PHENOTYPIC ANALYSIS OF SUBJECTS WITH UNCHARACTERIZED PLATELET FUNCTION DISORDERS

PHENOTYPIC ANALYSIS

OF

SUBJECTS WITH UNCHARACTERIZED PLATELET FUNCTION DISORDERS

By

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LAY ABSTRACT

Platelets are small blood cells that help stop bleeding. People who have platelets that do not work properly are more likely to bleed. Determining who has platelet problems can be challenging as there are limitations to diagnostic tests for these conditions. Additionally, the risks for bleeding in individuals with platelet problems are unknown. We looked at individuals with bleeding problems and found that a recommended test to assess platelet dense granule release, called lumi-aggregometry, wasn't able to reliably identify persons with bleeding problems. Based on this, we recommend that lumi-aggregometry should not be used to diagnose platelet function disorders. We also found that individuals with uncharacterized platelet function disorders have increased risks for wound healing problems and experiencing bruising, nosebleeds, menorrhagia, and excessive bleeding after dental or surgical procedures. These risks are common among other mild bleeding disorders and will be important to differentiate bleeding risk from other platelet disorders.

ABSTRACT

While some rare and severe forms of platelet function disorders are now well characterized, many common types of platelet function disorders are not yet characterized. My hypothesis was that uncharacterized platelet function disorders that impair platelet function in aggregation and/or dense granule ATP release assays are associated with increased bleeding risk. The main goal of the thesis was to study the phenotype and bleeding risks for uncharacterized platelet function disorders, through analysis of the results from clinical laboratory tests of platelet function and for a detailed analysis of their reported bleeding symptoms. First, I assessed if lumi-aggregometry provides useful diagnostic information on platelet function and can be used to help decide if an individual has a bleeding disorder. Two cohorts of individuals were studied that had dense granule ATP release assessed in response to multiple agonists as part of a work-up for a bleeding disorder. Cohort I was comprised of individuals tested between January 2007 and June 2013 and cohort II was comprised of subjects tested at least twice by this assay prior to September 2015. Among subjects tested more than once for dense granule release defects as part of the work up for a bleeding disorder (cohort I; n=133; cohort II; n=17), normal findings with all tested agonists were often confirmed by the second test (cohort I: 83%; cohort II: 100%), but impaired release with multiple agonists was not often confirmed (cohort I: 34%; cohort II: 54%) and even if it was present, the finding was not predictive of a bleeding disorder. Consequentially, it was recommended that lumi-aggregometry should not be used to diagnose platelet function disorders. Next, I studied the bleeding risks associated with uncharacterized platelet function disorders, by evaluating subjects who had abnormal findings by validated assays, namely subjects who had defective aggregation responses to two or more agonists and/or dense granule deficiency. Bleeding history was evaluated using the International Society for Thrombosis and Haemostasis bleeding assessment tool (ISTH BAT) and the likelihood for bleeding symptoms/ problems, was estimated using odds ratios (OR) collected using the clinical history assessment tool - platelet (CHAT-P) for all affected subjects, a subgroup family with a mutation *RUNX1*, unaffected family

members and general population controls. Individuals with platelet function disorders (n=29) and the affected members of the family with the RUNX1 mutation (n=6) had elevated ISTH BAT scores (median: 9; range:0-18 and median: 8.5, range 4-15, respectively) and an increased risk of abnormal bruising (OR 15-65 and 11-67), nosebleeds (OR 23-40 and 19-121), menorrhagia (OR 6.5-29) and excessive bleeding after trauma or dental/surgical procedures (OR 9.5-44 and 15-77) and wound healing problems (OR 13 and 38) compared to general population control (n=60) and unaffected (n=12) family members. Overall, the platelet function disorders in the study present with a significantly increased risk of mild, rather than severe bleeding problems. These findings are important for individuals and healthcare providers to promote evidence-based care of common uncharacterized inherited platelet function disorders for individuals with *RUNX1* mutations, dense granule deficiency and/or impaired aggregation responses.

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LIST OF ABBREVIATIONS AND SYMBOLS

The abbreviations used within the tables are defined in the tables. Additional symbols and abbreviations contained within this thesis include:

APC	Activated protein C
ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
α	Alpha
ANOVA	Analysis of variance
bp	Base pair
BSS	Bernard-Soulier syndrome
β	Beta
BAT	Bleeding assessment tool
COSMIC	Catalogue of somatic mutations in cancer
CHS	Chédiak–Higashi syndrome
CHAT-P	Clinical history assessment tool - platelet
GPVI	Collagen receptor
GPIa	Collagen-binding integrin
CI	Confidence interval
R^2	Correlation coefficient
dNTP	Deoxynucleoside triphosphate
DNA	Deoxyribonucleic acid
DAG	Diacylglycerol
dup	Duplication
GATA	Erythroid transcription factor 1, (gene)
ExAC	Exome aggregation consortium
FHL	Familial haemophagocytic lymphohistiocytosis
GPIIb	Fibrinogen binding integrin
fs	Frameshift
FLI1	Friend leukemia integration 1, (gene)
γ	Gamma
GT	Glanzmann thrombasthenia
GP	Glycoprotein
HPS	Hermansky-pudlak syndrome
HRLMP	Hamilton Regional Laboratory Program
OH^-	Hydroxide
IP_3	Inositol triphosphate
ISTH	International Society for Thrombosis and Haemostasis
Ile	Isoleucine
LTA	Light transmission aggregometry

MA	Maximal aggregation
MPV	Mean platelet volume
MK	Megakaryocyte
Met	Methionine
OR	Odds ratio
OCS	Open canalicular system
PRP	Platelet rich plasma
PIP_2	Phosphatidylinositol bisphosphate
PS	Phosphatidylserine
PLC	Phospholipase C
PLAU	Plasminogen activator, urokinase (gene)
PCR	Polymerase chain reaction
PAR	Protease activated receptor
H^+	Proton
$P2Y_1$	Purinergic P2 receptor 1, (ADP receptor)
$P2Y_{12}$	Purinergic P2 receptor 12, (ADP receptor)
QPD	Quebec platelet disorder
RNA	Ribonucleic acid
RUNX1	Runt-related transcription factor 1, (gene)
SSC	Scientific and standardization committee
Thr	Threonine
IIa	Thrombin
TxA_2	Thromboxane A2
U46619	Thromboxane analogue
TP	Thromboxane receptor
uPA	Urokinase plasminogen activator
VWD	von Willebrand Disease
VWF	von Willebrand Factor

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW OF PLATELETS

1.1.1. Role of platelets in hemostasis

Hemostatic pathway

Hemostasis is the process that controls and stops bleeding (Michelson, 2013; Versteeg et al., 2013; Gross et al., 2015). It prevents blood loss from damaged blood vessels by forming a hemostatic plug, keeps blood in a fluid state to allow the transport of vital nutrients and regulates the clearance of hemostatic plug after the repair of damaged vessel/tissue (Versteeg et al., 2013). The main components of the hemostatic system are blood vessels, platelets and plasma proteins (Gross et al., 2015).

Platelets are small blood cells derived from megakaryocytes in red bone marrow that circulate within blood in a resting state until agonist-induced activation, which promotes their hemostatic function (Nurden et al., 2009; Watson et al., 2010; Hayward, 2011; Italiano and Hartwig, 2013; Michelson, 2013). Platelets are the main blood cell involved in the formation of a hemostatic plug (Michelson, 2013). The three main phases of hemostasis involve: 1) the formation of a platelet plug, 2) the formation of a fibrin mesh that binds to the platelet plug forming a stable hemostatic plug and 3) the dissolution of the hemostatic plug, a process called fibrinolysis (Michelson, 2013).

Platelet plug formation

When platelets participate in hemostasis, they become activated, stick to one another and release the contents of their granules through exocytosis (Josefsson et al., 2013). Platelet plug formation involves three phases: 1) platelet adhesion to the damaged endothelium, 2) activation by collagen and soluble agonists and 3) aggregation with other activated platelets (Michelson, 2013). Following vascular injury, platelets adhere to

collagens and von Willebrand factor (VWF) in the exposed subendothelial tissue (Brass et al., 2013). The adherent platelets become activated by collagen, which induces rapid structural rearrangement of their cytoskeleton to maximize surface contact at the site of injury (Brass, 2010; Brass et al., 2013). Procoagulant phosphatidylserine (PS) is exposed on the exterior surface of the activated platelet membrane to accelerate thrombin generation (Bouchard, Silveira and Tracy, 2013). Thrombin, a serine protease, converts the abundant plasma glycoprotein fibrinogen to monomers of fibrin which spontaneously polymerize (Gross et al., 2015). Activated platelets accumulate by aggregation, a process that involves activation of the integrin, αIIbβ3 on their exterior membrane so that it binds the ligands fibrinogen, fibrin and VWF (Bledzka et al., 2013). Platelets activation also leads to the released of stored platelet granule contents via exocytosis which results in further local platelet activation (Flaumenhaft, 2013). Additional platelets are recruited to the site of injury to form a stable platelet aggregate (Israels et al., 2010; Gross et al., 2015). The result is the formation of a hemostatic plug that provides a physical barrier to further blood loss, a surface on which thrombin is generated and a surface for fibrin accumulation (Brass, 2010; Brass et al., 2013; Versteeg et al., 2013).

1.1.2. Platelet production, structure and function

Platelet formation and lifecycle

Circulating platelets are derived from megakaryocytes located in red bone marrow, which originate from megakaryocyte-erythroid progenitors (Italiano and Hartwig, 2013; Bianchi et al., 2016). Megakaryopoiesis is the term used to describe development of mature megakaryocytes from their progenitor cells, which takes approximately 9-12 days to yield cells that release platelets (Lordier et al., 2012; Italiano and Hartwig, 2013). During megakaryopoiesis, megakaryocytes undergo multiple incomplete cycles of cell division – a process referred to as polyploidization (Italiano and Hartwig, 2013). This produces megakaryocytes cells with multiples of normal diploid chromosome content (i.e., 4N, 16N, 32N, 64N). Although the number of cycles can range from two to six, the majority of megakaryocytes undergo three to attain a DNA content of 16N

(Lordier et al., 2012; Italiano and Hartwig, 2013). Cytoplasmic maturation also occurs during megakaryopoiesis in which the cytoplasm fills with platelet-specific proteins, organelles, and membrane systems that will ultimately be subdivided and packaged into platelets (Goldfarb, 2007; Italiano and Hartwig, 2013).

Mature megakaryocytes produce platelets through a process that is called thrombopoiesis (Italiano and Hartwig, 2013; Bianchi et al., 2016). During thrombopoiesis, megakaryocytes form cytoplasmic extensions (protoplatelets) that protrude into marrow sinusoids where platelets then detach and are released into the bloodstream as mature platelets (Italiano and Hartwig, 2013). Megakaryocytes produce 5000-10000 platelets throughout their lifespan, and an average healthy adult can produce approximately 10^{11} platelets per day (Josefsson et al., 2013; Ghoshal and Bhattacharyya, 2014; Bianchi et al., 2016).

The majority (approximately two thirds) of platelets circulate within blood at a concentration of 150-400 x 10^9/L (White, 2013) and the remainder are sequestered in the spleen (White, 2013; Ghoshal and Bhattacharyya, 2014). Platelet lifespan typically varies between 5-9 days, and is shortened when large numbers of platelets are recruited to participate in hemostasis (Josefsson et al., 2013). The spleen and liver clear senescent platelets from the circulation via phagocytosis (Josefsson et al., 2013; Ghoshal and Bhattacharyya, 2014).

Platelet Structure

Mature platelets lack a nucleus and are composed of a phospholipid membrane, cytoskeleton, open canalicular system (OCS), closed dense tubular system and various organelles and secretory vessels (White, 2013; Ghoshal and Bhattacharyya, 2014). Platelets are typically 2.0-5.0 µm in diameter, 0.5 µm thick with a mean cell volume of 6 to 10 femtolitres (White, 2013; Ghoshal and Bhattacharyya, 2014). The phospholipid membrane contains various surface receptors and lipid rafts involved in signalling (White, 2013; Ghoshal and Bhattacharyya, 2014). The platelet cytoskeleton maintains the inactive platelets' discoid shape (Hartwig, 2013).

The platelet cytoskeleton is composed of a spectrin membrane skeleton, actin cytoskeleton and a marginal microtubule coil (Hartwig, 2013; Ghoshal and Bhattacharyya, 2014).The OCS is a series of channels derived from exterior membrane inholdings that connect the interior of the platelet and the external milieu (Hartwig, 2013; White, 2013). In the activated platelet, the OCS facilitates the import of external substances and the export of released storage granule contents as well as a source of surface membrane for cell spreading (White, 2013). The dense tubular system is composed of endoplasmic reticulum and sequesters Ca^{2+} for release into the cytoplasm with intra-platelet signalling events (White, 2013; Ghoshal and Bhattacharyya, 2014).

Platelet Storage Granules

Platelets contain 2 types of storage granules: α granules and dense granules. α granules store proteins and there are typically 50-80 α granules per platelet (White, 2013; Ghoshal and Bhattacharyya, 2014). α granules range in size from 200-500 nm (10% platelet volume) and vary in content (White, 2013; Ghoshal and Bhattacharyya, 2014). α granules store a variety of proteins including adhesive proteins (e.g. fibrinogen and vWF), proteins involved in coagulation (e.g. Factor V), fibrinolytic proteins (e.g. plasminogen), growth factors and immune mediators (Flaumenhaft, 2013; White, 2013). Dense granules are less abundant and smaller than α granules (White, 1969, 2013) and contain electron dense substances such as Ca^{2+} and Mg^{2+}, polyphosphates, adenine nucleotides (ADP and ATP) and bioactive amines (serotonin and histamine) (White, 2013; Golebiewska and Poole, 2013, 2015). ADP and serotonin are known to participate in platelet activation, which triggers platelet shape change, platelet aggregation and further exocytosis (Flaumenhaft, 2013; Ghoshal and Bhattacharyya, 2014).

The release of platelet granules is an important step in the signalling events leading to platelet aggregation (Flaumenhaft, 2013). Granules are produced from the Golgi apparatus in developing megakaryocytes (Masliah-Planchon, Darnige and Bellucci, 2013; Boilard, Duchez and Brisson, 2015).Once platelets become activated, they release the intracellular contents of their granules (Flaumenhaft, 2013;

Matthews, 2013). These effector molecules in turn, affect platelet function in a variety of ways including binding receptor molecules on surrounding platelets, promoting platelet aggregation and stabilizing clot formation (Flaumenhaft, 2013).

Signalling pathways in platelet activation, secretion and aggregation

Platelet activation ultimately leads to the propagation and amplification of signalling that contributes to the growth of the platelet plug at sites of vessel injury (Brass et al., 2013). Activated platelets release the contents of their granules, including ADP and serotonin (Flaumenhaft, 2013). The response to these agonists lead to the recruitment of additional platelets and amplification of the signalling cascade (Nurden, Freson and Seligsohn, 2012; Brass et al., 2013; Rao, 2013a). This is a coordinated process involving the OCS, signalling receptors, various intracellular effector molecules and the α and dense granule secretory granules (Brass et al., 2013; Golebiewska and Poole, 2015). Platelet activation is facilitated through stimulation by a variety of soluble agonists (e.g., thrombin, ADP, thromboxane $A_{2,}$ and epinephrine), as well as the exposure of subendothelial collagens (Stalker et al., 2012; Clemetson and Clemetson, 2013). The potency of the platelet response is governed by the G-proteins and their receptors, the number of receptors found on platelets, the efficiency of activation and degree of platelet inhibition (Stalker et al., 2012).

Thrombin is a strong platelet agonist (Stalker et al., 2012; Zhang, Covic and Kuliopulos, 2013; Golebiewska and Poole, 2015). Thrombin facilitates platelet activation by binding PAR (protease-activated receptor)-1 and PAR-4 and it also binds to GPIb-IX-V on the platelet plasma membrane(Clemetson and Clemetson, 2013). PARs are expressed at a few thousand copies per platelet (Stalker et al., 2012; Clemetson and Clemetson, 2013). The interaction of thrombin with the G protein-coupled receptors (GPCRs) PAR-1 and PAR-4 stimulate the activity of phospholipase Cβ (PLCβ), which hydrolyzes phosphosphadtidylinositol 4,5 bisphosphate (PIP_2) into 1,4,5- inositol trisphosphate (IP_3) and 1,2-diacylglycerol (DAG) (Brass et al., 2013; Zhang et al., 2013). IP_3 causes Ca^{2+} release from the dense tubular system, which interacts with calmodulin,

leading to myosin light chain phosphorylation and subsequent platelet shape change (Nurden et al., 2012; Zhang et al., 2013; Golebiewska and Poole, 2013; Gross et al., 2015). DAG stimulates protein kinase C (PKC), which phosphorylates pleckstrin, resulting in platelet aggregation and exocytosis (Brass et al., 2013; Golebiewska and Poole, 2013).

Collagen can also trigger platelet activation and aggregation (Pollitt, Hughes and Watson, 2013). When collagen is exposed to flowing blood as a result of vascular injury, platelets that become adherent to collagen are activated through platelet GPVI interactions with collagen molecules, leading to phosphorylation of the platelet immunoglobulin FcRγ_2 portion of the GPVI- FcRγ_2 complex (Pollitt et al., 2013). This leads to subsequent phosphorylation of PLCγ_2 (Brass et al., 2013; Pollitt et al., 2013). Phosphorylated PLCγ_2 hydrolyzes PIP_2, leading to an increase in cytosolic Ca^{2+} and subsequent activation of downstream signalling events. This signalling cascade culminates in platelet activation and granule secretion (Stalker et al., 2012; Brass et al., 2013).

Arachidonic acid or arachidonate is an agonist that is formed when platelets are activated and PLC A_2 cleaves membrane phospholipids that are then converted through multistep enzymatic processes into thromboxane A_2 (TxA_2), a potent platelet agonist (Clemetson and Clemetson, 2013). Cyclooxygenase-1 (COX-1) is the primary enzyme involved in the production of TxA_2, and first converts arachidonic acid into PGG_2 and PGH_2 (Patrono, 2013). Aspirin exhibits antiplatelet effects through the inhibition of COX-1, which prevents the conversion of arachidonic acid to TxA_2 (Brown, Wilkerson and Love, 2015). After conversion of arachidonic acid to PGG_2 and PGH_2, thromboxane synthetase metabolizes PHG_2 into TxA_2 (Patrono, 2013). Once formed, TxA_2 is able to diffuse across the plasma membrane and activate platelets via the thromboxane (TP) receptor (Stalker et al., 2012; Clemetson and Clemetson, 2013). This in turn activates PLCβ to promote the hydrolysis of PIP_2, similar to the signaling that occurs when platelets are activated through PAR receptors (Brass et al.,

2013). However, unlike PARs, TP does not inhibit adenylyl cyclase or control its negative feedback on platelet activation (Brass et al., 2013).

ADP is primarily stored in platelet dense granules and is released upon platelet activation (Stalker et al., 2012; Cattaneo, 2013a) where it likely helps trigger further sustained platelet activation (Stalker et al., 2012; Cattaneo, 2013a). Once released, ADP binds platelet surface GPCRs $P2Y_1$ and $P2Y_{12}$ (Cattaneo, 2013a), activating these receptors (Cattaneo, 2013a; Rao, 2013a). Their activation leads to an inhibition of adenylate cyclase which causes a decrease in cAMP and activation of PLCβ. These activities promote the hydrolysis of PIP_2 and lead to platelet aggregation and exocytosis, similar to the effects of PAR-1 and PAR-4 (Brass et al., 2013).

Epinephrine is a weak activator of human platelets that potentiates responses to other weak agonists (Stalker et al., 2012; Brass et al., 2013). Epinephrine binds to α_{2A}-adrenergic receptors and exerts platelet stimulating effects by inhibiting adenylyl cyclase and cAMP formation (Stalker et al., 2012; Brass et al., 2013). Unlike other agonists, epinephrine does not appear to directly activate PLC or induce platelet shape change (Cattaneo, 2009; Stalker et al., 2012).

Transcription Factors

Hematopoietic transcription factors are known to be important in the production and developmental regulation of megakaryocytes, platelets and as well as playing a regulatory role in the function of platelets (Goldfarb, 2007; Songdej and Rao, 2015). Transcription factors influence a number of important processes such as cytoskeletal rearrangement and intracellular vesicle trafficking in megakaryocytes (Songdej and Rao, 2015; Bianchi et al., 2016). Some transcription factors, such as Runt-related transcption factor (*RUNX1*), Friend leukemia integration 1 (*FLI1*), and GATA-binding factor 1 (*GATA*-1) can act in combination in megakaryocytes (Songdej and Rao, 2015).With the aid of recruited co-effector molecules, these transcription

factors can specifically and selectively regulate the transcription of molecules involved in megakaryocyte production, platelet production and function (Tijssen and Ghevaert, 2013). Consequently, deleterious mutations in these genes can lead to impaired expression of downstream genes which can affect diverse cellular pathways and manifest as abnormalities in both platelet number and function (Rao, 2013a; Songdej and Rao, 2015; Bianchi et al., 2016).

1.1.3. Defects in platelet function

Defective platelet function can result from acquired or inherited disorders. In both cases, the dysfunction may arise from a variety of mechanisms, as there are many redundant and overlapping pathways governing platelet function (Bunimov, Fuller and Hayward, 2013).

Acquired platelet dysfunction, due to drugs, is commonly seen in clinical situations (Konkle, 2011; Koneti Rao, 2013). There are more than 100 drugs, foods and other supplements that have been reported to inhibit platelet function, and some have an unclear mechanism of action (Harrison et al., 2011; Konkle, 2011; Koneti Rao, 2013). Some commonly used drugs that inhibit platelet function include COX-1 inhibitors such as aspirin and nonsteroidal anti-inflammatory drugs (NSAIDs), thienopyridines (which block $P2Y_{12}$) and proton pump inhibitors (Harrison et al., 2011; Konkle, 2011; Koneti Rao, 2013). Other substances that inhibit platelet function include alcohol (McEwen, 2014), caffeine, beta-lactam antibiotics (Shattil et al., 1980), nitrates, selective serotonin reuptake inhibitors (SSRIs) (Gärtner et al., 2010) and ginkgo based herbal supplements (Yagmur et al., 2005; Konkle, 2011; McEwen, 2014).

