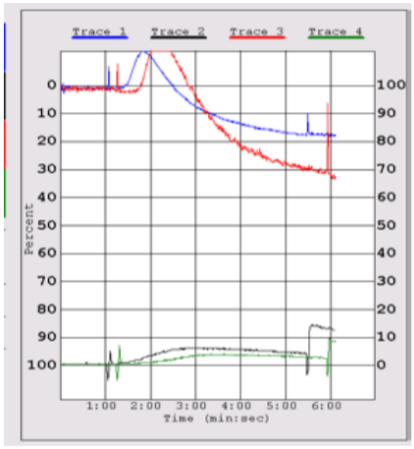


PAR-1 Peptide 100 µM

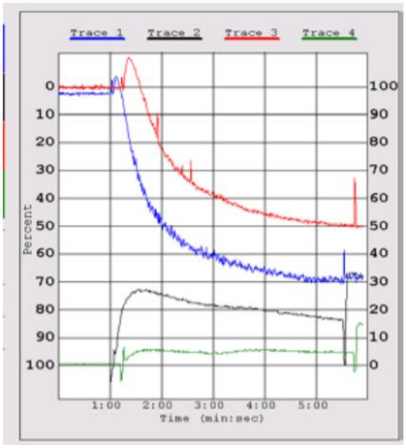
B



AA 1.5 mM



Collagen 10 µg/ml



U46619 3 µM

Figure 3.6. Representative lumi-aggregometry traces from patients recruited to the GAPP study who were also included in the trialling of the platelet spreading assay. (A) Patient 39.1 (represented by the blue line) shows reversed aggregation in response to ADP 10 µM, reduced aggregation and secretion (black line) to collagen 3 µg/ml and absent aggregation in response to 30 µM PAR-1 peptide however full aggregation in response to 100 µM PAR-1 peptide. These results might indicate a secretion defect. (B) Patient 39.2 (represented by the blue line) showed absent aggregation in response to high concentrations of arachidonic acid with no secretion (black line) and reduced aggregation and secretion in response to collagen. However, normal responses to U46619 and collagen-related peptide were observed. These findings suggest a defect in the cyclooxygenase pathway or aspirin intake.

3.7.2. T-TAS

The Total Thrombus-formation Analysis System (T-TAS) is an automated system that measures thrombus formation using microchip flow chambers. The flow chambers are pre-coated with specific agonists to mimic *in vivo* conditions; in addition, the flow pressure and conditions can be altered to mimic the different, variable flows within a vessel. T-TAS analysis was only carried out in a small number of patients in this study due to this being a trial of the T-TAS assay. Representative T-TAS graphs, showing normal platelet thrombus formation are shown in Figure 3.7.

The T-TAS is advantageous for use in this platelet testing setting as only small volumes of blood are required for the assay to be carried out successfully. Furthermore, the assay is relatively quick, easy to carry out, and can yield results approximately 10 minutes after the assay was started. The patient blood tested using the T-TAS provided useful insights into the possible future uses for the assay (Al Ghaithi et al., 2019). Analysis by T-TAS carried out in the laboratory demonstrated good agreement with LTA when the techniques were carried out in parallel (Al Ghaithi et al., 2019). This suggests that from our work, the T-TAS assay could be useful to screen patients with potential platelet function defects (Al Ghaithi et al., 2019). This could save time to diagnosis and would not require a skilled worker to perform, unlike LTA. It was also concluded that the T-TAS could be valuable for patients who are prescribed antithrombotic therapies and require regular monitoring. In addition to this, we showed the utility of the T-TAS in animal models of disease. The properties of the assay make it very suitable for quickly testing animal blood and has the potential to reduce the numbers of animals used for particular disease models in research laboratories (Al Ghaithi et al., 2019).

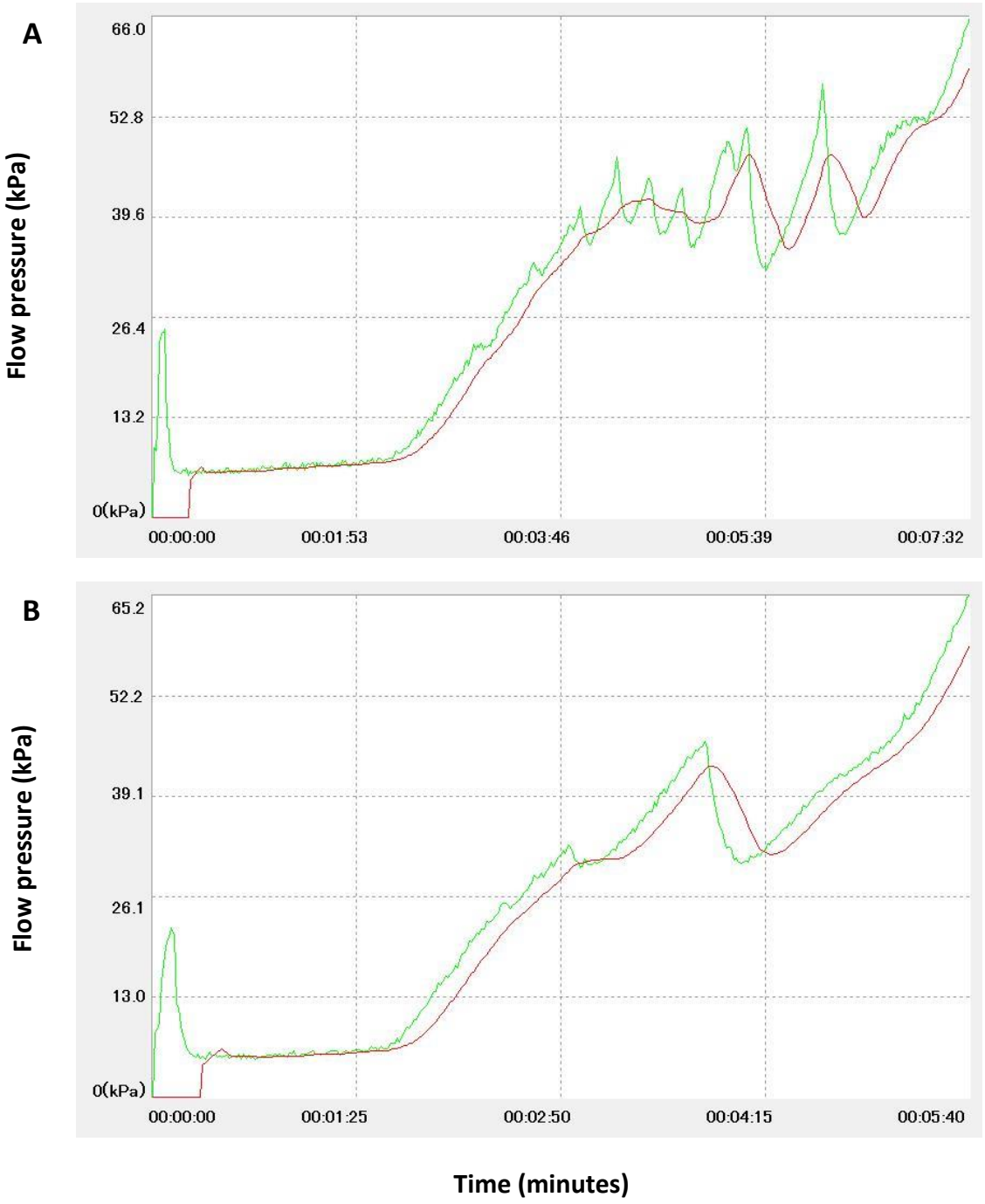


Figure 3.7. Measurements of thrombus formation using the T-TAS PL- chip with whole blood samples from patients. Thrombus formation in this assay was measured under low shear rates (1000 s^{-1}) to generate representative flow pressure curves. The time taken for the growing thrombus to fully occlude channels within the chip was measured. This process involved platelet adhesion, aggregation and granule secretion and hence thrombus growth. (A and B) show normal flow pressure curves and the time taken to reach full occlusion. The green lines show the actual reading of flow pressure, the red lines show the calculated mean measurement of the flow pressure.

3.8. Platelet spreading

During the process of platelet activation, platelets extend filopodia and develop lamellipodia. This cytoskeletal reorganisation results in a dramatic increase in the platelet size; platelets transition to a spherical shape to adhere to exposed or damaged subendothelial matrix of the blood vessels (Cuenca-Zamora et al., 2019). Here, they can become fully spread and aggregate to promote the cessation of unwanted blood flow. These platelet morphological changes can be affected if the individual has abnormal platelet function or is prescribed medication which can alter this. Furthermore, defects in components of the platelet cytoskeleton are not routinely screened following the diagnosis of a suspected platelet function defect. However, platelet spreading *in vitro* is a widely used laboratory technique to assess platelets and platelet proteins using blood from animal models and specific antibodies.

During this workflow, platelets were spread on fibrinogen for 45 minutes as described in Chapter 2, Section 2.19. The platelets could then be stained with fluorescent antibodies to actin and tubulin. The platelets can then be visualised using confocal microscopy and images can be acquired for later analysis.

3.8.1. Healthy donor volunteer spreading

Healthy donor volunteer spreading was also analysed in order to provide values for comparison with the patients' platelet spreading values. Confocal microscopy derived images of control platelet spreading can be seen in Figure 3.8.

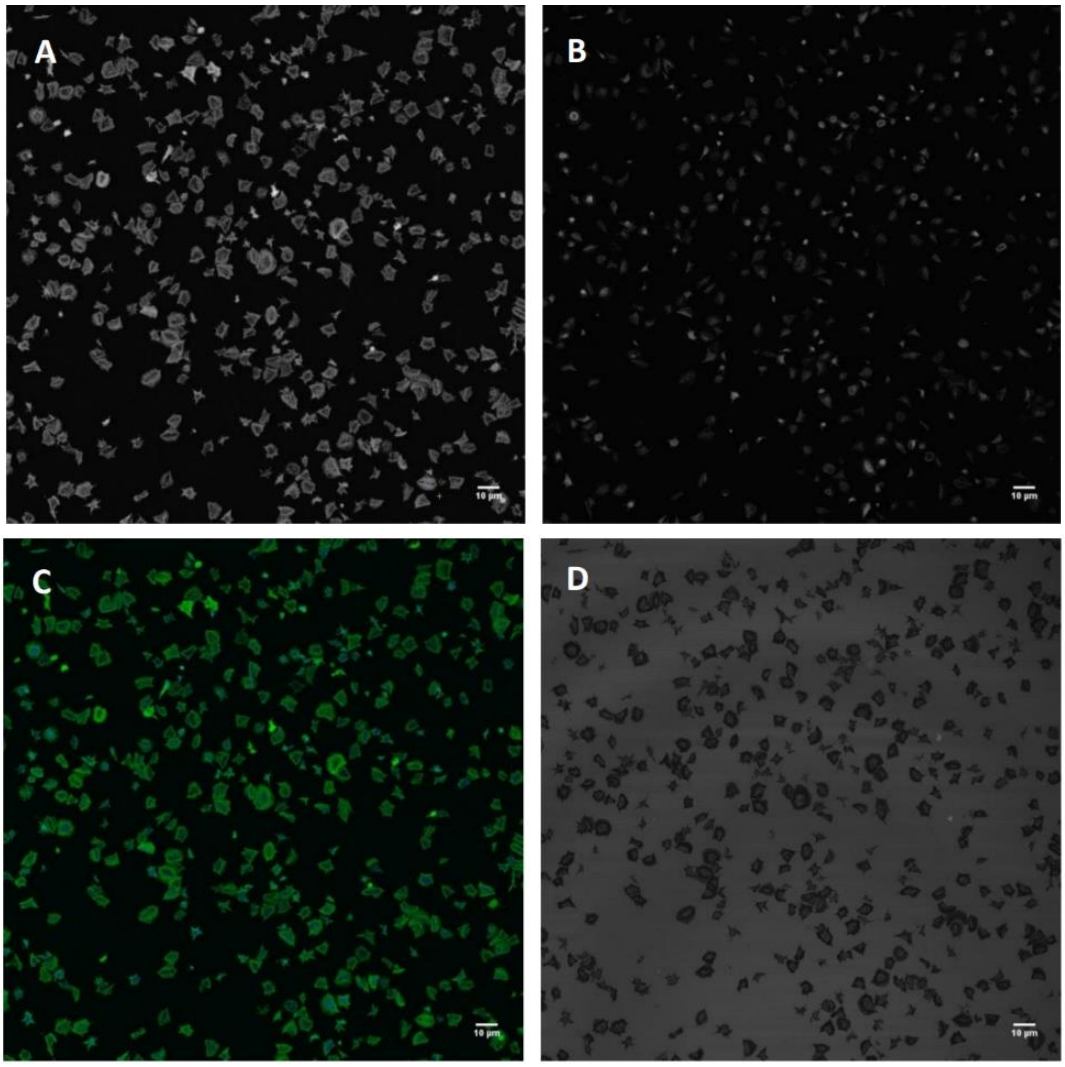


Figure 3.8. Wide field images of healthy volunteer control platelets after spreading on fibrinogen for 45 minutes. (A) Actin fluorescence. (B) Tubulin fluorescence. (C) Merged actin (green) and tubulin (blue) fluorescence. (D) Reflectance image. Scale bar, 10 µm.

3.8.2. Patient spreading

Example confocal microscopy derived images of a selection of patient platelet spreading can be seen in Figure 3.9. In these images, platelets were spread on fibrinogen for 45 minutes. Images were acquired at 63× magnification and at 2× and 8× zoom from the wide field image in order to view the actin and tubulin staining clearly. The patient platelets imaged, suffered from bleeding symptoms from an early age and currently have no diagnosis as the results from laboratory testing with lumi-aggregometry showed no apparent platelet defect.

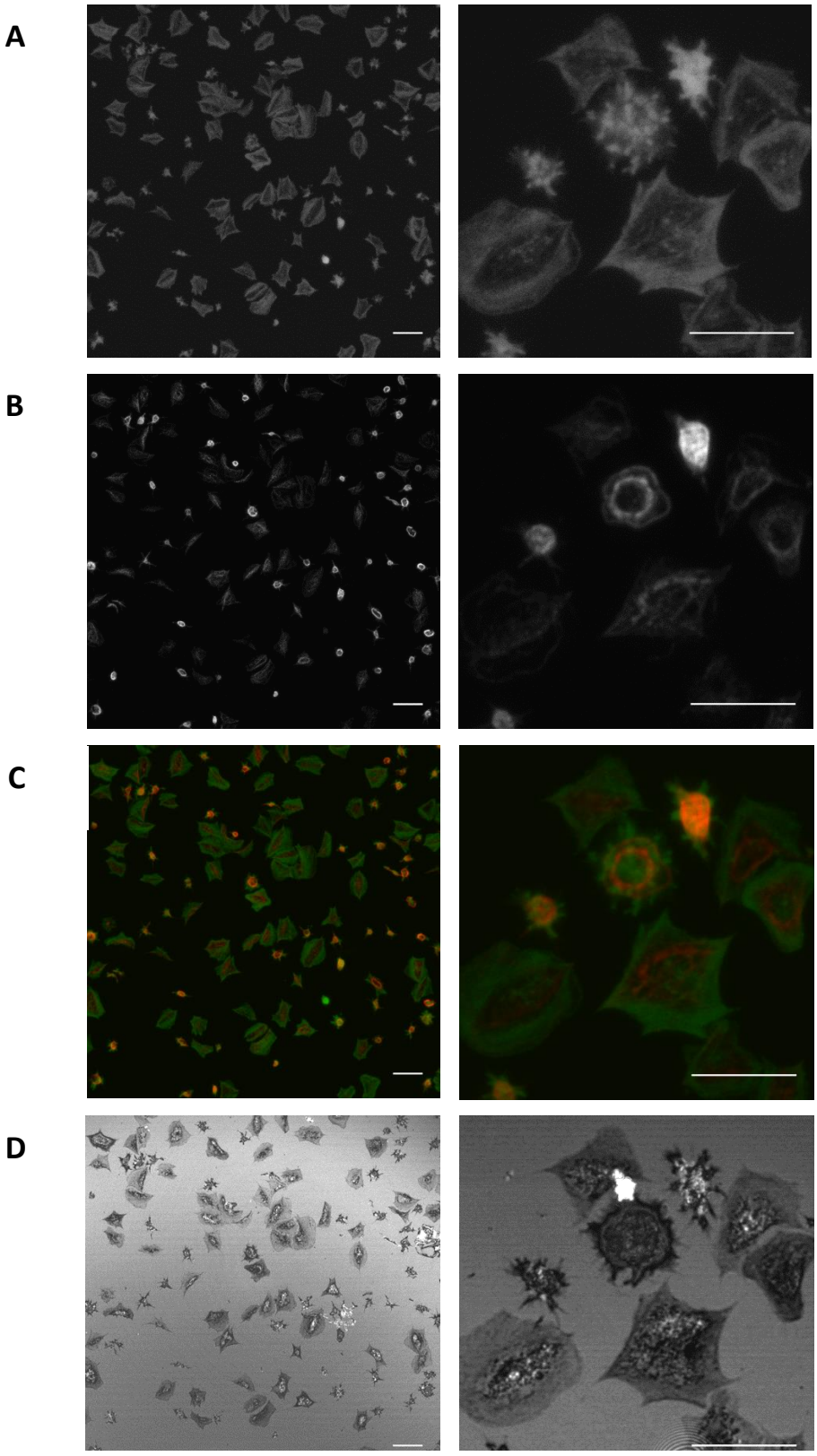


Figure 3.9. Confocal microscopy images of spread platelets after spreading on fibrinogen for 45 minutes from patient 55.1. Left column shows a 2× zoom and the right column shows a 8× zoom of a different field of view. (A) Actin fluorescence. (B) Tubulin fluorescence. (C) Merged actin (green) and tubulin (red) fluorescence. (D) Reflectance image. Scale bar, 10 µm.

3.9. Methods of image analysis

The field of image analysis in science is evolving rapidly and there are now many methods of analysis employed by different groups. There are also a multitude of software and programming techniques available in order to analyse the highly detailed and complex images we are able to acquire using microscopy techniques.

3.9.1. ImageJ

ImageJ is a Java-based image processing and analysis program. ImageJ was originally developed by Wayne Rasband at the National Institute of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin-Madison); it was first released in 1997. ImageJ was designed with an open architecture intended to make adding, upgrading or exchanging components easy. This provides ImageJ with extensibility therefore allowing custom plugins to be used or developed to deliver solutions to the image processing and analysis challenges that are routinely encountered by biologists.

ImageJ can calculate area and pixel value statistics of user-defined regions and create intensity-thresholded areas. In addition, the software can measure microscopic distances, and angles between points in an image. The image processing functions include, but are not limited to: convolution, sharpening, smoothing, and standard geometric transformations. In this thesis, ImageJ was used to create average intensity projection images from Z-stacks or multiple images taken at different focal distances in a user-defined field of view. This provided a composite image with a greater depth of field useful for analysing 3D cells such as platelets. ImageJ is an invaluable tool for the scientific community, and with the powerful tools it employs, users can develop and share reproducible analysis workflows.

3.9.2. KNIME and Ilastik

KNIME, the Konstanz Information Miner, is a free and open-source data platform that was created in 2004. The first version of KNIME was released in 2006 and was used by several pharmaceutical companies. By 2012, KNIME was being used by thousands of operators across sectors not only within life sciences but by banks, publishers, manufacturers and other industries. KNIME's popularity grew as the platform allows users to create bespoke data flows, selectively perform some or all chosen analysis steps and then later, view the results, models and interactive views. KNIME allows for the processing of large data volumes that are only limited by the available hard disk space. The results of the KNIME workflows can then be used to create report templates and can be exported to document formats. Furthermore, KNIME's additional plugins allow for the integration of methods for text mining and more importantly, image mining. The KNIME Image Processing Plugin allows for many methods of analysis to be run on images. Feature vectors identified can then be used to apply machine learning methods in order to train and apply a classifier.

This method of image analysis for this thesis was performed using a custom segmentation algorithm developed by Jeremy Pike at the University of Birmingham using the software, Ilastik. Ilastik is an open source software for image classification and segmentation. Ilastik allows users to annotate images with set classes using a mouse interface. This can be done to a number of images in order to train the machine learning component. Overall, this improves accuracy of the segmentation when the classifier is applied to further images or large sets of images as in this case. Commercial and open source platforms mentioned, focus on the ease-of-use value and broad coverage of image processing tasks.

3.10. High-throughput platelet spreading analysis

The large-scale platelet spreading data set for this high-throughput analysis was generated by taking images from 10 representative fields of view from each slide of patients' or healthy volunteer controls' spread platelets. The spread platelet samples were stained with phalloidin conjugated to a fluorophore to detect actin filaments in the cells. Probing for this cytoskeletal component allows for the accurate delineation of platelet morphology within the high signal/noise ratio that is required for successful image capture and robust high-throughput analysis.

In order to isolate fluorophore signal from the background signal, a pixel classifier within Ilastik was trained on a smaller subset of the images (Sommer et al., 2011). Once the training set was defined, this was run on the whole dataset within KNIME to produce binary images; any gaps smaller than 10 μm^2 within the binary images were automatically filled (Berthold et al., 2009). Inevitably, within the images, some platelets were overlapping or touching; as such, these cells were separated by manually placing identifying points within the centre of each one using the interactive annotator node within the KNIME workflow (Khan et al., 2019a). Following this, the previously identified points were used as seeds for a watershed transformation of the binary images, subsequently producing a labelled segmentation of individual platelets within the image (Khan et al., 2019a). Any image artefacts smaller than 1 μm^2 were removed from the image.

Once all the images had been analysed by the semi-automated workflow, the labelled segments within each image and for each patient could be processed by the programs. Measurements for each platelet were collected, which included, the area covered by the platelet (A), the perimeter of the spread platelet (P), platelet circularity (C) and mean

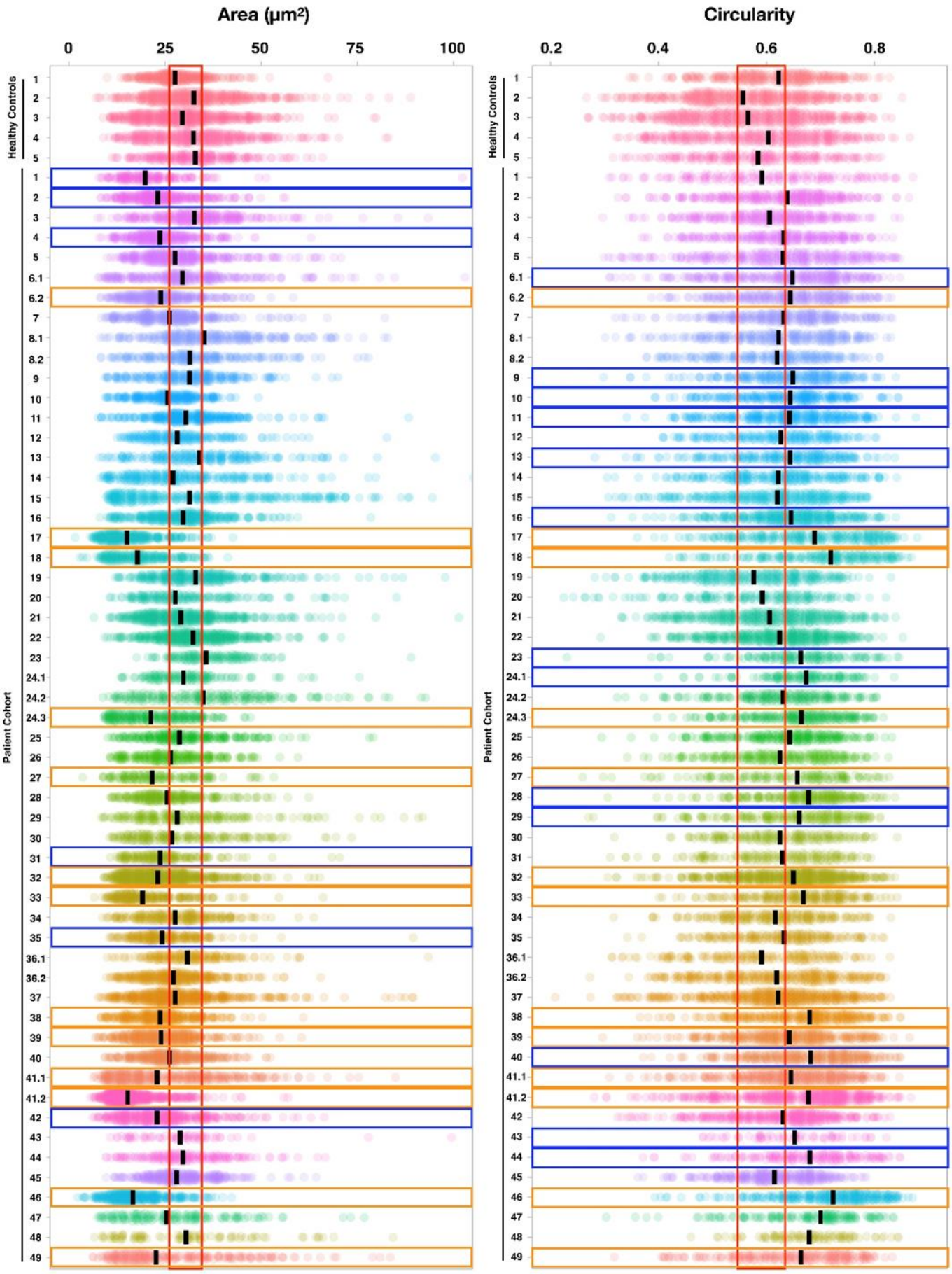
fluorescent intensity. Circularity is defined as the equation below and has a maximum value of 1 for perfect circularity.

$$C = \frac{4\pi A}{P^2}$$

3.10.1. High-throughput platelet spreading analysis results

Platelet area and circularity data measurements are displayed in Figure 3.10. This data was plotted using with the median of the data set generated for each patient or control and visualised using PlotsOfData which is a freely available, open-source, web app (Postma and Goedhart, 2019).

Firstly, it was necessary to calculate a normal range for spread platelet area and circularity as this is a novel assay and normal range values for these measurements had not been established. Control data set was used to establish the normal range from the healthy volunteer control samples that were treated identically, which included travelling with the patient blood from the referring centre to the laboratory in Birmingham where the assay took place. To create the normal range, the median value of the data set for each control was calculated; the 95% confidence interval (CI) was also calculated. The normal range was calculated by adding or subtracting the CI from the median value. The normal range for spread area and circularity were calculated. Platelet data which lay outside of the normal range were flagged as outliers which potentially have a function defect or a defect affecting the platelets' ability to spread normally.


Area (µm²)
0
25
50
75
100
Circularity
0.2
0.4
0.6
0.8
Healthy Controls
Patient Cohort
1
2
3
4
5
6.1
6.2
7
8.1
8.2
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24.1
24.2
24.3
25
26
27
28
29
30
31
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33
34
35
36.1
36.2
37
38
39
40
41.1
41.2
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43
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45
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48
49

Figure 3.10. Area and circularity data generated from automated image analysis were classified according to the control range established by spreading data from healthy control volunteers (n = 5). Patient samples with medians and confidence intervals outside of the control range, shown by the red box, were classified as positive hits (blue boxes) for either a defect in platelet area or circularity, while samples which fall outside both normal ranges were classified as double positives (orange boxes).

Overall, platelet spreading abnormalities were counted in 58% (32 of 55) of the patients tested (Khan et al., 2019a). This consisted of patient platelets displaying defects in area only (n = 6), circularity only (n = 13), defects in both platelet area and circularity (n = 13) and no platelet defects observed (n = 23). The defect distribution of patients double positive for area and circularity abnormalities is displayed in Figure 3.11. Abnormal platelet area and circularity are synonymous with small, round and unspread platelets and consequently, these 'double hits' (defects in both area and circularity) were considered a strong indication of morphological spreading defects. Representative platelet spreading immunofluorescent and segmentation images of selected patients with clear defects in platelet area or circularity can be seen in Figure 3.12.

This data was then correlated with the results of the other platelet function tests that were also carried out on the patient blood as part of the normal GAPP study workflow, namely lumi-aggregometry. Spreading defects were found in 9 patients where we also found defects in platelet aggregation (Gi defects, n = 3; secretion defects, n = 5; COX defect, n = 1). Interestingly, 50% (16 of 32) of the patients who were positive for a spreading defect, did not demonstrate a platelet function defect during testing by lumi-aggregometry. This suggests that platelet defects have been identified by the platelet spreading assay that would not have been identified by lumi-aggregometry. In addition, these 16 patients all presented with high ISTH-BAT scores, with a median score of 11. 63% (10 of 16) patients had double positive hits for defects in platelet area and circularity. This data confirms that a platelet spreading assay could supplement the results obtained from lumi-aggregometry by confirming aggregation defects as well as highlighting platelet morphological abnormalities that would not be detected by the gold-standard approach applied to patients with a suspected platelet function defect.

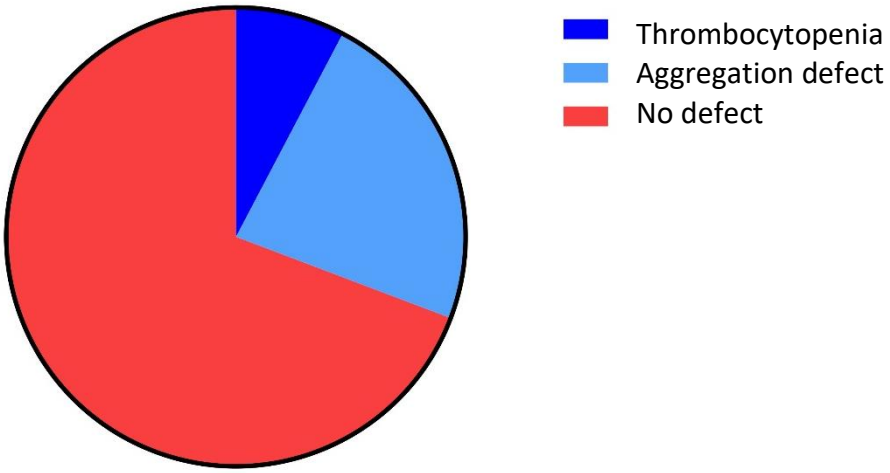


Figure 3.11. Pie chart indicating the defect distribution of patients double positive for platelet spreading defects (n = 13).

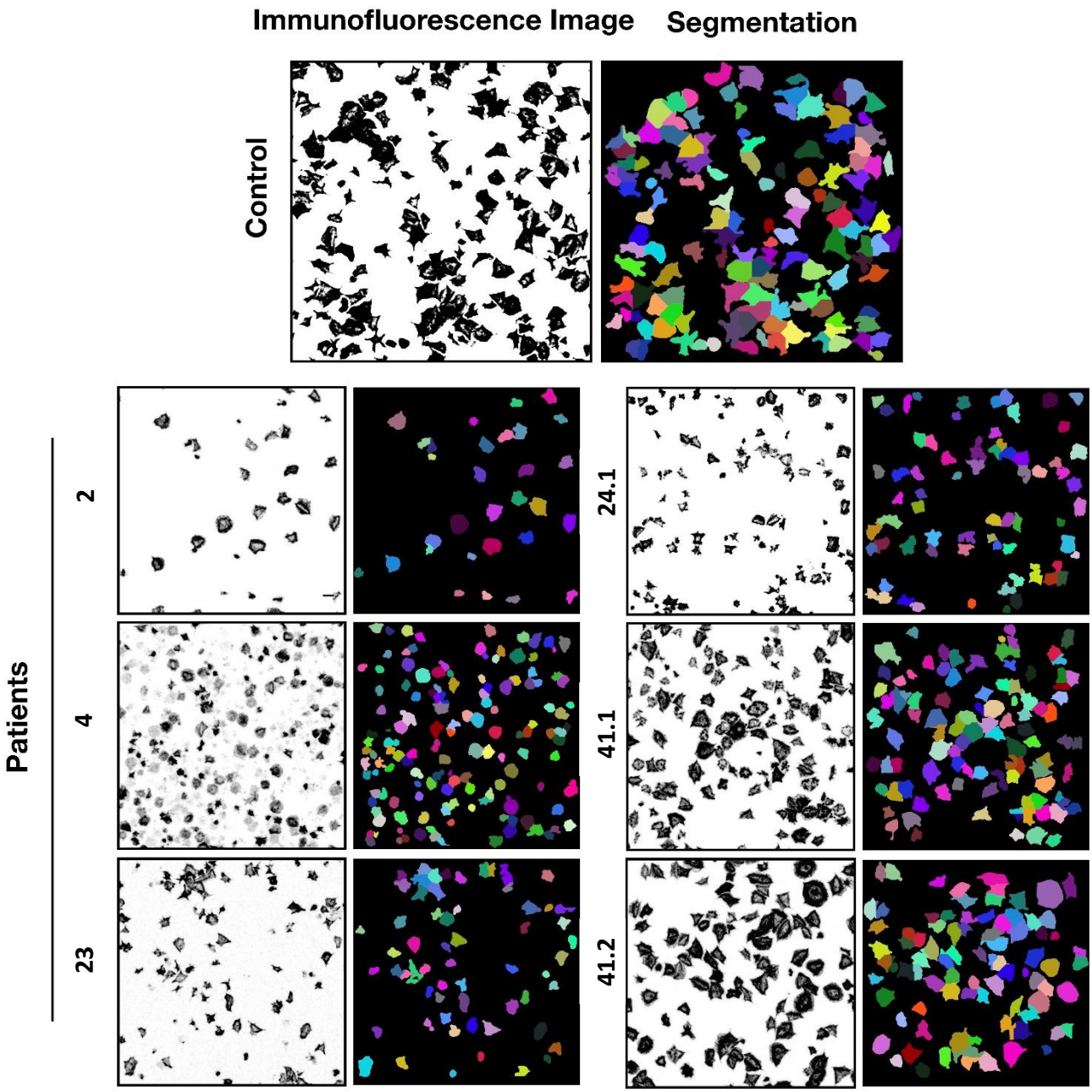


Figure 3.12. Representative platelet spreading immunofluorescent and segmentation images of selected patients with clear defects in platelet area or circularity. The top panels show a representative healthy volunteer control. Lower panels show patient images; patients 21 and 3 were found to have a platelet area spreading defect only, patients 13 and 31.1 had a platelet spreading circularity defect only, and patients 16.1 and 16.2 had both area and circularity spreading defects.

3.11. Discussion

From the work in this results chapter, high-throughput platelet spreading analysis was systematically correlated with clinical and lumi-aggregometry data to determine its utility and usability in the investigation of suspected platelet function disorders. The data showed that patients positive for both area and circularity abnormalities, as well as abnormal morphology are both positive and negative for defects in aggregation. Furthermore, 80% of patients with a spreading defect, but negative for an aggregometry defect presented with a high ISTH-BAT bleeding score. This is of particular interest as a large number of patients positive for a platelet spreading defect, and with a history of excessive bleeding, are negative for an aggregation defect. This all suggests that applying platelet spreading as part of a platelet testing panel can reveal problematic platelet morphological defects that might otherwise be missed by lumi-aggregometry alone.

As with all studies, this study is not without some limitations. The high-throughput method of analysis is indeed a rapid technique to analyse a large number of images in a standardised manner. However, it is the method of spreading, staining and image acquisition that are the rate limiting steps of this assay. This brings into question whether this assay could be used in a routine clinical testing laboratory or whether it is to be used more as a specialised research tool. The spreading step requires the coating of coverslips ahead of time as well as platelet resting steps and spreading steps. To get to this stage can take over 2 hours of relatively hands-on time. The staining steps again take longer than 2 hours. Lastly, the platelet image acquisition for this chapter was carried out by confocal microscopy. Not all centres especially Comprehensive Haemophilia Care Centres have confocal microscopes available with workers who are trained to carry out imaging on a regular basis; in addition, imaging to gain a set

number of representative fields of view also takes a long time. These are all limitations of this assay. The assay could be streamlined in order to speed up the process and it would benefit from set methods and standardised concentrations and values. An alternate method of imaging would also be beneficial to this assay for example, an EVOS® cell imaging system that could carry out the imaging independently. If we could then apply the images to the high-throughput workflow again mostly automatically, this would speed up the process of obtaining results and would require fewer hours of work by an individual in the laboratory and which might not have to be limited to a specialist site.