Inherited platelet disorders manifest as qualitative or quantitative defects in molecules or pathways that are important to platelet function (Cattaneo, 2013b; Rao, 2013a). By studying the molecular cases of some severe bleeding phenotypes, clinicians and scientists have characterized a variety of rare and syndromic platelet function disorders. However, less is known about the cause of more common types of platelet function

disorders (Hayward et al., 2012a). In the majority of individuals a with suspected platelet function disorder, the underlying molecular and genetic mechanisms are unknown (Cattaneo, 2013b; Rao, 2013a).

1.2. INHERITED PLATELET DISORDERS

Worldwide, over 14 000 people each year undergo testing for suspected inherited platelet function disorders, and just under half (5700 each year) are found to have platelet function abnormalities (Cattaneo, 2013b; Gresele et al., 2014). Uncharacterized platelet disorders represent one of the most common type of inherited bleeding disorder, that may be as common, or more common than von Willebrand disease (Israels et al., 1990; Pai et al., 2011; Castilloux et al., 2011). Since there are more than 1000 proteins contained in a single platelet, there are many potential causes of platelet disorders, which are common causes of abnormal bleeding (Hayward, 2011; Nurden et al., 2012; Cattaneo et al., 2013b).

1.2.1. Clinical phenotypes

Bleeding symptoms

Inherited platelet function disorders manifest as an altered ability to respond to hemostatic challenges and are highly variable in the presentation of bleeding symptoms (Hayward, Rao and Cattaneo, 2006; Israels et al., 2010; Rao, 2013a; Watson et al., 2013; Gresele et al., 2014). Some individuals with platelet disorders experience extensive and often unexplained bruising, soft tissue hematomas, excessive mucosal bleeding, epistaxis, menorrhagia (often presenting since menarche), excessive and prolonged bleeding with childbirth, and challenge-related bleeding triggered by surgical or dental procedures or trauma (Pai and Hayward, 2009; Rao, 2013a; Ghoshal and Bhattacharyya, 2014). Spontaneous bleeding into joints and deep hematomas are uncommon in individuals with platelet disorders (Rao, 2013a), but there are some exceptions (For example in QPD, joint bleeds are common (McKay et al., 2004)). When experiencing a bleeding episode from a minor

wound or intervention, the duration of bleeding is often prolonged in individuals with platelet disorders (Rao, 2013a).

There are many aspects of platelet function that may be impaired, which contributes to the considerable heterogeneity observed among inherited platelet disorders. Some of these aspects include defects in platelet: adhesion, aggregation, fibrinolysis or "secretion" ("release" or "activation"). Some of the characterized causes of secretion defects are recessive, syndromic disorders associated with dense granule deficiency and additional syndromic features, such as albinism (in the case of Hermansky Pudlak Syndrome) (Bunimov et al., 2013).

Laboratory investigations of platelets

Laboratory tests that evaluate platelet morphology and function are important in the diagnosis and characterization of inherited platelet disorders (Rodeghiero et al., 2010; Harrison and Lordkipanidzé, 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Inherited platelet disorders can result in thrombocytopenia (platelet concentration lower than 150 platelets/mL), reduced storage granule numbers, abnormal aggregation responses to agonists and/or an inability to release dense granule contents on activation of platelet rich plasma (PRP) (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015).

A full blood count is typically done as part of an assessment for a platelet disorders (Harrison and Lordkipanidzé, 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). An estimate of platelet numbers (using automated cell counters) is done in conjunction with blood film analysis (Harrison and Lordkipanidzé, 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). It is recommended that blood counts be assessed for any investigation of patients with abnormal bleeding (Harrison et al., 2011; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). A low

platelet count may indicate thrombocytopenia as the primary cause of bleeding manifestations, but further functional testing is still required (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015).

Light transmission aggregometry (LTA) is the present gold standard test for diagnosing defects in platelet function and it is also the most commonly performed platelet function test performed by diagnostic laboratories (Cattaneo et al., 2009; Hayward and Moffat, 2013; Gresele et al., 2014). LTA was first developed independently in 1962 by Born and O'Brien (Born, 1962; O'Brien, 1962). LTA measures the transmission of light through a sample of washed patient platelet-rich plasma (PRP) to which an agonist is added (Hayward and Moffat, 2013; Cattaneo et al., 2013a). The typical agonist panel used to assess platelet function contains: ADP, collagen, epinephrine, arachidonic acid and ristocetin (Cattaneo et al., 2013a; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015).

Responses to individual test agonists are typically classified as "abnormal" if the level of maximal aggregation falls outside of the established reference range. It is recommended that the reference range should be established internally and its performance validated with each reagent lot (Hayward et al., 2008; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Impaired platelet aggregation with two or more agonists is suggestive of some inherited platelet function disorders (Hayward et al., 2009b; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). A defective response to epinephrine is known to be frequent in routine screening and guidelines suggest that further studies should be conducted only if other abnormalities and/or strong clinical suspicion are present (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Aggregation testing can detect both severe and mild platelet function disorders (Hayward et al., 2008). Repeated platelet studies should be separated by at least 1 month, to

allow for disappearance of acquired interfering factors (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015).

Surveys of diagnostic laboratories indicate that a considerable number perform platelet dense granule release assays to assess for platelet function disorders (Hayward et al., 2012a; Gresele et al., 2014). Recent guidelines recommend that laboratories include an assay that assesses ATP and/or ADP release when assessing platelet granule release (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Commonly, testing for ATP release is done using a lumi-aggregometer, platelet rich plasma (PRP) and a luciferin/luciferase containing reagent (Hayward et al., 2012a; Gresele et al., 2014) to quantify the release of adenosine triphosphate (ATP) from platelets in response to an agonist. While there is a lack evidence-based guidelines to direct the performance interpretation and reporting of platelet dense granule release tests,(Mumford et al., 2015), impaired release can reflect dense granule deficiency or defects in signalling pathways that lead to dense granule release (Stalker et al., 2012; Glembotsky et al., 2014). However, some concern over the reproducibility of test findings has been raised as recent studies identified considerable variability (particularly with weak agonists) in a cohort of healthy control subjects (Hayward et al., 2009b). Uncertainties exist about whether this assay is reliable enough for the diagnosis of platelet function defects among individuals investigated for a bleeding disorder.

Collagen and thrombin are referred to as strong agonists as they directly to induce dense granule secretion and TxA_2 synthesis, even in the absence of extracellular calcium or fibrinogen binding (Cattaneo, 2009; Stalker et al., 2012). Strong agonists increase intracellular Ca^{2+} concentration in platelets through direct upregulation of PLC (Stalker et al., 2012). In contrast, thromboxane A_2 and ADP are considered weak agonists that induce dense granule secretion only when platelet aggregation has occurred (Cattaneo, 2009). The weaker agonists are thought to potentiate platelet activation and have very little impact on PLC formation and downstream intracellular Ca^{2+} release (Cattaneo, 2009).

Exome sequence analysis is being explored as a tool for evaluating platelet disorders but it presently is not a replacement for extensive platelet function testing (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015) as it is difficult to prove that a specific sequence variation is the cause of an inherited platelet function disorder in the absence of phenotypic information (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015).

1.2.2. Impaired pathways in platelet function

Inherited disorders of platelet function are often classified based on function or response found to be abnormal during the diagnostic work-up (Cattaneo, 2013b). However there are many overlapping and redundant aspects of platelet function that make it difficult to classify disorders based on functional tests alone (Cattaneo, 2013b). Even with a single disorder (such as BSS) patients may present with a wide range of bleeding symptoms and may vary in severity between patients (Israels et al., 2010; Cattaneo, 2013b; Nurden and Nurden, 2014). Common syndromic features may also aid in grouping related disorders, however not all platelet disorders are accompanied by these distinct features (Rao, 2013a). A summary of characterized platelet disorders, their key clinical features and bleeding phenotype is provided below (table 1).

Table 1. Summary of inherited platelet disorders

Type of defect	Name of disorder or affected protein	Key features	Bleeding symptoms and reference
Activation	GPVI	Impaired platelet activation by collagen	Mild to moderate bleeding phenotype (Hermans et al., 2009; Pollitt et al., 2013)
Activation	$P2X_1$	Impaired platelet activation by ADP and reduced Ca^{2+} mobilization	Severe bleeding phenotype; Spontaneous epistaxis occasionally requiring hospitalization and platelet transfusion (Oury et al., 2000)
Activation	$P2Y_1$	Impaired platelet activation by ADP	Mild bleeding phenotype; Bleeding following surgical procedures (Cattaneo, 2013b)
Activation	$P2Y_{12}$	Impaired platelet activation by ADP	Mild to severe bleeding phenotype (Remijn et al., 2007; Cattaneo, 2011)
Activation	TP	Impaired TxA_2-dependent aggregation and secretion	Mild to severe bleeding phenotype; Common symptoms include easy bruising and prolonged epistaxis (Ushikubi et al., 1987)
Activation	Thromboxane synthase	Impaired production of TxA_2	Moderate to severe bleeding tendency; Markedly prolonged bleeding time (Di Paola and Johnson, 2011)
Adhesion	Bernard-Soulier Syndrome (BSS)	Deficiency or functional defect in GPIbIXV; Macrothrombocytopenia; Absent agglutination with ristocetin	Mild to moderate bleeding tendency; Bleeding severity can range from mild to life threatening; Common symptoms include epistaxis, ecchymosis, menometrorrhagia, and gingival or gastrointestinal bleeding (Pham and Wang, 2007; Berndt and Andrews, 2011)
Adhesion	GPIV	Deficiency of platelet CD36 affecting thrombospondin binding; Associated with metabolic syndrome, atherosclerotic cardiovascular disease and cardiomyopathy	No known bleeding phenotype(Hirano et al., 2003)

Adhesion	Platelet type - Von Willebrand Disease (PT-VWD)	Gain-of-function defect in platelet GPIbα leading to increased binding to VWF and platelet clearance	Prolonged bleeding time; Epistaxis, ecchymosis, gingival bleeding and after dental procedures (Miller et al., 1991; Othman, 2011)
Adhesion	α2β1 defect	Thrombocytopenia and impairment of platelet aggregation and secretion to collagen	Mild bleeding disorder (Noris et al., 2006)
Aggregation	Glanzmann Thrombasthenia (GT)	Impaired platelet aggregation with all agonists due to reduction or loss of αIIbβ3, which binds fibrinogen	Mucocutaneous bleeding and bleeding after trauma or surgery; Postpartum bleeding may be frequent and severe; Heterozygotes have no bleeding phenotype (George, Caen and Nurden, 1990; Nurden et al., 2011b)
Aggregation	Leukocyte adhesion deficiency (LADIII)	Impaired platelet and leukocyte integrin activation due to defects in kindlin 3; Impaired platelet aggregation with various agonists; Associated with immune deficiency	Severe bleeding phenotype; Mucocutaneous bleeding requiring platelet transfusions, intracranial hemorrhage (Malinin et al., 2009; Svensson et al., 2009)
Fibrinolysis	Quebec Platelet Disorder (QPD)	Gain-of-function defect in fibrinolysis due to an increase in the platelet stores of urokinase-type plasminogen activator (uPA)	Moderate bleeding phenotype; Bleeding severity is moderate to severe with delayed bleeding; Common symptoms include easy bruising epistaxis, menorrhagia, hematuria, muscle and joint bleeds and excessive, delayed bleeding after trauma or surgical procedures.(Hayward et al., 1996; McKay et al., 2004; Diamandis et al., 2009)
Platelet number	Congenital amegakaryocytic thrombocytopenia	Thrombocytopenia related to a deficiency of the thrombopoetin receptor	Common symptoms include petechiae, purpura, and easy bleeding with a few reporting intracranial bleeding (Ballmaier and Germeshausen, 2011)
Platelet number	Congenital amegakaryocytic thrombocytopenia with synostosis of the radius and ulna	Neonatal amegakaryocytic thrombocytopenia and proximal fusion of the radius and ulna related to mutations in HOXA11	Common symptoms include easy bruising and GI bleeding (Horvat-Switzer and Thompson, 2006; Eto and Kunishima, 2016)

Platelet number	Filaminopathy	Varying thrombocytopenia related to a defect in filamin A; Impaired platelet aggregation to collagen; Presence of large and spheroid-like platelets	Mild to moderate bleeding phenotype (Nurden et al., 2011a)
Platelet number	Glanzmann Thrombasthenia-like syndromes	Macrothrombocytopenia and impaired aggregation associated with activating mutations in αIIbβ3	Minimal to severe bleeding phenotype; (Nurden et al., 2011c)
Platelet number	Macrothrombocytopenia with platelet expression of glycophorin A	Macrothrombocytopenia and platelets expressing surface glycophorin A	Mild bleeding phenotype (Gilman et al., 1995)
Platelet number	MYH9 related disorders (MYH9-RD)	Macrothrombocytopenia, leukocyte inclusions (Döhle-like bodies), with or without deafness, cataracts and nephritis	Bleeding tendency related to platelet count, from asymptomatic to severe; Common symptoms include easy bruising, menorrhagia, epistaxis and/or gum bleeding; Bleeding occasionally requires platelet transfusion; Rare severe post-partum hemorrhage (Althaus and Greinacher, 2009; Balduini, Pecci and Savoia, 2011)
Platelet number	Thrombocytopenia associated with absent radii syndrome (TAR)	Thrombocytopenia associated with the absence of radii and the presence of thumbs	Bleeding phenotype in the first year of life which diminishes in frequency and severity with age (Thompson et al., 2001; Albers et al., 2012)
Platelet number	Thrombocytopenia (THC2-linked)	Thrombocytopenia and hypolobated megakaryocytes	Mild bleeding phenotype; Increased bruising frequency (Di Paola and Johnson, 2011)
Platelet number	Thrombocytopenia and giant platelets	Thrombocytopenia and giant platelets related to an impairment in tubulin β-1 chain	Absent to mild bleeding tendency; Common symptoms include epistaxis, gum bleeding, easy bruising and menorrhagia (Millikan et al., 2011)
Platelet number	Thrombocytopenia Cargeeg	Thrombocytopenia, increased apoptosis and dysregulated megakaryopoesis related to mutations in cytochrome c (CYCS)	Mild or absent bleeding phenotype; Frequent bruising and epistaxis (Cramer Bordé et al., 2011)

Platelet number	Thrombocytopenia with or without syndromic features	Defects in filamin A resulting in thrombocytopenia with or without periventricular nodular heteropenia or otopalatodigital syndromes	Mild bleeding phenotype; Common symptoms include easy bruising, hematomas, heavy menstrual bleeding, gum bleeding, epistaxis, impaired wound healing, GI bleeding and bleeding after surgery (Nurden et al., 2011a)
Platelet number	Velo-cardio-facial syndrome (VCF)	Cardiac anomalies, cleft palate, hypocalcaemia, thymic aplasia, and typical facies; Macrothrombocytopenia	Common symptoms include epistaxis, severe post-partum hemorrhage (Van Geet et al., 1998)
Platelet number	Wiskott-Aldrich syndrome (WAS) and X-Linked Thrombocytopenia (XLT)	Mild to severe thrombocytopenia with significantly reduced platelet volumes, recurrent infections, autoimmune disease and often eczema	Common symptoms include petechiae and GI bleeding with occasional central nervous system hemorrhages (Thrasher, 2009)
Platelet number and storage granule secretion	Familial haemophagocytic lymphohistiocytosis (FHL)	Hyperinflammatory syndrome with thrombocytopenia, neurologic alterations and high mortality; Associated with fever, splenomegaly, bicytopenia, high triglycerides/low fibrinogen, hemophagocytosis, high ferritin, low natural killer (NK)–cell cytotoxicity, and high soluble CD25	Increased bleeding tendency presents in some patients; Severity ranges from trivial to severe; Bleeding is primarily cutaneous and occurs with anything from minor wounds and surgical procedures (zur Stadt et al., 2005; Meeths et al., 2010)
Platelet number, dense or α granule storage, aggregation and secretion	White Platelet Syndrome (WPS)	Larger than normal mitochondria and half normal-sized dense bodies with 1/3 grey platelets; Impaired platelet aggregation and secretion in response to thrombin, low collagen, ADP, epinephrine and AA	Mild to moderate bleeding phenotype; Prolonged bleeding time (White et al., 2004)
Procoagulant function	Scott Syndrome	Impaired maintenance of the procoagulant phospholipids in activated platelets	Common symptoms include excessive bleeding following trauma or surgery, epistaxis, severe postpartum bleeding, long-lasting menorrhagia and defective wound healing (Weiss, 2009; Lhermusier, Chap and Payrastre, 2011)
Procoagulant function	Stormorken Syndrome	Enhanced procoagulant activity in resting platelets;	Common symptoms include easy bruising, epistaxis requiring cauterization, excessive

		Reduced platelet aggregation and secretion with all agonists except collagen	bleeding following trauma (Stormorken et al., 1985, 1995)
Signalling	Cyclooxygenase-1 deficiency (COX-1)	Absent TxA_2 production; Aspirin-like defect	Mild bleeding tendency (Dubé et al., 2001)
Signalling	Cytoplasmic phospholipase A_2 deficiency (cPLA2)	Impaired AA production due to defects in cPLA2	Severe bleeding phenotype; Associated with idiopathic intestinal ulcers (Adler et al., 2008)
Signalling	PLC-β2	Impaired platelet aggregation and secretion associated with a reduction in PLC-β2	Mild bleeding phenotype; (Lee et al., 1996)
Signalling	G-protein pathway defect	Defective G-protein signalling; Reduced platelet aggregation and secretion	Mild bleeding phenotype; Common symptoms include mucosal bleeding and easy bruising (Van Geet et al., 2009)
Signalling	Gs platelet defect	Reduced sensitivity to Gs stimulation resulting in reduced cAMP production; enlarged, round platelets with abnormal α granules	No known bleeding phenotype (Noé et al., 2010)
Signalling	York Platelet Syndrome (YPS)	Thrombocytopenia associated with a gain of function mutation in the Ca^{2+} sensor STIM1; Large ER-derived inclusion bodies in platelets; Immune deficiency and non-progressive myopathy	Absent to moderate bleeding phenotype; Common symptoms include excessive bruising, epistaxis, heavy menses, occasional GI bleeding (Markello et al., 2015)
Transcription factor	Familial platelet disorder with propensity to acute myeloid leukemia and myelodysplastic syndrome (FPD/AML/MDS)	Thrombocytopenia associated with impaired platelet function and hereditary predisposition to myelodysplastic syndrome and myeloid leukemia related to mutations in *RUNX*1	Absent to moderate bleeding tendency; (Yamagata, Maki and Mitani, 2005; Stockley et al., 2013; Latger-Cannard et al., 2016)
Transcription factor	Macrothrombocytopenia with dyserythropoesis/anemia/beta-thalassemia (*GATA1*)	Thrombocytopenia associated with thalassemia, neutropenia and megakaryoblastic leukemia with or without Down syndrome	Common symptoms include easy bruising, petechiae, spontaneous nosebleeds, occasional hematuria (Millikan et al., 2011)
Transcription factor	Paris-Trousseau-Jacobsen Syndrome	Thrombocytopenia, giant platelets, and α granules, mental retardation, cardiac and facial defects associated with mutations in *FLI1*	Mild bleeding phenotype; Common symptoms include abnormal bleeding and easy bruising.

			(Favier et al., 2003; Raslova et al., 2004)
α granule storage	Arthrogryposis Renal Dysfunction and Cholestasis Syndrome (ARC)	Arthrogryposis multiplex congenita, renal dysfunction, and cholestasis associated with absent platelet α granules; mortality within the first few years of life	Severe bleeding phenotype; Recurrent and life-threatening episodes of hemorrhage, primarily epistaxis, requiring frequent hospitalizations and PICU admissions (Abu-Sa'da et al., 2005)
α granule storage	Grey Platelet Syndrome (GPS)	Thrombocytopenia associated with severe α granule protein deficiency and enlarged platelets; Platelets appear grey in peripheral blood smear due to reduction in α granules; Impaired aggregation in response to ADP and low concentrations of thrombin and collagen	Mild to moderate bleeding phenotype; Rare intracranial hemorrhage and postsurgical bleeding (Gunay-Aygun et al., 2010)
α granule storage	Medich Platelet Syndrome (MPS)	Macrothrombocytopenia with markedly decreased α granules; Platelets contain membranous cigar-shaped inclusions	Moderate bleeding phenotype; Easy bleeding and menorrhagia requiring platelet transfusions and hormonal therapy (White, 2004; Gunning et al., 2013)
Dense granule storage	Chediak-Higashi (CHS)	Dense granule deficiency, neutropenia, lymphoma, inclusion bodies in myoblasts and promyelocytes; Various degrees of oculocutaneous albinism; Progressive neurological dysfunction and recurrent infections	Bleeding severity ranges from mild to severe; Bleeding symptoms include mild bruising and mucosal bleeding; Bleeding from surgical or dental procedures can be minimized with prophylaxis (Lozano et al., 2014)
Dense granule storage	Griscelli syndrome	Dense granule deficiency associated with immunological and central nervous system defects, lymphohistiocytosis and hypopigmentation	No known bleeding phenotype (Van Gele, Dynoodt and Lambert, 2009)
Dense granule storage	Hermansky-Pudlak (HPS)	Dense granule deficiency and defects in melanosomes and lysosomes; Oculocutaneous albinism	Bleeding severity ranges from mild to severe; Common symptoms include easy bruising, epistaxis, severe menorrhagia and prolonged bleeding after a surgical or dental procedure (Ray, Ray and Matthew, 2013)
Dense granule storage	Multidrug resistance protein (MRP4) deficiency	Deficiency of platelet adenine nucleotides in dense granules associated with a defect in the adenine nucleotide transporter, MPR4	Easy bruising, frequent epistaxis, menorrhagia, and bleeding after dental extractions and surgery (Jedlitschky et al., 2004, 2010)

α and dense granule storage	α-dense storage pool disease (SPD)	Most cases are moderately deficient in both granules and dense bodies; Granules and dense bodies become connected to channels of the open canalicular system (OCS) and lose their contents to the exterior without prior activation of the cells	Mild bleeding phenotype; Common symptoms include easy bruising during childhood, menorrhagia and post-partum hemorrhage (White et al., 2007)
Miscellaneous	Montreal platelet syndrome (MPS)	Thrombocytopenia associated with giant platelets, and spontaneous platelet aggregation in vitro	Mild to severe bleeding phenotype; Common symptoms include easy bruising, epistaxis and in some cases severe postoperative bleeding, postpartum hemorrhage, and GI bleeding (Jackson et al., 2009)
Miscellaneous	Primary secretion defect (PSD)	Heterogenous group of ill-defined abnormalities in platelet secretion; Not associated with platelet granule deficiencies	Absent to moderate bleeding phenotype (Rao, 2013a)

Platelet disorder that impair activation

Disorders that impair platelet activation often present as an inability to respond to agonists (Cattaneo, 2013b; Rao, 2013a). Some inherited platelet disorders affect receptors that are known to be important for the activation of platelets (Cattaneo, 2013b; Rao, 2013a). Some disorders include qualitative or quantitative defects in responses to: thromboxane A_2 (TP), ADP ($P2Y_1$ and $P2_{12}$) and collagen (GPVI) (Rao, 2013a). In the case of inherited disorders that involve defects in the platelet receptors $P2Y_1$, $P2_{12}$ or TP, responses to other agonists, including collagen and thrombin, may also be impaired due to the role of ADP and TxA_2 in signal amplification (Rao, 2013a).

Platelet disorder that impair adhesion

Defective platelet adhesion impairs platelet-vessel wall interactions (Cattaneo, 2013b) and the ability of platelets to bind to subendothelial proteins and adhere at sites of vessel wall damage (Cattaneo, 2013b; Nurden and Nurden, 2014). Bernard-Soulier syndrome (BSS) is a rare disorder caused by inherited defects in the GPIb-IX-V complex that result in giant platelets, thrombocytopenia and impaired platelet adhesion to VWF (Berndt and Andrews, 2011; Nurden and Nurden, 2014; Bianchi et al., 2016) (Pham and Wang, 2007; Berndt and Andrews, 2011; Andrews and Berndt, 2013). BSS is associated with an absent or markedly impaired platelet aggregation response to ristocetin (Berndt and Andrews, 2011). There are over 100 mutations already identified as causes of BSS and the majority are recessively inherited and impair GPIb-IX-V complex assembly (Berndt and Andrews, 2011). Patients with BSS present early in life with bleeding symptoms such as epistaxis, extensive bruising, gingival, and gastrointestinal bleeding (Pham and Wang, 2007; Berndt and Andrews, 2011). The severity of bleeding symptoms in BSS can range from mild to life-threatening and may vary with age (Pham and Wang, 2007; Andrews and Berndt, 2013).

Platelet disorders that affect aggregation

Disorders that affect the ability of platelets to aggregate impair platelet-platelet interactions (Bavry, 2013; Cattaneo, 2013b). The causes of aggregation defects include severe plasma fibrinogen deficiency or severe deficiency or dysfunction of the platelet membrane GP$\alpha_{IIb}\beta_3$ complex, which binds fibrinogen when platelets are activated at low shear, to bridge adjacent platelets so that they form an aggregate (Inoue, Suzuki-Inoue and Ozaki, 2008; Cattaneo, 2013b). In cases of dysfunctional aggregation, disruptive mutations in $\alpha_{IIb}\beta_3$ (or a lack of fibrinogen) alter the platelets ability to bind fibrinogen, preventing stable platelet aggregation (Inoue et al., 2008; Cattaneo, 2013b). Severe GP$\alpha_{IIb}\beta_3$ deficiency or dysfunction is an autosomal recessive disorder known as Glanzmann thrombasthenia (GT) (Nurden et al., 2011b). Mutations that cause GT are more prevalent among certain ethnic groups (e.g., Iraqi Jews, Palestinian Arabs, French Gypsies) (Nurden et al., 2011b). GT impairs platelet aggregation responses to all agonists although ristocetin-induced platelet agglutination is still present (Harrison et al., 2011; Cattaneo, 2013b). As in BSS, patients with GT present early in life (typically in infancy or before the age of 5) (Cattaneo, 2013b; Rao, 2013a) with mucocutaneous bleeding symptoms (Nurden et al., 2011b) that can range from minimal bruising to potentially fatal hemorrhages (George et al., 1990). Bleeding risk is increased during surgical and dental procedures as well as during childbirth (George et al., 1990).

Platelet disorders that alter fibrinolysis

Clot lysis is important to allows normal blood flow to proceed once the injury has healed (Mutch, 2013). Accelerated clot lysis can result in bleeding complications due to a premature dissolution of the clot (Mutch, 2013). Quebec Platelet Disorder (QPD) is a unique platelet function disorder that affects clot lysis (Hayward et al., 1996; Diamandis et al., 2009; Paterson et al., 2010; Cattaneo, 2013b). QPD is a unique autosomal dominant disorder that results in a gain of function defect in fibrinolysis, due to an increase in the platelet stores of urokinase-type plasminogen activator (uPA). The disorder is cause by a tandem duplication mutation that

includes *PLAU*, the uPA gene (Paterson et al., 2010; Rao, 2013a). In contrast to the immediate bleeding that is typical of platelet disorders, QPD bleeding is delayed in onset after hemostatic challenges, in keeping with a defect in clot lysis(McKay et al., 2004). A standardized bleeding history assessment tool has been used to assess the bleeding risks for QPD, through comparisons of symptoms reported by affected and unaffected offspring of subjects with QPD (McKay et al., 2004). QPD was associated with higher mean bleeding scores ($P < .0001$) and a much higher likelihood of having bleeding that led to lifestyle changes, bruises that spread lower or as large or larger than an orange or both, joint bleeds, hematuria (OR 7.7), bleeding longer than 24 hours after dental extractions (OR 176) or deep cuts (OR 37), and received or been recommended other treatments (fibrinolytic inhibitors) for bleeding (OR 293) (McKay et al., 2004).

Platelet disorders that influence platelet number

Thrombocytopenia is the medical term for reduced platelet numbers (Lambert and Poncz, 2013). Most often (95%), cases of thrombocytopenia are acquired from drug interference or autoimmune disease (e.g. in the case of immune thrombocytopenia purpura (ITP)) however, some inherited disorders also cause thrombocytopenia (Lambert and Poncz, 2013). In some inherited disorders of platelet numbers, platelet function and morphology (e.g. platelet size) may also be altered (Lambert and Poncz, 2013). Bleeding symptoms associated with thrombocytopenia include superficial bruises, petechiae, and mucosal bleeding, epistaxis, gastrointestinal hemorrhages, and bleeding with tooth brushing (Lambert and Poncz, 2013).

Platelet disorders that affect secretion

Platelet secretion defects are a heterogenous but common type of platelet function disorder that impairs the ability of platelets to release their storage granule contents in response to agonist stimulation, particularly with weak agonists (Cattaneo et al., 2009; Pai et al., 2011; Watson et al., 2013; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Since there are many essential components in the platelet secretion process, there are many potential causes of these disorders

(Bunimov et al., 2013; Cattaneo, 2013b). The prevalence of these defects is currently unknown (Hayward, 2011; Rao, 2013b). Typical findings include abnormal aggregation responses, impaired dense granule release upon agonist stimulation (Nurden et al., 2012; Rao, 2013a).

Familial haemophagocytic lymphohistiocytosis (FHL) is a more recently described disorder with a bleeding predisposition linked to a disruption in in the mechanisms of dense granule secretion rather than the production of storage granules (zur Stadt et al., 2005). The disorder is a rare autosomal dominant auto-immune disease that also manifests as low red blood cell and platelet count with normal granule cargo amounts but abolished dense and α granule secretion (Ye et al., 2012; Golebiewska and Poole, 2015). It has been postulated that the low survival rate may account for a lack of bleeding symptoms in these patients (Ren et al., 2010).

Procoagulant function

Isolated defects in procoagulant function are rare (Rao, 2013a). Impaired platelet procoagulant function presents as an inability of platelets to support the enzymatic reactions in the coagulation system (Rao, 2013a). Scott syndrome is an inherited disorder in platelet procoagulant function associated with a decreased exposure of anionic phospholipids on the activated platelet surface (Cattaneo, 2013b; Rao, 2013a). Patients with Scott syndrome demonstrate reduced thrombin generation and defective wound healing (Cattaneo, 2013b). Mutations in a membrane-associated scramblase (TMEM16F) have been associated with inheritance of Scott syndrome (Rao, 2013a).

Signalling molecule defects

Platelet signalling defects represents a heterogenous group of disorders characterized by impairment in signal amplification or other processes involved in intracellular messages (Rao, 2013a). They may be caused by qualitative or quantitative defects in intracellular signalling molecules (e.g. PLC), GTP-binding proteins (α, β and γ subunits) or enzymes involved in TxA_2 production (e.g. COX-1 or thromboxane synthetase) (Cattaneo,

2013b; Rao, 2013b). These defects can present as an impairment in platelet aggregation and secretion with normal agonist receptors, storage granules and platelet morphology (Rao, 2013a).

Platelet disorders that alter storage granules

Defects in platelet storage granules a heterogeneous group of disorders that include deficiencies of the dense- and/or α granules, or their constituents (dense-, α,dense-, and α-storage pool deficiency) and other, less common defects of the α granules (Cattaneo, 2013b).A number of platelet disorders have been described that affect dense granules. Hermansky-Pudlak Syndrome (HPS) is a defect affecting platelet secretion that is caused by a disruption in granule biogenesis (Nurden et al., 2012; Golebiewska and Poole, 2015). Hallmarks of this disorder include: absent or impaired secondary platelet aggregation with weak agonists (e.g., epinephrine and ADP), oculocutaneous albinism and an associated lysosomal storage disease (Neunert and Journeycake, 2007). Bleeding symptoms are usually mild to moderate and manifest as spontaneous bruising, epistaxis, menorrhagia and prolonged bleeding after trauma or dental procedures (Golebiewska and Poole, 2015). Chediak–Higashi syndrome (CHS) is another dense granule storage defect with similar features but a much poorer prognosis than HPS (Nurden et al., 2012). Similarly, patients with CHS lack storage granules, present with bleeding and decreased pigmentation but, also are also accompanied by severe immunologic and neurological defects (Golebiewska and Poole, 2015). Mutations in genes known to be important in platelet granule production have been identified in platelet disorders where granules are affected (Bunimov et al., 2013; Cattaneo, 2013b; Westbury et al., 2015; Leo et al., 2015). Characterized disorders like HPS have been extensively studied and have revealed that HPS can result from mutations in *HPS1* to *HPS6*, *DTNBP1, BLOC1S3, and BLOC1S6* (*PLDN*) (Lentaigne et al., 2016).

In North America, electron microscopy (EM) is the predominant method for diagnosing dense granule deficiency (Hayward et al., 2012b). EM is prepared using whole mount preparations of PRP mounted on a nickel grid (Hayward et al., 2012b). Dense granules are then visualised and counted to determine if there is an

abnormal number of granules per platelet present as well as the biochemical properties of the contents of the granules. However, surveys indicate that EM analysis is not performed by most diagnostic laboratories (Gresele et al., 2014).

Platelet disorders caused by transcription factor mutations

Some platelet disorders involve mutations in genes encoding transcription factors important for hematopoiesis and megakaryopoiesis including: GATA-binding factor1 (*GATA1*), friend leukemia integration 1 (*FLI1*) and runt-related transcription factor 1 (*RUNX1*). *GATA1* (which binds the DNA sequence GATA) is regulates megakaryocyte and erythroid development (Songdej and Rao, 2015) and interacts with *RUNX1* and *FLI1* during megakaryopoiesis (Pimkin et al., 2014). Platelet defects due to inherited mutations in *GATA1* manifest with incomplete megakaryocyte maturation, thrombocytopenia and impaired aggregation responses to ristocetin and collagen (Hughan et al., 2005). *FLI1* is another transcription factor that influence megakaryocyte gene expression (including *ITGA2B, GP1BA, GP9* and *c-MPL* (Songdej and Rao, 2015)), and is mutated (deleted along with the rest of the long arm of chromosome 11) in Paris-Trousseau syndrome, which is characterized by macrothrombocytopenia, giant α granules and impaired granule release in response to thrombin (Songdej and Rao, 2015). *RUNX1*, which encodes a subunit of the polyomavirus enhancer-binding protein or core-binding factor transcriptional regulation complex (PEBP2/CBF) (Mikhail et al., 2006), works cooperatively with *FLI1* to influence gene expression in late stages of megakaryopoiesis (Huang et al., 2009; Tijssen et al., 2011). Abnormalities in *RUNX1* have been associated with familial predisposition to acute myeloid leukemia (AML) which may also present with thrombocytopenia (low platelet count), abnormal bleeding and platelet secretion defects (Rao, 2013b; Stockley et al., 2013; Leo et al., 2015; Bianchi et al., 2016). Most individuals with *RUNX1* defects have mild to moderate thrombocytopenia, impaired platelet function and a moderate bleeding tendency although some have normal platelet counts and minimal bleeding (Owen et al., 2008; Liew and Owen, 2011; Yoshimi et al., 2016).

It has been postulated that transcription factor mutations are more frequent causes of platelet function disorders than was previously appreciated. A recent article from the UK Genotyping and Phenotyping of Platelets (GAPP) study identified 6 transcription factor mutations (2 involving *RUNX1* and 4 involving *FLI1*) in 11 subjects (6 families each with a unique mutation) out of 13 index cases evaluated with impaired dense granule secretion or aggregation defects (Stockley et al., 2013). The prevalence of transcription factor mutations among subjects with unexplained platelet function disorders deserves further investigation.

1.3. BLEEDING ASSESSMENT TOOLS

1.3.1. Bleeding history and platelet disorders

The first step recommended in the diagnosis of a bleeding disorder is a careful clinical evaluation of the patient's medical history (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). To clinicians and researchers, the subject's medical history provides of the extent of dysfunction related to symptoms and may lead to insights into their potential relationship to the molecular causes. Related symptoms across groups of patients can indicate commonalities in pathology and may suggest a shared cause. In a clinical evaluation, it is important that not only a personal history, but also a family history be recorded as symptoms and traits shared within a family may indicate what symptoms a patient may expect to encounter, given the inheritance of the associated genes.

A history of excessive bleeding is a hallmark of a platelet function disorder (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Bleeding may occur in a variety of sites, vary in magnitude, frequency and can reflect a variety of contributing factors (Tosetto, Castaman and Rodeghiero, 2013), and some have suggested bleeding be classified as: 1) trivial 2) minor and 3) major. Trivial (or non-relevant) bleeding is a common occurrence and is considered a normal part of everyday life (Tosetto et al., 2013). An example of a trivial bleed would be bleeding from a minor wound or gum bleeds after brushing or flossing teeth, which do not often interfere with everyday life and do not require additional

medical intervention to cease (Tosetto et al., 2013). Minor bleeds, in contrast, do interfere with everyday life and require medical intervention to cease (Tosetto et al., 2013). An excessive nosebleed that requires cauterization to control would be considered an example of a minor bleeding event. Major bleeds may cause serious or permanent injury and are sometimes life threatening (Tosetto et al., 2013). They are site specific, can affect critical organs (e.g., brain), and require medical intervention, including transfusion of blood products, to manage.

One of the of the most challenging tasks for clinicians is the discrimination between normal and pathologic bleeding (Tosetto et al., 2013). Normal bleeds can be the result of a challenge and resolve with or without medical intervention (Tosetto et al., 2013; Elbatarny et al., 2014). In contrast, pathologic bleeding may become a serious concern and can often be attributed to an underlying cause (acquired or inherited) (Rodeghiero et al., 2005; O'Brien, 2012).

There is some debate about the influence of certain factors to the probability of experiencing bleeding events. Some studies suggest that bleeding increases exponentially with age (due to more exposures to challenges) and occurs more frequently in females (Tosetto et al., 2013; Miller et al., 2014). Alternatively, some studies argue that the incidence of bleeds does not increase with age, but rather, the ability to recall bleeding events that occurred in the past decreases (Mauer et al., 2011). In addition, once sex specific questions such as incidence of menorrhagia or post-pregnancy bleeding are removed, sex was found to have little influence on bleeding history (Mauer et al., 2011). Diet has also been shown to alter the occurrence of bleeding events and many compounds have been identified to alter platelet function (McEwen, 2014; Miller et al., 2014). Some bleeding within the general population has been attributed to antiplatelet or anticoagulant drugs (Filippi et al., 2011; Tosetto et al., 2013).

In the interests of providing meaningful comparison between normal and pathologic bleeding, there is a need for a detailed and accurate tool to document and assess clinically relevant bleeding. Such tools must be

capable of capturing a wide clinical spectrum of bleeding events as well as associated symptoms but also concise enough to be applied in a clinical or research setting. Standardization of these tools allows a consistent and accurate comparison between related conditions and may aid clinicians and researchers in identifying patterns to ultimately gain insight into possible causes. To date, many bleeding assessment tools have been used to document bleeding symptoms for a variety of bleeding disorders (McKay et al., 2004; Tosetto et al., 2006; Bowman et al., 2009; Rodeghiero et al., 2010; Fogarty et al., 2012).

1.3.2. Bleeding history assessment tools

The use of questionnaires has proven a useful tool in the documentation of clinical symptoms associated with a patient's bleeding history (Hayward, 2011). One of the first attempts at documenting bleeding history to individuals with and without a bleeding disorder (Srámek et al., 1995) identified bleeding problems after a trauma or intervention, or having a positive family history were informative but only outside of a referral setting as individuals referred for bleeding assessments had a high pretest probability of a bleeding disorder (Srámek et al., 1995). Since then, many iterations of bleeding assessment tools have been created with the hope of improving BAT diagnostic accuracy. However, even the more widely used tools have not been validated for use in diagnosing platelet function disorders amongst individual referred to a hematologist for a bleeding disorder assessment (Hayward, 2011). Nonetheless, bleeding assessment tools have proven useful to clinicians and researchers that wish to document bleeding histories in a standardized and comparable manner (Rydz and James, 2012; Cattaneo, 2013b).

ISTH BAT

In 2010, a Working Party sponsored by the VWF and Perinatal/Pediatric Hemostasis subcommittees of the Institute of Thrombosis and Hemostasis (ISTH), in collaboration with Women's Health Issues in Thrombosis and Hemostasis SSC, published the ISTH BAT (Rodeghiero et al., 2010). The ISTH BAT used

items from the Vincenza BAT to detect type 1 VWD (Rodeghiero et al., 2005), with modifications to reduce the time to administer the tool (Tosetto et al., 2006) and to incorporate sex specific (Philipp et al., 2008) and pediatric (Biss et al., 2010) questions.

The ISTH BAT questionnaire was designed to be administered by a physician or other trained health professional and takes around 20 minutes to complete (Rodeghiero et al., 2010; Rydz and James, 2012). It scores 14 categories of bleeding (including epistaxis, bruising, joint and muscle bleeds as well as bleeds after interventions such as surgery and dental procedures). Each these categories are scored from 0 (for trivial bleeds) to 4 (severe bleeds), based on the most severe bleeding event in each category. The scoring evaluated only symptoms and bleeding treatments, if any, that occurred before and/or at the time of diagnosis of a bleeding disorder. Scores are then tabulated across all categories to generate an overall bleeding score to estimate the overall burden of clinical bleeding symptoms experienced by an individual.

The ISTH BAT has been applied to a variety of populations that include normal healthy controls and persons with VWD and inherited platelet disorders (Tosetto et al., 2006; Rodeghiero et al., 2010; Elbatarny et al., 2014). The overall bleeding score has been suggested as a simple predictor of clinical outcomes of VWD and may be helpful to identify patients needing intensive therapeutic regimens (Federici et al., 2014). The normal range of ISTH BAT scores range from 0-4 for adult males, 0-6 fir adult females and 0-3 for children (Elbatarny et al., 2014). When the gender-specific symptoms of menorrhagia and postpartum bleeding are excluded, the normal range of the ISTH BAT score is the same for females and males (Elbatarny et al., 2014). One study found that individuals with VWD and a BS of >10 had the highest incidence of mucosal and non-mucosal bleeding (Tosetto et al., 2011). The researchers later identified that a BS >10 could predict which patient had bleeding events severe enough to require treatment with DDAVP and/or concentrates in large cohort of patients with VWD (Federici et al., 2014). A UK study of a cohort with inherited platelet function

disorders reported that affected individuals had a median ISTH bleeding score of 12 with an interquartile range [IQR] 8–16 (Lowe, Lordkipanidzé and Watson, 2013).

The ISTH BAT has proven useful to investigate a number of inherited bleeding disorder as it provides standardized information but it has some drawbacks (Rydz and James, 2012). The ISTH BAT scores each category based solely on the single worst bleeding episode in that category and the overall frequency of bleeding events is not recorded (Rydz and James, 2012). Furthermore, overlap has been noted between affected and unaffected subjects which the diagnostic useful of the ISTH BAT (Quiroga and Mezzano, 2012; Rydz and James, 2012).

Bleeding risks and an estimation of odds ratios

In 2004, a study was published that described the bleeding risks for individuals with QPD (McKay et al., 2004), by comparing the responses of affected and unaffected family members. Symptoms that were significantly different between the groups were then used to generate a summative bleeding symptom scores for each subject. The study identified that inheritance of QPD is associated with an increased likelihood (determined as an odds ratio) of experiencing a variety of bleeding symptoms including: bruising, minor wounds, excessive challenge related bleeding as well as spontaneous hematuria and joint bleeds (McKay et al., 2004). The estimation of bleeding risks as odds ratios allows each specific bleeding symptom to be compared between affected and unaffected subjects, instead of doing a general grouping of symptoms with assigned quantitative severities. Additionally, the odds ratio method takes into consideration that some general population controls experience some bleeding symptoms and it quantitatively estimates how much more likely that an affected subject will experience a symptom/problem. For example, QPD was identified to be associated with an increased risk of bleeding from surgical or dental procedures (unless treatments were given) but not with an increased risk of bleeding from uncomplicated childbirth (McKay et al., 2004). The observations served as a basis for recommending prophylaxis treatment (with fibrinolytic inhibitor drugs) for persons with QPD

undergoing surgical or dental procedures but not for uncomplicated childbirth. The estimates of bleeding risks are also easy to communicate to physicians, researchers and patients.

Recently, another BAT questionnaire was designed for determining bleeding risks for inherited platelet disorders called CHAT-P and I used this tool for part of my thesis project work.