Another major drawback of this assay is that it cannot diagnose specific disorders in isolation. This is because there are many platelet or bleeding disorders that could present with abnormalities in platelet spreading. If we could use patient blood from a group of patients who had already been diagnosed with a specific platelet function/bleeding disorder affecting platelet structure, it would be interesting to carry out the platelet spreading assay with their blood to observe if the area and/or circularity values would vary from a group of patients previously diagnosed with a different platelet disorder that affects platelet structure. If the values were to vary, it would be interesting to try to diagnose patients using these values. Furthermore, the diagnosis could be validated with genetic testing; this would allow for the assay to become more standardized and the normal range values to be improved.

A major advantage is that the platelet spreading assay is not dependent on platelet count unlike the aggregation-based platelet function tests (Dawood et al., 2007, Dawood et al., 2012). The spreading assay could therefore be a useful tool to test for platelet function defects in patients with thrombocytopenia as only a small number of platelets are required.

All that being said, this assay is a useful screen for patients with a strong bleeding history from which conventional assays have not been able to diagnose the disorder. The potential utility of this assay in a patient population is only just being explored but the platelet spreading assay approach described in this chapter validates the use of high-throughput platelet spreading analysis as an analytical tool to improve and enhance existing platelet function testing. As high content imaging approaches become increasingly accessible and commonplace, applying high-throughput platelet spreading strategies is likely to be an invaluable addition to the current range of platelet function tests.

3.12. Future work

Platelet counts, platelet parameters, and platelet sizes vary within an individual and there are many methods available to calculate all of these measures in patient blood samples. However, we do not currently have a robust assay that assesses activated platelet morphology. It would be interesting to combine other platelet parameters with the findings from the platelet spreading assay across a larger cohort of patients. If there were any correlations between platelet data and spreading data, we could characterise these disorders to hopefully provide the patients with a diagnosis for their previously undiagnosed platelet or bleeding disorder. In addition, it can be difficult to determine potential secondary defects in patients who are affected by bleeding symptoms. For example, if a patient was suspected to have a secondary qualitative defect, transmission electron microscopy could be carried out to confirm the presence or absence of alpha or dense granule deficiencies.

If the platelet spreading assay was to become more common place as a research tool or in specialist laboratories, a wider range of specialist antibodies could be investigated for use during the platelet staining stage. This would allow for an increased number of platelet

cytoskeletal components to be investigated during platelet activation and spreading. The semi-automated workflow could be altered for the analysis of these platelets, but the images could highlight aberrant proteins in patients with bleeding symptoms.

Whilst we are making advances in the field of platelet biology and our knowledge of platelet function has improved dramatically in recent years, there are still patients in this cohort without a clinical diagnosis despite having a clinically meaningful bleeding diathesis. In order to fully understand and hopefully find a diagnosis for these patients, a combination of phenotyping and genotyping is required, especially as these patients are suspected to harbour novel disorder with unknown genetic aetiology.

CHAPTER 4
Patient genotyping by whole exome sequencing

4. Patient genotyping by whole exome sequencing

4.1. Summary of background to this research

The investigation into an individual's genome and their genetics is now commonplace in hospitals and research centres across the world. Genetic testing has the ability to identify defects in chromosomes, genes or proteins. The results of a genetic test can either confirm or exclude a suspected genetic cause of disease, as well as helping to determine an individual's chance of developing or passing on a particular condition caused by a genetic change. Genetic testing is always voluntary due to the sometimes complex and life changing nature of the results. However, positive results can lead to disease prevention, disease monitoring and personalised treatment options.

Molecular genetic tests and the technology involved has rapidly progressed since the invention of Sanger sequencing over 40 years ago (Sanger et al., 1977). Today, a more contemporary version of Sanger sequencing using fluorescence and capillary array electrophoresis is widely used across all genetic diagnostic services. Following the development of next-generation sequencing technologies, there has been a shift in the usage of small-scale genetic sequencing applications, to larger scale studies involving whole exome and whole genome sequencing. This relatively new technology has led to the rapid and accurate ability to sequence large amounts of genetic material successfully (Ng et al., 2009, Ng et al., 2010, Bamshad et al., 2011).

The increase in clinical usage of next-generation sequencing is firstly due to dramatic improvements in the methods. This has allowed the sequencing to be performed more quickly but also allowed for specialised bioinformatic analysis tools to be created and utilised. This has decreased the clinical time frame overall for producing high quality and quality-controlled

results for patients. Secondly, the costs of next-generation sequencing have decreased. The total cost of sequencing the first human genome by the Human Genome Project was estimated to be approximately $3 billion. However, the cost of sequencing a whole genome is now approximately $1000 (Warr et al., 2015). This cost is set to decrease even further with many biotechnology companies competing to improve and refine their sequencing methods and products for clinical and research settings.

Such improvements have impacted the field of gene discovery in inherited bleeding and platelet disorders. NGS has aided in the discovery of novel, disease causing genetic variants as well as elucidated the genetic aetiology behind many previously known disorders (Albers et al., 2011, Kunishima et al., 2013, Maclachlan et al., 2017b). However, the use of genetic investigation is still mainly used for known diseases and well-classified disorders to confirm a diagnosis. The question is then whether the disease subset such as rare bleeding disorders in families with multiple affected family members, as well as single isolated cases would benefit from NGS by WES. WES and WGS could prove vital for discovering novel genetic causes of bleeding and in genes not previously implicated in bleeding.

Due to limitations of platelet function testing as discussed in Chapter 3, patient genotyping is an ideal approach to elucidate the cause of disease in patients with clinical bleeding of unknown aetiology. WES and Sanger sequencing were carried out on selected patients previously recruited to the GAPP study. The patients were included in this group if, following extensive platelet function testing and a normal platelet count, the overall laboratory phenotype results were normal despite the patient having an extensive bleeding history.

4.2. Aims of this chapter

This results chapter will aim to genotype individuals from 10 multiplex families within the GAPP study with bleeding of unknown cause. All individuals presented with normal platelet counts and normal platelet function but suffered from regular bleeding episodes. Up to two patients per family were sequenced by WES, to try to determine the genetic variant(s) likely to be causative of their disorder. The results will potentially lead to an overall diagnosis helping with treatment and management of the patients along with providing novel insights into bleeding physiology and potential novel genetic variants, with which to carry out further functional investigation and characterisation.

4.3. Patient characteristics

During the course of the research for this thesis, 18 patients with normal platelets but extensive bleeding histories were included in the WES cohort. This included 10 families with multiple affected members. Patients were consented to take part in the GAPP study that included consent regarding genetic research (Consent form, appendices Figure B).

The patients' ages were available for 16 of 18 patients, which included both paediatric and adult patients. The average age of the patients was 27 years, ranging from the youngest at 7 years to the oldest at 60 years (Figure 4.1). Gender information was available for all patients. The cohort consisted of 72% (n = 13) females and 28% (n = 5) males (Figure 4.1). The ethnicities of patients in this cohort were not made available by the referring clinicians.

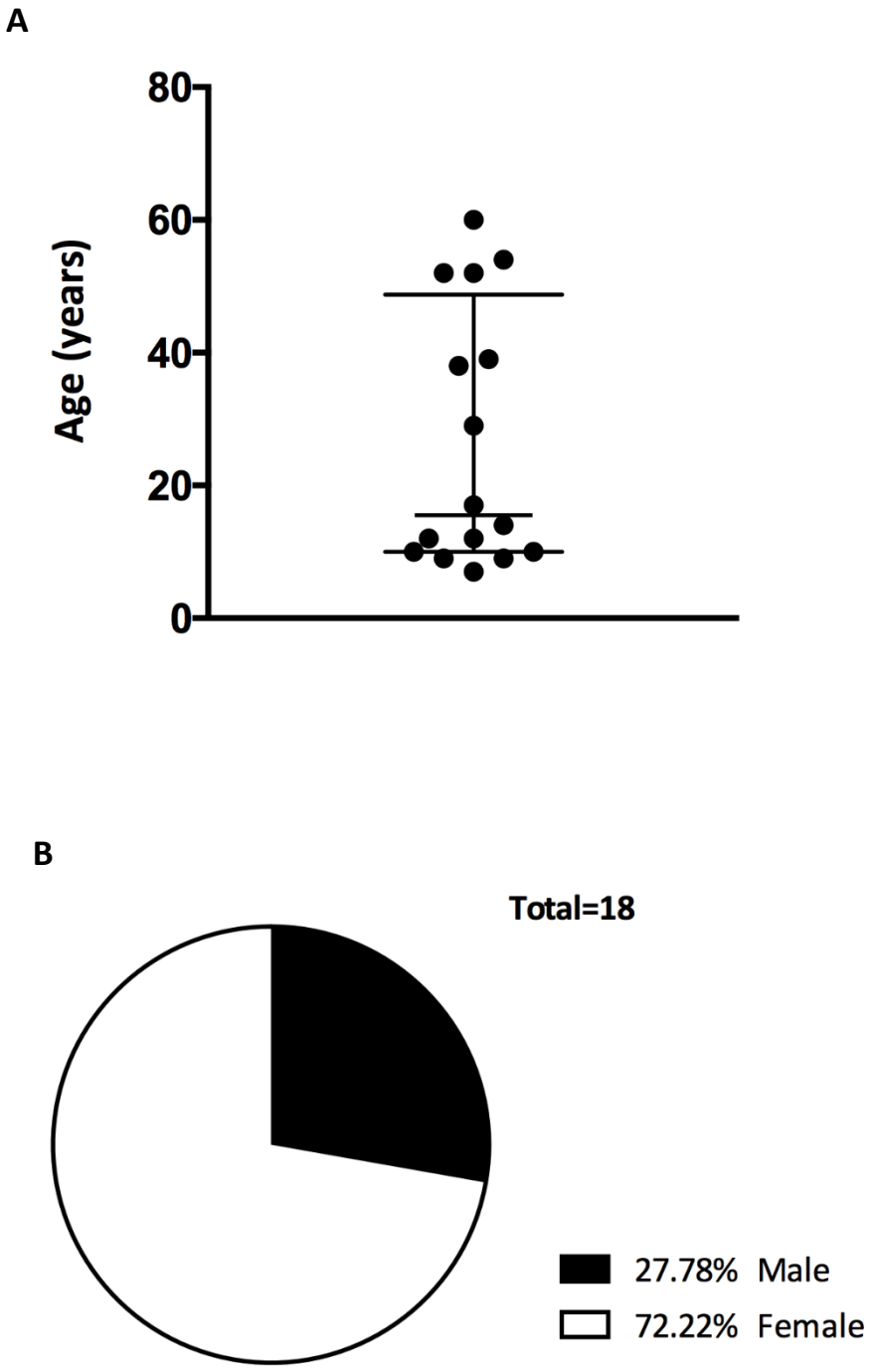


Figure 4.1. Demographics of GAPP study patients who underwent WES. (A) Ages of patients shown with median (27 years) and interquartile range. (B) Percentages of male and female participants.

The clinical findings and bleeding symptoms for all 18 patients can be seen in Table 4.1. Platelet count data from whole blood was only available for 5 of 18 patients as some patients were recruited before the acquisition of the Sysmex™ Haematology Analyser. Before this, platelet counts were measured in PRP. The platelet counts for all patients, regardless of the method of measurement, were within the normal range (150–400 × 10^9/l). MPV values were measured to examine platelet size and were available for 16 of 18 patients. MPV values ranged from 8 fL to 12.4 fL; the median value for the group was 9.8 fL. In addition, platelet secretion was measured by lumi-aggregometry (as described in Chapter 2, Section 2.9) and is available for 11 of 18 patients, the values are displayed in Table 4.1 where available.

The bleeding symptoms of the patients was provided by the referring clinicians for 17 of 18 patients. The patients all reported suffering from similar 'minor' bleeding symptoms. These were classed as minor, which can still have a big impact on the patients' lives, especially for the paediatric cases. The most common bleeding symptom reported was easy cutaneous bleeding with 71% (12 of 17) of patients reporting this occurring on a regular basis. Secondly, 47% (8 of 17) of patients reported suffering from frequent epistaxis (> 5 /year lasting more than 10 minutes); the third most common symptom reported was excessive bleeding from minor wounds with 41% (7 of 17) of patients reported regularly suffering from this. Furthermore, 58% (7 of 12) of females reported from suffering from menorrhagia often requiring time off work/school and requiring treatment by antifibrinolytics, hormonal therapy and/or iron therapy.

Table 4.1. Phenotypes of all patients recruited to the GAPP study that were included in WES investigations. The normal ranges for platelet count, MPV and secretion are shown in square brackets. F, Female; M, Male; MPV, Mean Platelet Volume
Code of bleeding phenotype:
1 – Epistaxis, 2 – Cutaneous bleeding, 3 – Petechiae, 4 – Oral cavity bleeding, 5 – Bleeding from minor wounds, 6 – bleeding after tooth extraction, 7 – Bleeding after surgery, 8 – Hematuria, 9 – GI bleeding, 10 – Ovulation bleeding, 11 – Menorrhagia, 12 – Post-partum haemorrhage, 13 – Muscle haematomas

Family/Individual	Age (Years)	Gender	Platelet count [150–400 $\times10^9$/l]	MPV [9.2–12.9 fL]	Secretion [> 0.65 nmol ATP/1$\times10^8$ platelets]	Main findings	Bleeding phenotype
Family 1 II:4	60	F		8		No defect	6, 7, 11
Family 1 III:2	39	M		10		No defect	2, 9, 13
Family 2 II:1	52	F		9.2		No defect	
Family 3 II:3	9	F	329	12.1	3.88	No defect	1, 2, 5, 7
Family 3 II:2	10	F	246	11.3	2.4	No defect	2, 5
Family 4 II:2	52	F		8.6		No defect	2, 11
Family 4 III:1	29	F		9.4		No defect	2, 4, 6, 11
Family 5 I:1	54	M				No defect	6, 7
Family 5 II:1	12	M				No defect	1, 2, 5, 13
Family 6 II:1	10	M		8.1	2.5	No defect	3
Family 6 II:2	14	F		7.8	2.01	No defect	1, 7
Family 7 I:2		F	284	12.1	0.72	No defect	1, 2, 4, 5, 11
Family 7 II:1		F	191	12.3	0.98	No defect	1, 2, 4, 5, 11,12
Family 8 II:1	12	F	221	12.4	2.74	No defect	1, 2, 5
Family 9 II:2	38	F		9.2	1.8	No defect	2, 4, 6, 11, 12
Family 10 II:2	9	M		8.9	1.59	No defect	1, 2
Family 10 II:3	7	F		8.8	1.91	No defect	1, 5
Family 10 II:1	17	F		8.9	1.96	No defect	2, 6, 7, 11

4.4. Whole exome sequencing overview

Whole exome sequencing was performed on 18 individuals following the GAPP phenotyping and platelet function testing workflow, as detailed in Chapter 1 and outlined in Figure 1.5. This included lumi-aggregometry in response to a panel of platelet agonists at multiple concentrations that are summarised in Chapter 2, Table 2.1.

4.4.1. Development of a bioinformatic pipeline

A bioinformatic pipeline was developed by the GAPP study team in order to determine plausible candidate variants from the WES data (Figure 4.2). Sequence alignment, annotation and variant calling were carried out by collaborators in London/Birmingham in addition to describing variant novelty and frequency of the variants in multiple populations. Bioinformatic analysis was then carried out on the variant called files (VCFs) for each individual.

Bioinformatic analysis was carried out by two different criteria (arms) performed in parallel. One arm compared variants to a gene panel created for this patient group which included genes implicated in platelet formation, count and function in addition to all genes implicated in blood coagulation, fibrinolysis and endothelial cell function. The second arm was used to analyse novel variants only.

Firstly, variants were compared to the gene panel of 856 known platelet, bleeding, coagulation and endothelial genes, a list developed by me and mentioned below. These variants were then filtered on the basis of exclusion criteria created by the GAPP study. A rare variant cut off value was used of 0.01 or 1% of the population as variants with a minor allele frequency (MAF) of < 0.01 were given priority when being analysed. Synonymous changes, not modifying the amino acid sequence, were excluded. Splice site variants occurring > 4 base pairs away from the exon

boundary were also filtered out. Where more than one affected family member was analysed by WES, the variants were compared and non-shared variants were also excluded.

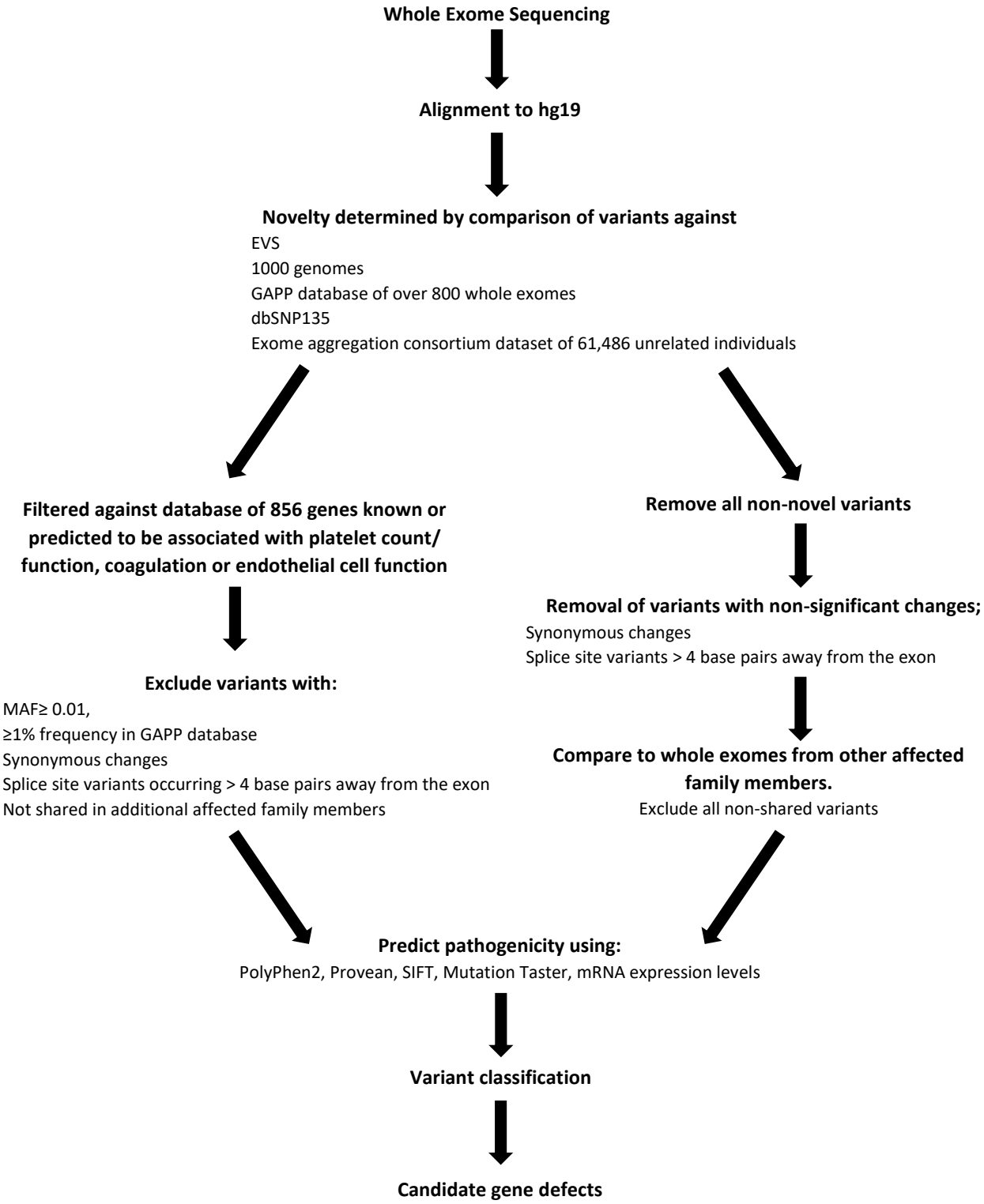


Figure 4.2. Bioinformatic pipeline for the analysis of WES data.

The second arm of analysis excluded all non-novel variants. Novelty was determined by comparison of the variants against multiple databases including EVS (Exome Variant Server), 1000 Genomes database, dbSNP135 (Single Nucleotide Polymorphism database) and ExAC (Exome Aggregation Consortium). Again, synonymous changes, not modifying the amino acid sequence, were also excluded. Splice site variants occurring > 4 base pairs away from the exon boundary were also filtered out. The remaining variants were compared to those from other affected family members and non-shared variants were excluded.

The remaining variants from both arms of analysis in the bioinformatic pipeline were further analysed by the same method. *In silico* pathogenicity prediction tools and software were employed. Individual tools are outlined and described in Chapter 2, Section 2.14.1. Variants that were predicted to alter the coding sequence and hence alter the length of the sequence for example, insertions, deletions and nonsense variants were analysed by MutationTaster (Schwarz et al., 2014). Single nucleotide variants that lead to missense variants were analysed by PolyPhen-2, Provean, SIFT and MutationTaster. Due to differences within the prediction tools, the pathogenicity prediction of certain variants was not always in agreement across all the tools used. This added difficulty to the classification of variants in the bioinformatic pipeline but is in keeping with previous studies (Leo et al., 2015, Richards et al., 2015). If 2 or more of the pathogenicity prediction tools suggested that a variant be pathogenic, the variant was taken forward for further analysis.

4.4.2. Development of inherited bleeding related gene panel

In order to create the gene panel used in the bioinformatic pipeline, genes were collected from a number of different sources and collated. Genes were collected from other previously used panels in the GAPP study such as the genes implicated in thrombocytopenia (Johnson et

al., 2016b). All genes implicated in coagulation and fibrinolysis-related genes were identified through previously published literature and findings, animal models and experts in their field. An endothelial-related genes list was obtained from Professor Roy Bicknell (Institute of Cardiovascular Sciences, Birmingham) in addition to literature searches/publications on expression data. The final gene panel contained 856 genes; these can be seen in Table A of the Appendices.

4.4.3. Analysis of candidate variants

The candidate gene variants that resulted from the bioinformatic pipeline analysis were then classified (outlined in Chapter 2, Section 2.14.2). This is one of the most challenging and complex tasks in the genetics field as for most novel variants, the true pathogenicity is unknown. It is also difficult to classify variants that have limited evidence and likely with novel variants. The ACMG guidelines were followed as recommended which provided a framework for evidence assessment for variants and is the current gold standard of variant interpretation (Richards et al., 2015). The 28 criteria that assess evidence from population data, functional data and allelic data to computational predictions are strictly defined and all assigned a code. The codes are weighted (stand-alone, very strong, strong, moderate or supporting) and assigned significance (benign or pathogenic). These evidence codes can be combined to produce a classification (pathogenic, likely pathogenic, variant of uncertain significance, likely benign, or benign) of the variant in question. If a variant does not have enough evidence to produce a classification, or the lines of evidence are contradictory, then the variant is classified as a variant of ‘uncertain significance’. This is the most common classification amongst novel variants. Overall, the ACMG guidelines provided much needed consistency when classifying variant pathogenicity.

4.5. Results of WES in families with multiple affected individuals

4.5.1. Family 1

A mother and son were recruited to the GAPP study with a lifelong bleeding tendency, including post traumatic bleeding, but presented with normal PRP counts (4.2×10^8 /ml and 3×10^8 /ml respectively) and normal platelet function. The family pedigree displays an autosomal dominant inheritance for the bleeding phenotype (Figure 4.3). An extensive bleeding history throughout the pedigree was evident. The index case, a male III:2, suffered from haemarthroses, soft tissue, and muscle bleeding in childhood and a spontaneous rectal sheath hematoma in 2006. His mother, II:4, was admitted to hospital for 3 weeks after wisdom teeth extraction, suffered bleeding after tonsillectomy and menorrhagia requiring hysterectomy further complicated by bleeding. His grandmother, I:1, suffered from bleeding after dental extraction requiring transfusion, easy bruising, and postpartum haemorrhage. His maternal aunt, II:3, suffered from extensive bleeding which resorted in a nephrectomy following a road-traffic accident. His maternal uncle II:2, lost an eye as a result of traumatic bleeding as a child, as well as suffering from bleeding post dental extraction and soft tissue and muscle haematomas. To study these patients further, a forward genetic approach by WES was implemented to establish the disease aetiology where laboratory testing had not identified a defect in platelet function.

The patients were evaluated and a clinical history was taken including bleeding scores using the International Society of Haemostasis and Thrombosis bleeding assessment tool (Lowe et al., 2013). Blood was taken from both patients, the PRP was isolated and platelet function was tested using lumi-aggregometry and a range of concentrations of agonists. Platelet ATP

secretion from dense granules was also measured using the luciferase assay (Dawood et al., 2012).

Patients' platelet aggregation traces showed normal responses to all the concentrations of agonists that were tested (Figure 4.4). Normal platelet granule secretion values were also observed in both patients in response to all agonists. Therefore, no defect in platelet aggregation or secretion was detected in either patient. Factor IX genetics were also normal; however, prothrombin consumption index was abnormal (both carried out by the Nottingham Haemophilia Centre).

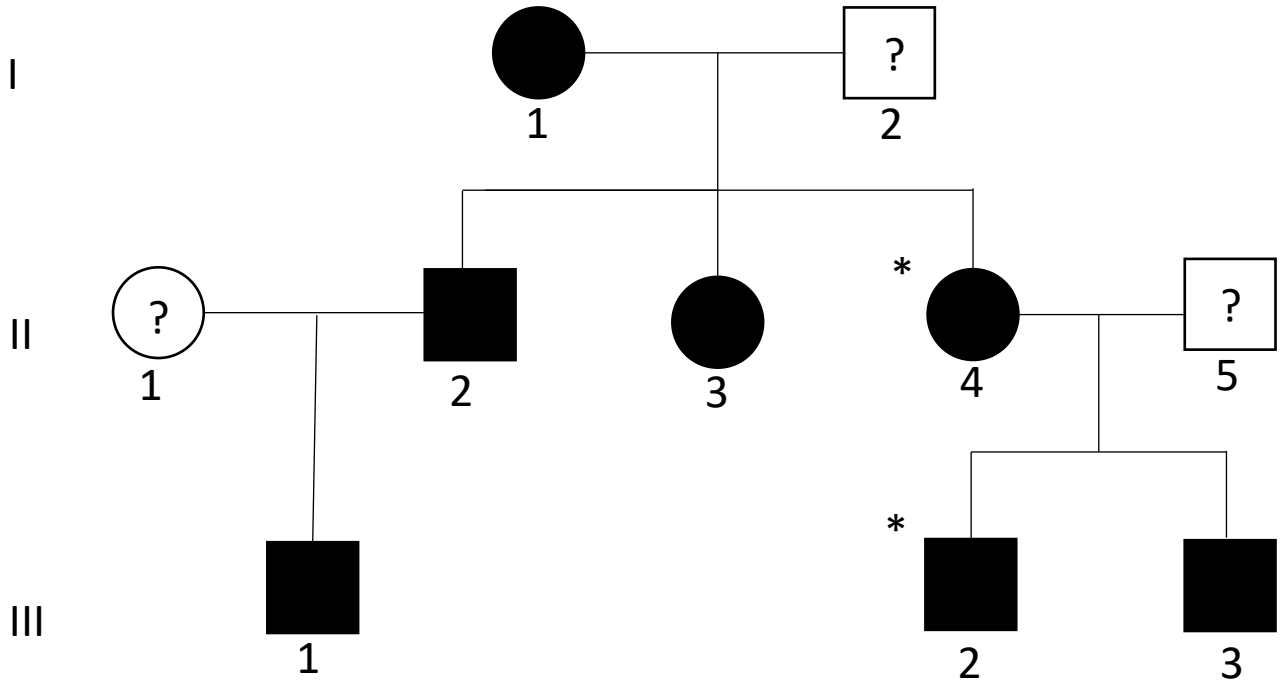


Figure 4.3. Family 1 Pedigree. This shows a dominant form of inherited bleeding. Pedigree shows affected individuals as shaded symbols and asterisks (*) indicate patients (II:4 and III:2) whose whole exomes were sequenced.

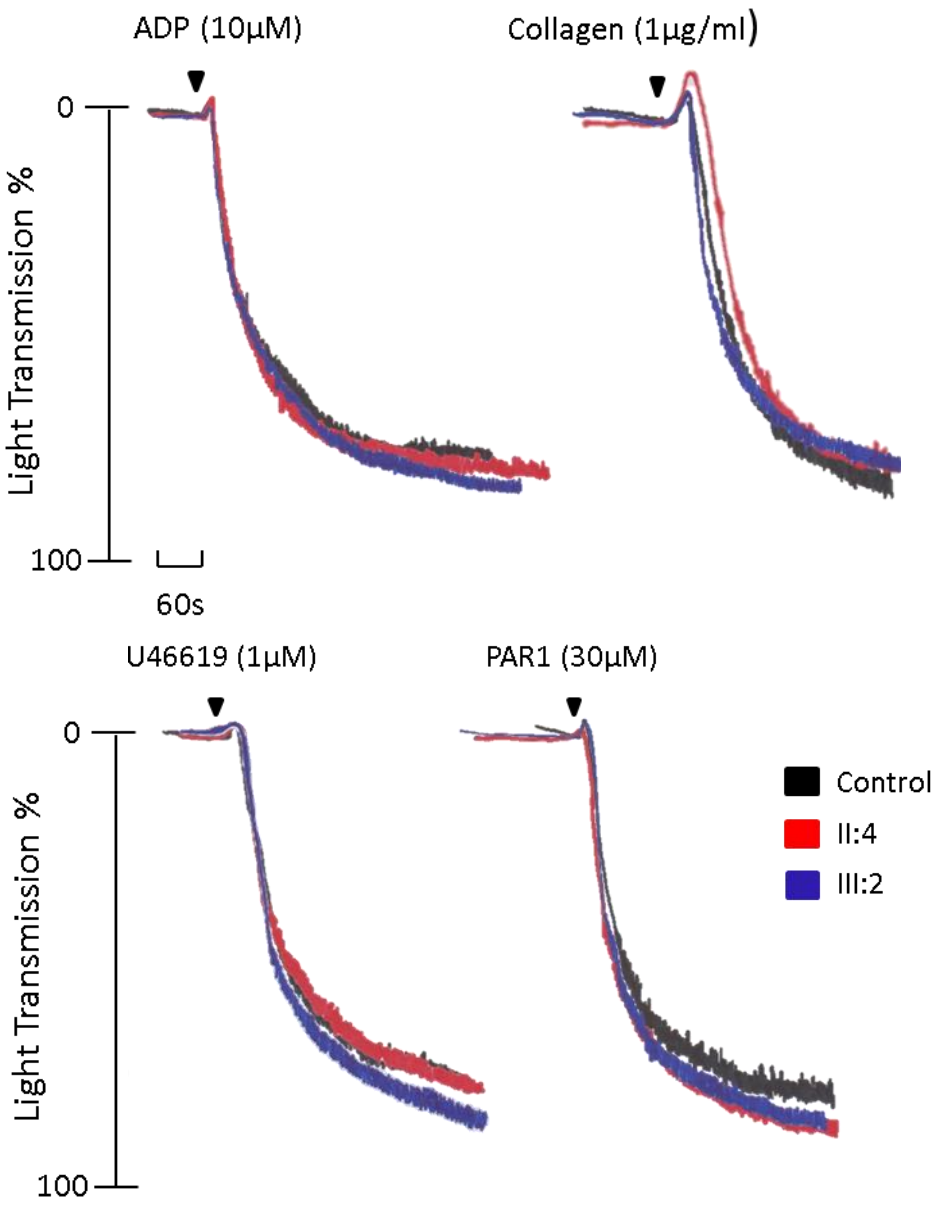


Figure 4.4. Representative light transmission aggregometry traces in platelet-rich plasma. The level of aggregation in response to stimulation was assessed with ADP (10 µM), collagen (1 µg/ml), U46619 (1 µm) and PAR1 peptide (30 µM) along with a multitude of other agonists and concentrations as summarised and shown in Chapter 2, Table 2.1.

WES was performed and identified 26,125 variants in II:4 and 25,969 variants in III:2. The bioinformatic pipeline was used to narrow down the variants with a series of filtering steps displayed in Table 4.2. Following the alignment step and determining the novelty of the variants, they were filtered against a panel of 856 genes known or predicted to be associated with platelet count or function, coagulation, fibrinolysis or endothelial cell function.

The remaining variants were then filtered by frequency; variants with a MAF of more than or equal to 0.01 were excluded as were any synonymous changes. Comparison of both patients with a MAF ≤ 0.01 reduced the number to a total of 17 sequence variants. Pathogenicity prediction tools (Mutation Taster, Phastcons, SIFT, Provean etc.) were then utilised and the variants were classified as outlined by the ACMG guidelines (Richards et al., 2015). Candidate sequence variants were selected and reduced based on a pathogenic prediction in at least three out of four of the prediction tools.

This left remaining candidate variants in five genes: *APC, PDE3A, ACE, OCLN*, and *THBD* where pathogenicity prediction details are shown in Table 4.3. All previously mentioned variants were classified as being of 'uncertain significance' by the ACMG guidelines except for the variants in *THBD* which was classified as 'pathogenic'. This classification led to further research into the function of the gene and the potential consequence of the *THBD* genetic variant. It was found to have been previously reported in the population, with two publications (Langdown et al., 2014, Dargaud et al., 2015). In both cases, the patients and their families suffered from similar bleeding histories as the patients in the GAPP study.

Sanger sequencing was then performed to confirm the variants thereby ruling out the possibility of false-positive results of WES. Sanger sequencing confirmed that the genetic variant in *THBD* (c.1611C>A) was present in both patients (Figure 4.5.).

Table 4.2. Genetic variants filtered by bioinformatic pipeline. A series of filtering steps were used to try to reduce variant numbers to elucidate potential causative genetic variants.

	II:4	**III:2**
Total number of variants identified by WES	26,125	25,969
Total number of variants (excluding synonymous) with a MAF ≤ 0.01	2,320	2,376
Shared significant variants from the panel of platelet and endothelial cell genes with a MAF ≤ 0.01	17	
Total number of novel variants	181	152
Total number of shared novel variants	50	
Total number of shared panel genes predicted to be pathogenic using all bioinformatic tools	5 (*APC, PDE3A, ACE, OCLN* and *THBD*)	

Table 4.3. Table of candidate genes for Family 1 predicted to be pathogenic by pathogenicity prediction tools. The table shows genomic variation and the protein effect as well as the prevalence of the variant in the population. ACMG criteria were assigned as appropriate leading to a classification for each variant.

Family 1, II:4 and III:2										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
ACE **NM_000789**	c.1142C>T	p.T381M	Missense	0.000756	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
APC **NM_001127511**	c.5162A>G	p.K1721R	Missense	8.29×10^{-6}	Disease causing	Probably damaging	Damaging	Neutral	PP3 PP1	Uncertain significance
OCLN **NM_002538**	c.70C>G	p.P24A	Missense	0.008548	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
PDE3A **NM_000921**	c.1535G>A	p.R512Q	Missense	3.39×10^{-5}	Disease causing	Probably damaging	Damaging	Neutral	PP3 PP1	Uncertain significance
THBD **NM_000361**	c.1611C>A	p.C537*	Nonsense	Novel	Disease causing				PM2 PP3 PP1 PM4 PS1 PVS1	Pathogenic

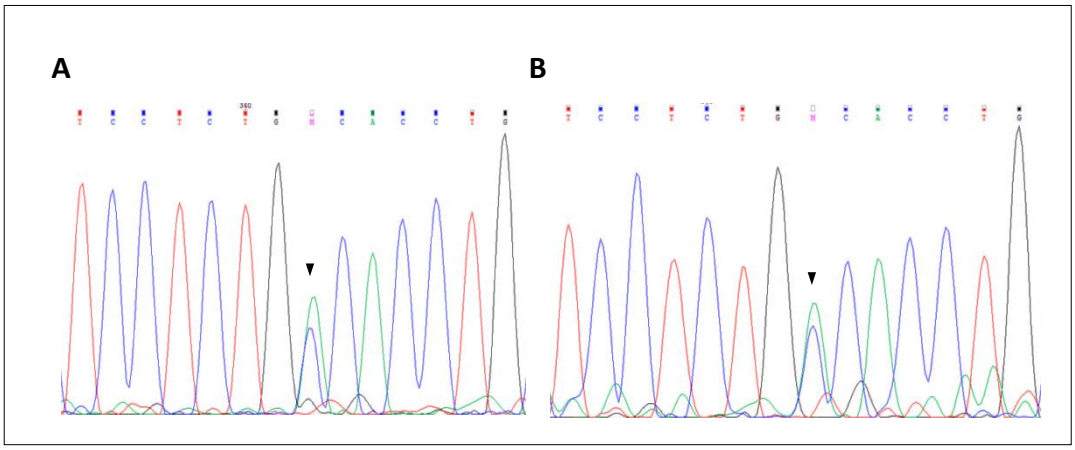


Figure 4.5. Sanger sequencing electropherograms confirming the c.1611 C>A change in (A) II:4 and (B) III:2.