1.4. IMPORTANT UNANSWERED QUESTIONS

This section outlines the question, hypothesis and specific objectives generated based on the background presented in the introduction, to study these aspects for common inherited platelet function disorders.

1.4.1. **Aim 1 - Diagnostic usefulness of platelet dense granule ATP release assays**

As uncharacterized inherited bleeding disorders are commonly found among individuals referred to a hematologist for abnormal bleeding, this has raised questions about how much testing is required to diagnose or exclude a platelet function disorder, particularly the forms that have an unknown molecular cause (Quiroga et al., 2007; Pai et al., 2011). Recent guidelines recommended a quantitative analysis of platelet dense granule release as part of the assessment for a suspected inherited platelet function disorder. However, there are concerns about these recommendations. Reduced ATP release has been associated with a bleeding disorder but that study did not evaluate if the findings were reproducible (Pai et al., 2011). Additionally, that study could have been biased as abnormal findings were considered in the criteria for which subjects had a bleeding disorder (Pai et al., 2011). Accordingly, there is a need to address whether reproducibly abnormal ATP release is predictive of a bleeding disorder.

At the present time, laboratories lack evidence-based guidelines to direct the performance, interpretation and reporting of platelet dense granule release tests. As the amount of platelet dense granule ATP release measured by lumi-aggregometry shows considerable variability amongst healthy control subjects (range of coefficients of variation [CV], for different agonists: 4.1% to 29.9%), (Pai et al., 2011; Miller et al., 2014), we considered that individual results might also be variable. Additionally, concerns have been raised about using tests with that have a CV above 20% for diagnostic purposes (Reed, Lynn and Meade, 2002).

Based on this knowledge, the following hypothesis was generated:

Dense granule ATP release measured using a lumiaggregometer provides useful diagnostic information on platelet function that can be used to help decide if an individual has a bleeding disorder.

To test this hypothesis, the specific objectives of this study were:

1. Evaluate the consistency of dense granule ATP release between tests by assessing between test findings in referred individuals who had multiple tests performed.

2. Evaluate consistency of test diagnosis by identifying proportion of subjects that showed normal, abnormal or inconsistent dense granule ATP release with individual agonists on multiple tests and the percentages of evaluated individual that had non-diagnostic, inconsistent and consistently abnormal findings.

3. Determine the finding of consistently abnormal platelet dense granule ATP release is associated with the presence of a bleeding disorder, using odds ratios and 95% confidence intervals.

1.3.3. Aim 2 - Phenotypic evaluation of common platelet disorders

The majority of individuals with inherited platelet function defects do not have a known underlying molecular mechanism to explain their increased predisposition to bleed (Rao, 2013a). In the last decade since the proliferation of next-generation sequencing technologies, much work has been done to identify genes associated with proper platelet production and function (Bunimov et al., 2013; Stockley et al., 2013). These discoveries provide an insight into possible genetic causes that could explain the underlying molecular defects in some of these cases.

In addition, it is recommended that in the diagnosis of a number of mild bleeding disorders, including platelet function disorders, a patient history of various bleeding symptoms be assessed (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). A

number of attempts have been made to standardize bleeding histories in an effort to improve their diagnostic accuracy as well as their ability to accurately capture the burden of bleeding symptoms experienced. The ISTH BAT is a recently developed tool designed to accurately record bleeding symptoms in hemorrhagic disorders in attempts to aid in the diagnosis of a possible bleeding disorder (Rodeghiero et al., 2010). It has been widely adopted for describing bleeding symptoms in affected populations (Lowe et al., 2013; Elbatarny et al., 2014; Kaur et al., 2016; Rashid et al., 2016). Previous studies using the ISTH BAT identified that common inherited platelet disorders are associated with epistaxis, cutaneous bleeding, menorrhagia and bleeding related to surgery (Lowe et al., 2013). However, because the questionnaire assigns a severity ranking to groups of symptoms (Rydz and James, 2012), it is not suitable to assess specific risks associated with an inherited platelet disorder.

In the past, a detailed questionnaire was developed to evaluate a diverse range of bleeding symptoms and it was applied to determine bleeding risks associated with Quebec Platelet Disorder. Since then, subsequent iterations have been used to identify bleeding symptoms in a variety of platelet function disorders (unpublished work). Features that are unique to this questionnaire include: i) platelet disorder specific symptoms, ii) the ability to be administered to the individual directly before review by a hematologist, iii) free text fields for elaboration and clarification of certain symptoms, iv) questions about family history, v) information about bleeding challenges before and after treatment.

Based on this knowledge, the following hypothesis was generated:

Uncharacterized inherited bleeding disorders that impair platelet aggregation responses to multiple agonists or have decrease dense granule numbers are associated with increased bleeding risk. These disorders are caused by mutations in genes that encode proteins that are important for platelet function.

To test this hypothesis, the specific objectives of this study were:

1. Determine the phenotype in a large family with an uncharacterized platelet disorder by:

a. Identifying the range of bleeding scores using the ISTH BAT.

b. Generating family specific bleeding risks for inheriting a platelet function disorder using the platelet CHAT to compare affected individuals to general population controls using OR and 95% confidence intervals.

c. Evaluate the type of abnormalities in platelet number, size, morphology and function that were identified in a cohort of individuals with suspected platelet function disorders due to uncharacterized cause.

d. Evaluate the molecular cause in selected families by using Sanger sequencing confirm potentially disease-causing mutations, identified through NGS, and how they might alter the gene product.

2. Determine the phenotype of families and sporadic cases with and uncharacterized inherited platelet disorders by:

a. Quantifying the bleeding scores of affected and unaffected subjects using the ISTH BAT.

b. Determining the bleeding risks for uncharacterized platelet function disorders, using platelet CHAT data with comparison of affected individuals to general population controls to determine OR and 95% confidence intervals.

c. Describe abnormalities in platelet phenotypes for subjects with confirmed platelet aggregation defects and/or dense granule deficiency due to an uncharacterized disorder.

CHAPTER 2

METHODS

The first aim was performed in accordance with the Hamilton Integrated Research Ethics Board requirements, which did not require informed consent for validating or evaluating clinical diagnostic assays performed by the Hamilton Regional Laboratory Program (HRLMP). The second aim was conducted with approval from the Hamilton Integrated Research Ethics Board, in accordance with the recently revised Declaration of Helsinki on human subject research. All subjects in the second aim provided written informed consent.

2.1. SUBJECTS

2.1.1. Subject cohorts evaluated for aim 1 studies

Two cohorts were evaluated for aim 1. Cohort I was comprised of all subjects tested at least twice for platelet dense granule ATP release abnormalities between January 2007 and June 2013. All subjects had been evaluated by a hematologist at Hamilton Health Sciences and had been tested for a bleeding disorder central, by the Hamilton Regional Laboratory Program. Cohort II were individuals, and their affected and unaffected relatives, who had been seen by one of four different hematologists at Hamilton Health Sciences and were included if they had been tested at least twice by this assay prior to September 2015. Each subject in cohort II provided written informed consent to participate in the study to evaluate uncharacterized platelet function disorders and 10 were included in aim 2.

2.1.2. Subject cohorts evaluated for aim 2 studies

Aim 2 involved three groups of subjects (defined below): 1) affected individuals, 2) unaffected family members of affected individuals and 3) general population controls.

Affected subjects

Subjects were recruited from a Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders by Dr. Hayward. Inclusion criteria for index cases were:

1. Bleeding problems compatible with an inherited platelet disorder, based on the opinion of the individuals' hematologist(s), obtained by medical record review
2. One or more of the following platelet function abnormalities:
    a. Impaired maximal aggregation responses to ≥2 agonists by light transmittance aggregation (LTA), not due to a well-characterized disorder (e.g., Glanzmann thrombasthenia, Bernard Soulier syndrome). LTA abnormalities were confirmed on another sample if dense granule deficiency was excluded.
    b. Confirmed dense granule deficiency (reference interval, RI, for average number of dense granules/platelet: 4.9-10.0), estimated by whole mount electron microscopy.

Initial subject recruitment included individuals with impaired dense granule ATP release with ≥2 agonists, measured by lumi-aggregometry. Impaired release was defined as a value below the RI lower limit, with confirmation by repeat dense granule ATP tests if LTA and dense granule numbers were normal. These criteria were revised, based on the results of the first (Badin et al., 2016); subjects who had been recruited that showed only impaired dense granule ATP releases were excluded as consistently impaired release did not show an association with a bleeding disorder.

General population controls

General population controls were recruited with "no known problem with bleeding or bruising problems". 60 individuals (40 female, 20 male) were recruited with similar ages to affected subjects determined by initial study criteria.

2.2. LABORATORY DATA

2.2.1. Collection of laboratory data for subjects in aim 1 studies

Each subject was assigned an anonymous study code and blinded study data was collected from electronic health records by Jodi Seecharan and Dr. Graff. I analyzed blinded study data in a Microsoft Excel spreadsheet (Microsoft, USA).

Laboratory testing for this study was performed in accordance with HRLMP standardized operating procedures and guidelines on platelet function testing that were published prior to 2015 (Christie et al., 2008; Hayward et al., 2009b; Pai et al., 2011). All subjects completed an HRLMP drug questionnaire before blood collection and those who had taken a nonsteroidal anti-inflammatory drug (NSAID) or a thienopyridine in the past 7 days were rescheduled to limit drug interference. All subjects had dense granule ATP release evaluated using a lumi-aggreometer (Chrono-Log, Havertown, PA), in accordance with the laboratory's published method and established reference interval (RI) (Hayward, 2011). Briefly, testing was performed using 400 µl of stirred platelet-rich plasma (adjusted to 250 x 109 platelets/L with autologous platelet poor plasma), 50 µl of agonist, and 50 µl of Chrono-Lume reagent (Chrono-Log; 0.2 nmol/l luciferin-luciferase, concentration reduced to 0.16 nmol/l if results with 2 nmol/l ATP standard exceeded the measurable range).

The agonist panel used to assess dense granule ATP release included (final concentrations): 1 U/mL thrombin, 6 µM epinephrine, 5.0 µg/ml Horm collagen, 1.6 mM arachidonic acid and 1 µM thromboxane analogue U46619. Repeat determinations were done to further evaluate all ATP release responses that fell below the lower limit of the established reference interval (RI) with an agonist(s), unless the sample volume was limited.

2.2.2. Analysis of laboratory data for subjects in aim 1 studies

Results were considered abnormal for a sample if the quantity of ATP released for an agonist was below the lower limit of the RI for that agonist on the majority of determinations. Test findings for an agonist were reported as inconsistent for a sample if there were similar numbers of normal and abnormal findings after

multiple determinations. Samples from subjects with reduced dense granule ATP release with thrombin and/or collagen were evaluated for dense granule deficiency using electron microscopy to quantify the average number of dense granules per platelet using platelet whole mount preparations, as previously reported (Hayward et al., 2009a).

Results with the panel of tested agonists were categorized as follows: if dense granule ATP release was within the assay reference interval (RI) with all agonists, or with all but one agonist, the findings were considered non-diagnostic. Tests showing reduced ATP release with 2 or more agonists were categorized as abnormal. The findings for the first and second test of a subject were classified as: consistently normal if both tests were non-diagnostic; consistently abnormal if both tests showed reduced ATP release with two or more agonists; and inconsistent if one test showed reduced ATP release with two or more agonists and the other test was non-diagnostic. Data for third and fourth tests was reviewed but not analyzed because few subjects had data for three or more tests. Additionally, the reported interpretative comments for each subject's tests were reviewed to determine if comments had been made about possible pre-analytical or analytical interferences (e.g., if it was noted that: the individual was a taking a drug or dietary supplement that might interfere with platelet function, if the sample collection had been difficult or if abnormalities suggested an aspirin-like defect, etc.).

2.2.3. Collection of laboratory data for aim 2 studies

Once subjects were enrolled in the study, each was assigned a unique, anonymous study code. All information was organized in a Microsoft Access database (Microsoft, USA).

Electronic health records of individual subjects were accessed to obtain: blood counts (white blood cell count, hemoglobin count, platelet count), mean platelet volumes (MPV), coagulation tests (INR, fibrinogen, APTT, thrombin time, FVIII, VWF:Ag, VWF:RCo and activity assay), blood type and findings for the

laboratory's validated platelet function assays for dense granule deficiency (Hayward et al., 2009b), LTA (Christie et al., 2008; Cattaneo, 2009) and dense granule ATP release (Pai et al., 2011). The agonist panel used to assess platelet aggregation in LTA included (final concentrations): 2.5 and 5.0 μM adenosine diphosphate (ADP), 1.25 and 5.0 μg/ml Horm collagen, 6.0 μM epinephrine, 1.6 mM arachidonic acid, 1.0 μM thromboxane analogue (U46619) and 0.5 and 1.25 mg/mL ristocetin. The agonist panel used to assess dense granule ATP release included (final concentrations): 1 U/mL thrombin, 6 μM epinephrine, 5.0 μg/ml Horm collagen, 1.6 mM arachidonic acid and 1 μM thromboxane analogue U46619. Results of bone marrow investigations, if performed, were obtained from medical records.

2.3. CONFIRMING MUTATIONS IDENTIFIED THROUGH EXOME SEQUENCING FOR AIM 2 STUDIES

In order to identify candidate mutations, DNA samples from affected subjects were prepared using methods elaborated below and submitted for exome sequencing to Dr. Guillaume Paré's laboratory, who evaluated the findings.

2.3.1. DNA isolation

5ml of blood was collected from affected and unaffected family members. Genomic DNA was isolated from EDTA anticoagulated whole blood using Qiagen QIAmp DNA blood Maxi Kit (QIAGEN, Courtaboeuf, France). Sample quality and quantity was assessed using Nanodrop 2000c (Thermo Fisher Scientific Inc., USA).

2.3.2. PCR and Sanger sequencing

The mutations identified by exome sequencing were confirmed by PCR and Sanger sequencing. PCR was performed on a Biometra thermocycler (Biometra, Göttingen, Germany) using 0.1-1 μg of template DNA, 25 μL Thermo Scientific Dream Taq PCR master mix (Thermo Fisher, Burlington, Canada) and 1 μL of 10 μM primers (Mobix, Hamilton, Canada).

Sets of forward (F) and reverse (R) primers for *RUNX1* c.583dupA mutation included: F: 5'-TCT GAG ACA TGG TCC CTGAG T -3' and R: 5'-TAT GTT CAG GCC ACC AAC CTC -3'). Sets of forward (F) and reverse R) primers for RUNX1 c.737T mutation: F: 5' AGA TGA TCA GAC AAG CCC G -3' and R: 5'-CTC CAT CGG TAC CCC TGC -3'.

After purification with MinElute PCR Purification Kits (QIAGEN, Courtaboeuf, France), and verification of purity on a Nanodrop 2000c (Thermoscientific, USA), PCR products were samples were submitted to MOBIX Lab, McMaster University (Hamilton, ON, Canada) for sequencing. DNA sequences were then visualized on sequence trace software using Finch TV (PerkinElmer, USA) and then compared to the reference *RUNX1* sequence NM_001754 (isoform AML1c).

2.4. BLEEDING HISTORY ASSESSMENT

2.4.1. Assessment of bleeding history for subjects in aim 1 studies

Subjects' charts were reviewed by two hematologists (Dr. Graff and Dr. Hayward) to classify the subject's diagnosis, as done previously (Hayward et al., 2009b), into one of three categories: no bleeding disorder (e.g., bleeding symptoms considered trivial), a definite bleeding disorder or a possible bleeding disorder (e.g., some bleeding symptoms but few significant challenges, bleeding symptoms that may or may not have reflected a technical problem with surgery or a dental procedure, symptoms such as a significant post-partum hemorrhage that may not reflect an underlying bleeding disorder). Discrepancies were resolved through consensus before entering the diagnoses into the study database.

All subjects in cohort II had bleeding scores assessed using the International Society on Thrombosis and Haemostasis bleeding assessment tool (ISTH-BAT) (Rodeghiero et al., 2010), following recommendations for defining trivial/ non-significant bleeding (Rodeghiero et al., 2010). Bleeding associated with non-bleeding disorder related disorders were scored as 0 or no/trivial (e.g. GI bleeds associated with active Crohn's disease).

2.4.2. Assessment of bleeding history for subjects in aim 2 studies

The CHAT-P questionnaire was designed by Dr. Hayward and Dr. Rivard and included questions that had previously been used to assess bleeding from QPD (McKay et al., 2004) and questions about other bleeding, including items useful for determining ISTH-BAT scores. ISTH-BAT scores were determined as outlined in section 2.4.1 except that the bleeding symptoms were initially scored based on CHAT-P collected data, for final review with a hematologist (Dr. Hayward), who reviewed medical notes and flagged any details that required clarification from subjects before determining ISTH-BAT scores or entering CHAT-P data.

The CHAT-P questionnaire contained a total of 79 questions. 39/79 questions were close ended "yes/no/not applicable", 28/79 multiple choice and 12/79 open ended questions. All subjects in Aim 2 studies completed this questionnaire. Information on affected or unaffected status was kept blinded until all data entry was complete.

The proportions responding "yes" to questions that were not dependent on the subject facing exposure to challenge, the "don't know" or "not applicable" responses were considered negative responses. For calculation of the proportions responding "yes" to questions with challenge related bleeding, subjects were excluded if they had not experienced the challenge ("not applicable").

2.5. STATISTICAL ANALYSIS

2.5.1. Statistical analysis for aim 1 studies

Differences in ATP release with individual agonists between females and males, subjects with and without dense granule deficiency or thrombocytopenia or a definite bleeding disorder were assessed by student's t-test in aim 1. Odds ratios (OR) and 95% confidence intervals (CI) were used to estimate the association between consistent test abnormalities and the likelihood of having a definite bleeding disorder compared to no bleeding disorder.

In cohort II, one-way ANOVA was used to compare mean ISTH bleeding scores of subjects with definite, possible or no bleeding disorder, and of subjects with consistently abnormal versus normal or inconsistent ATP release findings.

2.5.2. Statistical analysis for aim 2 studies

A one-way ANOVA was used to compare the overall ISTH-BAT scores between the affected individuals, unaffected relatives and general population controls. Platelet count, dense granules per platelet, aggregation and dense granule ATP release between index and non-index cases were assessed by student's t-test.

Odds ratios (OR) with 95% confidence intervals (CI) were used to estimate likelihoods for bleeding symptoms/problems (McKay et al., 2004), using CHAT-P data for affected subjects and general population controls. OR were calculated using GraphPad 6.0 (GraphPad Software, Inc., USA), after adding 0.5 to all contingency table cells that contained 0 values (Glas et al., 2003). For each subject, a sum of all reported CHAT-P symptoms with an OR>1 was calculated. P-values< 0.05 were considered statistically significant. The number of items reported by affected subjects was compared with the corresponding numbers of responses for

general population controls and unaffected relatives using ANOVA. Correlation coefficients (R^2) were also used to determine associations between CHAT-P scores and age.

CHAPTER 3

RESULTS

3.1. RESULTS FOR AIM 1

For this aim, I sought to determine if lumiaggregometer estimates of dense granule ATP release could be used to help decide if an individual has a platelet disorder. The figures and content presented in this aim are reproduced from the published article (Badin et al., 2016) with permission from the publisher, John Wiley & Sons, Inc..

Clinical laboratory data was collected from HRLMP records and was anonymized by Karen Moffatt and Jodi Seecharan. Dr. Lukas Graff performed the paired analysis of agonists and identified the proportions of subjects with consistent diagnosis for cohort I. The findings were presented at the Thrombosis and Hemostasis Societies of North America (THSNA) conference in 2014. I revised the analysis of paired agonists for cohort I and extended the analysis to subjects with consistent diagnosis to cohort II. I also analyzed relationships between ATP release findings and clinical diagnosis (for cohorts I and II) as well as ATP release findings and bleeding scores (for cohort II only). These data were presented at the THSNA conference in 2016.

3.1.1. **Demographics of subjects evaluated for platelet ATP release defects**

The demographics of subjects in aim 1 are shown below (Table 2). During recruitment, only 5 subjects with documented platelet function abnormalities were excluded: 1 declined to participate and 3 were excluded because they had characterized disorders (e.g., Quebec platelet disorder). 5% of subjects in cohort I had no clinical record available to evaluate if the subject had a bleeding disorder. Cohort II included 3 unaffected family members of index cases. The total number of unique subjects was 150 as 6 subjects were common to both cohorts. Among the 150 unique subjects, 14 (9% had been included in a previously published study on the diagnostic usefulness of platelet dense granule ATP release (Pai et al., 2011).

The time between first and second tests for all subjects ranged between 2-4016 days, with a median of 1251 days. The majority of subjects undergoing re-testing had abnormal ATP release on their first assessment (cohort I: 78%; cohort II: 83%). The majority of subjects only had data for two but dense granule ATP release assessments (combined: 130/150, 87%; cohort I: 114/133, 86%; cohort II: 19/23, 83%). 129 subjects had been tested for dense granule deficiency, and among this group, 4 were found to have reduced dense granule numbers (ranges of dense granule numbers/platelet for deficient subjects: 1.2-4.2; RI: 4.9-10.0 dense granules/platelet).

3.1.2. **Bleeding phenotype of evaluated subjects**

Among recruited subjects, the proportions in cohorts I and II that were classified as having a definite, possible or no bleeding disorder, were respectively: definite bleeding disorders: 37% and 78%; possible bleeding disorders: 47% and 13%; no bleeding disorder: 11% and 9%; no clinical record available: 5%, cohort I only (Table 2).

Analysis of cohort II data indicated that the ISTH bleeding scores were significantly higher ($p=0.01$) for the subjects that were classified as having a definite bleeding disorder (median 13, mean 12, range: 3-21) compared to those that were classified as having no bleeding disorder (median 2, mean 2, range 0-4) or a possible bleeding disorder (median/mean 3; 0-8) (Figure 1).

Table 2. Demographics of subjects in aim 1.