The *THBD* sequence variant identified in this family encodes the protein thrombomodulin (c.1611C>A, p.Cys537Stop). Thrombomodulin, a glycoprotein, is expressed on the surface of endothelial cells and normally serves as a cofactor for thrombin (Weiler and Isermann, 2003).

In a normal state, thrombomodulin forms a complex with thrombin reducing blood coagulation by the activation of protein C, therefore inactivating factors Va and VIIIa, cofactors highly involved in the coagulation cascade, and promoting fibrinolysis. However, the genetic variant in these patients leads to a stop codon in the sequence, resulting in premature termination of transcription and hence a truncated form of the protein. Due to the truncation, the protein is shed from the endothelial cell membrane into the blood plasma. The increase of soluble thrombomodulin promotes protein C activation and is also seen in several pathologic conditions associated with endothelial dysfunction (Dahlback and Villoutreix, 2005, Kurosawa et al., 2008). This therefore reduces thrombin generation within a potential thrombus, resulting in the phenotype of posttraumatic bleeding (Langdown et al., 2014). Furthermore, this concurs with the abnormal prothrombin consumption index observed when the patients were tested. This is a rare example of where a gain-of-function mutation in a procoagulant protein rather than platelet dysfunction, causes a bleeding disorder (Dargaud et al., 2015).

The discovery of a variant in the gene encoding thrombomodulin demonstrates that the bioinformatic pipeline is working to be applied to other exomes in the same way. In other words, this is proof of principle for the method that has been developed as part of the GAPP study, especially in those patients with non-classic platelet disorders such as those included in this chapter of this thesis. As for the patients, appropriate monitoring and preventative measures can be taken in light of their genetic diagnosis and has already been used to cascade

genetic screening for further members of the extended family to aid clinical management of this relatively severe bleeding disorder.

4.5.2. Family 2

Two sisters were recruited to the GAPP study at the ages of 41 and 36 years. Both sisters suffered from bleeding episodes for the duration of their lifetimes. Both sisters had normal platelet counts in PRP (4×10^8/ml and 4.6×10^8/ml) and normal MPV values (9.2 fl and 9.9 fl). There is no known history of bleeding in other family members. The family pedigree can be seen in Figure 4.6.

Platelet aggregation studies were performed using PRP against a wide range of agonists. All platelet responses were within the normal range. Platelet secretion was tested using lumi-aggregometry that resulted in normal values for both patients. Overall, no defect in aggregation or secretion was detected in the two sisters however, their bleeding disorder still remained undiagnosed. WES was carried out with DNA from II:1 only, this was because the concentration of DNA from extracted from II:2 was too low to perform accurate WES despite efforts to concentrate the samples.

WES followed by bioinformatic analysis revealed five potential disease-causing variants. These variants were in genes*: EPHA4*, *PLA2G4C*, *PRKACA*, *PTPRF* and *SELPLG*. The variants and their classifications can be seen in Table 4.4. The five variants were all classified as variants of 'uncertain significance' due to the lack of evidence for both the novel and rare variants. The variant in the gene *SELPLG* (c.245C>G, p.T82S) could be interpreted as the prime candidate for these patients due to its association as a ligand for P-selectin expressed on the surface of endothelial cells and activated platelets. However, the other variants cannot be ruled out by biases towards certain genes until the correct variant is proven to cause the disease.

Further work needs to be carried out to confirm this variant in this family however this work goes beyond the scope of this thesis.

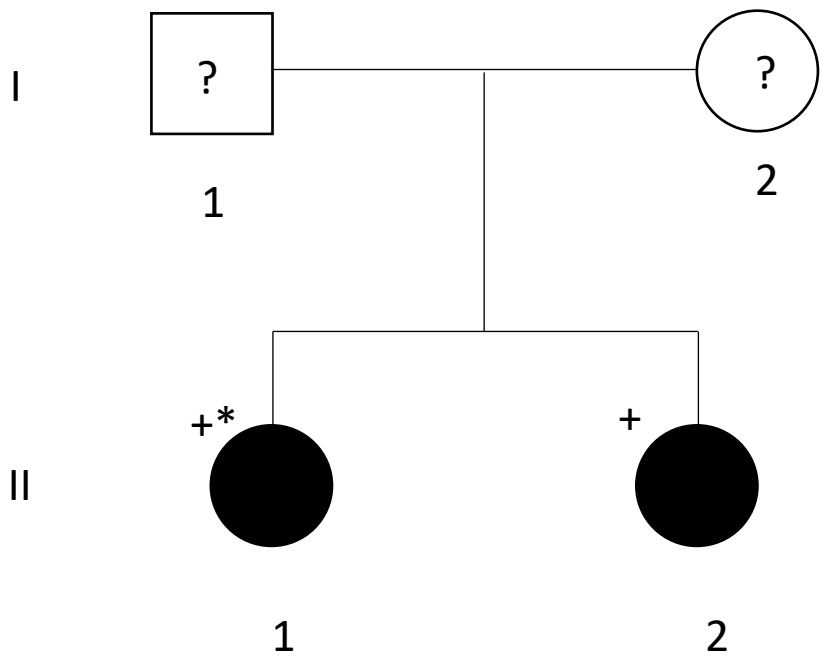


Figure 4.6. Family 2 Pedigree. Pedigree shows affected individuals as shaded symbols. Crosses (+) indicate patients recruited to the GAPP study and asterisks (*) indicate patients (II:1) whose whole exomes were sequenced.

Table 4.4. Table of candidate genes for Family 2 predicted to be pathogenic by pathogenicity prediction tools. The table shows genomic variation and the protein effect as well as the prevalence of the variant in the population. ACMG criteria were assigned as appropriate leading to a classification for each variant.

Family 2										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
EPHA4 **NM_004438**	c.1105T>C	p.C369R	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
PLA2G4C **NM_001159323**	c.889delC	p.P297Gfs*13	frameshift deletion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
PRKACA **NM_002730**	c.304C>A	p.P102T	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
PTPRF **NM_130440**	c.4324C>T	p.R1442W	Missense	8.57×10^{-6}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
SELPLG **NM_003006**	c.245C>G	p.T82S	Missense	4.94×10^{-5}	Disease causing	Probably damaging	Tolerated	Neutral	PP3 PP1	Uncertain significance

4.5.3. Family 3

A young girl (index case II:3), aged four years at the time of recruitment, was referred to the GAPP study for further phenotyping of her undiagnosed bleeding disorder. She was referred due to a strong history of bleeding, highly unusual in young children. She suffered from frequent epistaxis, cutaneous bruising and prolonged bleeding times from minor wounds. However, the possibility of a bleeding disorder was raised after she bled excessively following an adenotonsilectomy to the extent of becoming haemodynamically unstable. She required further surgery, packing and received blood products and other clinical treatments.

The index case has two sisters, one of whom was also recruited to the GAPP study (II:2) at the age of six years. She suffers from cutaneous bruising and prolonged bleeding times from minor wounds. There is no family history of bleeding apart from the mother who has always had heavy menstrual bleeding however never received any treatment for this. The family pedigree can be seen in Figure 4.7.

The GAPP study investigative workflow was carried out on both patients (II:2 and II:3). Both patients had normal platelet counts (II:2, 246 × 10^8/l and II:3, 329 × 10^8/l) and normal mean platelet volumes (II:2, 11.3 fl and II:3, 12.1 fl). Lumi-aggregometry performed showed normal platelet aggregation and secretion results in both II:2 and II:3 (2.4 nmol ATP/1 × 10^8 platelets and 3.88 nmol ATP/1 × 10^8 platelets respectively). Normal coagulation factor screen results were also gathered by the referring hospital. There was no obvious platelet defect found to explain the participants' bleeding history therefore WES was employed to further investigate their cause of bleeding.

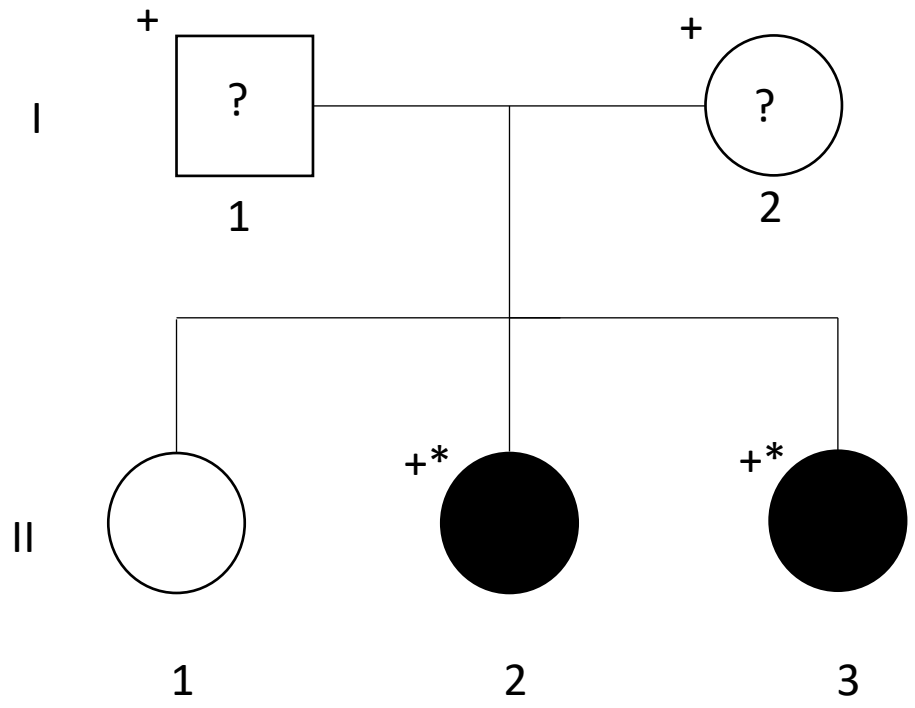


Figure 4.7. Family 3 Pedigree. Pedigree shows affected individuals as shaded symbols. Crosses (+) indicate patients recruited to the GAPP study and asterisks (*) indicate patients (II:2 and II:3) whose whole exomes were sequenced.

The genetic variants that resulted from sequencing and bioinformatic scrutiny were analysed. This resulted in nine potential disease causing genetic variants in genes: *ACPT*, *CD109*, *GLIS2*, *MEAF6*, *PLA2G3*, *PPP1R14A*, *TARS2* classified as variants of 'uncertain significance', and two variants in *TTLL10* classified as 'likely pathogenic'. The *TTLL10* variants are both frameshift variants (c.462delG and c.745_746insG). Table 4.5 shows the variants, their pathogenicity prediction results and classifications.

The 'likely pathogenic' classification of the *TTLL10* variants was due to the evidence collated for the variants. They are both frameshift variants therefore alter the length of the protein; they are also not present in population databases which together allows for two moderate evidences for pathogenicity criteria to be given. As there is more than one affected family member with the variants and because there is more than one line of computational evidence that supports a deleterious effect of the variants, two supporting lines of evidence for pathogenicity criteria are given. When these criteria are added together, the rules for combining criteria to classify sequence variants as 'likely pathogenic'. This classification led to further investigations.

Blood from the mother and father was obtained after consenting for genetic testing in order to strengthen the genetic analysis to show disease segregation with the genotyping. The use of true nuclear parent-offspring trios in genetics is highly valuable especially when assessing rare variants (Teo et al., 2009). DNA from I:1, I:2, II:2 and II:3 was used for Sanger sequencing to confirm the presence of the variants (Figure 4.8). Sanger sequencing confirmed both c.462delG, p.P154Rfs*38 and c.745_746insG, p.V249Gfs*57 variants were present in the index case, her sister (II:2) and their father (I:1). The mother was wild-type for both variants.

Table 4.5. Table of candidate genes for Family 3 predicted to be pathogenic by pathogenicity prediction tools. The table shows genomic variation and the protein effect as well as the prevalence of the variant in the population. ACMG criteria were assigned as appropriate leading to a classification for each variant.

Family 3										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
ACPT **NM_033068**	c.932C>T	p.A311V	Missense	8.28×10^{-6}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
CD109 **NM_133493**	c.763A>G	p.T255A	Missense	8.29×10^{-6}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
GLIS2 **NM_032575**	c.16G>A	p.E6K	Missense	Novel	Disease causing	Probably damaging	Damaging	Neutral	PM2 PP1 PP3	Uncertain significance
MEAF6 **NM_001270876**	c.359C>G	p.T120R	Missense	Novel	Disease causing	Probably damaging	Tolerated	Deleterious	PM2 PP1 PP3	Uncertain significance
PLA2G3 **NM_015715**	c.592T>C	p.Y198H	Missense	1.65×10^{-5}	Disease causing	Probably damaging	Tolerated	Deleterious	PP3 PP1	Uncertain significance
PPP1R14A **NM_033256**	c.256G>C	p.E86Q	Missense	0.00243	Disease causing	Probably damaging	Neutral	Deleterious	PP3 PP1	Uncertain significance
TARS2 **NM_025150**	c.T827T>G	p.V276G	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
TTLL10 **NM_153254**	c.462delG	p.P154Rfs*38	Frameshift deletion	Novel	Disease causing				PM2 PP3 PP1 PM4	Likely pathogenic
TTLL10 **NM_153254**	c.745_746insG	p.V249Gfs*57	Frameshift insertion	Not known	Disease causing				PM2 PP3 PP1 PM4	Likely pathogenic

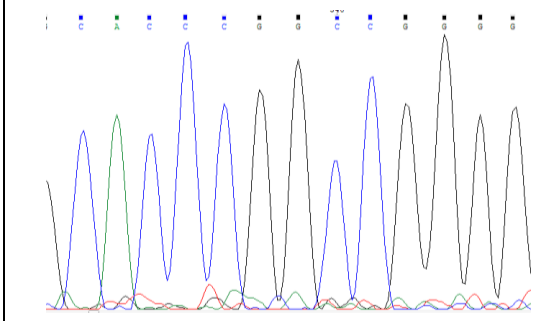
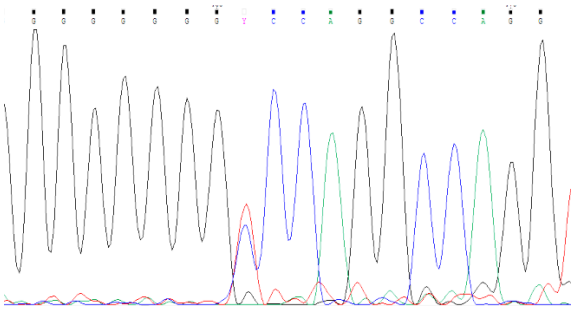
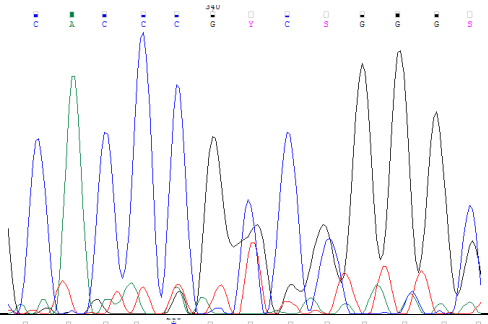
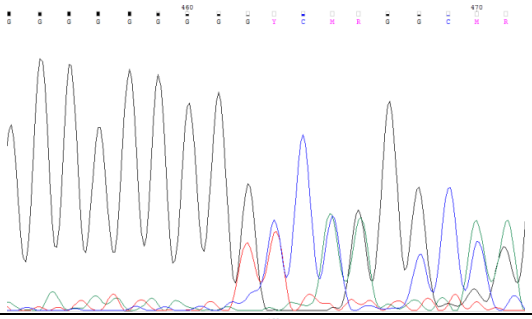
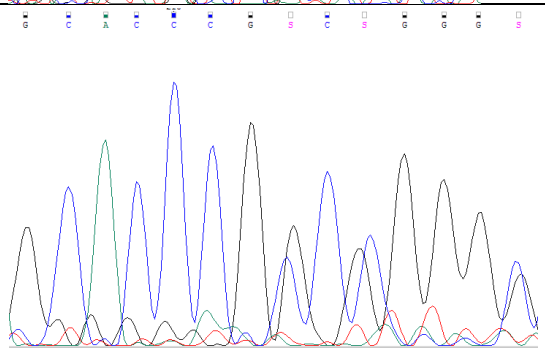
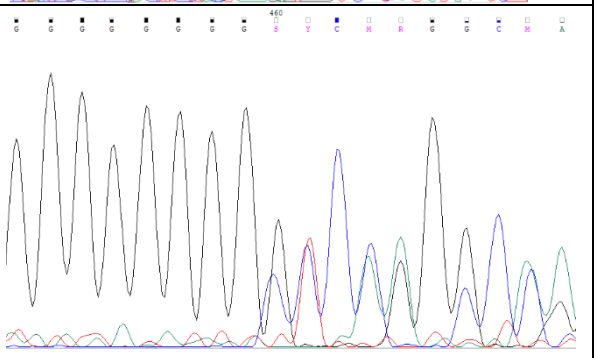
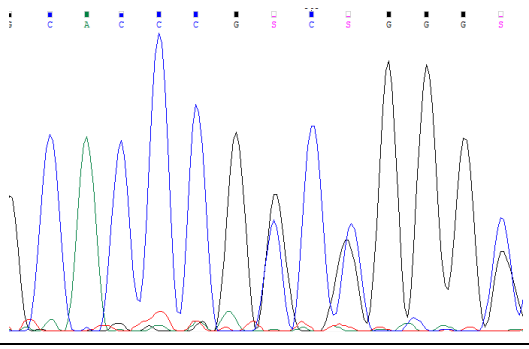
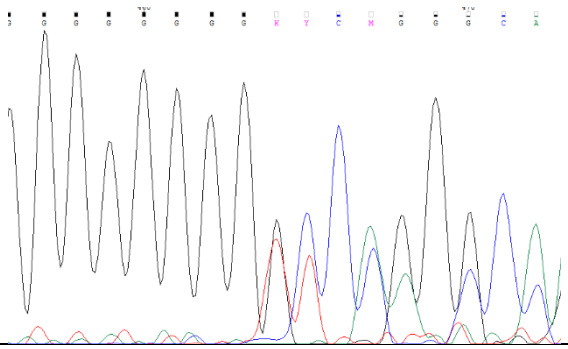
	c.462delG	c.745_746insG
Mother (I:2)		
Father (I:1)		
II:3		
II:2		

Figure 4.8. Sanger sequencing electropherograms for each of the family members recruited. The mother (I:2) is WT. The father (I:1) and both children (II:2 and II:3) have both *TTLL10* variants. c.462delG and c.745_746insG can be seen to cause a frameshift. *TTLL10* gene transcript NM_001130045.

Ensuring a candidate genetic variant is well characterised and proven to be functionally disrupting is challenging. It is not yet known whether *TTLL10* is the disease-causing gene in this family. It can be said that the variants are inherited in an autosomal dominant manner, but they may not necessarily be the cause of their bleeding. Furthermore, the mother does not have the *TTLL10* variants as seen in the Sanger sequencing. This family will be discussed further in Chapter 5.

4.5.4. Family 4

A mother and her daughter were recruited to the GAPP study with a suspected bleeding disorder of unknown cause. The mother was 41 years old at the time of recruitment and her daughter was 19 years old. The mother had a lifelong history of bleeding episodes including heavy menstrual bleeding, epistaxis, cutaneous bruising and excessive bleeding following a tooth extraction. At first, a diagnosis of VWD was suspected but later this was shown to be incorrect. Her daughter also suffered from bleeding episodes and experienced heavy menstrual bleeding, bleeding after tooth extraction and excessive bleeding from wounds (bleeding for two days following a superficial dog bite). After examining the family history, it emerged that her maternal grandmother had also suffered from bleeding episodes. The family pedigree can be seen in Figure 4.9.

Both mother and daughter had normal platelet counts in PRP (4.8×10^8/ml and 4.2×10^8/ml) and normal MPV values (8.6 fl and 9.4 fl). Platelet aggregation studies were carried out which showed responses within the normal range. Furthermore, secretion from dense granules was normal. Therefore, no defects in platelet aggregation or secretion were detected in the two patients. Unfortunately, the mother (II:2) died during the investigations of causes unrelated to bleeding.

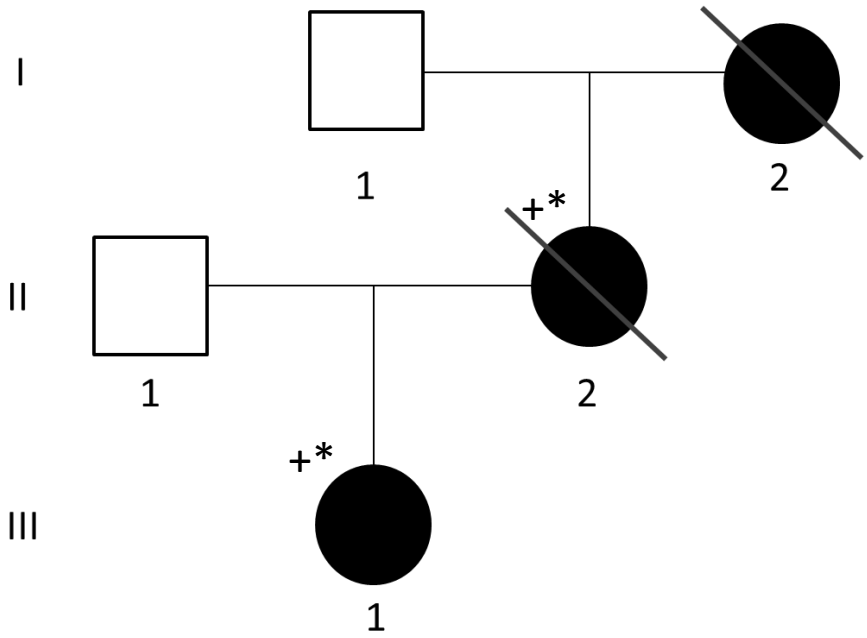


Figure 4.9. Family 4 Pedigree. Pedigree shows affected individuals as shaded symbols. Crosses (+) indicate patients recruited to the GAPP study and asterisks (*) indicate patients (II:2 and II:3) whose whole exomes were sequenced.

WES was performed for these two patients due to the strong family history and unknown aetiology of their disease. WES and bioinformatic analysis led to the discovery of 11 possible causative variants. Variants in genes: *ARRDC2*, *GRIN3B*, *GSN*, *ITFG1*, *PHF20*, *PIK3C2B*, *RAB40B*, and *ZNF749* were classified as variants of 'unknown significance'. Variants in genes: *ASTE1*, *STARD7* and *TTLL10* were classified at 'likely pathogenic'. Interestingly, we found the same frameshift insertion (c.745_746insG, p.V249Gfs*57) in the gene *TTLL1*0 that was also found in members of Family 3. The full table of candidate variants can be seen in Table 4.6.

4.5.5. TTLL10 variant in patients of Family 3 and 4

TTLL10 encodes a protein, tubulin tyrosine-like ligase 10, which is able to add glycine side chains to tubulin as a method of post-translational modification therefore the enzyme is known as an elongase (Ikegami and Setou, 2009). Little is known about the physiological role of TTLL10 however, evidence suggests that the genetic variants my abrogate protein function. Investigation into whether these *TTLL10* genetic variants are disease causing will be discussed in Chapter 5.

Table 4.6. Table of candidate genes for Family 4 predicted to be pathogenic by pathogenicity prediction tools. The table shows genomic variation and the protein effect as well as the prevalence of the variant in the population. ACMG criteria were assigned as appropriate leading to a classification for each variant.

Family 4										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
ARRDC2 **NM_015683**	c.212A>G	p.Y71C	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
ASTE1 **NM_001288950**	c.832A>T	p.K278X	Nonsense	Novel	Disease causing				PM2 PP1 PP3 PM4	Likely pathogenic
GRIN3B **NM_138690**	c.1672C>T	p.R558W	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
GSN **NM_001127664**	c.322G>A	p.G108S	Missense	Novel	Disease causing	Possibly damaging	Tolerated	Deleterious	PM2 PP1 PP3	Uncertain significance
ITFG1 **NM_030790**	c.1726G>A	p.G576S	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
PHF20 **NM_016436**	c.1951G>A	p.E651K	Missense	8.28×10^{-6}	Disease causing	Probably damaging	Damaging	Neutral	PP1 PP3	Uncertain significance
PIK3C2B **NM_002646**	c.3556G>T	p.V1186L	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
RAB40B **NM_006822**	c.592C>T	p.R198W	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
STARD7 **NM_020151**	c.1066C>T	p.R356*	Nonsense	Novel	Disease causing				PM2 PP1 PP3 PM4	Likely pathogenic
TTLL10 **NM_153254**	c.745_746insG	p.V249Gfs*57	Frameshift insertion	Novel	Disease causing				PM2 PP3 PP1 PM4	Likely pathogenic
ZNF749 ***NM_001023561***	c.1836delT	p.Y612*	Nonsense	8.25×10^{-6}	Disease causing				PP3 PP1 PM4	Uncertain significance

4.5.6. Families 5-10

Family 5 comprises a father and son kindred both of whom were affected by bleeding symptoms. The family pedigree is displayed in Figure 4.10. The father was recruited to the GAPP study at the age of 54 having suffered from heavy bleeding after teeth extraction, and rehospitalisation following both a tonsillectomy and appendectomy due to bleeding. His son, age 12 at the time of recruitment, suffers from bleeding episodes including: frequent cutaneous bleeding and epistaxis, prolonged bleeding from minor wounds and he suffers from haematomas in his calf muscles after trauma (playing football). As both patients had normal platelet function, their DNA was sent for WES. Following analysis of the variants, 14 candidate variants of 'uncertain significance' and 2 variants with the classification 'likely pathogenic' remained (Table 4.7.). One of the variants with a 'likely pathogenic' classification was in the gene *ADAMTS13* which encodes a metalloproteinase enzyme that cleaves von Willebrand factor. However, patients previously reported with variants in *ADAMTS13* usually have a reduced platelet count that is not seen in this family, the syndrome is also reported to be inherited in an autosomal recessive manner, which again is not the case for this family. This cause of disease has not been ruled out and further investigation is needed to find a cause of disease.

Family 6 are a brother aged 10 (index case) and his older sister aged 14, who have both suffered from a number of bleeding symptoms since birth. The family pedigree is displayed in Figure 4.10. He (I:1) has suffered from petechiae and excessive bruising after vaccinations. She (II:2) has suffered from frequent epistaxis requiring nasal cautery and experienced bleeding after tonsillectomy requiring readmission to hospital. An additional older sister, not recruited to the GAPP study, is also affected by bleeding symptoms such as menorrhagia and

experienced bleeding post-surgery requiring re-suturing. Their mother and maternal grandmother both experienced severe menorrhagia and underwent hysterectomies aged 38 and 33 respectively. This strong bleeding history and inheritance suggests an autosomal dominant disease. Both recruited individuals' platelet function was normal therefore DNA from II:1 and II:2 was sent for WES. The bioinformatic analysis that followed resulted in 15 candidate genetic variants classified as variants of 'unknown significance' (Table 4.7.). One of these variants was in the gene *ACTN1* which has been shown to cause macrothrombocytopenia in patients with some variants however, the platelet counts and MPVs for these patients are within the normal ranges (Kunishima et al., 2013). Patients with variants in *ACTN1* have also been reported without a thrombocytopenia (Gueguen et al., 2013). This cause of bleeding cannot be confirmed at this stage and as there are 14 other candidate variants for this family, further investigation is needed. It would be interesting and beneficial to recruit their mother and older sister of the children and carry out Sanger sequencing to try to narrow down the list of candidate genetic variants.

Family 7 included a mother and her daughter; the family pedigree is displayed in Figure 4.10. The mother (II:1) who's age is unknown, has a strong history of bleeding including: epistaxis, cutaneous bleeding, bleeding from minor wounds and oral cavity bleeding. She required packing following the extraction of 2 teeth and blood transfusions after her 3 successful pregnancies. Her daughter (II:2) suffers from the same symptoms in addition to menorrhagia requiring antifibrinolytic therapy and also experienced post-partum haemorrhage after her only pregnancy. Both females' platelets showed normal function with no explanation to their bleeding histories. DNA from both patients was sent for WES. 10 candidate genetic variants of 'uncertain significance' remained following the bioinformatic pipeline and these can be seen in Table 4.7. 7 of 10 of these variants are novel, therefore the cause of disease in these patients

could be a novel genetic variant. This family requires further work to try to elucidate their cause of disease. To strengthen the genetic investigation, additional siblings/children could be recruited to the GAPP study.

Family 8 comprises a family with 4 children born to a mother and father biologically related as first-degree cousins. The family pedigree is displayed in Figure 4.10. The eldest child, a female, was the only child whose whole exome was sequenced at the time of writing this thesis. The female (II:1), aged 12 at the time of recruitment to the GAPP study had a significant bleeding history including bruising, prolonged bleeding from cuts and grazes and recurrent nose bleeds. Her 3 brothers suffer from the same symptoms. Their father is also reported to suffer from bleeding episodes however; he was not available for recruitment to the GAPP study. When analysing the genetic variants for this female, special attention was given to any homozygous variants. This is because the phenomenon of consanguinity increases the level of homozygotes therefore 2 carriers of a recessive disorder can pass autosomal recessive disorders onto offspring. Heterozygous genetic variants were not excluded in the analysis of this family. 12 variants of 'uncertain significance' remained after the bioinformatic pipeline and series of filtering steps were followed. These variants can be seen in Table 4.7. This family may have a novel genetic cause of their bleeding which will need further work to resolve. It is hoped that the DNA of the further 3 children will be investigated in the future.

Family 9 were recruited to the GAPP study together as 2 sisters; the family pedigree is displayed in Figure 4.10. Patient II:1 was 44 at the age of recruitment at II:2 was 38. Both sisters have extensive bleeding histories including: easy bruising, excessive oral cavity bleeding, menorrhagia requiring hormonal and antifibrinolytic therapies and serious post-partum haemorrhages, one of which lasted 8 weeks for II:2. Due to a lack of high quality,

extracted DNA from II:1, only DNA from individual II:2 was sent for WES. Following WES and the bioinformatic pipeline, 11 candidate genetic variants were classified as variants of 'unknown significance' and can be seen in Table 4.7. Again, these individuals may have a novel genetic cause of their bleeding which requires further investigation. Sanger sequencing of the genetic variants in question for individual II:1 will help to decrease candidate variant numbers although WES would be ideal to strengthen the investigation.

Family 10 are 3 siblings affected by bleeding episodes. The eldest, a female, was 17 at the age of recruitment to the GAPP study and her siblings were 9 (male) and 7 (female). The eldest sister is believed to have a different father to the younger children and is therefore biologically their half-sister. The family pedigree is displayed in Figure 4.10. The 17-year-old girl has suffered from bleeding episodes since birth inducing: easy bruising, menorrhagia treated by hormone therapy, tooth extractions requiring packing and at the age of 7 she suffered from prolonged postoperative bleeding following a tonsillectomy. Both younger children have suffered from easy bruising since birth and frequent epistaxis. WES was carried out on the DNA from all 3 children. The candidate genetic variants can be seen in Table 4.7. There are fewer genetic variants (4 of 'uncertain significance') for this family due to the filtering steps; only variants that are shared between all 3 individuals have been included. This family requires further investigation. The genetic variants may need to be re-analysed if the mother of the children is also recruited for WES.

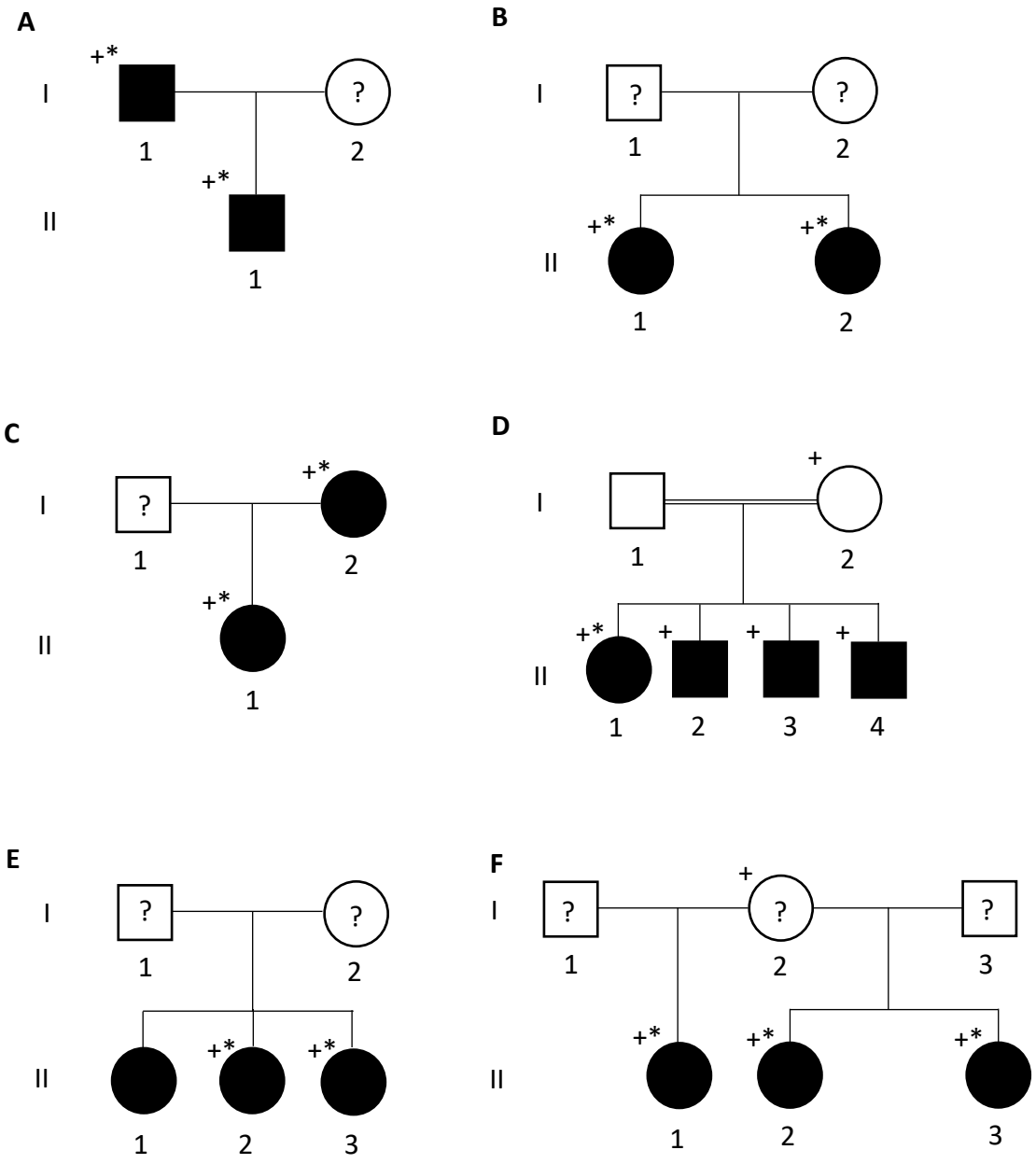


Figure 4.10. Family pedigrees for families 5–10. (A) Pedigree for Family 5. (B) Pedigree for Family 6. (C) Pedigree for Family 7. (D) Pedigree for Family 8. (E) Pedigree for Family 9. (F) Pedigree for Family 10. +, denotes the individual has been recruited to the GAPP study; *, denotes that WES has been carried out; =, denotes a relationship of consanguinity.

Table 4.7. Tables of candidate genes for Families 5-10 predicted to be pathogenic by pathogenicity prediction tools. The table shows genomic variation and the protein effect as well as the prevalence of the variant in the population. ACMG criteria were assigned as appropriate leading to a classification for each variant.