Cohort	Number of subjects	Female (%)	Median age in years (range)	Definite Bleeding Disorder (%)	Possible Bleeding Disorder (%)	No Bleeding Disorder (%)	Dense Granule Deficiency (%)	Low Platelet Count (%)
I	133	80	38 (5-76)	37	47	11	3	1
II	23	87	60 (10-80)	78	13	9	9	0

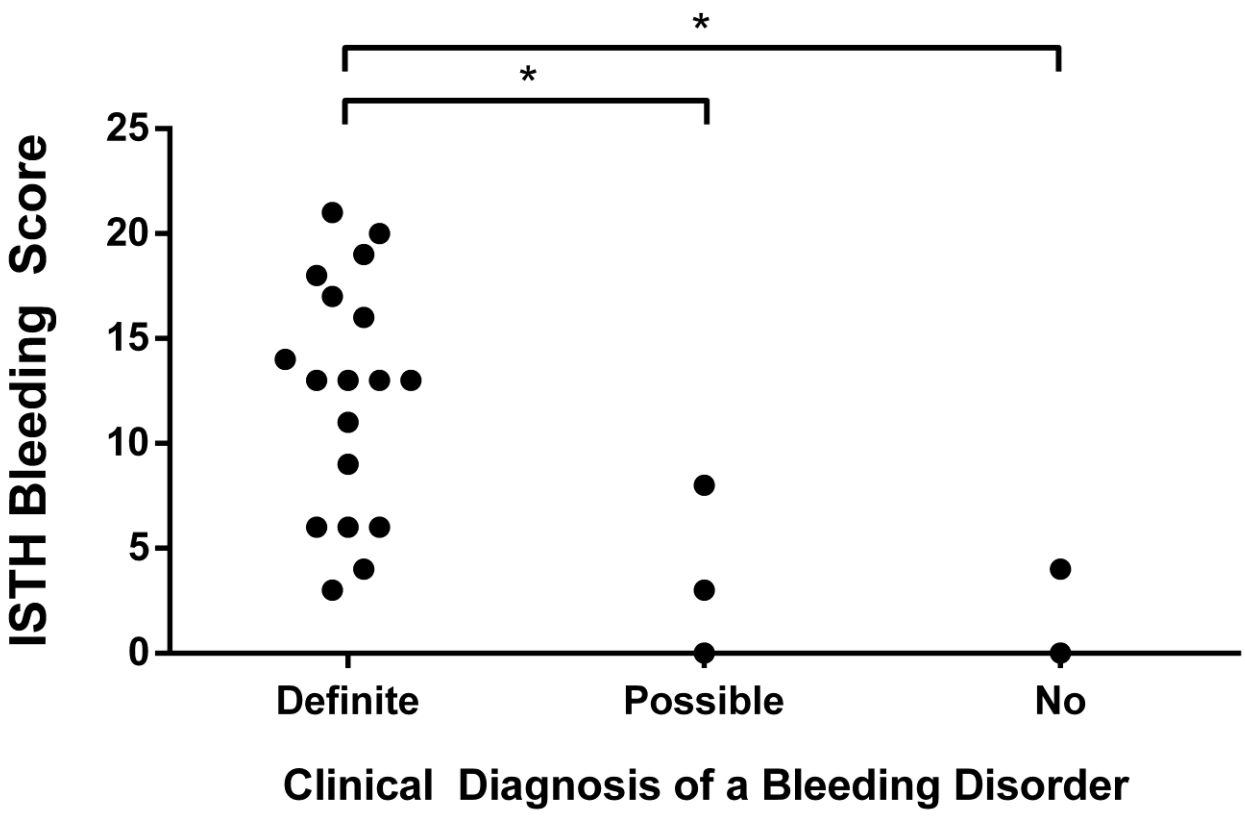


Figure 1. Bleeding scores of subjects in cohort II and their relationship to the clinical diagnosis. Bleeding scores were determined using the ISTH-BAT, for subjects that were classified as having a definite, possible or no bleeding disorder. * indicate significantly different results (p values= 0.02).

3.1.3. Inconsistent ATP release findings for single agonists between tests

Many individual subjects had inconsistent findings for dense granule ATP release with individual agonists (Figure 2 shows this data for subjects in cohort I). There were no significant differences between males and females in the mean quantity of ATP release with individual agonists (p values: 0.28-0.94).

The percentage of subjects in each cohort that showed inconsistent results for individual agonists varied from 22-32% for cohort I and from 13-35% for cohort II (Figure 3). Amongst subjects whose platelet dense granule numbers were evaluated by EM, the amount of ATP release with individual agonists did not show significant association to the numbers of dense granules/platelet (range of R^2: 0.0005-0.13).

With most agonists, there was no significant association between inconsistent ATP release findings and average dense granule numbers/platelet (p=0.06-0.57) however, with thrombin, post hoc analysis indicated that dense granule numbers (means ±SEM for both cohorts combined) were significantly different (p=0.007) between subjects with inconsistent (6.0±0.3) versus consistently normal ATP release (6.8±0.1). Only one of the four dense granule deficient subjects had reduced ATP release with all agonists on each test, while the others had inconsistent findings with thrombin and/or other agonists between tests.

Review of the interpretative comments indicated that two subjects were noted to have inconsistent findings with one or more agonists within a test, and two others had insufficient sample to confirm abnormal agonist response(s) for one of their tests. The possibility of NSAID exposure for individuals that had not recorded any recent exposures on their pre-test drug history questionnaire, had been raised in 4 subject's test interpretations, but only 2 of these individuals had inconsistent findings for agonist responses. Potential interference from other drugs or dietary supplements (clopidogrel n=1; fish oil n=1; citalopram n=1), or a difficult sample draw (n=1) was raised as a potential cause of abnormalities in 4 other subjects and 2 of these individuals had inconsistent agonist responses.

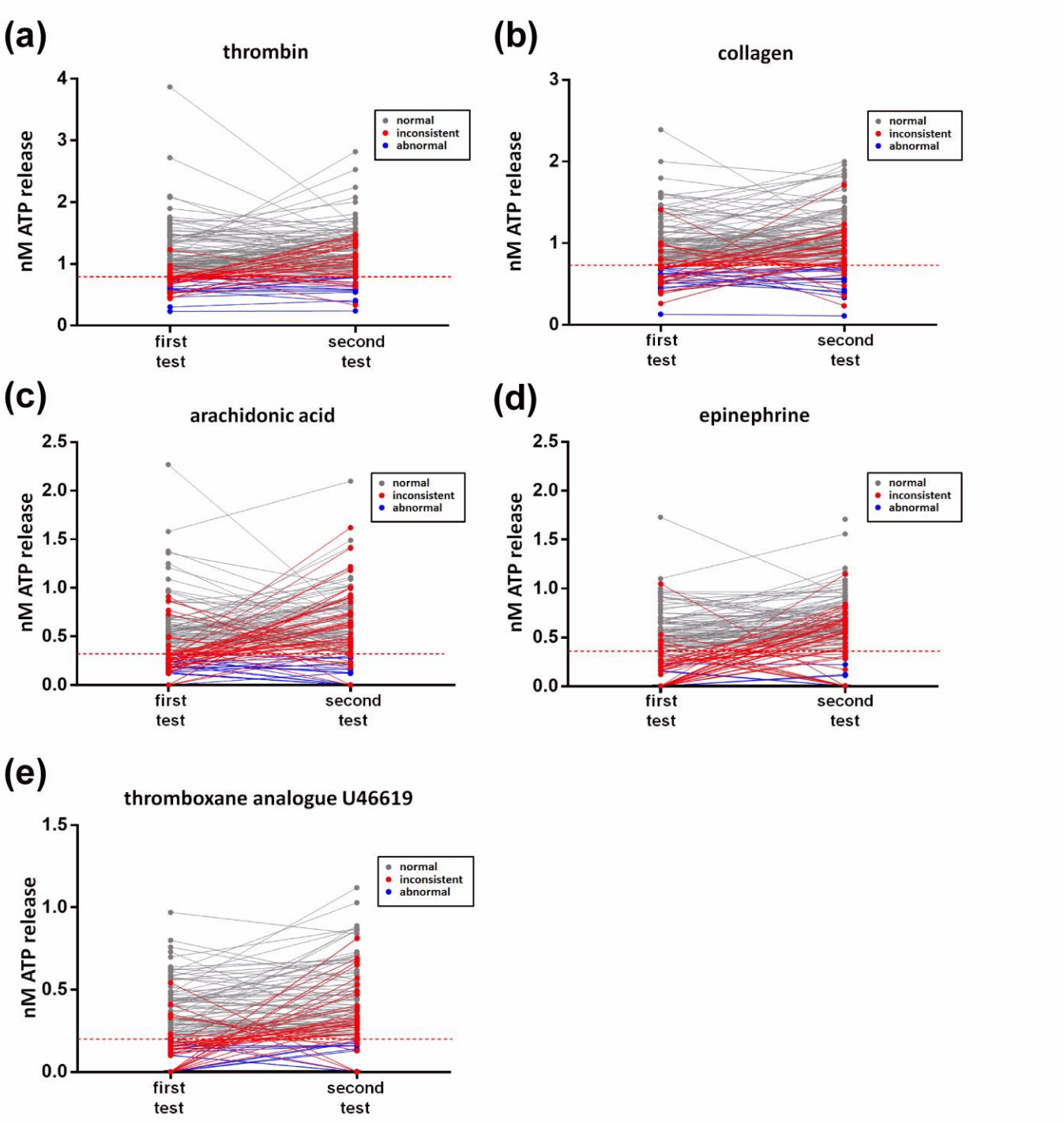


Figure 2. ATP release findings for individual agonists for subjects in cohort I. Graphs for each agonist show the nM of dense granule ATP release recorded for each subject on their first and second tests. Responses are shown for: (**a)** 1 U/mL thrombin (**b)** 5.0 µg/ml Horm collagen (**c)** 1.6 mM arachidonic acid (**d)** 6 µM epinephrine (**e)** 1 µM thromboxane analogue U46619.

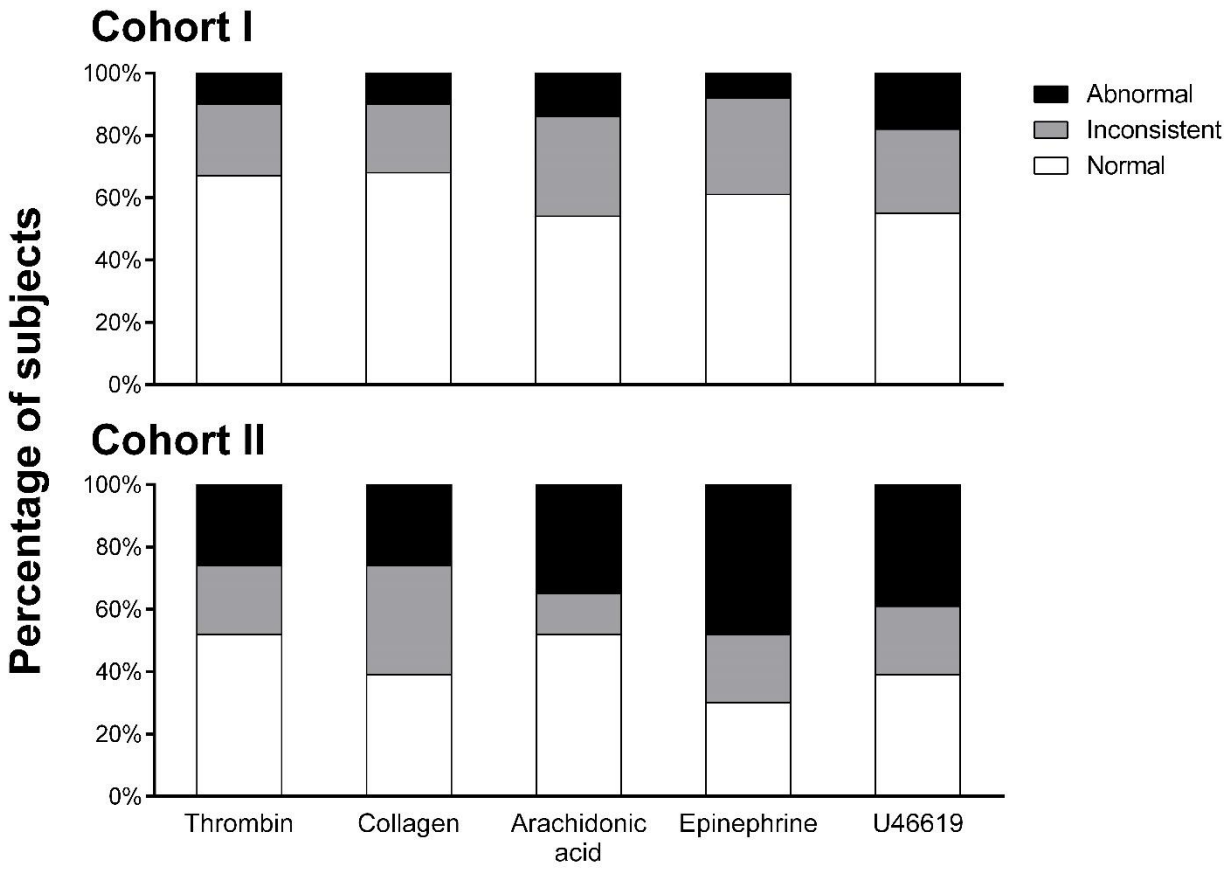


Figure 3. Proportion of subjects that showed normal, abnormal or inconsistent dense granule ATP release with each agonist on their two tests. Results are shown separately for cohort I and cohort II.

3.1.4. Inconsistency in ATP release findings for multiple agonists between tests

When responses to all agonists were considered, most subjects with normal ATP release findings on their first test had normal findings (cohort I: 83%; cohort II: 100%) or reduced release with single agonist (cohort I: 10%; cohort II: 0%) on their second test. Few subjects with normal ATP release on the first test showed multiple agonist abnormalities on the second test (cohort I; 7%; cohort II: 0%) (Figure 4A).

Many subjects with reduced release with a single agonist on their first test had normal findings on their second test (cohort I: 65%; cohort II: 50%), although some had multiple agonist abnormalities on their second test (cohort I: 14%; cohort II: 50%) (Figure 4A).

Among subjects in cohort I with impaired ATP release with multiple agonists on their first test, only 34% had multiple agonist abnormalities confirmed on the second test, as many had normal responses (46%) or only single agonist abnormalities (20%) on their second test (Figure 4A). Similarly, in cohort II, 56% of subjects that had impaired ATP release with multiple agonists on their first test showed impaired release with multiple agonists on their second test, whereas others showed normal findings (25%) or a single agonist abnormality (19%) on their second test (Figure 4A).

Consistent with the differences in findings for individual agonists between tests, there was variation in the overall findings between tests (Figure 4B). The proportion of subjects with inconsistent findings (i.e., abnormal responses with ≥2 agonists in only one test) was 36% for cohort I and 39% for cohort II. Within the subgroup of 4 subjects with reduced dense granule numbers, 2 had consistently abnormal findings whereas the others showed normal (n=1) or inconsistent (n=1) findings. No significant associations were found between subject age, or platelet count, and whether overall findings were consistently abnormal, normal or inconsistent (p values ≥0.11).

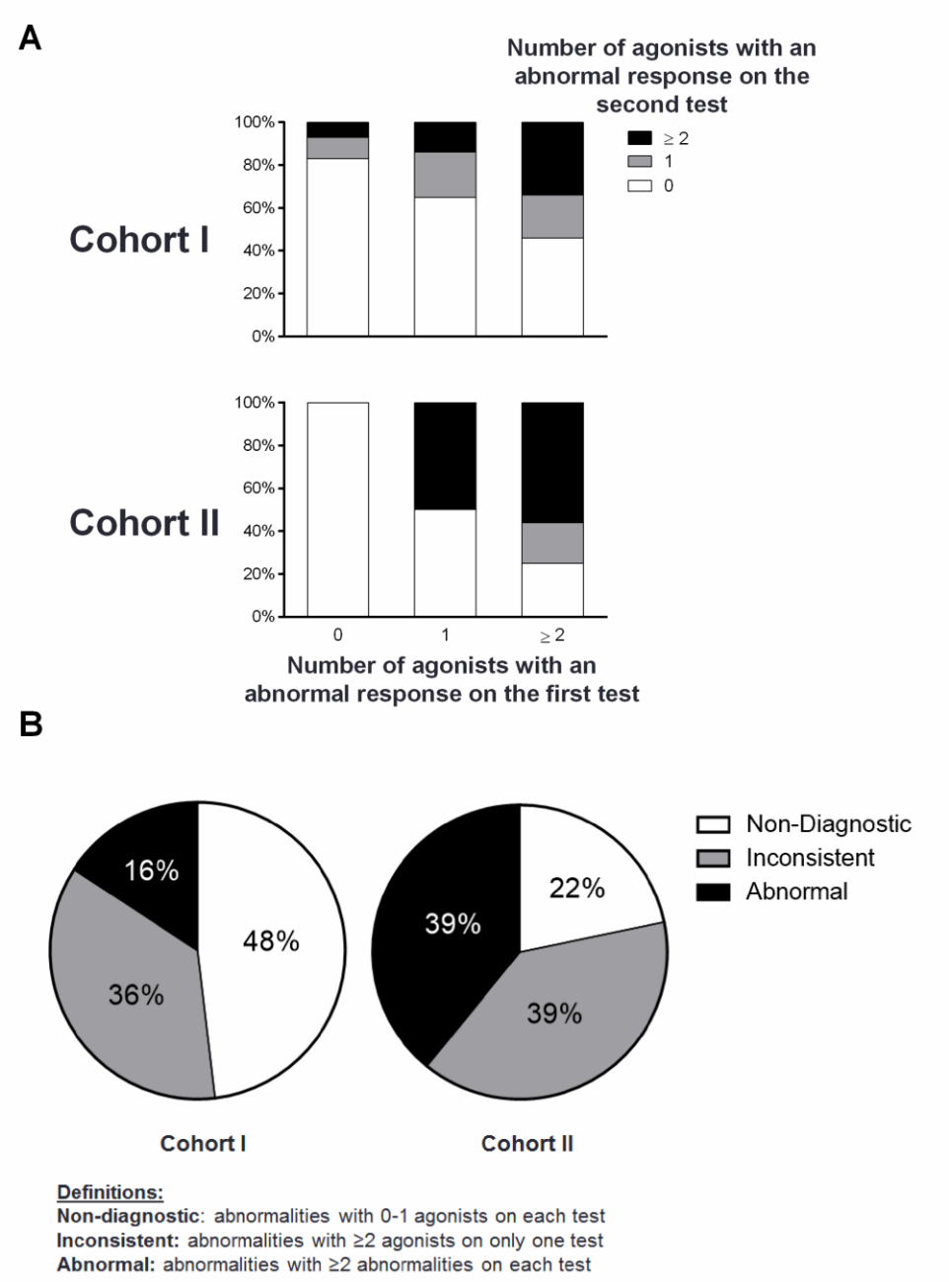


Figure 4. Inconsistencies in findings for dense granule ATP release tests. (a). Number of abnormal agonist responses observed on first and second tests for subjects in each cohort. (b). Percentages of subjects in each cohort that had non-diagnostic findings (impaired release with 0- 1 agonists on each test), inconsistent findings (reduced release with ≥2 agonists on only one test) and consistently abnormal findings (impaired release with ≥2 agonists on each test).

3.1.5. Relationships between ATP release findings, clinical diagnosis and bleeding scores

To identify associations between ATP release findings and a bleeding phenotype, consistently abnormal, inconsistent and consistently normal individuals were compared with their ISTH bleeding score (for cohort II) and their clinical diagnosis. There were no significant differences in the mean bleeding scores for subjects that had consistently normal, inconsistent or consistently abnormal ATP release findings (Figure 5A). Consistent ATP release abnormalities did not show a significant association to having a definite bleeding disorder for either cohort as the odds ratio crossed 1 (cohort I: $p=0.71$; cohort II: $p=0.50$) (Figure 5B). Accordingly, we decided that we could not use ATP release assay findings to help define which subjects had a bleeding disorder (affecteds) for the subsequent aim 2 of my thesis study.

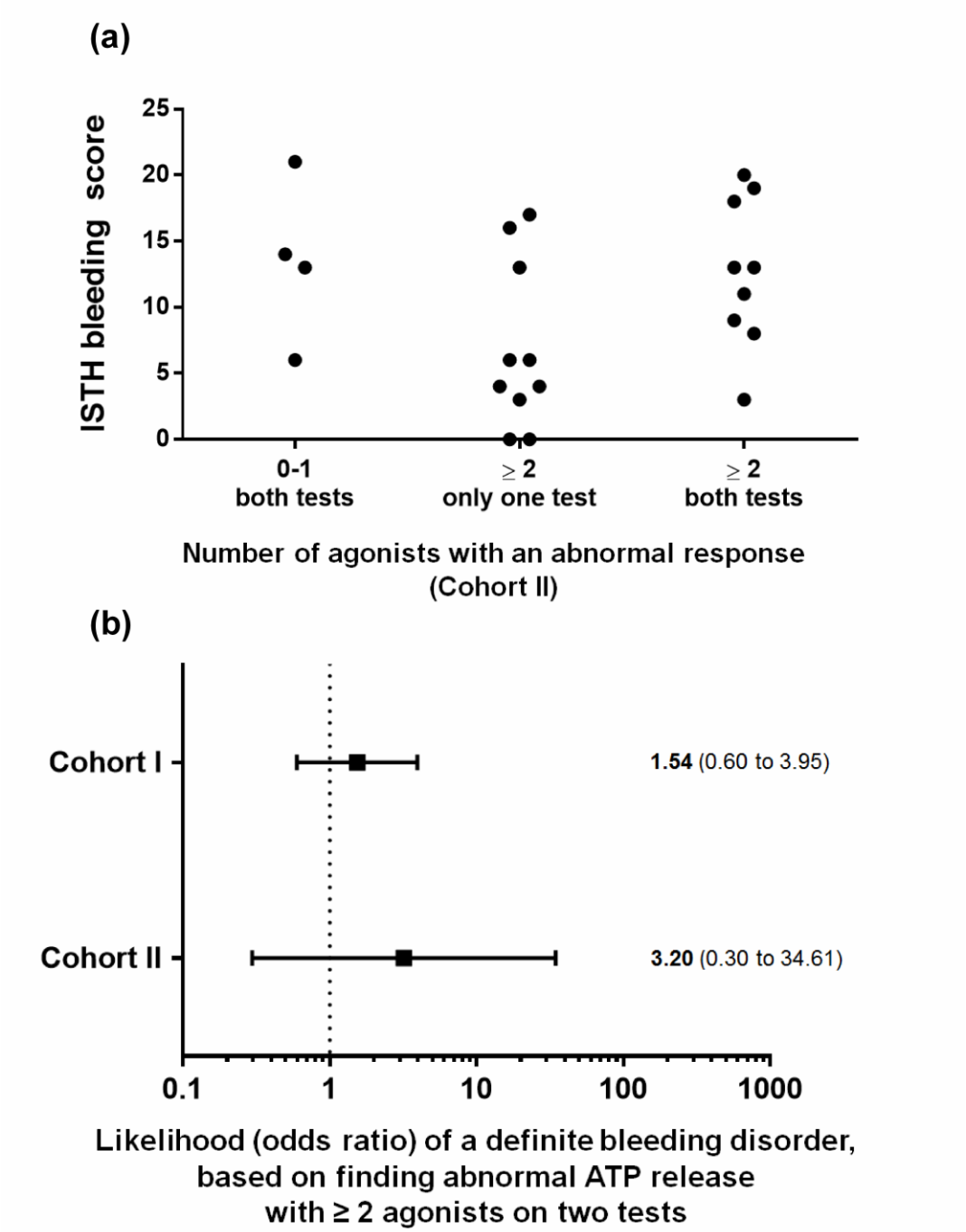


Figure 5. Clinical associations for ATP release findings. (a). Relationship between symptoms quantified by the ISTH-BAT with the number of agonist responses that were impaired in both ATP release tests. **(b).** The likelihood of a bleeding disorder, based on whether ATP release was consistently normal or consistently abnormal with multiple agonists, is shown as odds ratios, with 95% confidence intervals, for cohorts I and II.