Family 5										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
ADAMTS13 **NM_139025**	c.1315G>T	p.E439*	Nonsense	Novel	Disease causing				PM2 PP3 PM4 PP1	Likely pathogenic
B3GNT4 **NM_030765**	c.991C>T	p.R331W	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
BBS9 **NM_001033605**	c.114T>G	p.D38E	Missense	Novel	Disease causing	Possibly damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
CCDC168 **NM_001146197**	c.18301C>T	p.Q6101*	Nonsense	Novel	Disease causing				PM2 PP3 PM4 PP1	Likely pathogenic
FAM81B **NM_152548**	c.92delA	p.N31Ifs*9	Frameshift deletion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
KRTAP5-9 **NM_005553**	c.407C>G	p.S136*	Nonsense	8.269×10^{-6}	Disease causing				PP3 PP1 PM4	Uncertain significance
LIG3 **NM_013975**	c.2927C>T	p.T976M	Missense	8.24×10^{-6}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
LRPPRC **NM_133259**	c.3306_3307 insG	p.L1102fs	Frameshift insertion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
MYO5B **NM_001080467**	c.3191G>A	p.R1064Q	Missense	2.49×10^{-5}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
OR11G2 **NM_001005503**	c.413_414in sA	p.Q138fs	Frameshift insertion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
OR2T34 **NM_001001821**	c.234_235in sC	p.Y78fs	Frameshift insertion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance

SNRNP200 **NM_014014**	c.3551T>C	p.L1184S	Missense	Novel	Disease causing	Possibly damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
TDP1 **NM_001008744**	c.1459C>T	p.R487C	Missense	3.32×10^{-5}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
VILL **NM_015873**	c.2126_2127 insAA	p.K709fs	Frameshift insertion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
ZNF491 **NM_152356**	c.399_400in sT	p.C133fs	Frameshift insertion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
ZSCAN20 **NM_145238**	c.2396G>A	p.C799Y	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
Family 6										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
ACTN1 **NM_001130005**	c.2582C>T	p.P861L	Missense	Novel	Disease causing	Possibly damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
DACT3 **NM_145056**	c.1856G>A	p.R619H	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
DLEC1 **NM_007335**	c.5144G>T	p.R1715M	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
ETS1 **NM_005238**	c.485A>G	p.Y162C	Missense	8.276×10^{-6}	Disease causing	Benign	Damaging	Neutral	PP3 PP1	Uncertain significance
ETS1 **NM_005238**	c.754T>A	p.F252I	Missense	8.818×10^{-6}	Disease causing	Possibly damaging	Tolerated	Neutral	PP3 PP1	Uncertain significance
FAN1 **NM_014967**	c.2774_2775 del	p.L925Pfs*2 5	Frameshift deletion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
GIPC2 **NM_017655**	c.895T>G	p.F299V	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
GOLGA5 **NM_005113**	c.820T>C	p.S274P	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
HELZ2 **NM_001037335**	c.1325A>G	p.E442G	Missense	0.0000256	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance

NKAIN4 **NM_152864**	c.203G>C	p.W68S	Missense	2.487×10^{-5}	Disease causing	Possibly damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
PLXDC2 **NM_032812**	c.838C>G	p.L280V	Missense	2.486×10^{-5}	Disease causing	Possibly damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
POLD2 **NM_001256879**	c.874G>A	p.V292M	Missense	0.02006	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
RPN1 **NM_002950**	c.1544G>A	p.G515D	Missense	0.002285	Disease causing	Benign	Damaging	Neutral	PP3 PP1	Uncertain significance
TLDC2 **NM_080628**	c.413T>G	p.L138R	Missense	0.0003141	Disease causing	Possibly damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
ZNF292 **NM_015021**	c.4493G>T	p.G1498V	Missense	3.296×10^{-5}	Disease causing	Probably damaging	Damaging	Neutral	PP3 PP1	Uncertain significance
Family 7										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
BRD8 **NM_006696**	c.1516G>A	p.V506M	Missense	0.0000508	Disease causing	Probably damaging	Damaging	Neutral	PP3 PP1	Uncertain significance
FKBP15 **NM_015258**	c.390T>A	p.F130L	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
ILDR1 **NM_175924**	c.486G>C	p.K162N	Missense	Novel	Disease causing	Possibly damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
NME9 **NM_178130**	c.317C>T	p.P106L	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
PALMD **NM_017734**	c.178G>C	p.E60Q	Missense	0.001896	Disease causing	Probably damaging	Damaging	Neutral	PP3 PP1	Uncertain significance
SDK1 **NM_152744**	c.2932C>G	p.L978V	Missense	Novel	Disease causing	Possibly damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
SLC25A23 **NM_024103**	c.1090C>T	p.L364F	Missense	0.0000353	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
TBX21 **NM_013351**	c.1171_1172 insC	p.R391fs	frameshift insertion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance

TNFRSF12A **NM_016639**	c.329T>G	p.F110C	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
TTC39A **NM_001144832**	c.377A>C	p.H126P	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
Family 8										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
CER1 **NM_005454**	c.428C>T^{Hom}	p.P143L	Missense	4.943×10^{-5}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
DYSF **NM_001130987**	c.1966A>G	p.K656E	Missense	0.0008823	Disease causing	Possibly damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
FLNB **NM_00295956**	c.4058C>G	p.T1353S	Missense	0.0005618	Disease causing	Benign	Damaging	Deleterious	PP3 PP1	Uncertain significance
GFI1B **NM_0339463**	c.503G>T	p.C168F	Missense	0.0006011	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
MYO5B **NM_001080467**	c.638G>A	p.R213H	Missense	0.0001408	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
PIGT **NM_015937**	c.1247C>T	p.P416L	Missense	0.0002564	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
ROBO4 **NM_019055**	c.1028C>A	p.P343Q	Missense	0.0001476	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
RPN1 **NM_002950**	c.1673T>G	p.V558G	Missense	Novel	Disease causing	Possibly damaging	Tolerated	Deleterious	PM2 PP3 PP1	Uncertain significance
SOAT1 **NM_003101**	c.485T>C^{Hom}	p.I162T	Missense	0.0002311	Disease causing	Possibly damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
ST5 **NM_213618**	c.1831C>T^{Hom}	p.R611C	Missense	0.001044	Disease causing	Possibly damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
STXBP4 **NM_178509**	c.1287G>C	p.K429N	Missense	0.0003742	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
UBE3C **NM_014671**	c.1001C>T	p.S334F	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance

Family 9										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
ANKLE1 **NM_001278444**	c.1910_1911 insTT	p.V637fs	Frameshift insertion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
BCL9L **NM_182557**	c.3328A>T	p.I1110F	Missense	1.651×10^{-5}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
ECSIT **NM_001142465**	c.245G>A	p.R82H	Missense	6.606×10^{-5}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
MMP25 **NM_022468**	c.688G>A	p.V230M	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
PARP4 **NM_006437**	c.447T>G	p.F149L	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
PLCZ1 **NM_033123**	c.1658G>A	p.R553H	Missense	8.284×10^{-6}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
RIF1 **NM_001177664**	c.2150G>A	p.R717H	Missense	3.295×10^{-5}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
SENP7 **NM_001282803**	c.1395_1399 del	p.465_467d el	Frameshift deletion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
SH3TC2 **NM_024577**	c.G2602G>A	p.E868K	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP3 PP1	Uncertain significance
TAS2R39 **NM_176881**	c.767_777de l	p.256_259d el	Frameshift deletion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
ZNF229 **NM_014518**	c.1091delT	p.L364fs	Frameshift deletion	Novel	Disease causing				PM2 PP3 PM4 PP1	Uncertain significance
Family 10										
Gene/ Accession number	**Genomic variation**	**Protein effect**	**Variation type**	**Prevalence**	**Mutation taster**	**PolyPhen-2**	**SIFT**	**Provean**	**ACMG criteria**	**Classification**
MYO19 **NM_025109**	c.1247A>G	p.Y416C	Missense	6.025×10^{-5}	Disease causing	Probably damaging	Damaging	Deleterious	PP3 PP1	Uncertain significance
SETD4 **NM_001007261**	c.830G>T	p.G277V	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance

TJP3 **NM_001267560**	c.413C>G	p.S138C	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance
TLDC1 **NM_020947**	c.1349G>A	p.R450Q	Missense	Novel	Disease causing	Probably damaging	Damaging	Deleterious	PM2 PP1 PP3	Uncertain significance

4.6. Discussion

Presented within this chapter is the identification of genetic variants in patients with inherited bleeding disorders and normal lumi-aggregometry by the application of WES and bioinformatic analysis.

Overall, genetic variants within genes previously known to cause a bleeding phenotype were identified in one family (1 of 10). The majority of the other variants were missense and most of those were novel (not previously described in relevant databases). When considering pathogenicity, WES analysis revealed a positive prediction call (classification of 'pathogenic' or 'likely pathogenic') in 8.2% (8 of 98) of variants across all the families. 91.8% (90 of 98) of the variants were unknown and classified as variants of 'uncertain significance' due to a lack of evidence suggesting their pathogenicity. There were no variants where a negative pathogenic prediction classification was given. These figures reflect the sample of patients that were taken forward for WES as by only recruiting patients with an unknown aetiology of disease this excluded patients where a clinical diagnosis might be more obvious. This might be the case with, for example, the haemophilias where platelets on their own otherwise function normally in non-severe cases; or a thrombocytopenic cohort whereby the genetic causes are more well-known and therefore easier to target.

The genetic cause of bleeding in two individuals from Family 1 recruited to the GAPP study has been elucidated as a genetic variant in the gene *THBD* using next-generation sequencing. The same genetic variant in *THBD* (c.1611C>A, p.C537*) has previously been shown to cause a bleeding phenotype within affected individuals. This can therefore explain the cause of bleeding in II:4 and III:2 and suggests that it may be a recurrent mutation in the population. In addition to the further reports by Dargaud *et al.* and Langdown *et al.,* a recent finding has

shown the same mutation in an extended French pedigree and not thought to be related to the previous reports (personal communication, Dr Marie-Christine Alessi, Aix-Marseille University) (Langdown et al., 2014, Dargaud et al., 2015). Furthermore, this discovery highlights the importance of not restricting only platelet causes of bleeding to the investigation of bleeding related disorders. Here, is it shown that this is not a true platelet dysfunction however, a protein, thrombomodulin, involved in the coagulation cascade which indirectly effects thrombus formation; the mechanism of action of which can be seen in Figure 4.11. The loss of thrombin generation is also assumed to reduce the number of activated platelets and the platelet-platelet positive feedback activation of surrounding platelets.

WES in one member of Family 2 revealed multiple potential disease causing genetic variants. Currently, none of the variants have been proven to be the causative of the disease. Out of the five variants, they are all feasible candidates, but a mechanism of disease must be elucidated before any of them can be ruled out. *EPHA4* (c.1105T>C, p.C369R) belongs to the ephrin receptor subfamily of the protein-tyrosine kinase family whereas *PLA2G4C* (c.889delC, p.P297Gfs*13) encodes a protein which is a member of the phospholipase A2 enzyme family producing precursors in the production of signalling molecules. *PTPRF* (c.4324C>T, p.R1442W) encodes a protein of the protein tyrosine phosphatase family, signalling molecules known to regulate a variety of cellular processes. *PRKACA* (c.304C>A, p.P102T) encodes one of the catalytic subunits of protein kinase A (PKA) that with other subunits, binds to cAMP; cAMP-dependant phosphorylation of proteins by PKA is also important for many cellular processes (Turnham and Scott, 2016). Finally, *SELPLG* (c.245C>G, p.T82S) encodes a glycoprotein that functions as a high affinity counter-receptor for the cell adhesion molecules P-, E- and L-selectin. None of the genes mentioned above have been associated with bleeding and they are all functionally very different. Further work must be carried out in order to narrow down

the variants even more. Functional work regarding novel variants can be challenging and may not provide the answers to the questions being asked.

Family 3 presented with an interesting case. WES revealed variants in a number of genes including two frameshift variants in *TTLL10* (c.462delG, p.P154Rfs*38; c.745_746insG, p.V249Gfs*57). Variants in other genes included: *CD109* (c.763A>G, p.T255A) which encodes a glycosyl phophatidylinositol linked glycoprotein that localises on the surface of platelets activated T-cells and endothelial cells, *PLA2G3* (c.592T>C, p.Y198H) which encodes a protein that belongs to the secreted phospholipase A2 family and *PPP1R14A* (c.256G>C, p.E86Q) which encodes a protein that belongs to the protein phosphatase 1 inhibitor family. These variants and the others not mentioned were all confirmed in both II:2 and II:3 by Sanger sequencing. The mother and father were recruited to the GAPP study and Sanger sequencing for the *TTLL10* variants was carried out. This revealed that the mother was wild-type for both variants but the father as well as the two children possessed both frameshift variants. This added some questions as the father had not suffered from any bleeding symptoms. However, this is often common in males who have never been challenged with injury or surgery and they often present with symptoms later in life. With this family, WES sequencing could be carried out on the mother and father to make the genetic analysis more powerful as is known with parent-offspring trio investigations. There may also be more than one cause of bleeding within this family, the multifactorial nature of bleeding disorders is well known and other causes in each of the family members cannot be excluded (Peyvandi et al., 2013).

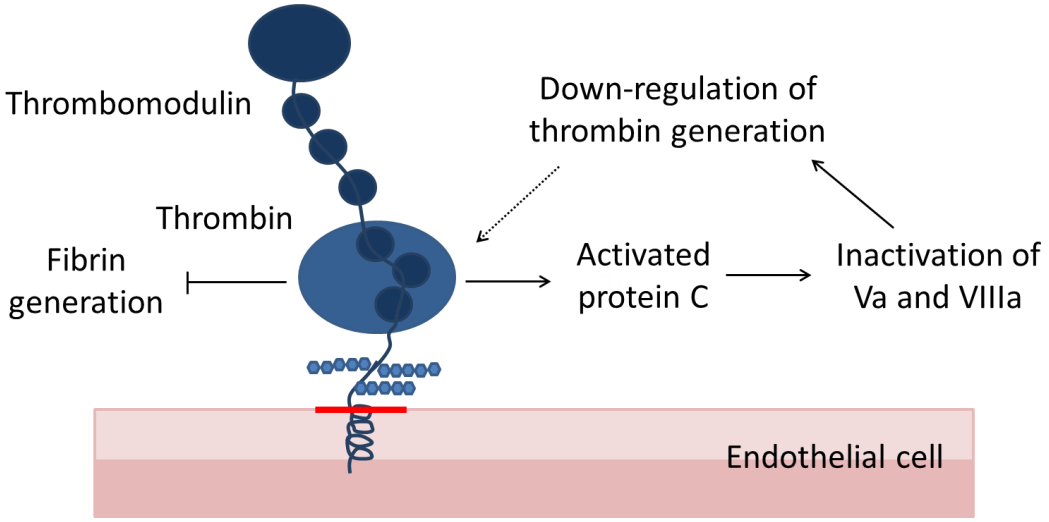


Figure 4.11. A gain of function mutation in thrombomodulin causes a bleeding phenotype. The c.1611C>A, p.C537X variant causes thrombomodulin to be shed from the endothelial surface (shown by red line) leading to an increase of soluble thrombomodulin in the circulation. This promotes protein C activation therefore inactivating factors Va and VIIIa and reducing thrombin generation within a potential thrombus. A loss of thrombin formation is also presumed to result in a decrease in overall platelet activation.

The candidate variants of Family 4 presented in a number of genes including the same frameshift deletion in *TTLL10* (c.745_746insG, p.V249Gfs*57) as was found in Family 3. This was particularly interesting as both families suffered from similar bleeding symptoms, normal platelet counts and normal platelet function. Other genes in which variants were found were: *GSN* (c.322G>A, p.G108S) which encodes a protein that binds to the plus ends of actin monomers and filaments to prevent monomer exchange, *PIK3C2B* (c.3556G>T, p.V1186L) which encodes a protein that belongs to the phosphoinositide 3-kinase family involved in cell signalling pathways and *STARD7* (c.1066C>T, p.R356X) which encodes a phospholipid transfer protein which promotes uptake of phosphatidylcholine by mitochondria (Yang et al., 2017, Kiuru et al., 2000). Variants in other genes were found and are not being excluded until a mechanism of disease is concluded. As the same variant was found in the same gene in two separate, unrelated families, further investigation into *TTLL10* expression and function was carried out.

The 5 further families again all present with interesting cases. Family 5, a father and son, both present with clinically relevant bleeding diathesis which is rarely seen in males due to the lack of haemostatic challenges. Family 6 present with a strong family history of bleeding going back 3 generations with 5 affected family members. This mode of inheritance is thought to be autosomal dominant. Further study into this family could provide novel insights into *ACTN1* variants that here may present differently to previously reported cases (Westbury et al., 2017). Both women of Family 7 have an extensive bleeding history that may be caused by a novel genetic variant. For this family, recruitment of additional unaffected family members would strengthen the investigation as variants not present in unaffected individuals could be excluded and therefore decrease the list of candidate genetic variants. Family 8, 5 children with bleeding episodes, also have a potential novel genetic cause of their disease. However,

the cause the disease could also be recessive. Children of consanguineous unions can be at increased risk of genetic disorders due to expression of autosomal recessive variants inherited from a common ancestor; first cousins share an average of 12.5% of their DNA (Wright, 1922). The DNA from the 4 boys will be investigated further by Sanger sequencing in this family in order to try to elucidate their cause of disease. Recruiting the father of the children to the GAPP study would also be beneficial if the confirmation of a recessively inherited disorder is needed.

The women in Family 9 both suffered from life threatening post-partum haemorrhage however, a cause of disease has still not been established. This could be because WES was only completed for one of the sisters. In order to find the cause of disease, WES in the second sister would be ideal to study the genetics in this family. Family 10, 3 young girls are all affected by bleeding symptoms. WES was carried out for all the sisters, giving rise to only 4 candidate genetic variants. However, as the older sister is a half-sibling of the other two girls, she could have a different disorder to her siblings. This is unlikely but the possibility cannot be ruled out. Therefore, the genetic variants could be reanalysed in a way in which takes into account the differences in genetics. This could be achieved by keeping her genetic variants separate and only comparing the variants of her sisters. This would allow any variants which are not shared by her sisters to be identified. Further investigation is needed for all the families mentioned above. This is very important to provide the necessary care and preoperative preventative measures to benefit the health of the patients. All the families could present very interesting cases for the clinical and research community investigating bleeding disorders.

Whole exome sequencing is of great value in this type of investigation but as with many methods, there are some drawbacks of using WES rather than WGS for this investigation.

Firstly, and most obviously, only the exons are sequenced which leaves intronic regions, where mutations can have pathogenic consequences and therefore would be unsequenced. This also affects possible splice site variants which may be missed or even regulatory variants deep within introns or promoter/enhancer regions of the genome. In addition to this, some exons are not detected including those buried in repeated regions towards the end of the chromosomes which are not normally part of exome sequencing chips. A few other genetic manifestations can also be missed, for example, copy number variants where they do not change the sequence but can increase the disease risk, and structural variants (translocations and inversions) where DNA can be dramatically altered. Furthermore, WES cannot assess neither epistatic interactions nor epigenetic changes as it only picks up DNA sequences rather than gene expression. These are all important considerations to remember when analysing genetic variants and when trying to link them to the disease state in question.

The biggest challenge faced in this investigation is with potential non-platelet genes causing bleeding. These are much more difficult to investigate and often these are novel discoveries as with the *SLFN14* variants *(Fletcher et al., 2015)*. However, with the updated panel of non-platelet and endothelial related genes, WES variants can now be readily screened against them all. As more data is accumulated about the genetics of bleeding disorders, the list of candidate variants for each patient will hopefully decrease. Therefore, the likelihood of finding a disease causing mutation will be improved with increased positive variant discovery rates even within this challenging area of genetic discovery. Powerful WGS will probably soon be used in place of WES across the field.

4.7. Future work

Next generation sequencing is a very powerful tool for investigating the genetic causes of unexplained bleeding. It is all very well classifying potential variants and in the presence of well-known disease causing variants NGS and Sanger sequencing are enough to confirm the variant. However, when an individual has multiple possible disease causing novel variants these can remain ambiguous and uncertain and usually these require further investigation to confirm or exclude.

As many of the genetic variants in question may have no previous associations to haemostasis and thrombosis, it can then be difficult to link the variant to the disease. Thus, functional work for each variant is challenging and time consuming often without *in vitro* or *in vivo* methods for investigation therefore requires work to develop, validate and improve with no reagents such as antibodies available. Often the best way to investigate this is overexpressing the dysfunctional gene in an immortalised cell line but as well as advantages, this comes with some disadvantages. Animal models of disease are also a strong method of investigation, but this is time consuming, expensive and comes with its own set of challenges namely regarding interspecies disparities.

Work is still needed to functionally confirm all the variants within the 9 families investigated as without this, the mechanisms of their disease will remain unknown. If successful, this has the potential to develop our current knowledge of haemostasis mechanisms and creates scope for potential new therapeutics for thrombosis. Furthermore, it will allow the patients to have a categorised diagnosis, which is highly important for some individuals and for their clinical treatment and management later in life.

CHAPTER 5
Determining the functional effects of TTLL10

5. Determining the functional effects of TTLL10

5.1. Summary of background to this research

Since next-generation sequencing became widely available, its usage has exponentially increased. Consequently, there are now growing numbers of genetic variants identified in individuals affected by all diseases and syndromes. Whether this is in a clinical or research setting, it brings with it a set of challenges. The biggest challenge researchers face in this field is the examination of candidate genetic variant lists in order to determine each variant's functional effect, if it is unknown, and potential disease causing status.

In the previous results chapter, Chapter 4, WES and a bleeding disorders specific bioinformatic pipeline identified a number of potential disease causing sequence variants in 10 unrelated pedigrees. These variants are potentially pathogenic and therefore causative of the bleeding phenotypes seen in the affected individuals. The classification of these variants has been based solely on computational analysis software and related algorithms and predictions. These software tools are highly valuable; however, the classification calls should be approached with caution. Rarely, but of high importance within this field, variants have been incorrectly classified; this demonstrates that these programs should not be used in isolation (Tchernitchko et al., 2004). Misclassification of a variant could be detrimental to the health of the patients, cause problems from a genetic counselling perspective, as well as raising questions surrounding medical negligence. In clinical settings, this is an area where only highly trained scientists operate and where multiple clinical scientists check final results which would lead to a diagnosis by a practicing clinician.

As many of the variants identified through this project, as well as similar projects, are potentially novel, further study of the variants is required to elucidate their functional

relevance and link the variation to a disease state. It has become well known that many disorders are not simple in their presentation. This is because there can sometimes be phenotypic variance in patients with the same disorder; for example, if two patients with the same disorder or even the same genetic variant present with very different phenotypes. It can be difficult to identify these from other disorders due to the differential presentation. However, genetic investigation, twinned with functional investigation, could provide the answers and hopefully link a genetic variant to a specific role. This also provides solid evidence for the investigation of future patients who may have slightly differing phenotypes of an already established disorder.

Functional investigation can be approached using a multiplicity of techniques which all assess function by employing different methods. It is also important to investigate and have a sound understanding of the gene and protein expression in humans as well as animal models including mouse models, if applicable. The information gained from further investigations can be used to assess whether the genetic variant will detrimentally affect the protein in question and cause serious health problems for human patients.

5.2. Aims of this chapter

This chapter will follow on from work on previous results from Chapter 4 where variants from WES were analysed in a number of families with inherited bleeding. The aim of this chapter is to investigate novel genetic variants in the gene *TTLL10,* as a likely genetic candidate in two unrelated families with bleeding and to categorise the novel variants within *TTLL10* focusing on the expression of TTLL10 protein.

5.3. Patient characteristics

Patients who are recruited to the GAPP study undergo phenotyping and genotyping with the aim to elucidate the cause of their previously undiagnosed cause of bleeding as outlined in Chapter 2, Section 2.9 and 2.14.

5.3.1. Family 3 & 4

A frameshift deletion (c.462delG, p.Pro155Argfs*38) and frameshift insertion (c.745_746insG, p.Val249Glyfs*57) were initially found in Family 3; the same frameshift insertion (c.745_746insG, p.Val249Glyfs*57) was found in unrelated Family 4 using WES analysis. Other candidate genetic variants were identified from WES in both families, though the *TTLL10* variants were the most plausible. All affected individuals had clinical bleeding symptoms of unknown cause after extensive phenotyping. Further members of Family 3 were recruited and lumi-aggregometry was carried out along with other phenotyping tests such as total thrombus-formation, platelet spreading and DNA was extracted for further genetic studies. The platelets aggregated normally in response to all agonists tested and the platelet secretion values were within the normal range. Sanger sequencing was carried out to verify the WES on all recruited family members, namely the mother, father and two of their three daughters. The Sanger sequencing results showed an autosomal dominant inheritance pattern. The mother was wild type for the *TTLL10* variants; however, the father had both *TTLL10* genetic variants (c.462delG, c.745_746insG). The two daughters recruited to the GAPP study also have both *TTLL10* variants therefore both variants are paternally inherited and also on the same allele. The frequency of the variants in genetic databases is important to take into consideration. The frameshift deletion variant is novel, which means that is has never been reported before in any population. The frameshift insertion is not described as novel however,

it has not been found at a relatively low frequency in the Exome Aggregation Consortium (ExAC) database.

5.3.2. Additional family 11 with a *TTLL10* sequence variant

Following this finding in Family 3 and 4, 95 patients previously recruited to the GAPP study, with normal platelet counts and with no obvious platelet dysfunction, were sequenced using specifically designed primers for PCR amplification of all exons of *TTLL10* followed by Sanger sequencing.

A missense variant was found in exon 11 of the *TTLL10* gene in a female patient with a moderate bleeding history but normal platelet count; this was a heterozygous variant (c.1018C>T), resulting in an amino acid substitution (p.R340W) (Figure 5.1). This variant was found to be classified as a variant of 'uncertain significance' by the ACMG guidelines as there was not sufficient evidence to suggest pathogenicity, however, multiple lines of computational evidence suggested this prediction of pathogenicity.

This female patient (age unknown), has suffered from lifelong bleeding tendencies. She has easy cutaneous bleeding, excessive bleeding from minor wounds, and suffers from menorrhagia. Following a tooth extraction, she bled for 7 days requiring packing and resuturing. She also suffered from post-partum haemorrhage following the births of both her children. It was noted by the referring clinician, that her grandfather also suffered from easy cutaneous bruising and excessive bleeding from minor wounds. The family pedigree is shown in Figure 5.2.

During the investigations into her platelet function, the results from lumi-aggregometry with all agonists showed normal platelet responses. Platelet secretion values were also normal. Other investigations including a clotting factor screen as well as vWF and factor VIII genetic

testing which all showed normal results (carried out at Sheffield Haemophilia and Thrombosis Centre).

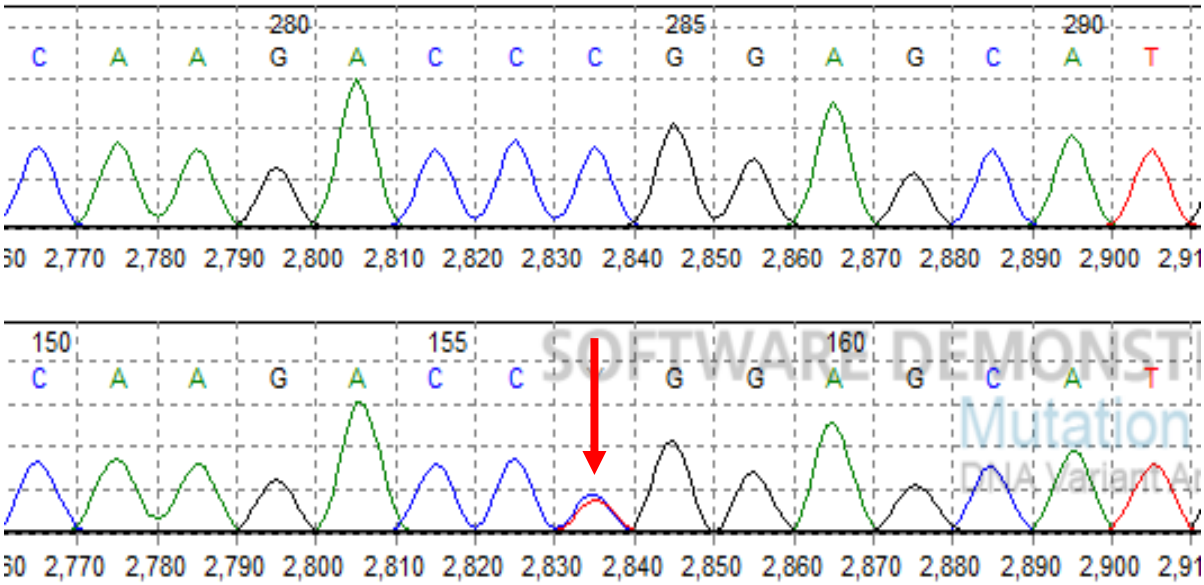


Figure 5.1. Sanger sequencing electropherograms in Family 11. Upper panel shows the reference sequence and the lower panel shows the patient with a heterozygous variant, c.C1018T, p.R340W in the *TTLL10* gene (red arrow).

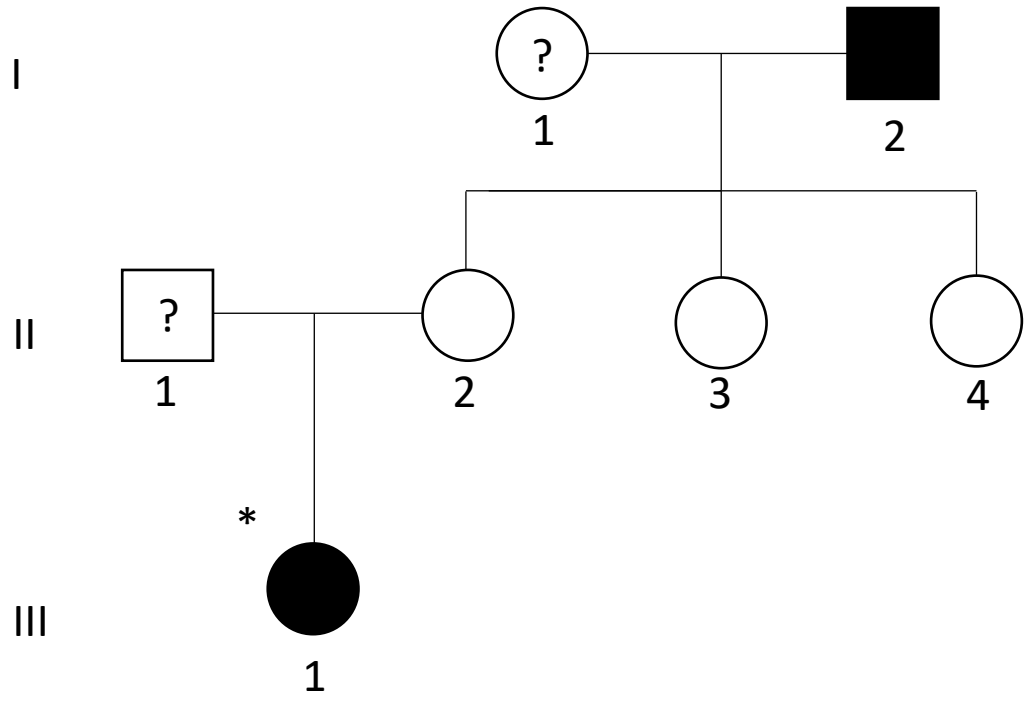


Figure 5.3: Family 11 Pedigree. Pedigree shows affected individuals as shaded symbols and asterisks (*) indicate patients whose whole exomes were sequenced.

5.4. TTLL10 background

Tubulin tyrosine ligase like 10, TTLL10, had been hypothesised to be involved in the polyglycylation of microtubules formed from tubulin, a major component of the cytoskeleton in eukaryotic cells (Ikegami and Setou, 2009).

Microtubules play an essential role in cells' normal function, from maintenance of cell morphology, to cell motility and intracellular transport (Gadadhar et al., 2017a, Gadadhar et al., 2017b). As tubulin is present in almost all cell types, it has adapted to provide a wide variety of different cellular functions (Janke, 2014). Tubulin sequences are highly conserved across species and abnormalities in structure or function are not well tolerated and often lead to significant, detrimental consequences (Luduena, 2013). Tubulin heterogeneity is generated by the expression of different isoforms of the tubulin gene. There are nine isoforms of tubulin monomers in mammals; combinations of these monomers then join to form a functionally distinct microtubule; isoforms α- and β-tubulin are the most common (Ludueña, 1993).

5.4.1. Microtubule post-translational modifications

It is also known that tubulin isoforms undergo PTMs in order to interact with other microtubules, motor proteins and accessory proteins (Gadadhar et al., 2017a). There are many PTMs that occur as the general mechanism for the regulation of microtubule function (Gadadhar et al., 2017a, van Dijk et al., 2007, Yu et al., 2015). Classical methods of PMT include microtubule phosphorylation and acetylation; there are also others that have mostly been found to regulate tubulin monomers which include: tyrosination/detyrosination, polyglutamylation and polyglycylation (Janke, 2014, van Dijk et al., 2007).

Phosphorylation of proteins is one of the most important and hence most studied form of PTM. Phosphorylation occurs by several kinases typically on serine 172 on β-tubulin which

allows for microtubules involved in the cell cycle to carry out their specialised function (Fourest-Lieuvin et al., 2006).

Acetylation has been found to play a greater role in cell biology than just transcriptional regulation. It typically targets lysine 40 of α-tubulin which is mediated by tubulin acetyl transferase enzyme (αTAT1) (Friedmann et al., 2012). The acetylation can be reversed by either SIRT2 or a histone deacetylase (HDAC6) (Friedmann et al., 2012). Neuronal microtubules are highly acetylated which has been shown to be important in neuronal survival and maintenance of synaptic transmission; decreased levels of acetylation have been linked to diseases including Huntington's disease and Charcot-Marie-Tooth syndrome (Magiera et al., 2018, Dompierre et al., 2007).

Tyrosination and detyrosination occur on α-tubulin by the addition or removal of tyrosine which has been shown to be ATP-dependent and tubulin tyrosine ligase (TTL) mediated. During detyrosination, an exposed terminal glutamate residue is left, therefore altering the stability of the microtubule (Maruta et al., 1986). Additional amino acids can then be removed, altering the microtubule further. Detyrosination of microtubules is thought to be a consequence of stability as the exposed residues on the microtubules are targeted by certain motor proteins often allowing for specific cellular transport (Liao and Gundersen, 1998). These interactions are vital for neuronal development; as such, TTL knock-out mice die prematurely due to the disorganisation of neuronal networks (Erck et al., 2005). Furthermore, a loss of detyrosination can affect muscle tissue, in particular, the normal function of cardiomyocytes as the microtubule transportation can be reduced therefore affecting cardiomyocyte contraction (Chen et al., 2018).

All tubulin glutamylation and glycylation is catalysed by enzymes which form the tubulin tyrosine ligase-like (TTLL) family of enzymes; this is a large family of proteins all with a TTL homology domain (Janke et al., 2005). To date, 13 distinct types have been found in humans (Garnham and Roll-Mecak, 2012, Bosch Grau et al., 2017). *TTLL3* and *TTLL8* are initiating polyglycylases whereas *TTLL10* is an elongating polyglycylase (Rogowski et al., 2009). Polyglutamylation results in the addition of glutamate residues and has been observed in axenomes, mitotic spindles, centrioles and neurons (Gadadhar et al., 2017a). Polyglycylation results in the addition of glycine residues and has also been observed in axenomes (Gadadhar et al., 2017a). As both of the PTMs mentioned above occur in axenomes, this suggests a role for these specific PTMs in regulating ciliary function (Gadadhar et al., 2017b). Ciliopathies can affect a wide range of biological processes which is shown by the number of diseases in which ciliopathies are implicated in (Waters and Beales, 2011).

The platelet cytoskeleton has been of great importance with regard to understanding platelet physiology and how it is impacted in disease states (Hartwig and DeSisto, 1991, Boyles et al., 1985). Therefore, further investigation of TTLL10 and its involvement in the platelet cytoskeleton is warranted and especially in the context of the novel *TTLL10* variants found in the GAPP patients mentioned earlier.