3.2. RESULTS FOR AIM 2

For this aim, I analyzed laboratory and bleeding history data from all affected subjects in the Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders and for a family (within the study) that we identified had an inherited mutation in the hematopoietic transcription factor, *RUNX1*.

For this aim, I analyzed data for all subjects recruited to the Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders by April 2016. Dr. Guillaume Paré's laboratory identified the *RUNX1* mutations in the index case through exome sequencing. I performed the follow-up Sanger sequencing to determine if other members of the family possessed the mutations in *RUNX1*. I entered laboratory data on all subjects in the Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders with oversight from Dr. Hayward. I analyzed ISTH BAT and CHAT-P questionnaires collected by Dr. Hayward and Dr. Graff. The figures and content presented in this aim are reproduced from an article currently in press (Badin et al., in press)

3.2.1. Demographics of all affected subjects with uncharacterized platelet function disorders

In the Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders, the cohort studied before April 2016 was comprised of 29 affected subjects (11 males, 18 females; median age at recruitment: 44 years; range 3-77) from 7 families and 10 sporadic cases (index cases with no other recruited family members), 12 unaffected family members (5 males, 7 females median age at recruitment: 46 years; range: 0-69) from 5 families and 60 general population controls (20 males, 40 females; median age at recruitment: 50 years; range 14-78). 12 index cases had exome sequencing completed by April 2016 and one was identified to have mutations in a hematopoietic transcription factor, *RUNX1*.

The family of the index case with the *RUNX1* mutations is French Canadian. 8 family members in total were recruited as participants (5 males 3 females; median age at recruitment: 25.5 years; range 1-69) (Figure 6). All family members provided blood samples for DNA, platelet function testing and complete blood counts.

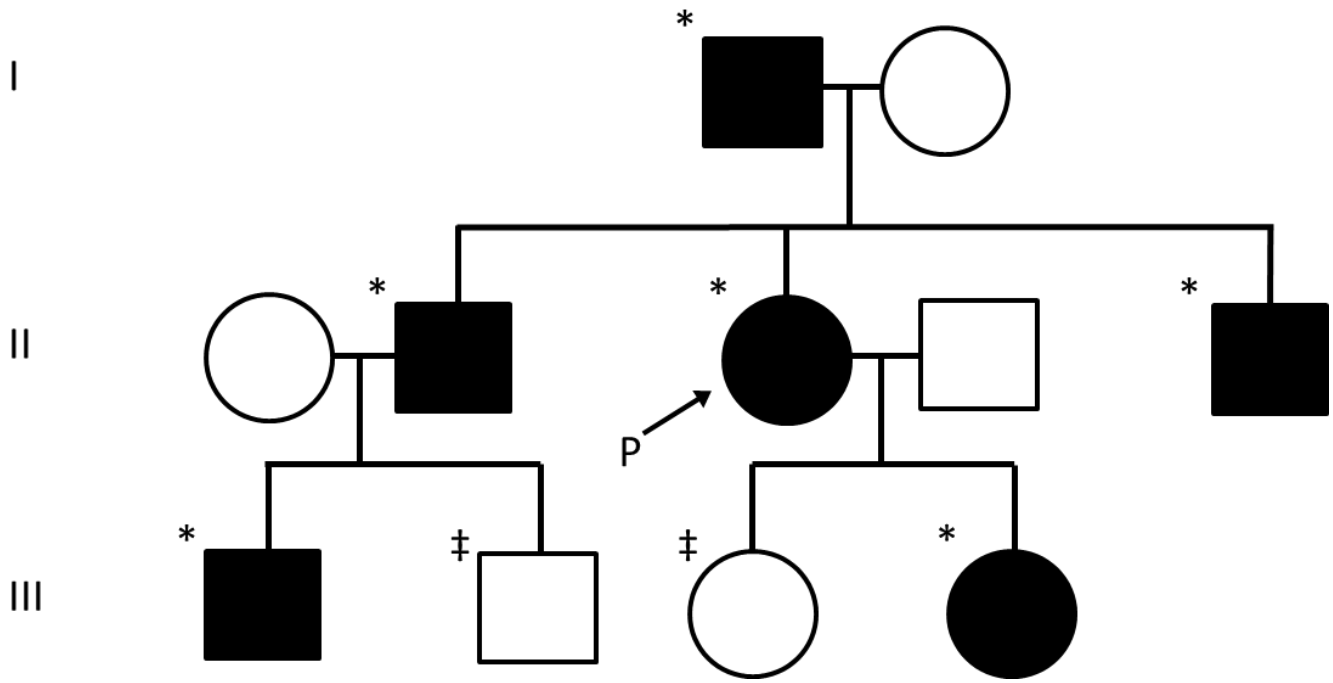


Figure 6. Inheritance of *RUNX1* mutations and bleeding problems in the family of the proband with *RUNX1* mutations. Individuals with (solid symbols) or without (open symbols) known bleeding problems in the proband's (P) family. Symbols indicate those that tested positive (*) or negative (‡) for the proband's *RUNX1* mutations.

3.2.2. Confirmation of c.583dup and c737C>T mutations identified through exome sequencing

Before analysis of the laboratory and bleeding phenotype, the mutations identified through exome sequencing were confirmed through Sanger Sequencing. Through exome sequencing, it was identified that the proband had two *RUNX1* sequence changes identified through exome sequencing. The first was a single base pair duplication (A) in exon 6 (c.583dup) on chromosome 21 at base position 34,859,504 of the Genome Reference Consortium Human Build 38 (http://www.ensembl.org/) (Fig 7), predicted to result in a reading frame shift beginning at position Ile195 and introducing a premature termination codon 17 positions downstream (p.I1e95fs*18), with *RUNX1* truncation at amino acid 211 instead of amino acid 480. A similar mutation (AAAA insertion) at this site, in an individual with chronic myelomonocytic leukaemia, was demonstrated to cause *RUNX1* haploinsufficiency (Nakao et al., 2004). The c.583dup variant was not found in the Exome Aggregation Consortium (ExAC) (Sugawara and Nikaido, 2014) or Catalogue of somatic mutations in cancer (COSMIC) (Forbes et al., 2015) databases.

The second was a single base pair substitution (G>A) in exon 7 (21:34834478 within the Genome Reference Consortium Human Build 38) (Figure 7), predicted to change amino acid 246 from Thr to Met (p.T246M), rs555366994 (Pruitt et al., 2014), with an allele frequency of <0.0001 based on Exome Aggregation Consortium data (Sugawara and Nikaido, 2014). This mutation was identified in ClinVar (accessible at: http://www.ncbi.nlm.nih.gov/clinvar/variation/239054/) and was observed once in Luhya Webuye, Kenya in population from 1000 genomes, as well as in 6 heterozygotes in 60,642 from ExAC. The OMIM number associated with this mutation is 601399 and the condition associated is aspirin-like thrombocytopenia, familial, with propensity to acute myelogenous leukemia; FPD/AML (accessible at: https://www.omim.org/entry/601399).

Sanger sequencing indicated that six family members, including the proband, were heterozygous for c.583dup and rs555366994 (Figure 7) whereas the other two family members studied had neither sequence

changes (Figure 7). This indicates that both sequence changes are on the same haplotype and implicated c.583dup as the pathologic mutation as it introduces a stop codon upstream of rs555366994.

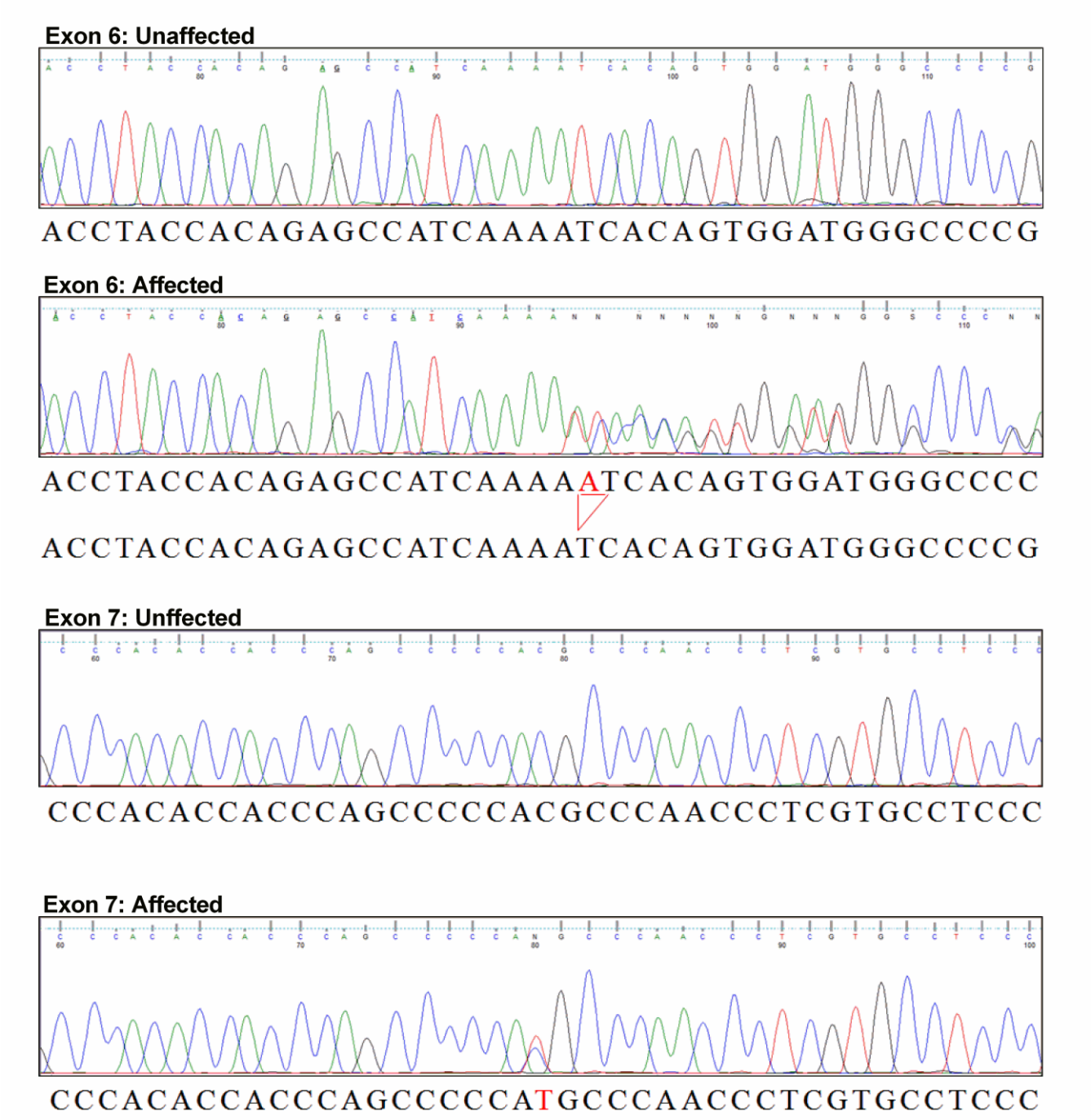


Figure 7. Sanger sequencing of the subjects with c.583dup and c737C>T mutations. *RUNX1* DNA sequences for representative family members, showing the non-mutated sequence in exon 6 and 7 of an unaffected family member and heterozygosity for the 1 base pair (A) duplication mutation in exon 6, and the 1 base pair (C>T) substitution in exon 7 of the proband (both denoted in red below the image).

3.2.3. Laboratory phenotype of all affected subjects with uncharacterized platelet function disorders

Among the 29 affected subjects that met the updated study criteria, five had thrombocytopenia (platelets <150 X 10^9 /L; affected members, median counts: 241; range: 108-378) (Figure 8) and 9 had dense granule deficiency (lower limit RI: 4.9; range for affecteds: 0.5-8.4). There was no difference between index cases and non-index cases with regard to platelet count (median index: 245, non-index:167; p=0.24) or the average number of dense granules per platelet (median index: 5.7, non-index: 5.5; p=0.95).

Many of the affected subjects had reduced MA with thromboxane analogue U46619 (23/29), 1.25 µg/mL collagen (22/29) and arachidonic acid (18/29). Most had a normal MA with 2.5 µM (24/29) and 5.0 µM (24/29) ADP and with 6 µM epinephrine (20/29) (Figure 9). There were no differences between MA responses with individual agonists between affected index and non-index case subjects, except that MA was lower with ristocetin for non-index cases for 1.25 mg/ml ristocetin (median; index: 84, non-index: 77; p= 0.01).

Most affected individuals had an impaired response to weak agonists including epinephrine (25/29), thromboxane analogue U46619 (25/29) and arachidonic acid (19/29). Some also had impaired response to strong agonists including thrombin (18/29) and collagen (20/29). There were no differences between dense granule ATP release responses in affected index and non-index case subjects with all agonists except for 1.0 µmol/L U66619 (median; index: 0.12, non-index: 0.0; p= < 0.01) (Figure 10).

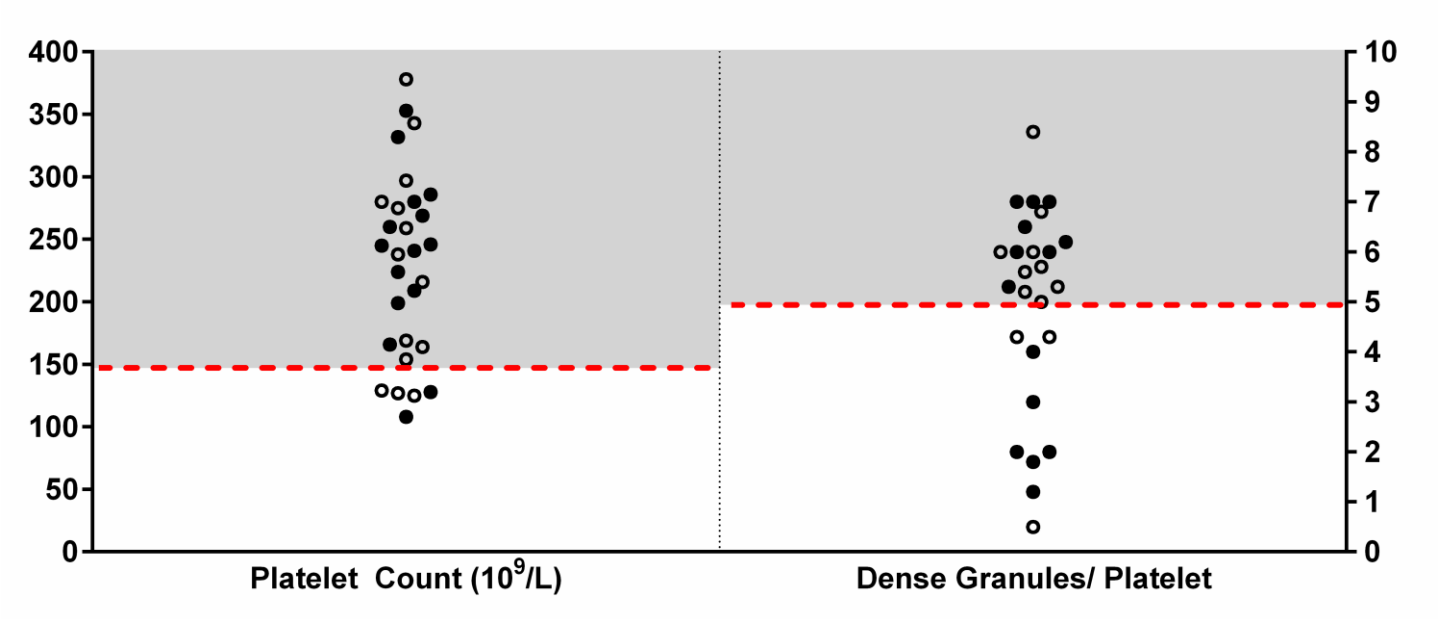


Figure 8. Platelet and dense granule count for all subjects. Platelet counts (RI: 150-400 X 10^9 platelets/L) and average number of dense granules/platelet (RI: 4.9-10) for all affected subjects (n=29). Affected index cases (n=17) are denoted by solid circles. Non-index case affected individuals (n=12) are denoted by open circles. Gray shading indicates normal results; red lines denote the reference interval lower limits.

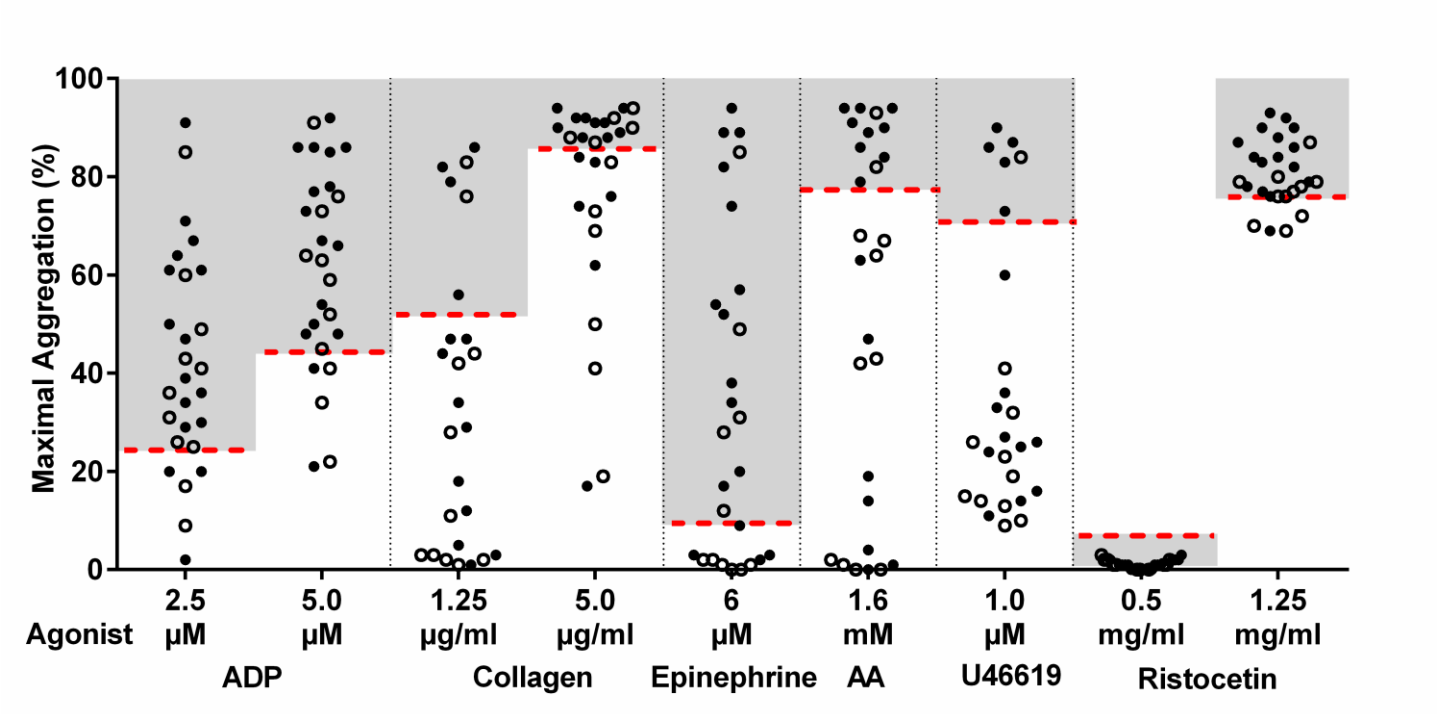


Figure 9. Light transmittance platelet aggregometry findings for all subjects. Results shown as the percent maximal aggregation in responses to indicated final concentrations of adenosine diphosphate (ADP), Horm collagen (collagen), epinephrine (Epi), arachidonic acid (AA), thromboxane analogue (U46619) and ristocetin. Affected index cases (n=17) are denoted by solid circles. Non-index case affected individuals (n=12) are denoted by open circles. Gray shading indicates normal results; red lines denote the reference interval lower limits, or upper limit for maximal aggregation with 0.5 mg/ml ristocetin.