5.4.2. Tubulin tyrosine ligase protein family

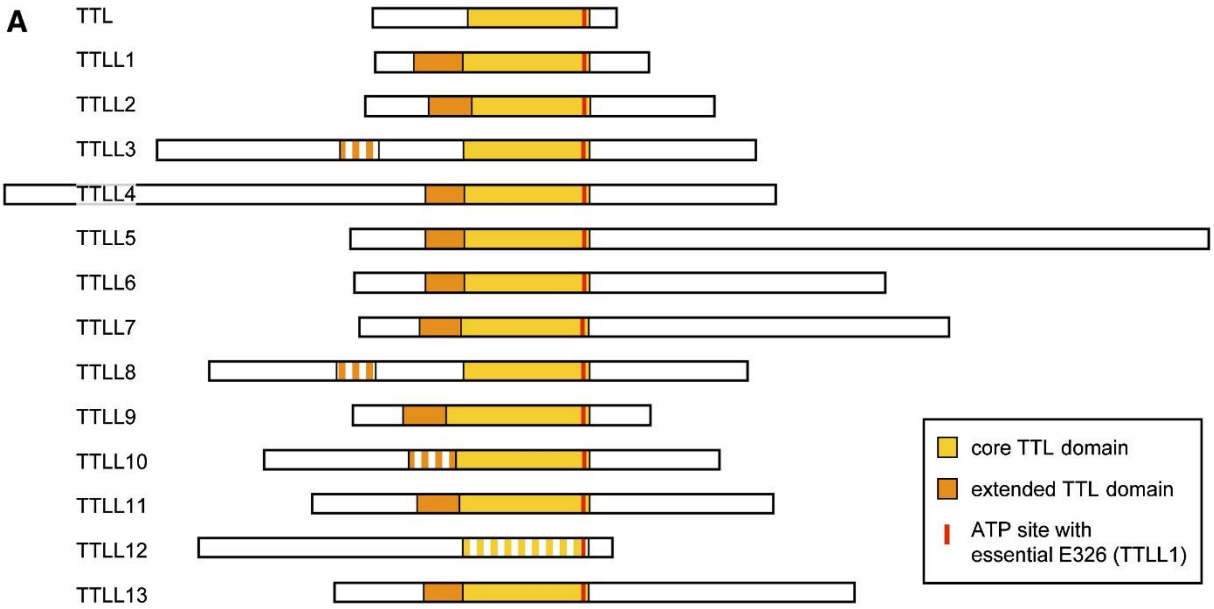
The study of protein regulation by PTM led to the discovery of tubulin polyglutamylation and subsequent investigation of the tubulin tyrosine ligase (TTL) family of proteins (Janke et al., 2008). The first polyglutamylase enzyme to be identified was polyglutamylase subunit 1 (PGs1) and further study identified another enzyme found to play a role in PTM, PGs3, which shared functionalities with the known protein TTL (Ersfeld et al., 1993, Janke et al., 2005). As such,

PGs3 is now called tubulin tyrosine ligase like-1 (TTLL1) and this finding discovered that polyglutamylase enzymes are members of the TTLL family (Janke et al., 2008).

Following this finding, systematic study of the TTLL family by a number of research groups showed the presence of 13 TTLL proteins in mammals. These were identified from sequence searches based on common regions present in the proteins. All 13 proteins differ in structure and function, however, they were all characterised by the presence of an identical TTL core domain and ATP binding domain. A summary of the 13 TTLL proteins' structure and function can be seen in Figure 5.3. TTLL1, 4–7, 9, 11 and 13 are glutamylases whereas, TTLL3, 8 and 10 are glycylases (Yu et al., 2015). The TTLLs function to initiate the incorporation of a specific amino acid (glutamic acid or glycine) to tubulin, or to elongate the modification by adding further amino acids to ones already present. Lastly, the TTLL proteins have shown preferences as to which isoforms of tubulin they work to modify; different TTLL proteins target either α- or β-tubulin and some can even work to modify both isoforms (Yu et al., 2015).

To date, despite significant progress in deciphering the tubulin code, it is not well known how tubulin PTMs are integrated into normal physiological processes (Yu et al., 2015). Further work is required to elucidate the mechanism of action of these PTMs as well as how they are regulated by differential expression and hence the localization of these enzymes in the human body (van Dijk et al., 2007).

A



B

Enzyme	Modification	Tubulin type preference	Initiase vs elongase activity
TTL	Tyrosination	α	Initiase
TTLL1	Glutamylation	α and β	Initiase
TTLL2	Glutamylation	Unknown	Unknown
TTLL3	Glycylation	α and β	Initiase
TTLL4	Glutamylation	β	Initiase
TTLL5	Glutamylation	α	Initiase
TTLL6	Glutamylation	α	Elongase
TTLL7	Glutamylation	β	Initiase/Elongase
TTLL8	Glycylation	α and β	Initiase
TTLL9	Glutamylation	α	Elongase
TTLL10	Glycylation	α and β	Elongase
TTLL11	Glutamylation	α	Elongase
TTLL12	Tyrosination	Unknown	Unknown
TTLL13	Glutamylation	α	Elongase

Figure 5.3. TTLL proteins (A) Schematic diagram to represent TTL and the 13 TTLL proteins. The core TTL domain is shared by TTL proteins (yellow rectangle) although TTL domain in TTLL12 is less conserved (striped yellow rectangle). It has also been shown that the ATP binding site (red bar) has high sequence homology between all TTLLs (van Dijk et al., 2007). (B) Summary of characteristics of TTL and TTLL family proteins.

5.4.3. Suspected function of TTLL10

It is believed that the novel protein, polyglycylase TTLL10, could be involved in the affected patients' bleeding disorder as described earlier in this thesis. As shown in Figure 5.4 A, the mechanism of action of the TTLL10 protein is to add glycine residues to the glycine chains already attached to tubulin, which is part of the PTM required for its normal function. The *TTLL10* genetic variants reported in the 3 unrelated families could lead to a loss-of-function of the protein and therefore a possibility is that little to no polyglycylation would occur. This suggests a role for the *TTLL10* gene, either in normal platelet function in a physiological setting, clot formation or thrombus stability.

Previous research has shown that by introducing point mutations into the core TTL domain of *TTLL10*, this renders the protein inactive and is sufficient for the enzyme to completely lose all of its enzymatic activity (Rogowski et al., 2009). As 2 of the genetic variants in the patients are predicted to result in a truncated protein and the other results in a missense change, and occur before the TTL core domain, the domain essential for ATP binding and to the function of the protein, we can predict that function maybe affected, and hence the reasoning behind the possibility that these variants may cause the patients' bleeding disorder. The positions of these variants are shown in Figure 5.4. B.

However, in order to further investigate the functional consequence of the TTLL10 protein, an understanding of the protein expression in humans is necessary. Furthermore, to be able to report the effects of the PTM on tubulin microtubules, a biological model is necessary which takes into account the complex regulatory network of enzymes that lead to PTMs. This model is required to be species specific to avoid any differences in evolutionary divergent mechanisms of PTM that occur in certain species (Rogowski et al., 2009).

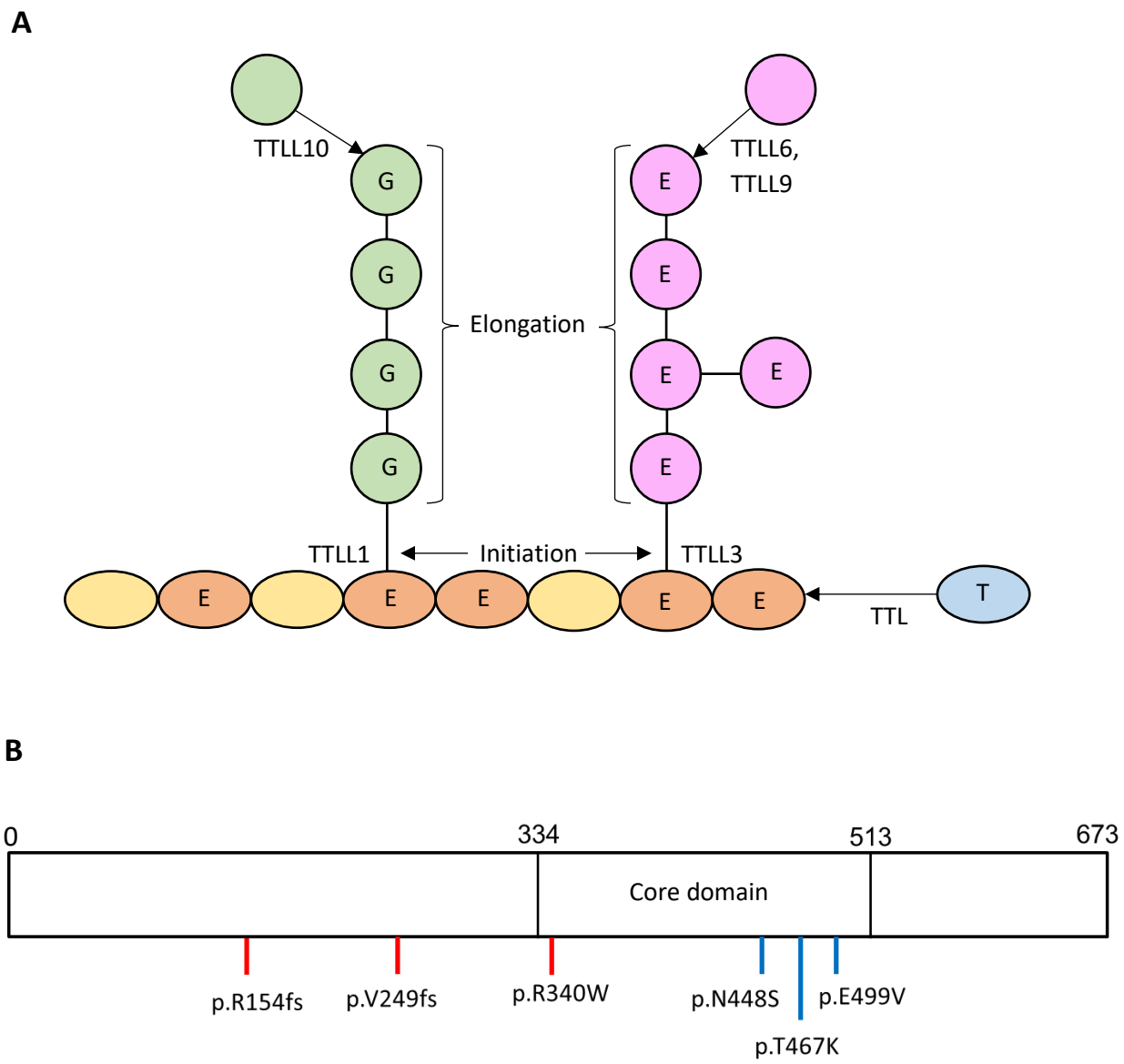


Figure 5.4. Schematic diagrams showing (A) TTL proteins mechanism of action on α-tubulin (B) the TTLL10 protein with annotated genetic variants. (A) The TTL family proteins catalyse adding or removing amino acids to and from tubulins; TTLL10 acts to elongate glycine sidechains, a polyglycylase and TTLL6 and TTLL9 act to elongate glutamic acid sidechains, polyglutamylases. (B) The TTLL10 protein is made up of 673 amino acids and contains a TTL core region from amino acid 334–513, which is highly conserved in the TTL protein family members. The red lines show the locations of the variants identified in patients of the GAPP study; the blue lines show variants that have been introduced *in vitro* and show that the enzyme activity was abrogated (Ikegami and Setou, 2009).

E, glutamic acid; G, glycine; K, lysine; N, asparagine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan.

5.5. TTLL10 and other TTL family protein expression

It is has been known since the 1960s that cells express different genes which allow them to develop into different cell types with specific physiological functions. It is also important to understand the level of protein expression within the different cell types. If protein expression is too high or too low for a specific cell type this can be detrimental to overall health and is a common cause of many disease states. In order to further understand the function of TTLL10, we investigated its expression within a number of tissue and cell types. To do this initially we used different online databases and tools. Most of these are freely available online and the valuable information they offer is frequently used by scientists.

5.5.1. Open access protein expression databases for TTLL10 and other TTL family members

The first version of the Human Protein Atlas (HPA) was released in 2005 when it covered 650 protein-coding genes (Uhlen et al., 2010). Today, all of the approximately 20,000 human protein-coding genes have been analysed within over 40 tissue types and 60 cell lines (Uhlen et al., 2015). There is still some protein expression data missing and their aim is to "provide a finite list of human protein-coding genes and generate firm protein evidence and expression characteristics for all those genes" (Uhlen et al., 2015). The latest release of the HPA is complimented with RNA-data from the Functional Annotation of Mammalian Genomes 5 (FANTOM5) project (http://fantom.gsc.riken.jp/). The HPA's Tissue Atlas shows the expression and the localisation of proteins within tissues and organs. This is based on deep-sequencing of RNA (RNA-seq); there is also antibody-based protein data which provides cell-type specificity and the relevant abundance of the protein in the tissues. The expression of the TTL-family of proteins in different tissues can be seen in Table 5.1. The Cell Atlas contains mRNA

expression profiles from a diverse set of human derived cell lines from different germ layers and tissues. This atlas also contains immunofluorescence images of cells detailing the subcellular distribution of the proteins in question. The expression of the TTL-family of proteins in different cell lines can be seen in Table 5.2.

Using the data from the HPA, we were able to see which of the TTL-family of proteins was most highly expressed in certain tissues and cell lines. TTLL3, TTLL5 and TTLL12 were moderately expressed across most tissues (range 2.6–104 transcripts per million [TPM]). TTLL4, TTLL7 and TTLL11 were expressed at low levels across most tissues (range 0.3–46.8 TPM). The most highly expressed protein was TTLL5 in testis tissue (104 TPM); the second most highly expressed protein was TTLL12 in oesophagus tissue (74.3 TPM). With regards to TTLL10 expression in tissues, it seems to be expressed at very low levels throughout a range of different tissues with its highest expression in the testis (27.9 TPM), fallopian tube (14.2 TPM) and lung tissues (2.7 TPM). TTLL12 is highly expressed across all cell lines (range 108–8.4 TPM) whereas TTLL3, TTLL4 and TTLL5 were lowly expressed across all cell lines (range 0.9–35.2 TPM). TTLL10 expression was negligible in most cell lines but showed low levels of expression in HaCaT (human keratinocyte) cells (0.4 TPM), HEL (human erythrolukaemia) cells (0.4 TPM) and RPMI-8226 (human multiple myeloma) cells (0.2).

We also used Haemosphere (https://www.haemosphere.org/) as a tool for investigating TTL family gene expression. Haemosphere is an online data portal where users can search for genes of interest, view their expression profiles within cell lines of the haematopoietic stem cell lineage, run differential expression analyses and handle multiple sets of genes (Choi et al., 2019). Unlike the HPA, Haemosphere focusses only on key specific cells across normal

haematopoiesis; in addition, it has curated datasets which cover mouse expression information (de Graaf et al., 2016).

Furthermore, the Blueprint project (http://www.blueprint-epigenome.eu/) which ran from October 2011 to September 2016, has provided data which has been utilised by many research groups across the world. This project aimed to further understand epigenetic and transcriptomic profiles of normal and malignant haematopoietic cells. The Blueprint Consortium sequenced RNA from human progenitor cell populations of the haematopoietic lineage (Chen et al., 2014). The expression of the TTL family of genes can be observed in Figure 5.5. This shows that TTL, TTLL1, TTLL3, TTLL4, TTLL5, and TTLL12 are expressed in the main progenitor cell types of the haematopoietic cell lineage. TTLL10 is lowly expressed until the common lymphoid progenitor cell stage where expression increases. TTLL10 expression is also increased in the granulocyte-monocyte progenitor cell population; however, expression decreases when cells progress through to the megakaryocyte-erythroid progenitor population and is decreased again in megakaryocytes.

Lastly, the human platelet proteomic database described by Burkhart *et al*. was scrutinised to assess the expression of the TTL proteins in platelets alone (Burkhart et al., 2012). The database showed low levels of TTLL12 protein expression in platelets. This fits with the data shown by the HPA as TTLL12 was the most expressed protein in both the tissue types and the cell lines. In addition, the TTLL12 platelet expression is also mirrored by the Blueprint data which shows TTLL12 expression all through the haematopoietic progenitor cells, down to megakaryocytes. This database aimed to provide a 'starting point' for platelet proteomics therefore not all proteins may be included; further studies have been initiated and the database will then be updated (Burkhart et al., 2012).

Table 5.1. The Human Protein Atlas dataset showing TTL protein expression in tissue. RNA-seq tissue data is reported as mean TPM (transcripts per million) corresponding to mean values of different individual samples from each tissue. Colour coding is based on TPM values; white represents the lowest values up to red representing the highest.

	TTL	TTLL1	TTLL2	TTLL3	TTLL4	TTLL5	TTLL6	TTLL7	TTLL8	TTLL9	TTLL10	TTLL11	TTLL12
Adipose tissue	1.1	3.5	0	15	4	5.2	0	4.6	0	0	1.3	4.5	11.3
Bone marrow	1.2	1.5	0	19.7	3.6	7.4	0	0	0	0	0	0.8	16.6
Breast	0.8	6.2	0	17	7.8	8.9	0	1.2	0	0.6	0.1	4.4	10.8
Cerebral cortex	4.4	18.3	0	18.3	12.4	10.8	0.5	46.8	0	1.3	0	15.7	19.9
Cervix	0.9	7.9	0	26	5	11.1	0	2.5	0	0.1	0.4	5.9	12.4
Colon	0.7	3	0	11.2	5.4	5.7	2.8	1.9	0	0	0.1	4.2	34.9
Oesophagus	1.3	4.5	0	11.8	7	8.7	0	3.5	0	0	0.1	7.8	74.3
Fallopian tube	0.9	17.1	0	40.3	11.7	18.7	5.8	6.7	0	7.1	14.2	8.1	22.7
Heart muscle	1.1	6.3	0	10.1	1.7	3.7	0	1.7	0	0	0	3.4	4.5
Kidney	0.9	4.7	0	7.1	3.6	6.7	1	0.8	0	0.1	0.1	3	6.6
Liver	0.3	0.8	0.1	2.6	2.2	2.1	0	0.6	0	0	0	2.8	5.7
Lung	2.1	5.9	0	22.4	8.4	9.7	0.7	4.6	0	1.1	2.7	5.7	11.5
Parathyroid gland	1.3	9.7	0	22.3	9.5	20.4	0	6.4	0	1.5	0	9.3	10.7
Prostate	1	8.8	0	30.1	7.7	11.3	0.1	8.9	0	0.1	0.1	7.2	23.4
Skin	1.7	5.1	0	47.8	9.3	8.6	0	0.3	0	0	0.1	4.7	31.8
Small intestine	0.7	2.3	0	11.7	6.1	5	1.8	1.9	0	0	0.1	2.8	25.4
Testis	1.2	20.2	34.1	52.5	46.8	104	26	9.2	4.2	6.3	27.9	21	31.4
Thyroid	1.5	12.6	0	20.6	5.7	13.1	0	2.7	0	0.6	0	5.8	10.8
Urinary bladder	1.3	4.8	0	17.3	5	7.1	0	3.7	0	0	0.1	6.1	15.5

Table 5.2. The Human Protein Atlas dataset showing TTL protein expression in cell lines. The cell lines in the Human Protein Atlas have been analysed by RNA-seq to estimate the transcript abundance of each protein-coding gene. The RNA-seq data is measured in TPM.

	TTL	TTLL1	TTLL2	TTLL3	TTLL4	TTLL5	TTLL6	TTLL7	TTLL8	TTLL9	TTLL10	TTLL11	TTLL12
ASC diff	2.2	13.8	0	12.8	5.5	7.5	0.2	1.5	0	0	0	3.7	19
HaCaT	1.7	8	0	2.6	17.9	18.9	0	0.1	0	0	0.4	5.1	69.3
HAP1	5	1.6	0	7	35.2	12.7	0	5.1	0	0	0	7	80
HBEC3-KT	1.8	1.2	0	0.9	12	15.7	0	1.8	0	0	0	3.8	47.3
HBF TERT88	6	3.4	0	2.1	22.2	28.5	0	3.6	0	0	0	4.6	8.4
HEK 293	2.5	2.8	0.1	17.8	28.9	6.8	0	3.2	0	0	0	5.1	112.8
HEL	2	1.7	0	12.4	17	11.4	0.1	3.6	0	0	0.4	2.3	23.8
HeLa	2.1	0.7	0	10.4	12.3	11.6	0	3.4	0	0	0	3.5	49.9
Hep G2	2.3	1.2	0	5.9	34.1	7.7	0	0	0	0	0	2.5	44.9
HMC-1	1	2.1	0	20.7	11.8	10.3	0.1	0.4	0	0	0	2.6	23.2
HUVEC TERT2	3.7	9.8	0	5.2	15.8	28.3	0	2.4	0	0	0	2.3	30.5
NB-4	1.1	4.9	0	12.3	10	8	0	0	0	0	0	2.3	41
REH	2.7	9.9	0	6	27.9	8.8	0	0	0	0.2	0	3.1	76.9
RPMI-8226	1	0.7	0	3.4	13.3	6.1	0	0	0	0	0.2	5.8	108
RPTEC TERT1	1.2	4.9	0	4.1	14.9	11.4	0	0.3	0	0	0	3	21.9
SiHa	4.1	5.2	0	3.5	9.9	29.7	0	4	0	0	0	2.9	81.6
THP-1	2.1	5.3	0	5.6	8	6.7	0	0.1	0	0	0	2.7	35.6
TIME	3.6	9.2	0	16.3	6.6	31.6	0	0.3	0	0	0	2.6	28.7
U-698	1.5	0.4	0	10.9	22.6	10.2	0	0.1	0	0	0	1.5	54.6
U-87 MG	6.6	9.3	0	7.6	9	20.5	0	5	0	0	0	3.2	25.2

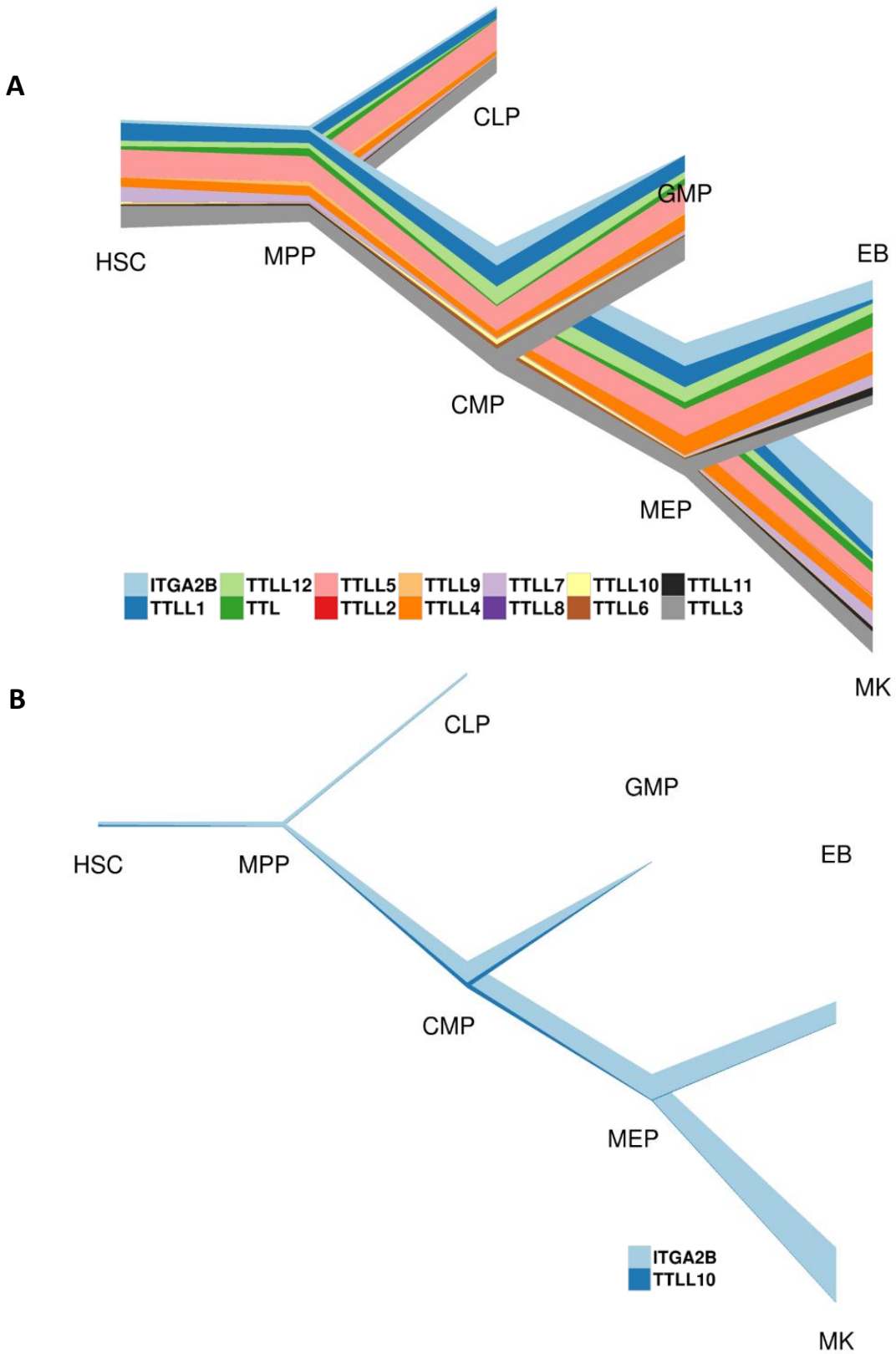


Figure 5.5. In silico Blueprint River Plot for visualising the level of expression genes. (A) Shows each TTL family gene within the haematopoietic stem cell lineage cell types. (B) Shows and TTLL10 specifically. ITGA2B is also shown as a comparison due to its known high expression throughout the lineage. HSC, Haematopoietic stem cell; MPP, Multipotent progenitor; CLP, Common lymphoid progenitor; CMP; Common myeloid progenitor; GMP, Granulocyte-monocyte progenitor; MEP, Megakaryocyte-erythroid progenitor; EB, Erythroblast; MK, Megakaryocyte.

5.6. Further investigation into expression levels of TTLL proteins

Further expression analysis was carried out using platelet samples in order to elucidate the expression of the TTLL proteins a different stages of platelet development, as well as to see if there was any difference between expression in resting and activated platelets.

5.6.1. qRT-PCR to investigate the expression of the TTLL family of genes

A qRT-PCR panel was designed in the laboratory to examine the expression of the 13 TTLL proteins expressed in mammals. RNA was extracted from inducible pluripotent stem cell (iPSC) derived megakaryocytes; all cell culture and cell differentiation were carried out by Dr Abdullah Khan. These cells were harvested at differing stages of their differentiation process; day 1 cells (d1) are representative of haematopoietic stem cells and cells committed to progressing to become progenitors of megakaryocytes, day 5 cells (d5) are comprised of approximately 60% megakaryocytes and lastly, heparin was added to d5 cells to induce proplatelet formation (d5 + Hep). These latter cells were included in the investigation because it is not known whether TTLLs are either up- or down-regulated upon proplatelet formation.

The results showed that the TTLL family of proteins vary in expression during the differentiation process and maturation of the cells (Figure 5.6 A). GAPDH was used as a housekeeping gene for this experiment and showed similar amplification across all 3 cell sample types (Figure 5.6 A). A number of the TTLLs that showed expression were taken forward for quantification across replicate experiments that required multiple differentiations of the cell types (figure 5.6. B). There was found to be a significant increase in expression of *TTLL1* ($p = 0.00181$), *TTLL2* ($p = 0.0105$), *TTLL4* ($p = 0.026$), and *TTLL10* ($p = 0.0004$) in the d5 + Hep cells when compared to d1 cells using the Delta-Delta Ct method to calculate relative fold

gene expression. This data shows that a number of TTLLs are upregulated during the process of platelet production including *TTLL10*.

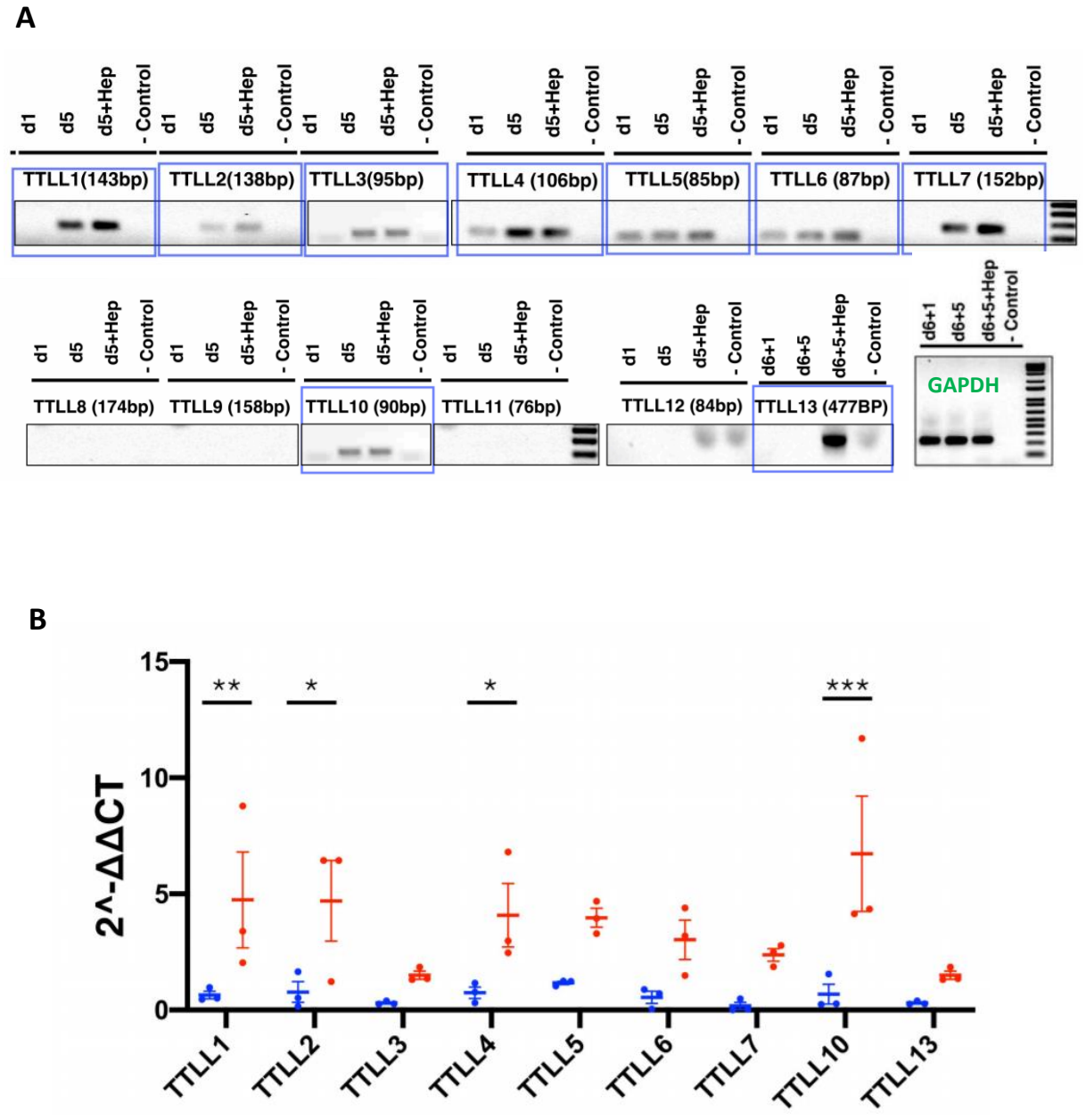


Figure 5.6. IPSCs, megakaryocytes and proplatelets differentially express TTLL proteins through their development cycle. (A) Cell samples were amplified with TTLL1–13 specific primers or GAPDH housekeeping primers. A few of the TTLLs appeared to be upregulated in the more mature and proplatelet forming cells. (B) The samples from the qRT-PCR were taken forward and quantified using the delta-delta Ct ($2^{\Delta\Delta Ct}$) method using d1 cells as controls to determine if there was any upregulation in TTLL expression during the process of platelet production. The expression of TTLL1, 2, 4, and 10 was found to be significantly upregulated in d5 + Hep proplatelets (* p = 0.026, ** p = 0.0105, *** p = 0.0081, **** p = 0.0004).

5.7. Western blot analysis of TTLL10

Western blots were carried out in order to determine protein expression in different cell types and platelet protein samples as described in Methods section 2.18.

5.7.1. Available TTLL10 antibodies

When planning the western blot investigations, several TTLL10 antibodies were found to be commercially available. Three different anti-TTLL10 antibodies were ordered from different suppliers (a table of antibodies used in this thesis can be seen in Table 2.2). The 3 different antibodies were chosen based on the regions of the protein that they were directed against; they all targeted a different section of amino acids. Anti-TTLL10.3 was raised to a region within amino acids 242–455 of human TTLL10 and anti-TTLL10.4 corresponded to a region within the N terminal amino acids 73–122 of human TTLL10.

We also tested a further anti-TTLL10 antibody provided by a collaborator as well as an antibody that detected global polyglycylation, a polyclonal anti-polyglycine antibody generated by using Cys-$(Gly)_9$ as an immunogen (Ikegami et al., 2008).

5.7.2. Western blots with cell lines

Firstly, cell types that were readily available and used regularly in the laboratory were used for the western blots in addition to platelet lysate samples from numerous healthy volunteer donors were tested. Cell lysates were also produced from mouse testes as following the investigation of TTLL10 expression, high expression was seen in testes tissue. It was thought that the testes lysates could act as a positive control however remaining cautious about the species difference which could affect levels. The different TTLL10 antibodies were used on these cells types in order to try to elucidate which antibody might be the most specific to the protein and hence the likely best antibody to use for further investigations (Figure 5.7).

All the antibodies that were used in the western blots were found to be non-specific to TTLL10. It was very difficult to make out a specific TTLL10 protein band at the molecular weight the protein was predicted to be by the respective providers' data sheets.

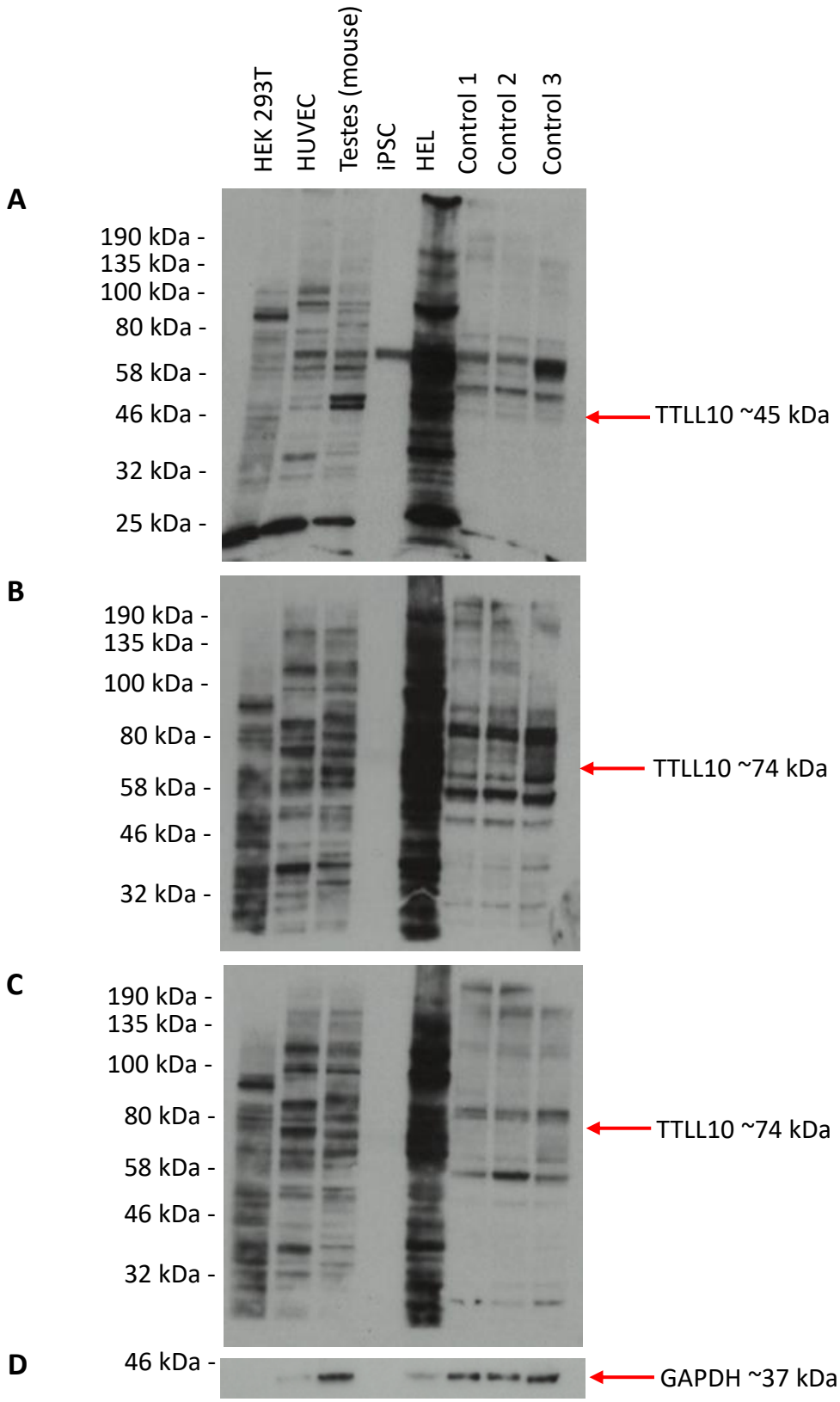


Figure 5.7. Western blot to trial different anti-TTLL10 antibodies. TTLL10 expression compared to GAPDH loading control. TTLL10 expression is difficult to identify therefore the antibodies are not specific to TTLL10. (A) anti-TTLL10.2, 5 minute exposure. (B) anti-TTLL10.3, 1 minute exposure. (C) anti-TTLL10.4, 20 second exposure. (D) GAPDH housekeeping protein.