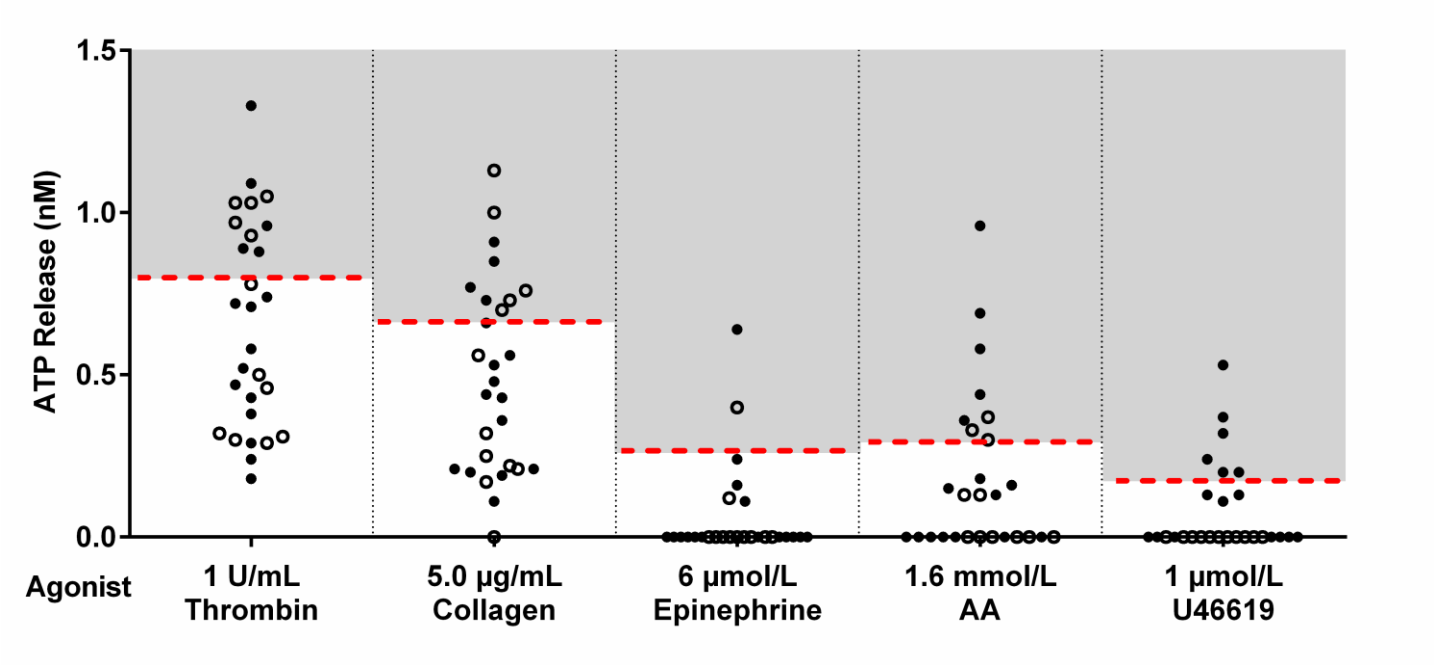


Figure 10. Platelet dense granule ATP release in all subjects. ATP release is shown in response to indicated final concentrations of: thrombin (IIa), Horm collagen, arachidonic acid, thromboxane analogue (U46619) and epinephrine (Epi). Affected index cases (n=17) are denoted by solid circles. Non-index case affected individuals (n=12) are denoted by open circles. Gray shading indicates normal results; red lines denote the reference interval lower limits.

3.2.4. Laboratory phenotype of the family with the *RUNX1* mutations

Compared to the entire cohort, the affected family members with the *RUNX1* mutations had a similar laboratory phenotype. Two (of 6) affected family members had thrombocytopenia (platelets <150 X 10^9 /L; affected members, median counts: 164; range: 125-169) (Figure 11) and three had mild dense granules deficiency (lower limit RI: 4.9; range for affected: 4.0-6.0; unaffected: 7.5). None had severe dense granule deficiency (Figure 11). All affected family members had a low MPV, as did the unaffected family members (lower RI limit 7.4; ranges, affected: 6-7.3; unaffected: 6.9-7.0).

LTA was performed on all but the two youngest family members (one affected; III.1, one unaffected; III.2). All five affected individuals evaluated by LTA (Figure 12; all tested at 250 X10^9 platelets/L) had normal aggregation with ADP but reduced MA with 1.25 µg/mL and 5.0 µg/mL collagen and 1.0 µM thromboxane analogue U46619 and absent secondary aggregation with epinephrine and most (4/5) had reduced MA with arachidonic acid. Only one affected family member had reduced MA with ristocetin (Figure 12).

All affected family members (6/6, 5 tested with all agonists) had reduced dense granule ATP release with all evaluated agonists, unlike unaffected family members (Figure 13).

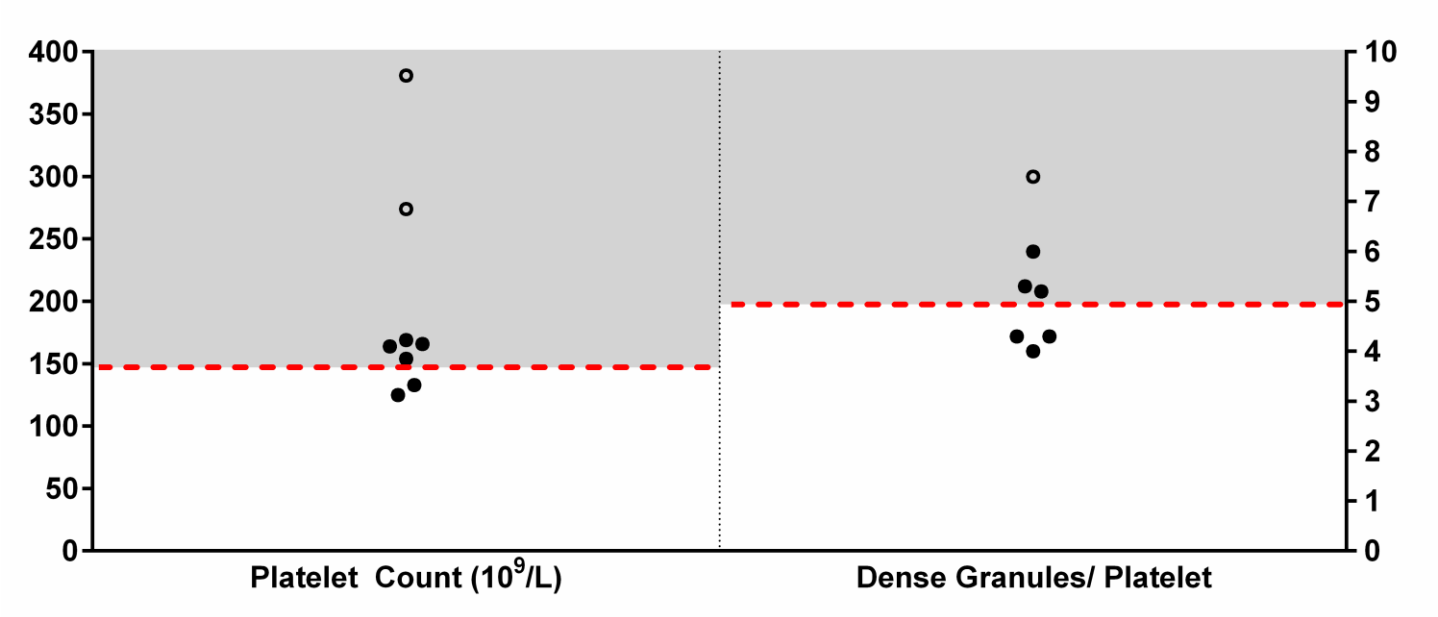


Figure 11. Platelet and dense granule count in the family with *RUNX1* mutations. Platelet counts (RI: 150-400 X 10^9 platelets/L) and average number of dense granules/platelet (RI: 4.9-10). Data for individuals that tested positive (closed symbol) or negative (open symbols) for the *RUNX1* mutation are indicated. Gray shading indicates normal results; red lines denote the reference interval lower limits.

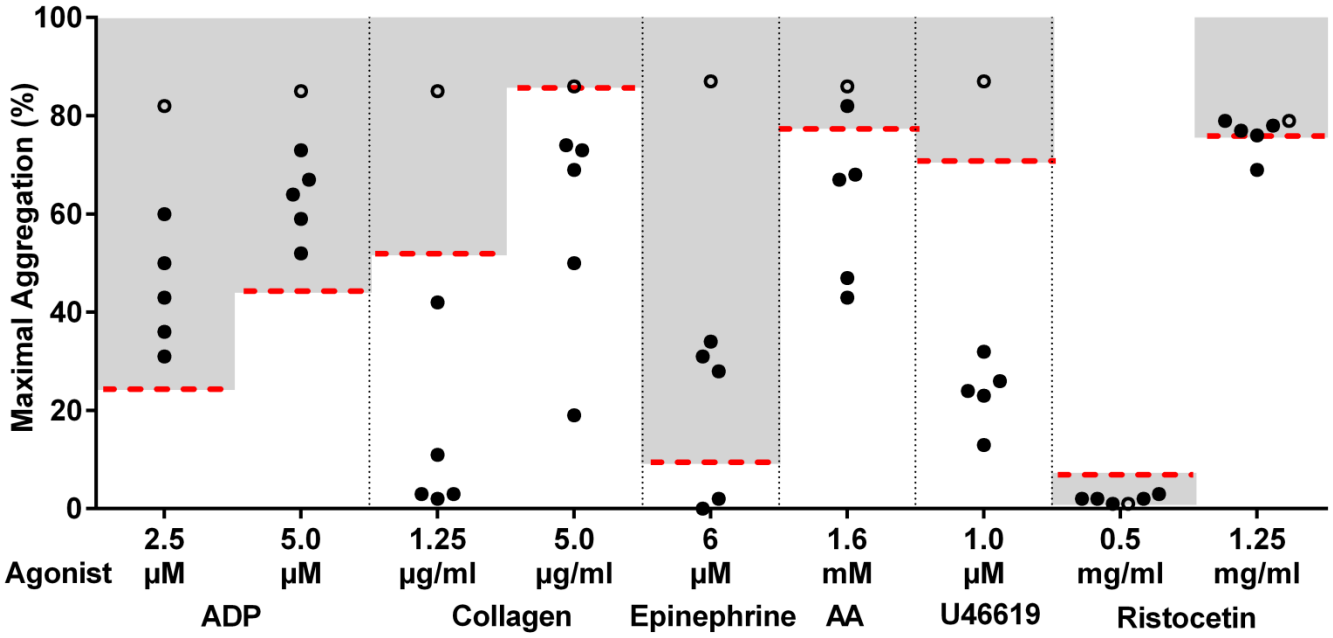


Figure 12. Light transmittance platelet aggregometry findings for the family with the *RUNX1* mutations. Results shown are the percent maximal aggregation responses to indicated final concentrations of adenosine diphosphate (ADP), Horm collagen (collagen), epinephrine, arachidonic acid (AA) thromboxane analogue (U46619) and ristocetin. Data for individuals that tested positive (closed symbol) or negative (open symbols) for the *RUNX1* mutation are indicated. Gray shading indicates normal results; red dashed lines denote the reference interval lower limits, or upper limit for maximal aggregation with 0.5 mg/ml ristocetin.

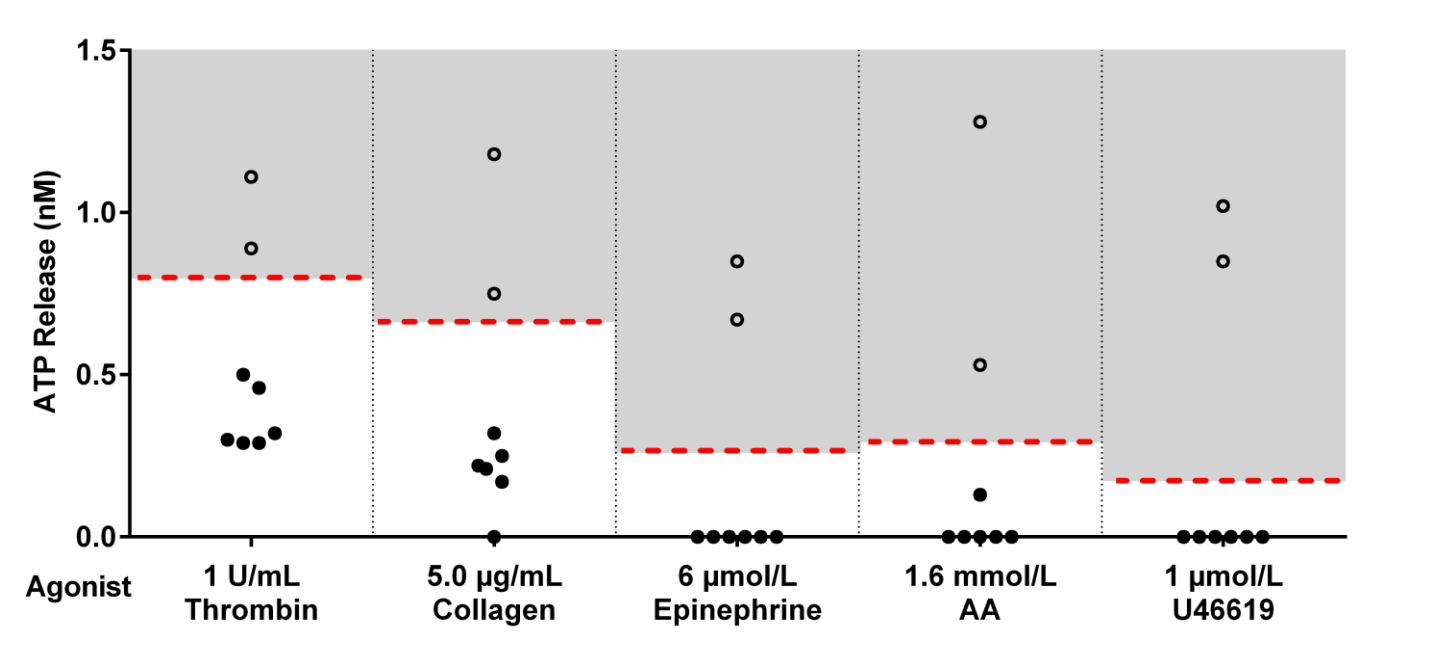


Figure 13. Platelet dense granule ATP release in the family with the *RUNX1* mutations. ATP release is shown in response to indicated final concentrations of: thrombin, Horm collagen (collagen), epinephrine, arachidonic acid (AA) thromboxane analogue (U46619). Data for individuals that tested positive (closed symbol) or negative (open symbols) for the *RUNX1* mutation are indicated. Gray shading indicates normal results; red dashed lines denote the reference interval lower limits.

3.2.5. ISTH BAT bleeding scores of all affected subjects with uncharacterized platelet function disorders

Mild bleeding phenotypes were observed in most affected subjects in the Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders. Most subjects had higher ISTH-BAT scores (median: 9, range: 0-18) than their unaffected family members (median: 0.5, range: 0-1) and general population controls (median: 0, range: 0-6) ($p < 0.01$) (Figure 14). However, 4 affected subjects, that were relatives of an index case, had bleeding scores below 4..

ISTH BAT bleeding scores of affected subjects were similar for index and non-index cases ($p = 0.060$), subjects with or without thrombocytopenia (median: 8 vs 9.5; $p = 0.22$) or with or without dense granule deficiency (median: 10 vs 8.5; $p = 0.41$).

Affected females reported higher ($p = <0.01$) ISTH-BAT scores (median: 11, range: 6-18) than males (median: 4.5, range: 0-16). However, when sex specific questions were removed, the difference was no longer significant ($p = 0.097$) between females (median: 8, range: 1-15) and males (median: 4.5 range: 0-16). Among affected subjects, the more severe ISTH-BAT symptom scores were for the problems of epistaxis, menorrhagia, bleeding from dental extractions, surgery, cutaneous bleeding and CNS bleeds (Figure 15), and the least severe (69% with score of 1) bleeding was for minor wounds.

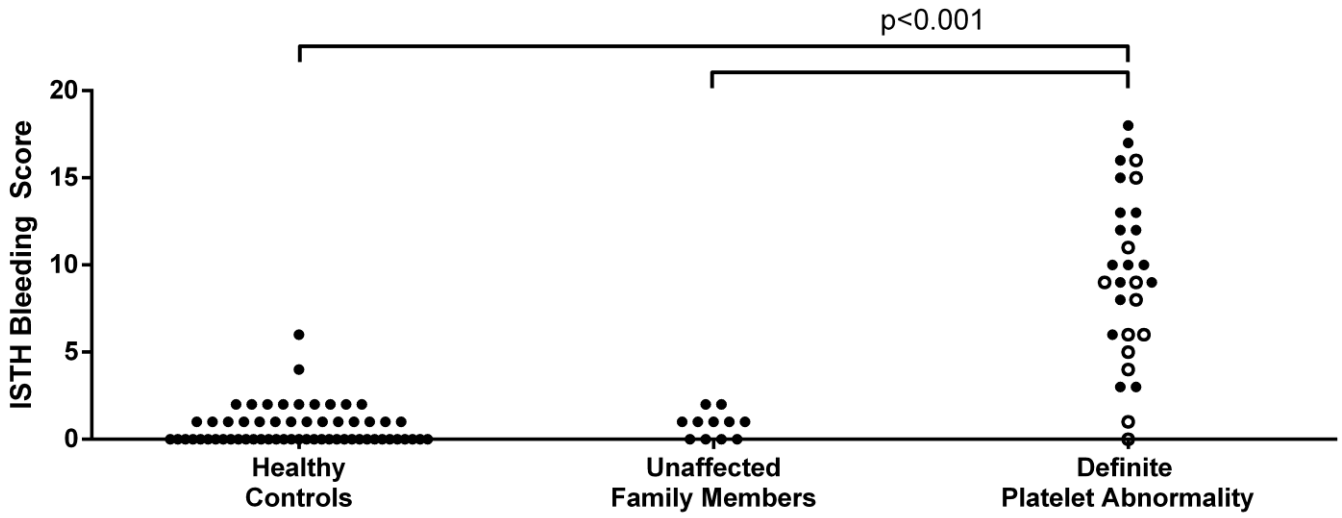


Figure 14. ISTH-BAT bleeding scores for family members with definite platelet abnormalities compared to general population controls and unaffected family members. Data for affected (n=29) and unaffected (n=12) family members and general population controls (n=60) are shown. Affected index cases (n=17) are denoted by solid circles. Affected non-index case individuals (n=12) are denoted by open circles. * indicate significantly different results (p values= <0.01).

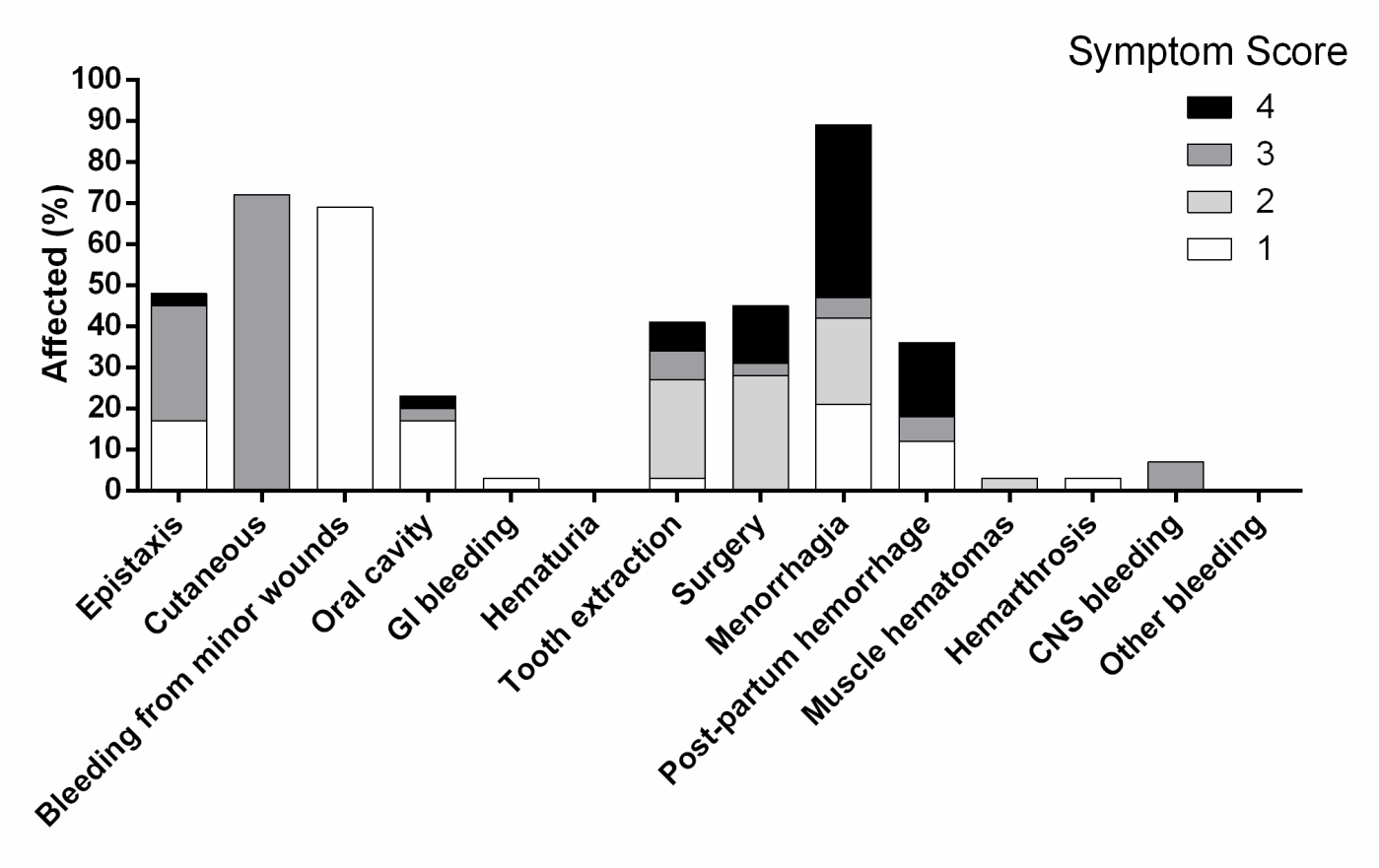


Figure 15. ISTH-BAT of bleeding symptoms for all affected subjects. Proportion of affected family members experiencing bleeding symptoms of different severity, based on ISTH-BAT data (4 indicates the most severe symptoms). Menorrhagia and post-partum hemorrhage were assessed for affected females (n=18).