5.7.3. TTLL10 protein expression in patient samples

Platelet protein and platelet lysates were prepared from patient samples when the patients were recalled and attended the clinic for investigations into their unexplained bleeding disorder; 3 members of Family 3 and 1 member of Family 11 were included in the western blot experiments. The patient samples were run in parallel to healthy volunteer control platelet protein samples and prepared in the same way. The membrane was probed with anti-TTLL10.3 polyclonal antibody as this antibody was thought to be the most specific as it showed a band at approximately the correct molecular weight as to what was described on the data sheet provided by the manufacturer (Figure 5.8 A). Additionally, the same samples were run on a gel but probed with the anti-polyglycine antibody, in order to identify polyglycylation in the platelet samples (Figure 5.8 B).

There appeared to be no difference in expression of TTLL10 in the patient samples compared to the controls. The loading control using GAPDH did show that there were varying levels of protein in the samples. Despite there not being an observable decrease in expression, the question of the antibody specificity comes into question as it cannot be concluded that the band highlighted is showing the TTLL10 protein. Similarly, for the anti poly-glycylation antibody, the specificity is unknown.

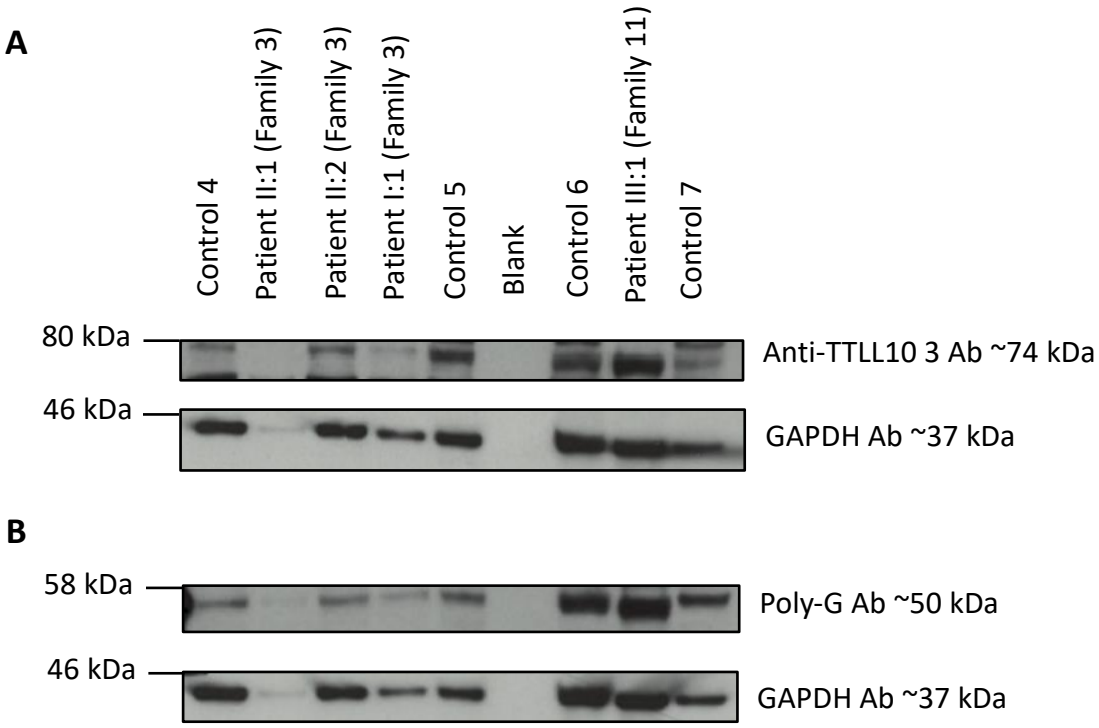


Figure 5.8. Western blot to determine the expression of TTLL10 and polyglycylation in patient platelet lysates. TTLL10 expression and the presence of polyglycylation was investigated in patients and healthy volunteer control platelet samples and compared to GAPDH as loading control. TTLL10 expression is difficult to identify therefore the antibodies are not specific to TTLL10. (A) TTLL10 expression using the anti-TTLL10 3 polyclonal antibody. (B) Expression of polyglycylation using an anti-polygycylation antibody which is not commercially available.

5.8. Platelet spreading analysis of 'TTLL10' patients

Platelet spreading was carried out as described in the Methods section 2.19. Platelets were spread on coverslips coated in fibrinogen and were dually stained with TTLL10 and tubulin antibodies. The platelet spreading assay could potentially be a method for investigating the role of TTLL10 in platelets as they undergo morphological changes upon activation.

5.8.1. Control platelet spreading

Firstly, the platelet spreading assay was carried out using healthy volunteer control platelets. This was because the antibodies had not previously been used for immunofluorescence using spread platelets and it was important to understand the normal pattern of TTLL10 staining seen in healthy platelets.

The spread platelets were imaged using confocal microscopy (Figure 5.9). The TTLL10 staining can be seen as a strong fluorescent signal in the non-spread platelets and what seems to be weaker signal in the fully spread platelets. The pattern changes from punctate when the platelets are not spread to more diffuse when the platelet is fully spread. The reason behind this pattern may be due to the cytoskeletal components, of which TTLL10 is part, spreading out to cover a larger surface area compared to non-spread platelets and hence the signal seems weaker in the spread platelets.

Immunofluorescence was also carried out on resting control platelets. These were not spread on fibrinogen but on poly-L-lysine therefore the platelets would not become activated. For the platelets to adhere to the cover slip, they were spun in a centrifuge for a short period of time. There was no difference in the staining pattern of TTLL10 in these resting platelets compared to the platelets spread on fibrinogen that were not spread.

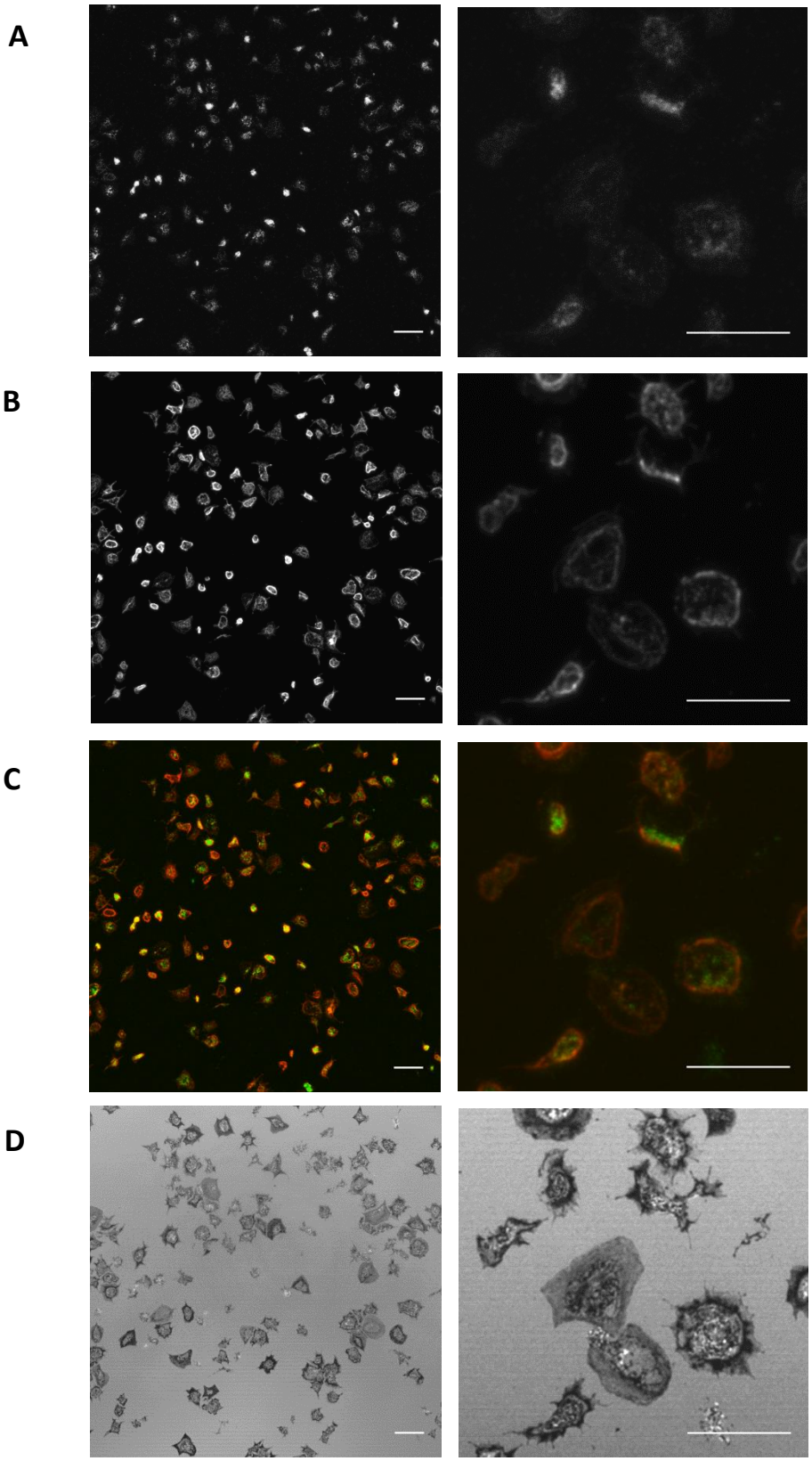


Figure 5.9. Confocal microscopy images of spread platelets from a healthy donor after spreading on fibrinogen for 45 minutes. Left column shows a 2× zoom and the right column shows 8× zoom at a different field of view. (A) TTLL10 fluorescence. (B) Tubulin fluorescence. (C) Merged TTLL10 (green) and tubulin (red) fluorescence. (D) Reflectance image. Scale bar, 10 µm.

5.8.2. Patient platelet spreading

Platelet spreading was carried out in family members from Family 3 as mentioned in Chapter 4. A father and his 2 daughters possess a frameshift deletion (c.462delG) and a frameshift insertion (c.745_746insG).

The platelet spreading images show that their platelets spread on fibrinogen which were stained with actin (green) and tubulin (red) (Figure 5.10). Previous work showed that the platelets from these affected family members are enlarged and it was thought that the genetic variants they harbour might affect the ability of the platelets to spread (Khan et al., 2019b).

The platelets were counted and categorised into the different stages of spreading. If the platelet was fully adherent, wide and circular it was classed as 'spread'. If the platelet was spikey, with microtubule extensions visible but starting to show a circular shape, this was classed as 'spreading'. If the platelet was small with only one or two small microtubule extensions this was classed as 'unspread'. The number of platelets per field of view was normalised by calculating percentages of each category of spreading. The in-house controls were added in order to get a range of normal spreading values that patient values could be compared to spreading and fully spread. A Dunnett's test of multiple comparisons showed that there were a statistically significant number of spread platelets when the in-house control spread platelets were compared to each patient separately. There was significance found between the control and patient, daughter II:2 ($p < 0.05$) and the father, patient I:1 ($p < 0.0001$) (Figure 5.11). This was an interesting finding as there was no significance between the control and the mother (I:2) however this is in keeping with the patient who is wild type for the *TTLL10* variants. However, this would not explain the lack of significance seen in the other daughter who does have the 2 *TTLL10* genetic variants mentioned above. This is of great

interest and may explain a platelet defect however further research still need to be done to pinpoint the exact disease mechanism.

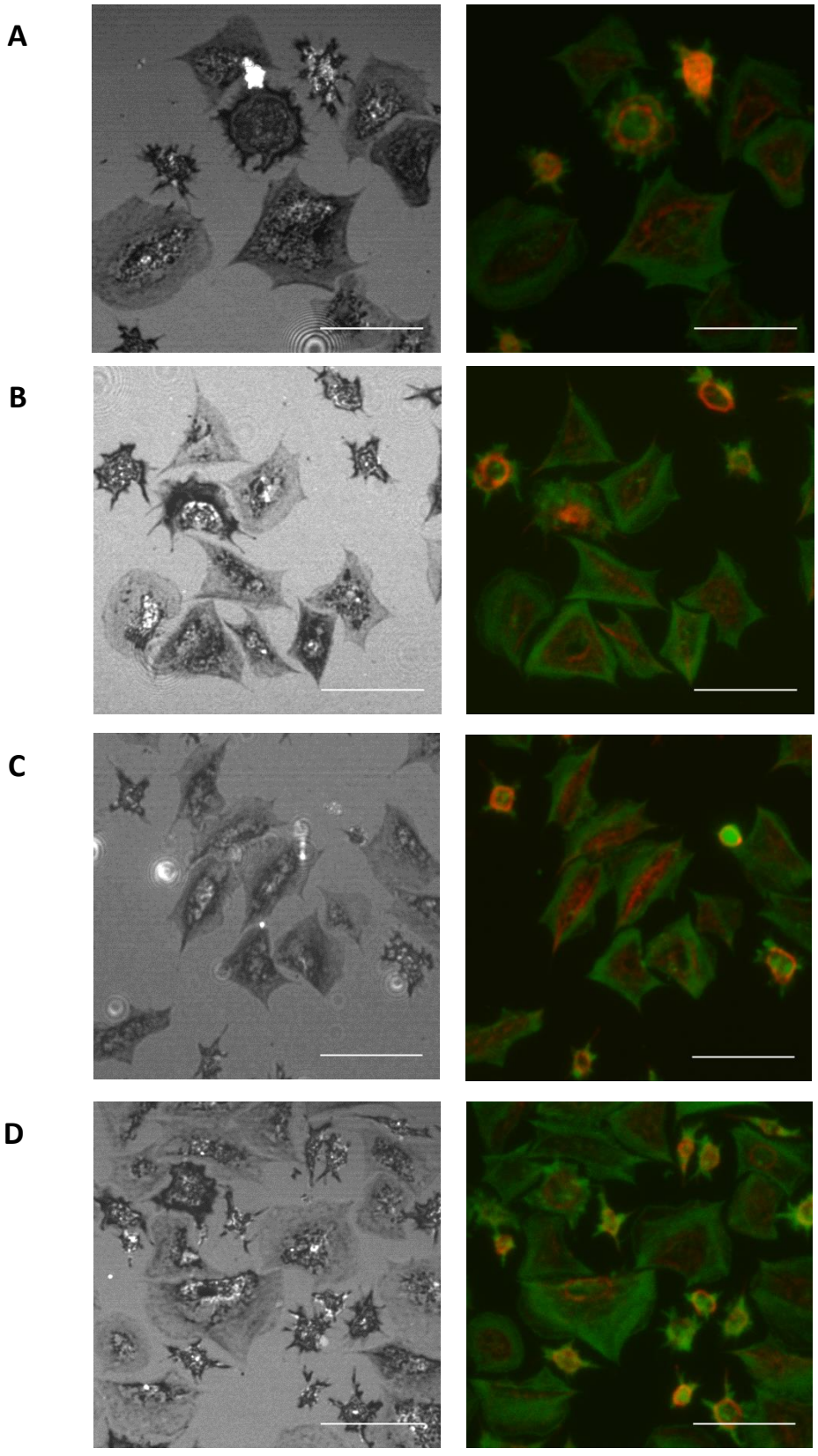


Figure 5.10. Confocal microscopy images of spread platelets from members of Family 3 with TTLL10 genetic variants. Left column shows reflectance images and the right column shows merged fluorescent images with actin in red and tubulin in green (8× zoom). Family 3 members (A) II:2. (B) II: 3. (C) I:2. (D) I:1. Scale bar, 10 µm.

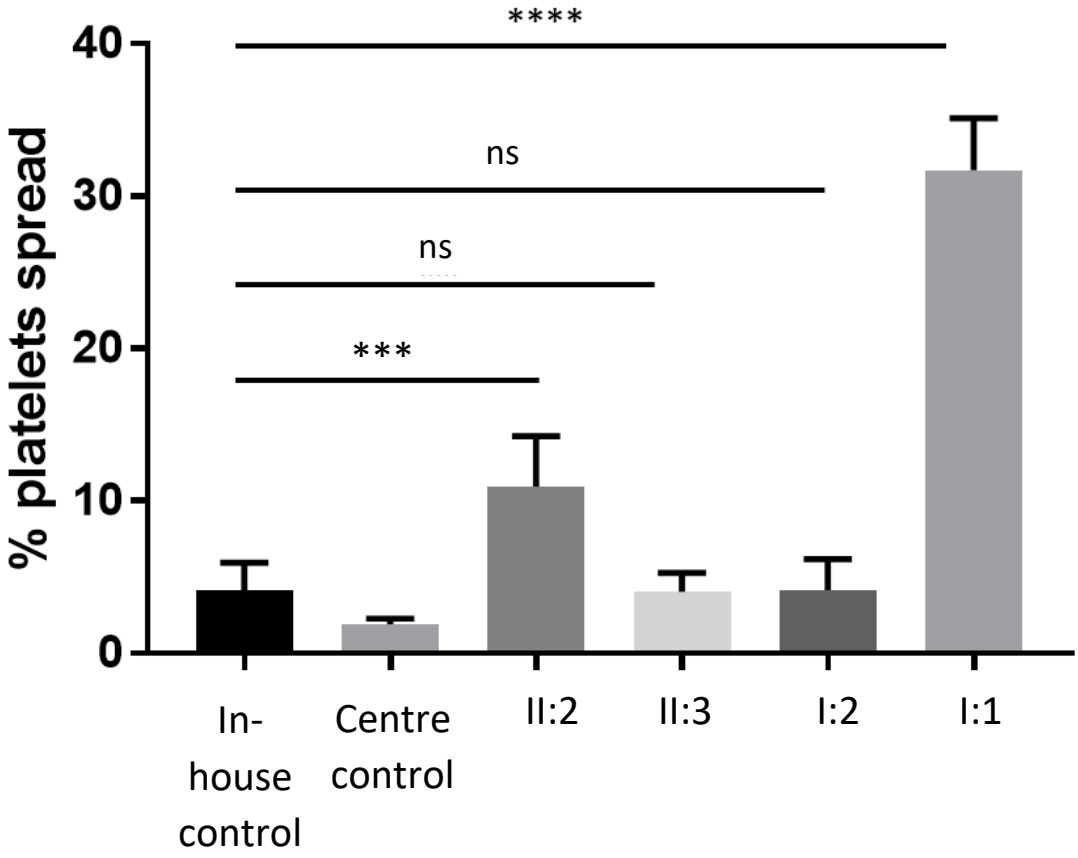


Figure 5.11. The percentage of spread platelets from the patients with TTLL10 variants and controls. Error bars show the mean with standard deviation. A Dunnett's test of multiple comparisons compared the multiple patients to a single control. There was found to be a statistical significance between the number of spread platelets in the in-house control group compared with patient II:2 and I:1 (*** $p < 0.05$, **** $p < 0.0001$; ns, not significant).

5.9. Discussion

The work in this chapter was carried out when genetic variants in the same gene, *TTLL10*, were discovered in 3 unrelated families recruited to the GAPP study. All the patients affected had similar bleeding diathesis and bleeding histories despite having normal platelet function following extensive laboratory phenotyping. We found that TTLL proteins are ubiquitously expressed and required for numerous physiological processes (Ikegami et al., 2008). We also found that the expression of many of the TTLL proteins is upregulated during platelet differentiation, including TTLL10 ($p = 0.0004$).

The TTLL10 protein expression using the patient platelet lysates by western blotting could benefit from repetition however, repeated patient samples are sometimes difficult to get hold of as the patients would be required to attend a clinic at the hospital. The antibodies that were detected to test for TTLL10 protein expression may not be reliable, although commercially available, the specificity can be variable leading to problems interpreting sound results and difficulty investigating the protein levels.

In addition, the investigation of platelet spreading with TTLL10 antibodies could be strengthened by repeating the assay with the patients' platelets. This would allow us to investigate any differences between normal healthy control platelets and the platelets from patients with the *TTLL10* genetic variants. Further image analysis could also be performed to further investigate the localization of the protein within the platelet in addition to any colocalisation with other proteins for example, tubulin or other TTLL proteins. It would also be interesting to measure area and circularity of the platelets as additional parameters to consider in addition to TTLL10. It is well known in the field that abnormalities of the platelet cytoskeleton can cause moderate to severe bleeding diatheses (Freson et al., 2007). From our

limited knowledge of the TTLL10 protein and our findings of enlarged platelets, the *TTLL10* variants are proposed to affect the formation of tubulin microtubules.

Due to time constraints during the course of the PhD, further work is required to fully understand the role of TTLL10 in platelets and confirm the cause of disease in the 3 families mentioned in this Chapter who all experience bleeding episodes.

5.10. Future work

It is notoriously difficult to generate the conditions and modification patterns that mimic those found in cells and to build complex microtubule arrays, assays or even effective and reproducible cell models. This is because there are many accessory proteins that will be involved in the process physiologically which would not be present in said assays *in vitro*. Furthermore, the cell dynamics and mechanical stressors are very difficult to recreate which also affect the formation of tubulin microtubules *in vivo*. Additional investigations are required though, to determine the functional relevance of the genetic variants in *TTLL10*.

The work that should take place following the genetic findings described in this chapter is generally in cell lines as a starting point as they are a robust and reproducible method of investigation. These investigations could use an erythro-myeloid related cell line for example Hel/Dami cells that were established from the blood of individuals with an erythro-leukaemia and megakaryoblastic leukaemia respectively and have been used routinely in a laboratory setting for over 30 years (Martin and Papayannopoulou, 1982, Greenberg et al., 1988). These cells are close in the hematopoietic stem cell differentiation lineage to megakaryocytes. These cells would provide material to examine the protein expression and function in a similar cell type that produces platelets. However, as platelets are essentially fragments of

megakaryocytes, they are notoriously hard to work on and a study model does not exist; platelet experiments nearly always require human donors.

In the last 5 years, massive progress has been made in the field of iPSCs. iPSCs are an ideal source of cells as they are pluripotent, self-renewing and widely available. In addition, there are fewer ethical issues surrounding iPSC work in the laboratory and they are relatively easy to grow and manipulate. A workflow has been designed to induce differentiation of iPSCs to produce megakaryocytes (Sugimoto and Eto, 2017). This system is now being used to produce reasonable numbers of megakaryocytes successfully. For future experiments, it would be interesting to take cells at multiple different time points through the differentiation process to build our understanding of the differential expression when progressing through the cell lineage to become platelets.

To investigate the *TTLL10* genetic variants, and their effects on platelets without the need for taking multiple patient blood donations, CRISPR-Cas9 genome editing technology could be implemented to create a TTLL10 disease model. CRISPR-Cas9 editing is now commonplace in most laboratories and even though it is still very experimental and often unreliable, this is a field that is rapidly evolving and improving (Ran et al., 2013, Khan et al., 2019b, Khan et al., 2018). The megakaryocytes produced from this model could be used to determine specific effects on multiple aspects of megakaryocyte/platelet function. However, this work would still be *in vitro* as the many different factors involved in haemostasis would not be present. To combat this, it could be argued that an animal model using transgenic mice could be developed to produce an *in vivo* disease model. Animal models are extremely useful and critical for research, but they do not come without limitations. For example, some human diseases cannot be modelled using mice, this is because there are fundamental biological

differences between the species. This has been identified in certain diseases that develop very differently in mice.

Lastly, previous work pertained that TTLL10 is a non-functional protein in humans (Rogowski et al., 2009). The work and analysis in this thesis suggest that this might not be the case. In the field of scientific research there are always contradictory findings and newer findings overriding older, previously believed ideas. Little is currently known about polymodifications in particular polyglycylation in platelets but it seems to have an important role. TTLL10 is an interesting protein and no doubt further work is warranted. This is currently being carried out and has opened up a window for investigation by future projects. *TTLL10* will now also be included on the GAPP study platelet and bleeding related gene sequencing bioinformatic panel.

CHAPTER 6
Discussion and Conclusions

6. Overall discussion

Inherited platelet and bleeding disorders are a heterogeneous group of disorders characterised by extensive bleeding symptoms following haemostatic challenges. The patients recruited to the GAPP study with bleeding symptoms vary extensively in terms of their clinical histories but also with regard to the demographic information. The female to male patient ratio and the age range of patients recruited to the GAPP study was similar to other large-scale studies of inherited bleeding disorders previously mentioned in this thesis. Furthermore, the variation of the patients' platelet characteristics also fit with other studies as the platelet size, platelet counts and severity of the bleeding, from mild to severe, was different across all patients. A unique attribute of the research undertaken in this thesis, highlighted in Chapter 4, was the extensive genetic investigation of patients with normal platelet parameters following laboratory testing despite having extensive bleeding diatheses. These patients were studied further as genetic investigation by WES is a powerful method of identifying and scrutinising potential disease causing variants. In addition, the patients who were included in the platelet spreading assay, in Chapter 3, whose platelet morphologies were deemed abnormal as a result of the platelet spreading assay, would have otherwise been missed. All of the patients included in investigations and research carried out for this thesis might have otherwise been overlooked if the usual platelet function testing standard cut-off values were applied. Therefore, the sub-set of patients described in this thesis have offered a unique opportunity to investigate and provide further insights into the genetic aetiology of inherited platelet and bleeding disorders and could lead us to novel findings regarding haemostasis and thrombosis mechanisms in the near future.

6.1. Patient platelet phenotyping using a platelet spreading assay

Despite the many different platelet function tests that are available, there is still a lack of a single and robust platelet function test, therefore diagnosis in this group of patients with inherited bleeding disorders can be difficult to achieve. To add to the difficulty, the phenotypes of patients with inherited bleeding disorders can be very heterogeneous despite similar genetic aetiologies. This variable phenotypic presentation suggests that patients should be individually considered instead of carrying out one particular method of diagnosis for all.

A platelet spreading assay was carried out to assess potential abnormalities in components of the platelet cytoskeleton. The platelet cytoskeleton is an important feature, enabling platelets to change morphology upon activation and hence perform their haemostatic role correctly. To date, 71 patients from the GAPP study cohort have been included for phenotyping using this novel platelet spreading assay (Khan et al., 2019a). The data showed that patients positive for both area and circularity abnormalities, as well as abnormal morphology are both positive and negative for defects in aggregation. Furthermore, 80% of patients that were found to have a spreading defect, but negative for an aggregometry defect, presented with a high ISTH-BAT bleeding score. This is of particular interest as a large number of patients positive for a platelet spreading defect, and with a history of excessive bleeding, are negative for an aggregation defect. This all suggests that applying platelet spreading as part of a platelet testing panel can reveal problematic platelet morphological defects that might otherwise be missed by lumi-aggregometry alone.

The drawbacks of this assay include the time it takes to complete, and the specialist laboratory and analytical skills and equipment required to fully implement the assay. In addition, this

assay serves more as a platelet spreading screen rather than an exact diagnostic assay as there are a number of possible causes for abnormal platelet spreading and cytoskeleton malformations. However, this would narrow down the possible causes of bleeding and provide an area to target with potential genetic investigations. Furthermore, the semi-automated system for analysing the images is becoming commonplace in laboratory settings across the world with machine learning technologies becoming widely available and some software are even open access. A method for automating the assay further would be advantageous and would possibly reduce the timeframe and the need for specialist scientists. More automated systems are becoming preferable for high-throughput analysis such as this. Herein lies the question however, if whether this more specialised spreading assay has a place in a routine haematology laboratory? Lastly, genetic studies are now the most robust method of patient diagnosis. The phenotyping by this platelet spreading assay would complement genetic investigations by narrowing down the potential disease causing variants in the affected patient. The challenge arises when the genetic variant is novel or of very low incidence in the general population. In order to fully understand and hopefully find a diagnosis for these patients, a combination of phenotyping and genotyping is required, especially as these patients are suspected to harbour novel variants or disorders with unknown genetic aetiology.

6.2. Patient genotyping by whole exome sequencing

Due to limitations of platelet function testing as discussed throughout this thesis, 60% of patients recruited to the GAPP study have no identifiable platelet defect (Watson et al., 2013). Patient genotyping is an ideal approach to elucidate the cause of disease in patients with clinical bleeding of unknown aetiology. WES and Sanger sequencing were carried out on 18 patients from 10 unrelated families, previously recruited to the GAPP study. These patients

were selected if their overall laboratory phenotyping results were all within the normal parameters despite having an extensive bleeding history. These patients were both paediatric and adult cases and all had bleeding symptoms of varying severities. Utilising WES in order to investigate these patients is a unique method within this patient sub-set however with the advent of the 100,000 Genomes Project, more and more patients are being referred for WES and/or WGS which overtime will strengthen investigations into all disease states (Turnbull et al., 2018). A custom designed bioinformatic pipeline was developed by the GAPP study researchers to efficiently and correctly narrow down and classify potential disease causing variants in affected patients.

Overall within this thesis, genetic variants within genes previously known to cause a bleeding phenotype were identified in one family (1 of 10). A mother and son with a lifelong bleeding tendency were found to have a heterozygous variant in *THBD* resulting in a stop codon which encoded a truncated form of the glycoprotein thrombomodulin. This was confirmed as their cause of disease and a rare example of a gain-of-function mutation in an anticoagulant factor causing bleeding (Maclachlan et al., 2017a, Dargaud et al., 2015). The majority of the other rare variants found were missense and most of those were novel (not previously described in the relevant databases). When considering pathogenicity, WES analysis revealed a positive prediction call (classification of 'pathogenic' or 'likely pathogenic') in 8.2% (8 of 98) of variants across all the families. 91.8% (90 of 98) of the variants were unknown and classified as variants of 'uncertain significance' due to a lack of evidence suggesting their pathogenicity, especially not being implicated in platelet or bleeding disorders previously. These numbers reflect the sample of patients that were taken forward for WES, as by only recruiting patients with an unknown aetiology of disease where patients with a clinical diagnosis might be more obvious, were not included. Additionally, the WES method might be preventing the discovery of

variants as WES does not guarantee to cover all exons and does not cover intragenic or intergenic regions or large-scale genomic rearrangements such are duplications or deletions. The sequencing method might have also missed potential variants embedded in a promoter region(s) which can only be made possible once a particular gene promoter has been sufficiently characterised. The other reasons for genetic variants not being identified include: multigenic disease, the incidence of disease predisposing genes or a combination of these genes or modified genes causing different phenotypes (Cooper et al., 2013, Wright et al., 2019). Furthermore, the multifactorial nature of disease in bleeding could be considered including the importance of environmental factors and as shown in Figure 6.1

The detection rate for disease causing variants in this thesis is low however this is in line with other studies with similar patient cohorts, which highlights the difficulty faced by researchers and clinicians alike. In order to identify more potential causative genetic variants, this work would be strengthened by the recruitment of additional family members for those patients where no plausible candidate variants were identified. This would help to define the variants by looking at genetic segregation. Secondly, the bioinformatic pipeline could be altered to increase sensitivity, however this might lead to inaccuracies. In addition, it is well known that the parent-child trio model is powerful for hereditary disorders (Chen et al., 2013). Furthermore, the application of WGS in patients with no candidate variants would be invaluable to look for variants occurring within the whole genome.

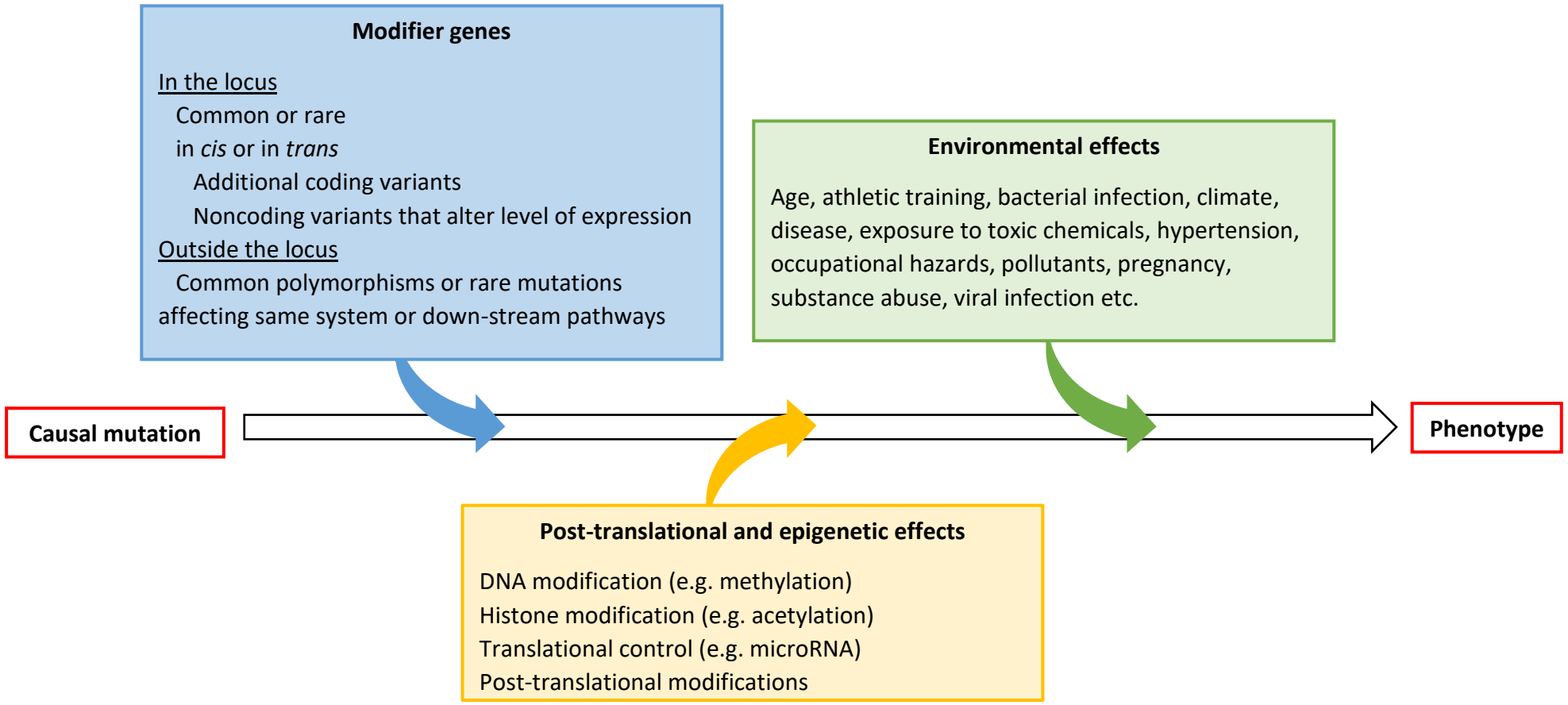


Figure 6.1: The complexities of the genotype-phenotype relationship in disease. The effect of a specific mutation in a gene is altered by other genetics, epigenetics and environmental stressors. Due to this, phenotypes can vary even within families of individuals with the same genetic mutation. There are many examples of the categories listed in this figure but as expected, there are likely many modifying factors that are unknown.

The ability to determine the genetic aetiology of unknown genes/proteins that cause bleeding is difficult due to the heterogeneity of disease as previously mentioned. Following genetic scrutiny of novel variants, functional characterisation is necessary to conclusively determine whether the variants are disease causing or benign. Some variants in the databases are said to be benign even when they have been previously associated with disease (Niroula and Vihinen, 2019). Another potential challenge arises with the inevitable discovery of incidental findings; clinicians could face difficulty with the question of should they disclose the incidental findings and any clinical relevance to their patients (Maclachlan et al., 2017b)? This is a contentious topic of discussion however the ACMG Working Group on Incidental Findings in Clinical Exome and Genome Sequencing have published a list of variants they recommend disclosing to patients if found during genetic screening. Hopefully, further studies in this field will strengthen overall detection rates for novel genetic variants.

With regards to the patients studied in this thesis who are still without a diagnosis for their unexplained bleeding, work is still needed to functionally confirm all the variants discovered. If successful, this has the potential to develop our current knowledge of haemostatic mechanisms and creates scope for potential new therapeutics for thrombosis. Furthermore, it will allow the patients to have a categorised diagnosis, which is highly important for some individuals and for their clinical treatment and management later in life.

6.3. Determining the functional effects of the novel candidate TTLL10

The work from this chapter follows after genetic variants (c.462delG, c.745_746insG and C.1018C>T) in the same gene, *TTLL10*, which were identified by WES in 3 unrelated families. The affected patients all presented with similar bleeding diatheses and after routine laboratory testing, showed results in the normal range for platelet aggregation and secretion. Research was carried out to investigate the functional effect of TTLL10 and investigate a potential role for this protein in platelet function.

From this work, it has been shown that in the affected patients there is likely a reduction or total loss of polyglycylation depending on the patients' genetic variant; the effects of glycine sidechains on tubulin in platelets are not currently known. However, as the variants only affect the TTLL10 protein function, the other types of PTM remain unaffected. The affected patients appear to have enlarged platelets as their mean platelet volumes are in the upper limit of the normal range and above. This suggests a potential role for an extending glycine sidechain in platelet size regulation with a downstream effect causing bleeding. This could be caused by the inability of the patients' platelets to fully change their morphology when undergoing activation. There is always some compensation from other pathways when complex cellular and molecular mechanisms are involved in a process, therefore the defects seen in the affected patients might be more subtle that previously thought. This is seen in the patients' normal platelet aggregometry results. Nevertheless, the 3 families with genetic variants in *TTLL10* all have the same bleeding symptoms.

TTLL10 still remains the most likely genetic candidate of disease for these patients as increasing lines of evidence suggest involvement of TTLL10 in platelets and bleeding. The phenotype of enlarged platelets is seen in many known platelet disorders, for example

Bernard-Soulier syndrome, which should be taken into account if additional patients with variants in *TTLL10* are identified (Watson et al., 2013). In addition, there are other known disorders, for example Wiskott- Aldrich syndrome, that affect platelet activation and platelet cytoskeletal arrangement (Paknikar et al., 2019). Further investigations are required to fully prove the cause of disease as it is still very difficult to prove if a variant is disease causing, especially if it is novel or has a very low incidence in the population. Extensive functional work is required in this case and there are many different methods to achieve this.

6.3.1. Future work to determine the functional relevance of candidate gene variants in novel genes including *TTLL10*

The work that should take place following the genetic findings described in this thesis is generally in cell lines as a starting point as they are a robust and reproducible method of investigation. These investigations could use an erythro-myeloid related cell line. These cells are close in the hematopoietic stem cell differentiation lineage to megakaryocytes. These cells would provide material to examine the protein expression and function in a similar cell type that produces platelets.

Of recent years, massive progress has been made in the field of scientific investigation using inducible pluripotent stem cells (iPSCs). IPSCs are an ideal source of cells as they are pluripotent, self-renewing and widely available. As mentioned in Chapter 5, a workflow has been designed to induce differentiation of iPSCs to produce megakaryocytes (Sugimoto and Eto, 2017). This system is now being used to produce reasonable numbers of megakaryocytes successfully.

To investigate the *TTLL10* genetic variants, CRISPR-Cas9 genome editing technology could be implemented to create a TTLL10 disease model. The megakaryocytes produced from this model could be used to determine specific effects on multiple aspects of megakaryocyte/platelet function when the genetic variant in question is inserted into the genome of the cell type. In addition, animal models using transgenic mice could be developed to produce an *in vivo* disease model. Animal models are extremely useful and critical for research, but they do not come without limitations previously mentioned in this thesis.

Lastly, previous work pertained that TTLL10 is a non-functional protein in humans (Rogowski et al., 2009). The work and analysis in this thesis suggest that this might not be the case. In the field of scientific research there are always contradictory findings and newer findings overriding older previously believed ideas. Little is currently known about polymodifications in particular polyglycylation in platelets but it seems to have an important role. TTLL10 is an interesting protein and no doubt further work is warranted.

6.4. Final conclusions

The work carried out in this thesis focussed on improving current methods of analysis for diagnosing inherited bleeding disorders and the aims highlighted the platelet spreading assay and the WES analysis in order to complete this. Firstly, the work has shown that the platelet spreading assay identifies previously undiscovered platelet disorders that would have otherwise been missed with the standard testing. This assay could be an invaluable addition to the current range of platelet function tests offered to patients with bleeding of unknown aetiology.

Secondly, this work has shown that WES was of great value in this investigation and for the patients who were included. The cause of a previously unknown disease was identified in one

family who were hence clinically diagnosed with a bleeding disorder due to a truncated form of the thrombomodulin protein. These patients are now undertaking suitable preventative measures before medical procedures and have been offered genetic counselling and a cascade genetic testing to identify at risk family members. In addition, a number of potential disease causing variants were identified in other families, however further investigations will now have to be carried out in order to functionally prove the variants are the cause of their disease.

Lastly, preliminary evidence from the work carried out for this thesis and from broader literature shows that the role of PTMs on the normal function of megakaryocytes and platelets is more important and more involved than was expected. Not a great deal is known about their exact functions in platelets specifically however establishing the changes in PTMs in platelets will be an exciting addition to the rapidly growing field and may provide insights to the understanding of platelet biology and the tubulin code.

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Appendices

8. Appendices

Figure A. The International Society of Thrombosis and Haemostasis Bleeding Assessment Tool, used to assist the diagnosis of bleeding disorders.

Annexe 1: ISTH/SSC Bleeding Assessment Tool

The clinical appreciation of the presence and severity of bleeding symptoms is a fundamental step in the evaluation of patients referred for a possible bleeding disorder. In an attempt to improve the collection and reproducibility of the bleeding history, several Bleeding Assessment Tools (BAT) have been proposed and used. Currently available BAT have some limitations, particularly regarding the lack of paediatric-specific symptoms in some of them and the predominance of the severity of bleeding symptoms over other potentially clinically important features, such as the frequency of symptoms.

To overcome the above-mentioned limitations and to promote the standardization of the available BATs, a Working Group was established within the framework of the ISTH/SSC Subcommittees on VWF and on Perinatal/Paediatric Haemostasis (ISTH/SSC-BAT) during the 53rd SSC Annual Meeting held in Geneva in 2007. Members of the group first met in Toronto on January 2008 and then regularly at each subsequent SSC meeting. This paper presents a structured questionnaire and its clinical use agreed on by the ISTH/SSC-BAT together with a proposal for a new BS system to undergo validity and reliability testing in future studies. This new BAT is intended for inherited bleeding disorders in children and adults. The questionnaire should be collected by a physician or another adequately trained health-professional. Only symptoms and related treatments, if any, before and/or at diagnosis should be reported. Refer to the full text for additional instructions.

Minimal criteria defining a significant bleeding

For each specific bleeding symptom, the ISTH/SSC joint working group proposed minimal criteria in order to classify a symptom as significant and thus receive a score of 1 or more (see also Table 1):

1. Epistaxis: Any nosebleed, especially occurring after puberty that causes patient concern (e.g., interference or distress with daily or social activities) is considered significant. In general, epistaxis should not be considered significant when it lasts less than 10 minutes, has a frequency of < 5 episodes/year, has a seasonal occurrence, or is associated with infections of the upper respiratory tract or other identifiable cause (e.g., dusty dry air).

2. Cutaneous bleeding: Bruises are considered significant when 5 or more (> 1cm) in exposed areas; petechiae when adequately described by the patient or relatives; or hematomas when occurring without trauma.

3. Minor cutaneous wound: Any bleeding episode caused by superficial cuts (e.g., by shaving razor, knife, or scissors) or that requires frequent bandage changes is considered significant. Insignificant bleeding from wounds includes those of duration < 10 minutes and lesions that usually require stitches in normal subjects (e.g., under the chin). Symptoms should also be manifest on more than one occasion to be considered significant.

4. Oral cavity bleeding: Gum bleeding should be considered significant when it causes frankly bloody sputum and lasts for 10 minutes or longer on more than one occasion. Tooth eruption or spontaneous tooth loss bleeding should be considered significant when it requires assistance or supervision by a physician, or lasts at least 10 minutes (bleeding associated with tooth extraction is considered

separately). Bleeding occurring after bites to lips, cheek, and tongue should be considered significant when it lasts at least 10 minutes or causes a swollen tongue or mouth.

5. Hematemesis, melena, and haematochezia: Any gastrointestinal bleeding that is not explained by the presence of a specific disease should be considered significant.

6. Haematuria: Only macroscopic haematuria (from red to pale-pink urine) that is not explained by the presence of a specific urologic disease should be considered significant.

7. Tooth extraction: Any bleeding occurring after leaving the dentist's office and requiring a new, unscheduled visit or prolonged bleeding at the dentist's office causing a delay in the procedure or discharge should be considered significant.

8. Surgical bleeding: Any bleeding judged by the surgeon to be abnormally prolonged, that causes a delay in discharge, or requires some supportive treatment is considered significant.

9. Menorrhagia: Any bleeding that interferes with daily activities such as work, housework, exercise or social activities during most menstrual periods should be considered significant. Criteria for significant bleeding may include any of the following: changing pads more frequently than every 2 hours; menstrual bleeding lasting 7 or more days; and the presence of clots > 1 cm combined with a history of flooding. If a patient has previously made a record of her menstrual loss using a pictorial blood loss assessment chart (PBAC), a PBAC score higher than 100 also qualifies for a score of 1.

10. Post-partum bleeding. Vaginal bleeding or uterine discharge (lochia) that lasts for more than 6 weeks. Any bleeding of lesser duration that is judged by the obstetrician as abnormally heavy or prolonged, that causes a delay in discharge, requires some supportive treatment, requires changing pads or tampons more frequently than every 2 hours, or causes progressive anaemia is also considered significant.

11. Muscle hematomas or haemarthrosis. Any spontaneous joint / muscle bleeding (not related to traumatic injuries) is considered significant.

12. CNS bleeding. Any subdural or intracerebral haemorrhage requiring diagnostic or therapeutic intervention is scored 3 or 4, respectively.

13. Other bleeding symptoms. When these bleeding symptoms occur during infancy, they are scored 1 or more. Their presence when reported by either the patient or a family member should always prompt detailed laboratory investigation.

Only symptoms and treatment BEFORE and AT diagnosis should be considered

1.	**Epistaxis**

1.1	Have you ever had spontaneous epistaxis?	□ Yes	□ No or trivial (skip to 2)
1.2	Have the symptom ever required medical attention?	□ Yes	□ No (resolve spontaneously; skip to 1.6)
1.3	If answer to 1.2 is yes, please specify	□ Consultation only □ Cauterization/ Packing □ Treatment with desmopressin / antifibrinolytics / iron therapy □ Treatment with plasma, platelet or factor concentrates □ Blood (RBC) transfusion	
1.4	How many times in your life did you receive any of the above treatments (# 1.3)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
1.5	At what age did you first have symptoms?	□ Before 1 year □ Between 1-5 years of age □ Between 6-12 years of age □ Between 13-25 years of age □ After 25 years of age	
1.6	Approximate number of episodes NOT requiring medical attention	□ less than 1 per year □ 1 per year □ 1-5 every six month □ 1-3 every month □ 1 every week	
1.7	Duration of average single episode (min.) NOT requiring medical attention	□ 1 minute or less □ 1 - 10 minutes □ more than 10 minutes	

2.	**Cutaneous bleeding (Bruising, ecchymoses, purpura, subcutanueos hematomas)**		
2.1	Have you ever had any of the above cutaneous bleeding?	□ Yes	□ No or trivial skip to 3
2.2	Have the symptom ever required medical attention?	□ Yes	No □ skip to 2.6
2.3	If answer to 2.2 is yes, please specify	□ Consultation only □ Treatment with desmopressin □ Treatment with plasma, platelets or factor concentrates □ Blood (RBC) transfusion	
2.4	How many times in your life did you receive any of the above treatments (# 2.3)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
2.5	At what age did you first have symptoms?	□ Before 1 year □ Between 1-5 years of age □ Between 6-12 years of age □ Between 13-25 years of age □ After 25 years of age	
2.6	Approximate number of episodes NOT requiring medical attention	□ less than 1 per year □ 1 per year □ 1-5 every six month □ 1-3 every month □ 1 every week	
2.7	Type of bleeding	□ Petechiae □ Bruises □ Hematomas	
2.8	Location	□ Exposed sites □ Unexposed sites □ Both	
2.9	Common size	□ ≤ 1 cm □ >1 cm □ Extensive (palm sized or larger)	
2.10	How many bruises >1 cm in exposed areas in the most severe manifestation?	□ ≤ 5 □ > 5	
2.11	Location of petechiae	□ Limited to lower limbs □ Diffuse	

3.	**Bleeding from minor wounds (not requiring stitches in the average patient)**

3.1	Have you ever had prolonged bleeding from minor wounds?	□ Yes	□ No or trivial skip to 4
3.2	Have the symptom ever required medical attention?	□ Yes	□ No skip to 3.6
3.3	If answer to 3.2 is yes, please specify	□ Consultation only □ Surgical hemostasis □ Treatment with desmopressin □ Treatment with plasma, platelet or factor concentrates □ Blood (RBC) transfusion	
3.4	How many times in your life did you received any of the above treatments (# 3.3)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
3.5	At what age did you first have symptoms?	□ Before 1 year □ Between 1-5 years of age □ Between 6-12 years of age □ Between 13-25 years of age □ After 25 years of age	
3.6	Approximate number of episodes NOT requiring medical attention	□ less than 1 per year □ 1 per year □ 1-5 every six month □ 1-3 every month □ 1 every week	
3.7	Duration of average single episode (min.)	□ 1 to 10 minutes □ more than 10 minutes	

4.	**Hematuria**

4.1	Have you ever had hematuria?	□ Yes	□ No skip to 5
4.2	If answer to 4.1 is yes, please specify		
	Presence of associated urologic disease	Yes □ (skip to 5) Specify: □ Stones □ Infection □ Kidney/ bladder disease	No □

Please answer the following questions only for SPONTANEOUS symptoms (answer No to 4.1)

4.3	Have the symptom ever required medical attention?	Yes □	No □ skip to 4.7
4.4	If answer to 4.3 is yes, please specify	□ Consultation only □ Surgery □ Treatment with desmopressin □ Treatment with plasma, platelet or factor concentrates □ Blood (RBC) transfusion	
4.5	How many times in your life did you received any of the above treatments (# 4.4)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
4.6	At what age did you first have symptoms?	□ Before 1 year □ Between 1-5 years of age □ Between 6-12 years of age □ Between 13-25 years of age □ After 25 years of age	
4.7	Approximate number of episodes NOT requiring medical attention	□ less than 1 per year □ 1 per year □ 1-5 every six month □ 1-3 every month □ 1 every week	

5.	**Gastrointestinal bleeding** (Hematemesis, Melena, Hematochezia)

5.1	Have you ever had gastrointestinal bleeding ?	□ Yes	□ No skip to 6
5.2	If answer to 5.1 is yes, please specify		
	Type of bleeding	□ Hematemesis □ Melena □ Hematochezia	
	Presence of associated GI disease	Yes □ Specify: □ Ulcer □ Portal hypertension □ Angiodysplasia	No □

Please answer to the following questions only for SPONTANEOUS symptoms

5.3	Have the symptom ever required medical attention?	Yes □	No □ skip to 5.7
5.4	If answer to 5.3 is yes, please specify	□ Consultation only □ Surgical haemostasis □ Treatment with desmopressin □ Treatment with plasma, platelet or factor concentrates □ Blood (RBC) transfusion	
5.5	How many times in your life did you received any of the above treatments (# 5.4)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
5.6	At what age did you first have symptoms?	□ Before 1 year □ Between 1-5 years of age □ Between 6-12 years of age □ Between 13-25 years of age □ After 25 years of age	
5.7	Approximate number of episodes NOT requiring medical attention	□ less than 1 per year □ 1 per year □ 1-5 every six month □ 1-3 every month □ 1 every week	

6. Oral cavity bleeding (Tooth eruption, spontaneous or after brushing/flossing, gum bleeding, bleeding after bites to lip & tongue)

6.1	Have you ever had oral cavity bleeding?	□ Yes	□ No or trivial skip to 7
6.2	Have the symptom ever required medical attention?	Yes □	No □ skip to 6.6
6.3	If answer to 6.2 is yes, please specify	□ Consultation only □ Surgery (dental packing, suture, cauterization) □ Treatment with desmopressin / iron therapy □ Treatment with plasma, platelet or factor concentrates □ Blood (RBC) transfusion	
6.4	How many times in your life did you received any of the above treatments (# 6.3)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
6.5	At what age did you first have symptoms?	□ Before 1 year □ Between 1-5 years of age □ Between 6-12 years of age □ Between 13-25 years of age □ After 25 years of age	
6.6	Approximate number of episodes NOT requiring medical attention	□ less than 1 per year □ 1 per year □ 1-5 every six month □ 1-3 every month □ 1 every week	
6.7	Duration of average single episode (min.)	□ 1 to 10 minutes □ more than 10 minutes	

7. Bleeding after Tooth/ Teeth extraction

7.1	Have you ever had bleeding after tooth (teeth) extraction?	□ Yes	□ No skip to 8
7.2	If answer to 7.1 is yes, please specify		
	Number of extractions	□□	

*Please fill in one of the following forms for **each** tooth extraction*

Age at extraction	□□	Type of extraction	□ Deciduous □ Permanent □ Molar
Actions taken to prevent bleeding		□ None □ Antifibrinolytics □ Desmopressin □ Plasma or clotting factor concentrates □ Platelet infusion	
Bleeding after extraction?		Yes □	No □
Actions taken to control bleeding		□ Resuturing □ Packing □ Antifibrinolytics □ Desmopressin □ Plasma or clotting factor concentrates □ Platelet infusion □ Blood (RBC) transfusion	

8. Bleeding after Surgery or Major Trauma

8.1 Have you ever had bleeding after surgery or major trauma? ☐ Yes ☐ No, skip to 9

8.2 If answer to 8.1 is yes, please specify

Number of interventions ☐☐

*Please fill in one of the following forms for **each** surgery or major trauma episode*

Age at intervention/trauma ☐☐

Type of surgery

☐ Tonsillectomy/Adenoids
☐ Pharynx/Nose
☐ Major-abdominal
☐ Major-thoracic
☐ Major-gynecology
☐ Other

Actions taken to prevent bleeding

☐ None
☐ Antifibrinolytics
☐ Desmopressin
☐ Plasma or clotting factor concentrates
☐ Platelet infusion

Bleeding after intervention? Yes ☐ No ☐

Actions taken to control bleeding

☐ None
☐ Resuturing
☐ Packing
☐ Antifibrinolytics
☐ Desmopressin
☐ Plasma or clotting factor concentrates
☐ Platelet infusion
☐ Blood (RBC) transfusion

9.	**Menorrhagia**

9.1	Have you ever had very heavy menstrual bleeding (menorrhagia)?	□ Yes	□ No or trivial skip to 10
	If answer to 9.1 is yes, please specify	□ Changing pads/tampons more frequently than every 2 hours □ Bleeding more than 7 days □ Clot and flooding	
		Impairment of daily activities (work, housework, exercise, social activities):	□ Never or rarely □ Most menses
9.2	Have the symptom ever required medical attention?	□ Yes	□ No skip to 9.6
9.3	If answer to 9.2 is yes, please specify	a □ Consultation only	
		b □ Pictorial Bleeding Assessment	Score _____
		c □ Antifibrinolytic therapy	
		d □ Iron therapy	
		e □ Hormonal therapy	
		f □ Combined antifibrinolytics & Hormonal therapy	
		g □ Hysterectomy / endometrial ablation / D & C	
		h □ Treatment with desmopressin, plasma or factor concentrates, platelet transfusion	
		i □ Blood (RBC) transfusion	
		l □ Hospital admission and emergency treatment	
9.4	How many times in your life did you received any of the above treatments (# 9.3 a-l)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
9.5	At what age did you first have symptoms?	□ At menarche □ Between 14-25 years of age □ After 25 years of age	
9.6	Have you had time off work/school for menorrhagia?	□ < twice a year □ > twice a year	
9.7	Duration of menorrhagia	□ Since menarche □ > 12 months □ < 12 months	
9.8	Have you had acute menorrhagia requiring emergency treatment/hospital admission	□ Yes How many times: ____	□ No

10.	**Post-partum hemorrhage**

10.1	Number of successful pregnancies (live births)	□□	
10.2	Have you ever had post-partum haemorrhage?	□ Yes	□ No or trivial skip to 11
10.3	Did it occur	□ In the first 24 hours after delivery (Primary) □ Between 24 hours and 6 weeks postpartum (Secondary) □ Both Primary and Secondary	
10.4	How long did vaginal discharge (lochia) last?	□ < 6 weeks □ > 6 weeks	
10.5	Did it require changing pads/tampons more frequently than every 2 hours?	□ Yes	□ No
10.6	Did this bleeding cause delay of hospital discharge/ readmission to hospital?	□ Yes	□ No
10.7	Have the symptom ever required medical treatment?	□ Yes	□ No
10.8	If answer to 10.7 is yes, please specify	□ Consultation only /oxytocin i.v. infusion □ Additional uterotonic medications □ Iron therapy □ Antifibrinolytic therapy □ Treatment with plasma or factor concentrates, platelet transfusion □ Blood (RBC) transfusion □ Any procedure requiring examination under anaesthesia □ Uterine balloon/package to tamponade the uterus □ Any procedure requiring critical care or surgical intervention (includes: hysterectomy, internal iliac artery legation, uterine artery embolization, uterine brace sutures)	
10.9	Number of deliveries that required any of the above treatments (# 10.8)?	□□	

11.	**Muscle hematomas or hemarthrosis (spontaneous)**		
11.1	Have you ever had muscle hematomas or hemarthrosis?	□ Yes	□ No or trivial skip to 12
11.2	Have the symptom ever required medical attention?	□ Yes	□ No skip to 11.6
11.3	If answer to 11.2 is yes, please specify	□ Consultation only □ Surgical draining □ Treatment with desmopressin □ Treatment with plasma, platelet or factor concentrates □ Blood transfusion	
11.4	How many times in your life did you receive any of the above treatments (# 11.3)?	□ 1 - 2 □ 3 to 5 □ 6 to 10 □ more than 10	
11.5	At what age did you first have symptoms?	□ Before 1 year □ Between 1-5 years of age □ Between 6-12 years of age □ Between 13-25 years of age □ After 25 years of age	
11.6	Approximate number of episodes NOT requiring medical attention	□ less than 1 per year □ 1 per year □ 1-5 every six month □ 1-3 every month □ 1 every week	

12	Other bleedings

12.1 Have you ever had one of the following?

Excessive umbilical stump bleeding	□ Yes	□ No
Cephalohematoma	□ Yes	□ No
Bleeding at circumcision	□ Yes	□ No
Venipuncture bleeding	□ Yes	□ No
Suction Bleeding	□ Yes	□ No
Ovulation bleeding(in women)	□ Yes	□ No

12.2 Have one of these symptoms ever required medical attention? □ Yes □ No

12.3 If answer to 12.2 is yes, please specify

- □ Consultation only
- □ Surgery
- □ Treatment with desmopressin
- □ Treatment with plasma, platelet or factor concentrates
- □ Blood (RBC) transfusion

12.4 How many times in your life did you receive any of the above treatments (# 12.3) for this symptom?

- □ 1 - 2
- □ 3 to 5
- □ 6 to 10
- □ more than 10

Table 1. Bleeding score

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

GI bleeding	No/trivial	Present (not associated with ulcer, portal hypertension, hemorrhoids, angiodysplasia)	Consultation only*	Surgical hemostasis, antifibrinolytic	Blood transfusion, replacement therapy or desmopressin
Hematuria	No/trivial	Present (macroscopic)	**Consultation only***	Surgical hemostasis, iron therapy	Blood transfusion, replacement therapy or desmopressin
Tooth extraction	No/trivial or none done	Reported in ≤25% of all procedures, no intervention**	**Reported in >25% of all procedures, no intervention****	Resuturing or packing	Blood transfusion, replacement therapy or desmopressin
Surgery	No/trivial or none done	Reported in ≤25% of all procedures, no intervention**	**Reported in >25% of all procedures, no intervention****	Surgical hemostasis or antifibrinolytic	Blood transfusion, replacement therapy or desmopressin
Menorrhagia	No/trivial	Consultation only* or - Changing pads	**- Time off work/school >**	- Requiring combined treatment with antifibrinolytics and hormonal	- Acute menorrhagia requiring hospital admission and emergency treatment

		more frequently than every 2 hours or - Clot and flooding or - PBAC score>100[#]	**2/year or - Requiring antifibrinolytics or hormonal or iron therapy**	therapy or - Present since menarche and > 12 months	or - Requiring blood transfusion, Replacement therapy, Desmopressin, or - Requiring dilatation & curretage or endometrial ablation or hysterectomy)
Post-partum hemorrhage	No/trivial or no deliveries	Consultation only* or - Use of syntocin or - Lochia > 6 weeks	- Iron therapy or - Antifibrinolytics	- Requiring blood transfusion, replacement therapy, desmopressin or - Requiring examination under anaesthesia and/or the use of uterin balloon/package to tamponade the uterus	- Any procedure requiring critical care or surgical intervention (e.g. hysterectomy, internal iliac artery legation, uterine artery embolization, uterine brace sutures)
Muscle hematomas	Never	Post trauma, no therapy	**Spontaneous, no therapy**	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring surgical intervention or blood transfusion
Hemarthrosis	Never	Post trauma, no therapy	**Spontaneous, no therapy**	Spontaneous or traumatic, requiring desmopressin or replacement therapy	Spontaneous or traumatic, requiring surgical intervention or blood transfusion

CNS bleeding	Never	-	-	Subdural, any intervention	Intracerebral, any intervention
Other bleedings^	No/trivial	Present	**Consultation only***	Surgical hemostasis, antifibrinolytics	Blood transfusion or replacement therapy or desmopressin

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

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

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

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

If already available at the time of collection

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

Figure B. Consent form for recruitment to the GAPP study.

UNIVERSITY OF BIRMINGHAM

Birmingham Platelet Group

INFORMED CONSENT FOR 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, ☎ 0121 415 8680. 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 leukaemia.

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

Table A. List of genes included in the GAPP study gene panel utilised during the bioinformatic screen.

A2M	Alpha-2-macroglobulin
ABCA12	ATP Binding Cassette Subfamily A Member 12
ABCB4	ATP Binding Cassette Subfamily B Member 4
ABCC4	ATP Binding Cassette Subfamily C Member 4
ABCG5	ATP Binding Cassette Subfamily G Member 5
ABCG8	ATP Binding Cassette Subfamily G Member
ABI1	Abl Interactor 1
ACE	Angiotensin I Converting Enzyme
ACP2	Acid Phosphatase 2, Lysosomal
ACSL4	Acyl-CoA Synthetase Long-Chain Family Member 4
ACTA1	Actin, Alpha 1, Skeletal Muscle
ACTN1	Actinin Alpha 1
ACTR3	ARP3 Actin-Related Protein 3 Homolog
ACVRL1	Activin A Receptor Like Type 1
ADAM15	ADAM Metallopeptidase Domain 15
ADAM17	ADAM Metallopeptidase Domain 17
ADAMTS13	ADAM Metallopeptidase With Thrombospondin Type 1 Motif 13
ADCY3	Adenylate Cyclase 3
ADCY6	Adenylate Cyclase 6
ADCY7	Adenylate Cyclase 7
ADORA2B	Adenosine A2b Receptor
ADRA2A	Adrenoceptor Alpha 2A
ADRA2B	Adrenoceptor Alpha 2B
ADRBK1	Adrenergic, Beta, Receptor Kinase 1
ADSS	Adenylosuccinate Synthase
AGT	Angiotensinogen
AGTR1	Angiotensin II Receptor Type 1
AK3	Adenylate Kinase 3
AKT1	Protein Kinase B Alpha
AKT2	Protein Kinase B Beta
ALDH1A1	Aldehyde Dehydrogenase 1 Family Member A1
ALOX12	Arachidonate 12-Lipoxygenase
ALOX5	Arachidonate 5-Lipoxygenase
ANGPT2	Angiopoietin 2
ANKRD12	Ankyrin Repeat Domain 12
ANKRD18A	Ankyrin Repeat Domain 18A
ANKRD18B	Ankyrin Repeat Domain 18B
ANKRD26	Ankyrin Repeat Domain 26
ANKRD33	Ankyrin Repeat Domain 33
ANTXR2	Anthrax Toxin Receptor 2
ANXA2	Annexin A2
ANXA5	Annexin A5
AP2B1	Adaptor Related Protein Complex 2 Beta 1 Subunit
AP3B1	Adaptor Related Protein Complex 3 Beta 1 Subunit
AP3D1	Adaptor Related Protein Complex 3 Delta 1 Subunit
AP3M1	Adaptor Related Protein Complex 3 Mu 1 Subunit
AP3S1	Adaptor Related Protein Complex 3 Sigma 1 Subunit
APC	Adenomatous Polyposis Coli
APLN	Apelin
APLP2	Amyloid Beta Precursor Like Protein 2
ARAF	A-Raf Proto-Oncogene, Serine/Threonine Kinase
ARHGAP1	Rho GTPase Activating Protein 1
ARHGAP17	Rho GTPase Activating Protein 17
ARHGAP18	Rho GTPase Activating Protein 18
ARHGAP24	Rho GTPase Activating Protein 24
ARHGAP32	Rho GTPase Activating Protein 32
ARHGAP6	Rho GTPase Activating Protein 6
ARHGDIA	Rho GDP Dissociation Inhibitor Alpha
ARHGDIB	Rho GDP Dissociation Inhibitor Beta
ARHGEF12	Rho Guanine Nucleotide Exchange Factor 12
ARHGEF15	Rho Guanine Nucleotide Exchange Factor 15
ARHGEF3	Rho Guanine Nucleotide Exchange Factor 3
ARID1A	AT-Rich Interaction Domain 1A
ARRB1	Arrestin Beta 1

ART4	ADP-Ribosyltransferase 4 (Dombrock Blood Group)
ASPN	Asporin
ATP5H	ATP Synthase, H+ Transporting, Mitochondrial Fo Complex Subunit D
AZIN1	Antizyme Inhibitor 1
BAG3	BCL2 Associated Athanogene 3
BAK1	BCL2 Antagonist/Killer 1
BCL2	B-Cell CLL/Lymphoma 2
BCL2L1	BCL2 Like 1
BCOR	BCL6 Corepressor
BET1L	Bet1 Golgi Vesicular Membrane Trafficking Protein Like
BGN	Biglycan
BIVM	Basic, Immunoglobulin-Like Variable Motif Containing
BLOC1S1	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 1
BLOC1S2	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 2
BLOC1S3	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 3
BLOC1S4	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 4
BLOC1S5	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 5
BLOC1S6	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 6
BMP4	Bone Morphogenetic Protein 4
BMP6	Bone Morphogenetic Protein 6
BMX	Non-Receptor Tyrosine Kinase
BRD2	Bromodomain Containing 2
BTBD9	BTB Domain Containing 9
BTK	Bruton Tyrosine Kinase
C10orf10	Chromosome 10 Open Reading Frame 10
C12orf11	Asunder, Spermatogenesis Regulator
C14orf133	VPS33B Interacting Protein, Apical-Basolateral Polarity Regulator, Spe-39 Homolog
C14orf78	AHNAK Nucleoprotein 2
C16orf30	Transmembrane Protein 204
C16orf63	FGFR1OP N-Terminal Like
C19orf55	Proline And Serine Rich 3
C20orf42	Fermitin Family Member 1
C4orf18	Family With Sequence Similarity 198 Member B
C6orf25	Immunoglobulin Receptor
C7	Complement Component 7
C9orf26	Interleukin 33
CALCRL	Calcitonin Receptor Like Receptor
CALU	Calumenin
CAP1	Adenylate Cyclase-Associated Protein 1
CASP7	Caspase 7
CAV1	Caveolin 1
CBARA1	Mitochondrial Calcium Uptake 1
CCDC99	Spindle Apparatus Coiled-Coil Protein 1
CCL2	C-C Motif Chemokine Ligand 2
CCL20	C-C Motif Chemokine Ligand 20
CCL5	C-C Motif Chemokine Ligand 5
CD226	Platelet And T Cell Activation Antigen 1
CD34	CD34 Molecule
CD36	CD36 Antigen (Collagen Type I Receptor, Thrombospondin Receptor)
CD55	CD55 Molecule (Cromer Blood Group)
CD93	Complement Component 1, Q Subcomponent, Receptor 1
CDC42	G25K GTP-Binding Protein
CDCA8	Cell Division Cycle Associated 8
CDH5	Cadherin 5
CEP135	Centrosomal Protein 135
CHD3	Chromodomain Helicase DNA Binding Protein 3
CLEC1B	C-Type Lectin Domain Family 1 Member B
CLEC4F	C-Type Lectin Domain Family 4 Member F
CLIC4	Chloride Intracellular Channel 4
CNN2	Calponin 2
CNO	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 4
CNP	2',3'-Cyclic Nucleotide 3' Phosphodiesterase
COL18A1	Collagen Type XVIII Alpha 1
COL3A1	Collagen Type III Alpha 1
COPG	Coatomer Protein Complex Subunit Gamma 1
CPB2	Carboxypeptidase B2
CAPRIN1	Cell Cycle Associated Protein 1
CS	Citrate Synthase

CSF2	Colony Stimulating Factor 2
CSF5	Colony Stimulating Factor 5
CSK	C-Src Tyrosine Kinase
CSTA	Cystatin A
CTGF	Connective Tissue Growth Factor
CTTN	Cortactin
CUL4B	Cullin 4B
CX3CL1	C-X3-C Motif Chemokine Ligand 1
CXCR5	C-X-C Motif Chemokine Receptor 5
CYCS	Cytochrome C, Somatic
CYR61	Cysteine Rich Angiogenic Inducer 61
DAAM1	Dishevelled Associated Activator Of Morphogenesis 1
DAB2	Clathrin Adaptor Protein
DAP	Death-Associated Protein
DCTN1	Dynactin Subunit 1
DDR2	Discoidin Domain Receptor Tyrosine Kinase 2
DDX5	DEAD (Asp-Glu-Ala-Asp) Box Helicase 5
DIAPH1	Diaphanous Related Formin 1
DIAPH2	Diaphanous Related Formin 2
DIAPH3	Diaphanous Related Formin 3
DLC1	Rho GTPase Activating Protein
DNAH11	Dynein Axonemal Heavy Chain 11
DNAJC10	DnaJ Heat Shock Protein Family (Hsp40) Member C10
DNM1L	Dynamin 1-Like
DNM2	Dynamin 2
DNM3	Dynamin 3
DRCTNNB1A	Family With Sequence Similarity 126 Member A
DTNBP1	Dystrobrevin Binding Protein 1
DUSP6	Dual Specificity Phosphatase 6
DYNC1I2	Dynein Cytoplasmic 1 Intermediate Chain 2
DYNLL1	Dynein Light Chain LC8-Type 1
DYSF	Dysferlin
ECE1	Endothelin Converting Enzyme 1
ECOP	Putative NF-Kappa-B-Activating Protein 055N
ECSM1	endothelial cell specific molecule 1
ECSM2	endothelial cell specific molecule 2
EDG1	Endothelial Differentiation, Sphingolipid G-Protein-Coupled Receptor
EDN1	Endothelin 1
EDN2	Endothelin 2
EDNRA	Endothelin Receptor Type A
EFEMP1	EGF Containing Fibulin-Like Extracellular Matrix Protein 1
EFNB1	Ephrin B1
EFTUD2	Elongation Factor Tu GTP Binding Domain Containing 2
EGLN2	Egl-9 Family Hypoxia-Inducible Factor 2
EHD2	EH Domain Containing 2
EIF2S3	Eukaryotic Translation Initiation Factor 2 Subunit Gamma
EIF4G2	Eukaryotic Translation Initiation Factor 4 Gamma 2
ELK3	ETS Transcription Factor
ELOVL5	Fatty Acid Elongase 5
ELTD1	Adhesion G Protein-Coupled Receptor L4
EMCN	Endomucin
EMP1	Epithelial Membrane Protein 1
ENC1	Ectodermal-Neural Cortex 1
ENG	Endoglin
ENPP2	Ectonucleotide Pyrophosphatase/Phosphodiesterase 2
EPHA4	Tyrosine-Protein Kinase Receptor SEK
EPHB1	Tyrosine-Protein Kinase Receptor EPH-2
ERG	V-Ets Avian Erythroblastosis Virus E26 Oncogene Homolog
ESM1	Endothelial Cell Specific Molecule 1
ETS1	ETS Proto-Oncogene 1, Transcription Factor
ETV6	ETS Variant 6
EVI1	AML1-EVI-1 Fusion Protein
EWSR1	Ewing Sarcoma Breakpoint Region 1
EXOC1	Exocyst Complex Component 1
EXOC6	Exocyst Complex Component 6
EXOSC10	Exosome Component 10
F10	Coagulation Factor X
F11	Coagulation Factor XI

F13A1	Coagulation Factor XIII A Chain
F13B	Coagulation Factor XIII B Chain
F2	Coagulation Factor II, Thrombin
F2R	Coagulation Factor II Thrombin Receptor
F2RL3	F2R Like Thrombin/Trypsin Receptor 3
F5	Coagulation Factor V
F7	Coagulation Factor VII
F8	Coagulation Factor VIII
F9	Coagulation Factor IX
FABP4	Fatty Acid Binding Protein 4
FAM43A	Family With Sequence Similarity 43 Member A
FAM62A	Extended Synaptotagmin Protein 1
FAM8A1	Family With Sequence Similarity 8 Member A1
FARP2	Pleckstrin Homology Domain-Containing Family C Member 3
FASLG	Fas Ligand
FAU	Finkel-Biskis-Reilly Murine Sarcoma Virus (FBR-MuSV) Ubiquitously Expressed
FBXL3	F-Box And Leucine-Rich Repeat Protein 3
FCER1G	Fc Fragment Of IgE Receptor Ig
FCGR2A	Fc Fragment Of IgG Receptor IIa
FERMT3	Fermitin Family Member 3
FES	FES Proto-Oncogene, Tyrosine Kinase
FGA	Fibrinogen Alpha Chain
FGB	Fibrinogen Beta Chain
FGD3	FYVE, RhoGEF And PH Domain Containing 3
FGD5	FYVE, RhoGEF And PH Domain Containing 5
FGF1	Fibroblast Growth Factor 1
FGG	Fibrinogen Gamma Chain
FGR	Proto-Oncogene, Src Family Tyrosine Kinase
FHOD1	Formin Homology 2 Domain Containing 1
FLI1	Fli-1 Proto-Oncogene, ETS Transcription Factor
FLII	Flightless I Actin Binding Protein
FLJ10815	Solute Carrier Family 38 Member 8
FLJ22746	Family With Sequence Similarity 124 Member B
FLJ39531	Chromosome 15 Open Reading Frame 54
FLNA	Filamin A
FLNB	Filamin B
FLT1	Fms Related Tyrosine Kinase 1
FMNL1	Formin Like 1
FMNL2	Formin Like 2
FMNL3	Formin Like 3
FN1	Fibronectin 1
FNBP1L	Formin Binding Protein 1 Like
FNTB	Farnesyltransferase, CAAX Box, Beta
FSTL1	Follistatin Like 1
FTH1	Cell Proliferation-Inducing Gene 15 Protein
FYN	FYN Proto-Oncogene, Src Family Tyrosine Kinase
FZD4	Frizzled Class Receptor 4
G3BP	GTPase Activating Protein (SH3 Domain) Binding Protein 1
GANAB	Glucosidase II Alpha Subunit
GATA1	GATA Binding Protein 1
GBE1	Glucan (1,4-Alpha-), Branching Enzyme 1
GBP4	Guanylate Binding Protein 4
GDI2	GDP Dissociation Inhibitor 2
GFI1	Growth Factor Independent 1 Transcription Repressor
GFI1B	Growth Factor Independent 1B Transcription Repressor
GGCX	Gamma-Glutamyl Carboxylase
GIMAP6	GTPase, IMAP Family Member 6
GJA1	Gap Junction Protein Alpha 1
GNA12	G Protein Subunit Alpha 12
GNA13	G Protein Subunit Alpha 13
GNAI1	G Protein Subunit Alpha I1
GNAI2	G Protein Subunit Alpha I2
GNAI3	G Protein Subunit Alpha I3
GNAQ	G Protein Subunit Alpha Q
GNAS	Guanine Nucleotide Binding Protein (G Protein), Alpha Stimulating Activity Polypeptide 1
GNAZ	G Protein Subunit Alpha Z
GNB2	G Protein Subunit Beta 2
GNB3	G Protein Subunit Beta 3

GNE	Glucosamine (UDP-N-Acetyl)-2-Epimerase/N-Acetylmannosamine Kinase
GNG11	G Protein Subunit Gamma 11
GNG12	G Protein Subunit Gamma 12
GNG13	G Protein Subunit Gamma 13
GNG5	G Protein Subunit Gamma 5
GP1BA	Glycoprotein Ib Platelet Alpha Subunit
GP1BB	Glycoprotein Ib Platelet Beta Subunit
GP5	Glycoprotein V Platelet
GP6	Glycoprotein VI Platelet
GP9	Glycoprotein IX Platelet
GPI	Glucose-6-Phosphate Isomerase
GPR177	G Protein-Coupled Receptor 177
GPR4	G Protein-Coupled Receptor 4
GPR56	Adhesion G Protein-Coupled Receptor G1
GRAP2	Growth Factor Receptor-Binding Protein
GRB2	Growth Factor Receptor Bound Protein 2
GRK5	G Protein-Coupled Receptor Kinase 5
GRK6	G Protein-Coupled Receptor Kinase 6
GRN	Granulin
GRWD1	Glutamate-Rich WD Repeat Containing 1
GSN	Gelsolin
GSTO1	Glutathione S-Transferase Omega 1
GTF3C4	General Transcription Factor IIIC Subunit 4
GTF3C5	General Transcription Factor IIIC Subunit 5
GTPBP4	GTP Binding Protein 4
GUCY1A3	Guanylate Cyclase 1, Soluble, Alpha 3
GUCY1B3	Guanylate Cyclase 1, Soluble, Beta 3
HBB	Hemoglobin Subunit Beta
HDAC6	Histone Deacetylase 6
HDLBP	High Density Lipoprotein Binding Protein
HEL308	Helicase, POLQ-Like
HEXB	Hexosaminidase Subunit Beta
HEY1	Hes Related Family BHLH Transcription Factor With YRPW Motif 1
HHIP	Hedgehog Interacting Protein
HNRPH1	Heterogeneous Nuclear Ribonucleoprotein H1 (H)
HOOK3	Hook Microtubule-Tethering Protein 3
HOXA11	Homeobox A11
HPS1	Hermansky-Pudlak Syndrome 1
HPS3	Hermansky-Pudlak Syndrome 3
HPS4	Hermansky-Pudlak Syndrome 4
HPS5	Hermansky-Pudlak Syndrome 5
HPS6	Hermansky-Pudlak Syndrome 6
HRG	Histidine Rich Glycoprotein
HSPA8	Lipopolysaccharide-Associated Protein 1
HSPD1	Heat Shock Protein Family D (Hsp60) Member 1
HTR2A	5-Hydroxytryptamine Receptor 2A
HYOU1	Hypoxia Up-Regulated 1
ICAM1	Intercellular Adhesion Molecule 1
ICAM2	Intercellular Adhesion Molecule 2
IFI16	Interferon Gamma Inducible Protein 16
IFI27	Interferon Alpha Inducible Protein 27
IFNB1	Interferon Beta 1
IKBKE	Inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cells, Kinase Epsilon
IL11	Interleukin 11
IL1B	Interleukin 1 Beta
IL1RL1	Interleukin 1 Receptor Like 1
IL3	Interleukin 3
IL6	Interleukin 6
IL7	Interleukin 7
INPP5D	Inositol Polyphosphate-5-Phosphatase D
INTS3	Integrator Complex Subunit 3
ITGA2	Integrin Subunit Alpha 2
ITGA2B	Integrin Subunit Alpha 2b
ITGA5	Integrin Subunit Alpha 5
ITGAV	Integrin Subunit Alpha V
ITGB1	Integrin Subunit Beta 1
ITGB3	Integrin Subunit Beta 3
ITPR1	nositol 1,4,5-Trisphosphate Receptor Type 1

JAK2	Janus Kinase 2
JAM2	Junctional Adhesion Molecule 2
JMJD1C	Jumonji Domain Containing 1C
KCTD15	Potassium Channel Tetramerization Domain Containing 15
KDELC1	KDEL Motif Containing 1
KIAA0174	Putative MAPK-Activating Protein PM28
KIAA0195	Transmembrane Protein 94
KIAA0652	Autophagy Related 13
KIAA0690	Ribosomal RNA Processing 12 Homolog
KIAA1109	Fragile Site-Associated Protein
KIAA1539	Family With Sequence Similarity 214 Member B
KIAA2018	Upstream Transcription Factor Family Member 3
KLK3	Kallikrein Related Peptidase 3
LAIR1	Leukocyte Associated Immunoglobulin Like Receptor 1
LAMA4	Laminin Subunit Alpha 4
LAT	Linker For Activation Of T-Cells
LCP2	Lymphocyte Cytosolic Protein 2
LDB2	LIM Domain Binding 2
LDLR	Low Density Lipoprotein Receptor
LENG4	Lysophosphatidylinositol Acyltransferase
LGALS1	Lectin, Galactoside Binding Soluble 1
LIFR	Leukemia Inhibitory Factor Receptor Alpha
LMAN1	Lectin, Mannose Binding 1
LMNA	Lamin A/C
LOC645638	
LOC646195	
LOC646999	Akirin 1 Pseudogene
LOC653105	
LOC653352	
LOC653949	
LOX	Lysyl Oxidase
LPAR1	Lysophosphatidic Acid Receptor 1
LRRC41	Leucine Rich Repeat Containing 41
LRRC8C	Leucine-Rich Repeat Containing 8 Family Member C
LTA4H	Leukotriene A4 Hydrolase
LTBP1	Latent Transforming Growth Factor Beta Binding Protein 1
LTBR	Lymphotoxin Beta Receptor
LY6G6F	Lymphocyte Antigen 6 Complex, Locus G6F
LYN	LYN Proto-Oncogene, Src Family Tyrosine Kinase
LYST	LYST
MAN2B1	Mannosidase Alpha Class 2B Member 1
MANSC1	MANSC Domain Containing 1
MAP1LC3B	Microtubule Associated Protein 1 Light Chain 3 Beta
MAP2K2	Mitogen-Activated Protein Kinase Kinase 2
MAP2K3	Mitogen-Activated Protein Kinase Kinase 3
MAP2K4	Mitogen-Activated Protein Kinase Kinase 4
MAP3K8	Mitogen-Activated Protein Kinase Kinase Kinase 8
MAPK1	Mitogen-Activated Protein Kinase 1
MAPK13	Mitogen-Activated Protein Kinase 13
MAPK14	Mitogen-Activated Protein Kinase 14
MAPK8	Mitogen-Activated Protein Kinase 8
MAPRE1	Microtubule Associated Protein RP/EB Family Member 1
MASTL	Microtubule Associated Serine/Threonine Kinase Like
MAT2A	Methionine Adenosyltransferase 2A
MBTPS1	Membrane Bound Transcription Factor Peptidase, Site 1
MCAM	Melanoma Cell Adhesion Molecule
MCFD2	Multiple Coagulation Factor Deficiency 2
MDS1	Myelodysplasia Syndrome-Associated Protein 1
MED28	Mediator Complex Subunit 28
MEF2A	Myocyte Enhancer Factor 2A
METAP2	Methionyl Aminopeptidase 2
MGAT1	Mannosyl (Alpha-1,3-)-Glycoprotein Beta-1,2-N-Acetylglucosaminyltransferase
MKL1	Megakaryoblastic Leukemia (Translocation) 1
MLK1	Mitogen-Activated Protein Kinase Kinase Kinase 9
MLPH	Melanophilin
MMP1	Matrix Metallopeptidase 1
MMP17	Matrix Metallopeptidase 17
MMP2	Matrix Metallopeptidase 2

MMP9	Matrix Metallopeptidase 9
MMRN1	Multimerin 1
MMRN2	Multimerin 2
MMS19L	MMS19 Homolog, Cytosolic Iron-Sulfur Assembly Component
MNX1	Motor Neuron And Pancreas Homeobox 1
MOAP1	Modulator Of Apoptosis 1
MOV10	Moloney Leukemia Virus 10 Protein
MOV10L1	Mov10 RISC Complex RNA Helicase Like 1
MPL	Myeloproliferative Leukemia Protein
MRPS34	Mitochondrial Ribosomal Protein S34
MT2A	Metallothionein 2A
MUC16	Mucin 16, Cell Surface Associated
MUC2	Mucin 2, Oligomeric Mucus/Gel-Forming
MUS81	MUS81 Structure-Specific Endonuclease Subunit
MUTED	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 5
MVP	Major Vault Protein
MYB	Proto-Oncogene, Transcription Factor
MYCT1	Myc Target 1
MYD88	Myeloid Differentiation Primary Response 88
MYH10	Myosin, Heavy Chain 10, Non-Muscle
MYH13	Myosin, Heavy Chain 13, Skeletal Muscle
MYH9	Myosin, Heavy Chain 9, Non-Muscle
MYL9	Myosin Light Chain 9
MYLK	Myosin Light Chain Kinase
MYLK2	Myosin Light Chain Kinase 2
MYO18B	Myosin XVIIIB
MYO1C	Myosin IC
MYO3A	Myosin IIIA
MYO5A	Myosin VA
MYO5B	Myosin VB
NAPA	NSF Attachment Protein Alpha
NAPG	NSF Attachment Protein Gamma
NBEA	Neurobeachin
NBEAL2	Neurobeachin Like 2
NCL	Nucleolin
NDRG1	N-Myc Downstream Regulated 1
NDUFS1	NADH:Ubiquinone Oxidoreductase Core Subunit S1
NFE2	Nuclear Factor, Erythroid 2
NFE2L1	Nuclear Factor, Erythroid 2 Like 1
NIPSNAP3A	Nipsnap Homolog 3A
NOD27	NLR Family, CARD Domain Containing 5
NONO	Non-POU Domain Containing, Octamer-Binding
NOS2	Nitric Oxide Synthase 2
NOS3	Nitric Oxide Synthase 3
NOSTRIN	Nitric Oxide Synthase Trafficking
NOTCH1	Notch 1
NOX1	NADPH Oxidase 1
NPPB	Natriuretic Peptide B
NPR1	Natriuretic Peptide Receptor 1
NQO1	NAD(P)H Quinone Dehydrogenase 1
NRCAM	Neuronal Cell Adhesion Molecule
NRG3	Neuregulin 3
NRP1	Neuropilin 1
NSF	N-Ethylmaleimide Sensitive Factor
NUCB1	Nucleobindin 1
NUMB	Endocytic Adaptor Protein
NUP54	Nucleoporin 54kDa
NUPL1	Nucleoporin 58kDa
NXF1	Nuclear RNA Export Factor 1
OCLN	Occludin
OGDH	Oxoglutarate Dehydrogenase
OGFOD1	2-Oxoglutarate And Iron Dependent Oxygenase Domain Containing 1
ORAI1	ORAI Calcium Release-Activated Calcium Modulator 1
OS9	Osteosarcoma Amplified 9, Endoplasmic Reticulum Lectin
P2RX1	Purinergic Receptor P2X 1
P2RY1	Purinergic Receptor P2Y1
P2RY12	Purinergic Receptor P2Y12
P2RY13	Purinergic Receptor P2Y13

P4HB	Prolyl 4-Hydroxylase Subunit Beta
PALMD	Palmdelphin
PAPSS1	3'-Phosphoadenosine 5'-Phosphosulfate Synthase 1
PARN	Poly(A)-Specific Ribonuclease
PCBP4	Poly(RC) Binding Protein 4
PCDH1	Protocadherin 1
PCDH12	Protocadherin 12
PCSK7	Proprotein Convertase Subtilisin/Kexin Type 7
PDE2A	Phosphodiesterase 2A
PDE3A	Phosphodiesterase 3A
PDE4D	Phosphodiesterase 4D
PDE5A	Phosphodiesterase 5A
PDPK1	3-Phosphoinositide Dependent Protein Kinase 1
PDZD3	PDZ Domain Containing 3
PDZK1	PDZ Domain Containing 1
PEAR1	Platelet Endothelial Aggregation Receptor 1
PECAM1	Platelet And Endothelial Cell Adhesion Molecule 1
PF4	Platelet Factor 4
PGD	Phosphogluconate Dehydrogenase
PGM2	Phosphoglucomutase 2
PGM3	Phosphoglucomutase 3
PHF8	PHD Finger Protein 8
PHOX2A	Paired Like Homeobox 2a
PI4K2B	Phosphatidylinositol 4-Kinase Type 2 Beta
PIGG	Phosphatidylinositol Glycan Anchor Biosynthesis Class G
PIGT	Phosphatidylinositol Glycan Anchor Biosynthesis Class T
PIK3CA	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Alpha
PIK3CB	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Beta
PIK3CD	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Delta
PIK3CG	Phosphatidylinositol-4,5-Bisphosphate 3-Kinase Catalytic Subunit Gamma
PIK3R1	Phosphoinositide-3-Kinase Regulatory Subunit 1
PIK3R3	Phosphoinositide-3-Kinase Regulatory Subunit 3
PIK3R5	Phosphoinositide-3-Kinase Regulatory Subunit 5
PITRM1	Pitrilysin Metallopeptidase 1
PLA1A	Phospholipase A1 Member A
PLA2G4A	Phospholipase A2 Group IVA
PLA2G4C	Phospholipase A2 Group IVC
PLAT	Plasminogen Activator, Tissue Type
PLAU	Plasminogen Activator, Urokinase
PLCB2	Phospholipase C Beta 2
PLCB3	Phospholipase C Beta 3
PLCG2	Phospholipase C Gamma 2
PLD2	Phospholipase D2
PLDN	Biogenesis Of Lysosomal Organelles Complex 1 Subunit 6
PLG	Plasminogen
PLOD1	Procollagen-Lysine,2-Oxoglutarate 5-Dioxygenase 1
PLOD3	Procollagen-Lysine,2-Oxoglutarate 5-Dioxygenase 3
PLS3	Plastin 3
PMM2	Phosphomannomutase 2
PODXL	Podocalyxin Like
POLR2L	Polymerase (RNA) II Subunit L
POLR3H	Polymerase (RNA) III Subunit H
POSTN	Periostin
PPIA	Peptidylprolyl Isomerase A
PPIL2	Peptidylprolyl Isomerase Like 2
PPM1F	Protein Phosphatase, Mg2+/Mn2+ Dependent 1F
PPM1G	Protein Phosphatase, Mg2+/Mn2+ Dependent 1G
PPP1CA	Protein Phosphatase 1 Catalytic Subunit Alpha
PPP1CB	Protein Phosphatase 1 Catalytic Subunit Beta
PPP1CC	Protein Phosphatase 1 Catalytic Subunit Gamma
PPP1R12A	Protein Phosphatase 1 Regulatory Subunit 12A
PPP1R12C	Protein Phosphatase 1 Regulatory Subunit 12C
PPP1R14A	Protein Phosphatase 1 Regulatory Inhibitor Subunit 14A
PPP1R2	Protein Phosphatase 1 Regulatory Inhibitor Subunit 2
PPP2R1A	Protein Phosphatase 2 Regulatory Subunit A, Alpha
PPP2R1B	Protein Phosphatase 2 Regulatory Subunit A, Beta
PPWD1	Peptidylprolyl Isomerase Domain And WD Repeat Containing 1
PRCP	Prolylcarboxypeptidase

PRG1	P53-Responsive Gene 1
PRKACA	Protein Kinase CAMP-Activated Catalytic Subunit Alpha
PRKACB	Protein Kinase CAMP-Activated Catalytic Subunit Beta
PRKACG	Protein Kinase CAMP-Activated Catalytic Subunit Gamma
PRKAR1A	Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha
PRKAR2A	Protein Kinase CAMP-Dependent Type II Regulatory Subunit Alpha
PRKCA	Protein Kinase C Alpha
PRKCB	Protein Kinase C Beta
PRKCD	Protein Kinase C Delta
PRKCQ	Protein Kinase C Theta
PRKCSH	Protein Kinase C Substrate 80K-H
PRKD1	Protein Kinase D1
PRKG1	Protein Kinase, CGMP-Dependent, Type I
PRKG2	Protein Kinase, CGMP-Dependent, Type II
PROC	Protein C, Inactivator Of Coagulation Factors Va And VIIIa
PROS1	Protein S (Alpha)
PRSS23	Protease, Serine 23
PSAP	Prosaposin
PSME3	Proteasome Activator Subunit 3
PTEN	Phosphatase And Tensin Homolog
PTGIR	Prostaglandin I2 (Prostacyclin) Receptor (IP)
PTGIS	Prostaglandin I2 (Prostacyclin) Synthase
PTGS1	Prostaglandin-Endoperoxide Synthase 1
PTK2	Protein Tyrosine Kinase 2
PTPN1	Protein Tyrosine Phosphatase, Non-Receptor Type 1
PTPN11	Protein Tyrosine Phosphatase, Non-Receptor Type 11
PTPN12	Protein Tyrosine Phosphatase, Non-Receptor Type 12
PTPN18	Protein Tyrosine Phosphatase, Non-Receptor Type 18
PTPN2	Protein Tyrosine Phosphatase, Non-Receptor Type 2
PTPN6	Protein Tyrosine Phosphatase, Non-Receptor Type 6
PTPN7	Protein Tyrosine Phosphatase, Non-Receptor Type 7
PTPN9	Protein Tyrosine Phosphatase, Non-Receptor Type 9
PTPRA	Protein Tyrosine Phosphatase, Receptor Type A
PTPRC	Protein Tyrosine Phosphatase, Receptor Type C
PTPRF	Protein Tyrosine Phosphatase, Receptor Type F
PTPRJ	Protein Tyrosine Phosphatase, Receptor Type J
PTRF	Polymerase I And Transcript Release Factor
PTTG1IP	Pituitary Tumor-Transforming 1 Interacting Protein
PXDN	Peroxidasin
QSER1	Glutamine And Serine Rich 1
RAB27A	GTP-Binding Protein Ram
RAB27B	Member RAS Oncogene Family
RAB38	Member RAS Oncogene Family
RAB4A	Member RAS Oncogene Family
RABGGTA	Rab Geranylgeranyltransferase Alpha Subunit
RAC1	Ras-Related C3 Botulinum Toxin Substrate 1
RAD54L2	Androgen Receptor-Interacting Protein 4
RAF1	Raf-1 Proto-Oncogene, Serine/Threonine Kinase
RAI1	Retinoic Acid Induced 1
RALA	Ras Family Small GTP Binding Protein RALA
RALB	V-Ral Simian Leukemia Viral Oncogene Homolog B
RANBP1	RAN Binding Protein 1
RAP1B	RAP1B, Member Of RAS Oncogene Family
RAP1GAP	RAP1 GTPase Activating Protein
RAP1GAP2	RAP1 GTPase Activating Protein 2
RAP1GDS1	Rap1 GTPase-GDP Dissociation Stimulator 1
RAPGEF3	Rap Guanine Nucleotide Exchange Factor 3
RASGRP2	RAS Guanyl Releasing Protein 2
RBM8A	RNA Binding Motif Protein 8A
RGS1	Regulator Of G-Protein Signaling 1
RGS10	Regulator Of G-Protein Signaling 10
RGS18	Regulator Of G-Protein Signaling 18
RGS19	Regulator Of G-Protein Signaling 19
RGS20	Regulator Of G-Protein Signaling 20
RGS4	Regulator Of G-Protein Signaling 4
RGS5	Regulator Of G-Protein Signaling 5
RGS9	Regulator Of G-Protein Signaling 9
RHOA	Ras Homolog Family Member A

RHOB	Ras Homolog Family Member B
RHOBTB1	Rho Related BTB Domain Containing 1
RHOBTB3	Rho Related BTB Domain Containing 3
RHOC	Ras Homolog Family Member C
RHOF	Ras Homolog Family Member F (In Filopodia)
RHOJ	Ras Homolog Family Member J
RNASE1	Ribonuclease A Family Member 1, Pancreatic
RNF4	Ring Finger Protein 4
RNF40	Ring Finger Protein 40
RNU2	RNA, U2 Small Nuclear 1
ROBO4	Roundabout Guidance Receptor 4
ROCK1	Rho Associated Coiled-Coil Containing Protein Kinase 1
ROCK2	Rho Associated Coiled-Coil Containing Protein Kinase 2
RPAP1	RNA Polymerase II Associated Protein 1
RPL13A	Ribosomal Protein L13a
RPL17	Ribosomal Protein L17
RPL19	Ribosomal Protein L19
RPL24	Ribosomal Protein L24
RPL26	Ribosomal Protein L26
RPL27A	Ribosomal Protein L27a
RPL30	Ribosomal Protein L30
RPL31	Ribosomal Protein L31
RPL32	Ribosomal Protein L32
RPL36	Ribosomal Protein L36
RPL37	Ribosomal Protein L37
RPL37A	Ribosomal Protein L37a
RPL41	Ribosomal Protein L41
RPL5	Ribosomal Protein L5
RPLP1	Ribosomal Protein Lateral Stalk Subunit P1
RPLP2	Ribosomal Protein Lateral Stalk Subunit P2
RPN1	Ribophorin I
RPS12	Ribosomal Protein S12
RPS13	Ribosomal Protein S13
RPS14	Ribosomal Protein S14
RPS15	Ribosomal Protein S15
RPS15A	Ribosomal Protein S15a
RPS18	Ribosomal Protein S18
RPS20	Ribosomal Protein S20
RPS24	Ribosomal Protein S24
RPS25	Ribosomal Protein S25
RPS26	Ribosomal Protein S26
RPS27	Ribosomal Protein S27
RPS27A	Ribosomal Protein S27a
RPS7	Ribosomal Protein S7
RPS8	Ribosomal Protein S8
RUNX1	Runt Related Transcription Factor 1
S100A6	S100 Calcium Binding Protein A6
S100A8	S100 Calcium Binding Protein A8
SCAMP2	Secretory Carrier Membrane Protein 2
SCAMP4	Secretory Carrier Membrane Protein 4
SCAMP5	Secretory Carrier Membrane Protein 5
SCARB2	Scavenger Receptor Class B Member 2
SCFD1	Sec1 Family Domain Containing 1
SDCBP	Syndecan Binding Protein
SDF4	Stromal Cell Derived Factor 4
SDPR	Serum Deprivation Response
SEC14L1	SEC14 Like Lipid Binding 1
SELE	Leukocyte-Endothelial Cell Adhesion Molecule 2 Selectin E
SELL	Leukocyte-Endothelial Cell Adhesion Molecule 1 Selectin L
SELP	Platelet Activation Dependent Granule-External Membrane Protein Selectin P
SELPLG	Selectin P Ligand
SEPN1	Selenoprotein N, 1
SERINC3	Serine Incorporator 3
SERP1	Stress-Associated Endoplasmic Reticulum Protein 1
SERPINB5	Serpin Family B Member 5
SERPINC1	Serpin Family C Member 1
SERPIND1	Serpin Family D Member 1
SERPINE1	Serpin Family E Member 1

SERPINE2	Serpin Family E Member 2
SERPINH1	Serpin Family H Member 1
SFRS1	PC4 And **SFRS1** Interacting Protein 1
SGK	Serum/Glucocorticoid Regulated Kinase 1
SH2B3	SH2B Adaptor Protein 3
SHC1	SHC (Src Homology 2 Domain Containing) Transforming Protein 1
SHQ1	H/ACA Ribonucleoprotein Assembly Factor
SIRPA	Signal Regulatory Protein Alpha
SLC29A1	Solute Carrier Family 29 Member 1 (Augustine Blood Group)
SLC35A2	Solute Carrier Family 35 Member A2
SLC35A5	Solute Carrier Family 35 Member A5
SLC35D3	Solute Carrier Family 35 Member D3
SLC35F2	Solute Carrier Family 35 Member F2
SLC39A9	Solute Carrier Family 39 Member 9
SLC7A7	Solute Carrier Family 7 Member 7
SLC9A3R1	SLC9A3 Regulator 1
SLC9A3R2	SLC9A3 Regulator 2
SLFN14	Schlafen Family Member 14
SMAD1	SMAD Family Member 1
SMAD6	SMAD Family Member 6
SMARCA4	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily A, Member 4
SMG6	Nonsense Mediated MRNA Decay Factor
SMURF2	SMAD Specific E3 Ubiquitin Protein Ligase 2
SNAP23	Synaptosome Associated Protein 23kDa
SNAP25	Synaptosome Associated Protein 25kDa
SNAP29	Synaptosome Associated Protein 29kDa
SNAPIN	SNAP Associated Protein
SND1	Staphylococcal Nuclease And Tudor Domain Containing 1
SNRK	SNF Related Kinase
SNX1	Sorting Nexin 1
SNX12	Sorting Nexin 12
SOD1	Superoxide Dismutase 1, Soluble
SOX7	SRY-Box 7
SPARC	Secreted Protein Acidic And Cysteine Rich
SPARCL1	High Endothelial Venule Protein
SPG20	Spastic Paraplegia 20 (Troyer Syndrome)
SPHK1	Sphingosine Kinase 1
SPINK5	Serine Peptidase Inhibitor, Kazal Type 5
SPRR3	Small Proline Rich Protein 3
SPTLC1	Serine Palmitoyltransferase Long Chain Base Subunit 1
SRA1	Steroid Receptor RNA Activator 1
SRC	SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase
SRF	Serum Response Factor
SRPX	Sushi Repeat Containing Protein, X-Linked
SRPX2	Sushi Repeat Containing Protein, X-Linked 2
STAB1	Stabilin 1
STARD4	StAR Related Lipid Transfer Domain Containing 4
STIM1	Stromal Interaction Molecule 1
STOM	Stomatin
STX11	Syntaxin 11
STX12	Syntaxin 12
STX2	Syntaxin 2
STX4	Syntaxin 4
STX6	Syntaxin 6
STX7	Syntaxin 7
STXBP1	Syntaxin Binding Protein 1
STXBP2	Syntaxin Binding Protein 2
STXBP3	Syntaxin Binding Protein 3
STXBP4	Syntaxin Binding Protein 4
STXBP5L	Syntaxin Binding Protein 5 Like
STXBP6	Syntaxin Binding Protein 6
SURF4	Surfeit 4
SUZ12	SUZ12 Polycomb Repressive Complex 2 Subunit
SYK	Spleen Tyrosine Kinase
SYTL3	Synaptotagmin Like 3
SYTL4	Synaptotagmin Like 4
TAF15	TATA-Box Binding Protein Associated Factor 15
TAL1	T-Cell Acute Lymphocytic Leukemia 1

TAOK1	TAO Kinase 1
TARDBP	TAR DNA Binding Protein
TBXA2R	Thromboxane A2 Receptor
TBXAS1	Thromboxane A Synthase 1
TCF4	Transcription Factor 4
TCF8	Zinc Finger E-Box Binding Homeobox 1
TEC	Tec Protein Tyrosine Kinase
TEK	TEK Receptor Tyrosine Kinase
TFPI	Tissue Factor Pathway Inhibitor
TFPI2	Tissue Factor Pathway Inhibitor 2
TGFBR3	Transforming Growth Factor Beta Receptor 3
TGM2	Transglutaminase 2
THBD	Thrombomodulin
THBS1	Thrombospondin 1
THPO	Thrombopoietin
THRAP4	Mediator Complex Subunit 24
TIE1	Tyrosine Kinase With Immunoglobulin Like And EGF Like Domains 1
TIMP1	TIMP Metallopeptidase Inhibitor 1
TINAGL1	Tubulointerstitial Nephritis Antigen Like 1
TLN1	Talin 1
TLR2	Toll Like Receptor 2
TM6SF1	Transmembrane 6 Superfamily Member 1
TMBIM1	Transmembrane BAX Inhibitor Motif Containing 1
TMCC2	Transmembrane And Coiled-Coil Domain Family 2
TMEM154	Transmembrane Protein 154
TMEM43	Transmembrane Protein 43
TMSB10	Thymosin Beta 10
TMSB4X	Thymosin Beta 4, X-Linked
TNF	Tumor Necrosis Factor
TNFRSF1A	Tumor Necrosis Factor Receptor Superfamily Member 1A
TNFSF10	Tumor Necrosis Factor Superfamily Member 10
TNFSF18	Tumor Necrosis Factor Superfamily Member 18
TNNC2	Troponin C2, Fast Skeletal Type
TNPO1	Transportin 1
TP53	Tumor Protein P53
TPD52L2	Tumor Protein D52 Like 2
TPM1	Tropomyosin 1 (Alpha)
TPM4	Tropomyosin 4
TPP1	Tripeptidyl Peptidase 1
TRAF4	TNF Receptor Associated Factor 4
TRAM2	Translocation Associated Membrane Protein 2
TREML1	Triggering Receptor Expressed On Myeloid Cells Like 1
TRPM7	Transient Receptor Potential Cation Channel Subfamily M Member 7
TSN	Translin
TTC37	Tetratricopeptide Repeat Domain 37
TTLL5	Tubulin Tyrosine Ligase Like 5
TTLL10	Tubulin Tyrosine Ligase Like 10
TUBA3C	Tubulin Alpha 3c
TUBB1	Tubulin Beta 1 Class VI
TUBB6	Tubulin Beta 6 Class V
TXNDC5	Thioredoxin Domain Containing 5
TXNRD1	Thioredoxin Reductase 1
TYK2	Tyrosine Kinase 2
TYMP	Thymidine Phosphorylase
UACA	Uveal Autoantigen With Coiled-Coil Domains And Ankyrin Repeats
UBA52	Ubiquitin A-52 Residue Ribosomal Protein Fusion Product 1
UBAP2	Ubiquitin Associated Protein 2
UBC	Ubiquitin C
UBE1C	Ubiquitin Like Modifier Activating Enzyme 3
UBE3C	Ubiquitin Protein Ligase E3C
UBL5	Ubiquitin Like 5
UBN1	Ubinuclein 1
UGDH	UDP-Glucose 6-Dehydrogenase
UNC13A	Unc-13 Homolog A (C. Elegans)
UNC13B	Unc-13 Homolog B (C. Elegans)
UNC45A	Unc-45 Myosin Chaperone A
VAMP2	Vesicle Associated Membrane Protein 2
VAMP3	Vesicle Associated Membrane Protein 3

VAMP7	Vesicle Associated Membrane Protein 7
VAMP8	Vesicle Associated Membrane Protein 8
VAT1	Vesicle Amine Transport 1
VAV1	Vav Guanine Nucleotide Exchange Factor 1
VAV2	Vav Guanine Nucleotide Exchange Factor 2
VAV3	Vav Guanine Nucleotide Exchange Factor 3
VCAM1	Vascular Cell Adhesion Molecule 1
VCP	Valosin Containing Protein
VEGFA	Vascular Endothelial Growth Factor A
VEPH1	Ventricular Zone Expressed PH Domain Containing 1
VIM	Vimentin
VIPAS39	VPS33B Interacting Protein, Apical-Basolateral Polarity Regulator, Spe-39 Homolog
VKORC1	Vitamin K Epoxide Reductase Complex Subunit 1
VPS11	VPS11, CORVET/HOPS Core Subunit
VPS16	VPS16, CORVET/HOPS Core Subunit
VPS18	VPS18, CORVET/HOPS Core Subunit
VPS33A	VPS33A, CORVET/HOPS Core Subunit
VPS33B	Late Endosome And Lysosome Associated
VPS39	VPS39, HOPS Complex Subunit
VPS41	VPS41, HOPS Complex Subunit
VPS4B	Vacuolar Protein Sorting 4 Homolog B
VPS52	VPS52, GARP Complex Subunit
VPS8	VPS8, CORVET Complex Subunit
VWF	Von Willebrand Factor
WARS	Tryptophanyl-TRNA Synthetase
WAS	Wiskott-Aldrich Syndrome
WASF2	WAS Protein Family Member 2
WBP2	WW Domain Binding Protein 2
WDR1	WD Repeat Domain 1
WDR46	WD Repeat Domain 46
WDR6	WD Repeat Domain 6
WDR66	WD Repeat Domain 66
WSB1	WD Repeat And SOCS Box Containing 1
WWP2	WW Domain Containing E3 Ubiquitin Protein Ligase 2
WWTR1	WW Domain Containing Transcription Regulator 1
XLKD1	Lymphatic Vessel Endothelial Hyaluronan Receptor 1
XPO6	Exportin 6
XRCC6	X-Ray Repair Complementing Defective Repair In Chinese Hamster Cells 6
ZFPMI	
ZNF207	Zinc Finger Protein 207
ZNF346	Zinc Finger Protein 346
ZNF521	Zinc Finger Protein 521
ZNF586	Zinc Finger Protein 586
ZNF827	Zinc Finger Protein 827