3.2.6. ISTH BAT bleeding scores for the family with the *RUNX1* mutations

Affected individuals from the family with the *RUNX1* mutation that tested positive for the mutation predicted to cause *RUNX1* haploinsufficiency (n=6) had bleeding symptoms typical of a platelet function disorder, reflected by higher ISTH-BAT scores (median: 8.5, range 4-15) than the two unaffected family members (median: 0.5, range: 0-1) and general population controls (median: 0, range: 0-6) ($p < 0.01$) (Figure 16). ISTH bleeding scores of affected family members were similar for those with or without thrombocytopenia (median: 8 vs 9; $p = 1.00$) or with or without dense granule deficiency (median: 9 vs 8; $p = 0.4$).

Among affected family members, the more severe ISTH-BAT symptom scores were for epistaxis, menorrhagia, bleeding from dental extractions, cutaneous bleeding and a subdural hematoma at birth (Figure 17). Only one individual in the family member (deceased maternal aunt of the proband's father, mutation status unknown) was known to have developed leukemia and none were known to have developed myelodysplasia.

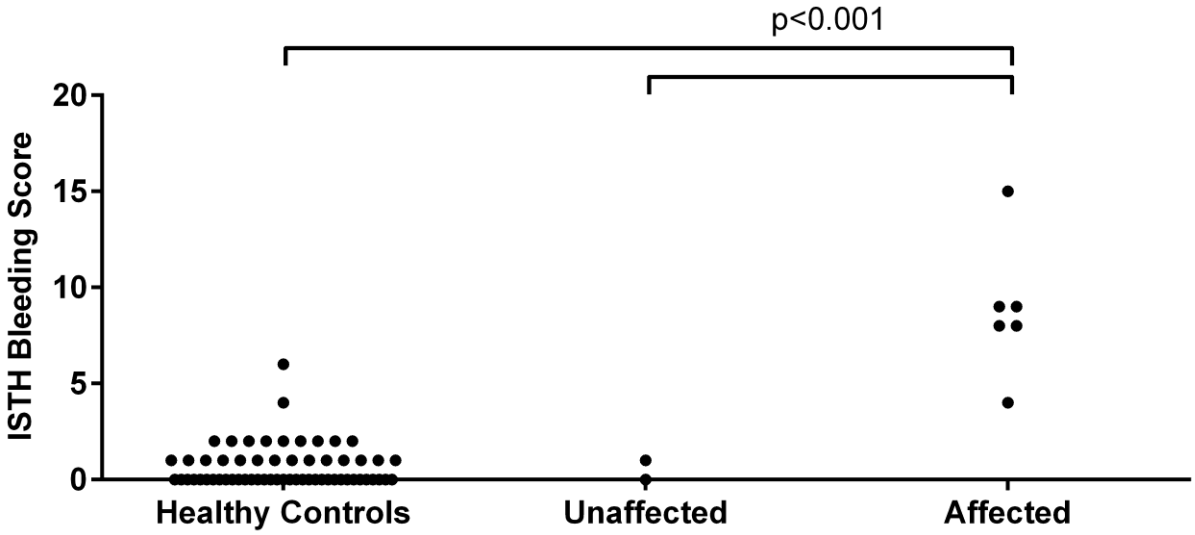


Figure 16. ISTH-BAT bleeding scores in the family with the *RUNX1* mutations. Results compare data for affected (n=6) and unaffected (n=2) family members and general population controls (n=60). * indicate significantly different results (p values= <0.01).

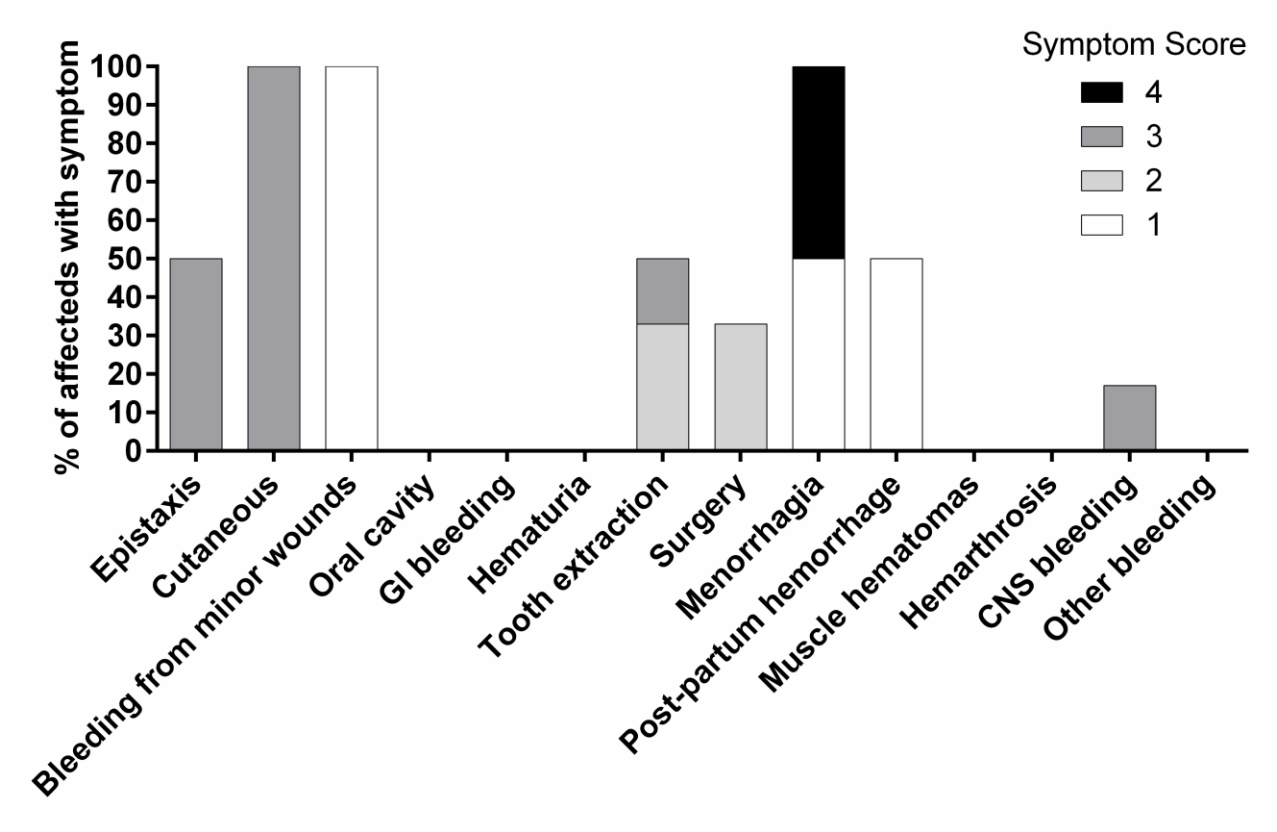


Figure 17. ISTH-BAT data for bleeding symptoms experienced by affected members of the family with the *RUNX1* mutations. Proportion of affected family members (n=6) experiencing bleeding symptoms of different severity, based on ISTH-BAT data (4 indicates the most severe symptoms). Menorrhagia and post-partum hemorrhage were assessed for the affected females (n=2).

3.2.7. CHAT-P bleeding risks for all affected subjects with uncharacterized platelet function disorders

The estimated bleeding risks for all affected subjects are summarized in Figures 18-21 and in the supplementary file (Figure A1) that shows all responses in the questionnaire. The OR estimates for bleeding symptoms that showed significant differences ranged from 3 to 360 and were consistent with bleeding problems that have been associated with mild platelet function disorders. Excluding the questions that were specifically for women, proportionally more female controls reported anemia, iron deficiency or treatment with iron than male controls (9/40 vs. 0/20; $p = 0.02$). No significant differences were found between all other symptoms reported by female and male controls (P values 0.10 - 1.00).

There were no significant differences in the proportion of subjects reporting general health related problems, including cancer, compared to general population controls and only one affected subject for each item reported a history of heart problems (e.g., heart attack or angina), thrombosis from deep veins or lungs and thrombosis in the brain (e.g., stroke or TIA) (Figure 18). Thrombocytopenia was reported by one general population control and two affected subjects. Bone marrow or white blood cell problems were reported by only one general population control and one affected subject.

Proportionately more affected subjects also reported a family history of (OR 7.6-44): bleeding complications causing serious complications or death, thrombocytopenia and leukemia and other bone marrow problems than general population controls (Figure 18). When the family with the *RUNX1* mutation was removed from the analysis, these 3 items remained significantly different from general population controls (OR: 6 – 138). Most (22/29) affected subjects reported that they had a first degree relative (parent or sibling) with a bleeding problem. The proportion of affected subjects reporting other family members with problems such as cancer (other than leukemia), albinism, birth defects hearing problems and kidney problems were not significantly different from general population controls.

Proportionately more affected subjects reported a personal history of excessive bleeding that necessitated lifestyle changes than general population controls (12/29 vs. 2/60; OR 20; 95% CI, 4.2-100;

P=<0.0001) (Figure 19). Some (9/29) affected subjects reported experiencing abnormal bleeding before the age of 18 (OR 260; 95% CI, 5.6-1800; P=<0.0001).

Proportionately more affected family members than general population controls reported prolonged bleeds from cuts and minor wounds that lasted longer than 10 minutes (OR 73; 95% CI, 8.8-600; P=<0.0001) or longer than an hour (OR 56; 95% CI, 3-1000; P=<0.0001) (Figure 19). Few (3/29) affected subjects reported bleeding from a minor wound that lasted longer than a day and only one reported minor wound bleeding that required medical attention or treatment.

While some general population controls reported abnormal bruising (7/60), the majority of affected subjects (25/29) reported some abnormal bruising (OR 15-65), including bruises that appeared without reason, were disproportionate to trauma, lumpy and/or left permanent marks. Bruises that tracked downward were reported less frequently by affected subjects (7/29) which was rarely reported by general population controls (give OR etc. to be consistent) (Figure 19).

Proportionately more affected subjects reported prolonged nosebleeds (>15 minutes) than general population controls (OR 23; 95% CI, 6-92; P=<0.0001) and one affected subject reported nosebleeds lasting between 5-7 hours (Figure 19). Some (11/29) affected subjects had required treatment for nosebleeds, including packing (OR 33; 95% CI, 2-620; P=0.0008) or cauterization (OR 27; 95% CI, P=0.0029), but none required transfusion. Only two affected subject had required admission to hospital to control a nosebleed. One subject reported a bleed in one of their eyes lasting 2-3 days.

Gastrointestinal bleeds were reported by some affected subjects (10/29), mainly due to conditions such as hemorrhoids or anal fissures, and this was also proportionally greater (OR 4; 95% CI, 1.3-12; P=0.0191) than the gastrointestinal bleeds reported by general population controls (Figure 19). Two affecteds had suffered a severe bleed from an ulcer that required hospital admission and in one case, platelet transfusions. None of the affecteds reported spontaneous hematuria or joint bleeds. In all cases, hematuria had occurred from kidney stones/disease or infections. Only one spontaneous muscle bleeds were reported by an affected subject. Three

affected subjects reported bleeding into the brain; one subject reported having experienced a spontaneous bilateral subdural hematoma, another had a bleed in association with a TIA and the third had experienced a subdural hematoma at birth. However, the proportion with bleeding into the brain was not significantly different from general population controls.

Proportionately more affected subjects (12/29) reported wound healing problems after an injury, wound, surgery or dental procedure (OR 13; 95% CI, 3.4-53; P=<0.0001) than general population controls (3/60) (Figure 20). Proportionately more affected family members had been given or has been recommended medications to prevent bleeding (22/29, OR: 363; 95% CI, 20-6600; P=<0.0001). Among the sixteen subjects recommended DDAVP, 4 were never given the drug, and among those who had received DDAVP to prevent or control a bleed, ten indicated that the medication worked while the 2 remaining subjects were unsure about its effectiveness. 4 affected subjects reported that they were given fibrinolytic inhibitors, with half o reporting that this treatment had worked and the others reporting that they were unsure about its effectiveness.

Excessive bleeding from serious injuries or trauma, including car accidents or serious sports injuries, was more commonly reported by affected subjects than general population controls (OR 9.5; 95% CI, 1-87; P=0.0293) (Figure 20). Proportionately more affected family members reported excessive oral or dental bleeding than general population controls (OR 44; 95% CI, 5.3-370; P=<0.0001) (Figure 20). Among the affected subjects that had excessive bleeding during dental procedures, most reported that this had occurred with every (4/13) or only some procedures (5/13), and that it had lasted longer than a day (7/13) and/or resulted in extensive bruising (3/13). One individual reported bleeding from a dental procedure lasting 3 weeks post procedure. A few subjects reported receiving packing or resuturing as treatment for oral or dental bleeds (4/13) and none required hospital admission, transfusions or subsequent surgeries to control an oral or dental bleed. One affected subjects who had received prophylactic treatment with desmopressin and tranexamic acid for a subsequent dental extraction reported no abnormal bleeding with that procedure.

Among 25 subjects who had undergone operations, proportionately more affected subjects experienced excessive bleeding than general population controls (OR 17; 4.1-68) (Figure 20). Of the 13 affected subjects who reported excessive bleeding due to an operation, most (5/13) had bled with every operation (including one subject who only reported having a single operation). Of the remaining 8/13 that bled with only some operations, 4 reported bleeding only during operations that were not performed with treatment to prevent bleeding. Most (10/13) subjects reported that the excessive bleeding occurred on the day of the operation. One subject with a history of multiple surgeries had reported bleeding that began a few days after a cholecystectomy and an appendectomy. Almost half (6/13) of the affecteds who reported excessive bleeding from an operation had required an extended stay in hospital, two individuals had to be re-admitted, but only one required a subsequent surgery for bleeding control. Transfusions during or after surgery were reported by six affected individuals; three had received platelets, two had received red blood cells and one had received whole blood. A few affected individuals (3/13) had received drug treatment to manage excessive surgical bleeding. Of the affecteds who had been given treatment and did not bleed during a surgical procedure, half had received DDAVP alone (4/8), one had received DDAVP and a fibrinolytic inhibitor, two had received platelets and one had received fibrinolysis inhibitor therapy only.

Among sex-specific items evaluated, significant differences were seen between affected subjects and general population controls for a number of bleeding symptoms (OR 4-41) (Figure 21). Of the 19 affected females who have reported that they had reached menarche, approximately half (9/19) reported that their typical menstrual period lasted longer than 7 days, and similar numbers (10/19) reporting having experienced flooding or gushing accidents. Many affected females also reported heavy flow that lasted longer than 2 days (15/19) or longer than 3 days (7/19), and soaking through tampons or pads within 2 hours (17/19) or in less than an hour (6/19). Clots greater than 1 cm with menses were also commonly reported by affected individuals (17/19), with some reporting clots under 3 cm (12/19) or over 3 cm (6/19). Only 2 affected subjects had required transfusions to treat menorrhagia, which was not significantly different from general population controls. Some (8/19)

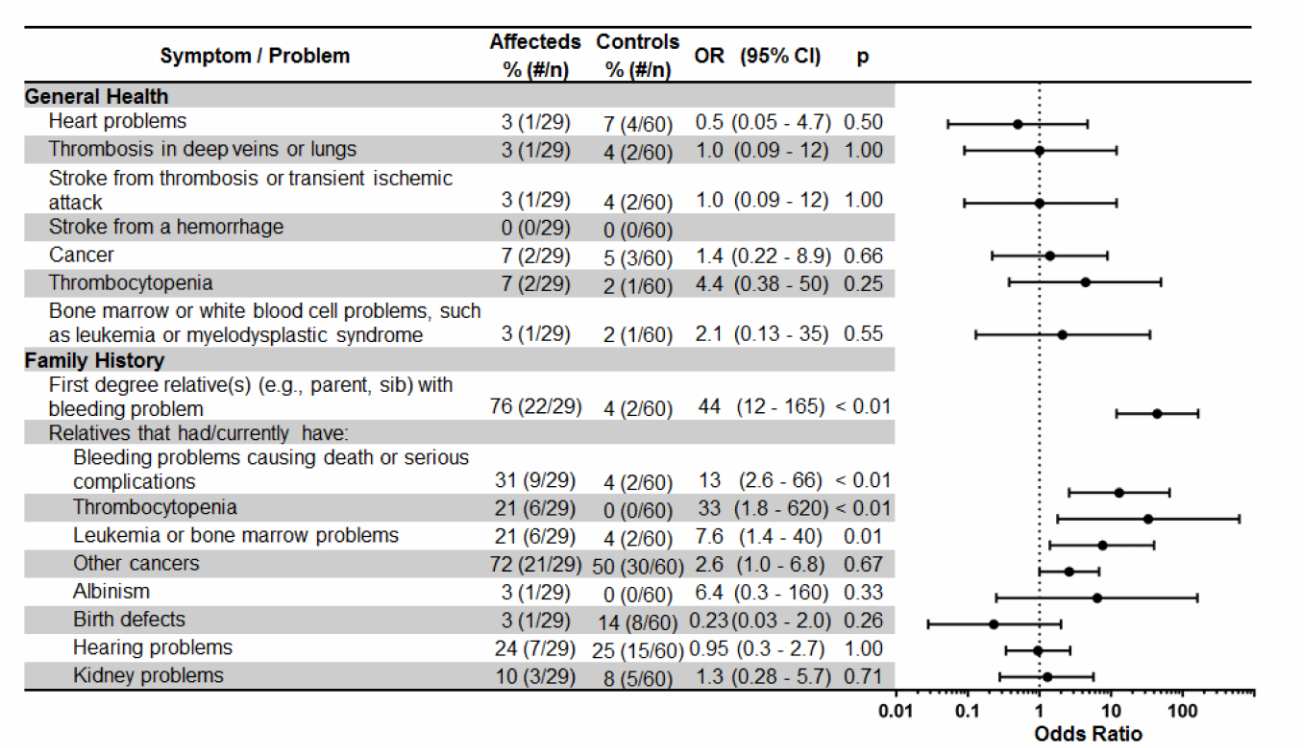
affecteds had required drug treatment for heavy periods and in all cases, the birth control pill was one of the treatments received. Three others had received fibrinolytic inhibitors and/or Depo-Provera for heavy periods. Only 7 affecteds had required surgical treatment for heavy periods, including hysterectomy (4/7), an endometrial ablation (3/7) or dilation and curettage (2/7). The two affecteds that reported having an dilation and curettage also reported having an endometrial ablation or hysterectomy. For the 8 affected subjects who had received treatment for heavy periods, 6 reported a decrease in the length of their periods, 4 reported a decrease in the length of heavy flow and 2 reported an increase in how long it took them to soak through tampons or pads during the periods with treatment.

Bleeding problems that affected sex life were more commonly reported by affected individuals than general population controls (OR 6.5; 95% CI, 1.1-38; P=0.037) (Figure 21). Thirteen (of 17) affected individuals and 23 (of 33) general population had been pregnant, and 76%and 90% of their respective pregnancies had been successful, which was not significantly different (p= 0.09). Further comparisons of affected subjects to general controls indicated that there was no difference in the mean numbers of successful pregnancies that they had experienced (mean ± SD [range]; affected, 2.9 ± 1.9 [1-8]; general population control, 2.7 ± 2.1 [1-8]; p = 0.8). While excessive bleeding at the time of childbirth was more common among affected individuals (8/13) than general population control (2/23) subjects, prolonged bleeding after childbirth (lochia >6 weeks) was not significantly different. Some affected subjects reported that they had received treatment to prevent bleeding on subsequent deliveries (5/13).

Some affected women had undergone surgical procedures (e.g. hysterectomy) to control a bleed during or after childbirth (6/13). The proportions of affected individuals that had required longer stays, readmission, transfusions, drug treatment for bleeding, or an ICU stay for childbirth related bleeding were not significantly different between affecteds and general population controls.

Among affected individuals, the bleeding reported by index and non-index cases were similar, as the only symptoms/problems that were reported more frequently by index cases were: transfusions (P=0.0018), bruises spreading lower (P=0.023), and heavy menstrual flow lasting longer than 3 days (P=0.037).

The number of CHAT-P symptoms/problems reported by individual subjects (maximums: 24; female: 34) was significantly higher for affected subjects (0-24, median 15) compared to unaffected family members (0-6, median 2) and general population controls (0-14, median 1) (P=<0.01) (Figure 22). There was no significant difference in the CHAT-P bleeding scores for affected family members with or without thrombocytopenia (median: 5 vs 15; p= 0.1) or dense granule deficiency (median: 14 vs 16; P= 0.8). CHAT-P bleeding scores were higher for affected index cases (median 16; range [2-24]) compared to non-index cases (median 9; range [0-20]) (P=0.008), despite the significant overlap in their scores.



Symptom / Problem	Affecteds % (#/n)	Controls % (#/n)	OR (95% CI)	p
General Health				
Heart problems	3 (1/29)	7 (4/60)	0.5 (0.05 - 4.7)	0.50
Thrombosis in deep veins or lungs	3 (1/29)	4 (2/60)	1.0 (0.09 - 12)	1.00
Stroke from thrombosis or transient ischemic attack	3 (1/29)	4 (2/60)	1.0 (0.09 - 12)	1.00
Stroke from a hemorrhage	0 (0/29)	0 (0/60)		
Cancer	7 (2/29)	5 (3/60)	1.4 (0.22 - 8.9)	0.66
Thrombocytopenia	7 (2/29)	2 (1/60)	4.4 (0.38 - 50)	0.25
Bone marrow or white blood cell problems, such as leukemia or myelodysplastic syndrome	3 (1/29)	2 (1/60)	2.1 (0.13 - 35)	0.55
Family History				
First degree relative(s) (e.g., parent, sib) with bleeding problem	76 (22/29)	4 (2/60)	44 (12 - 165)	< 0.01
Relatives that had/currently have:				
Bleeding problems causing death or serious complications	31 (9/29)	4 (2/60)	13 (2.6 - 66)	< 0.01
Thrombocytopenia	21 (6/29)	0 (0/60)	33 (1.8 - 620)	< 0.01
Leukemia or bone marrow problems	21 (6/29)	4 (2/60)	7.6 (1.4 - 40)	0.01
Other cancers	72 (21/29)	50 (30/60)	2.6 (1.0 - 6.8)	0.67
Albinism	3 (1/29)	0 (0/60)	6.4 (0.3 - 160)	0.33
Birth defects	3 (1/29)	14 (8/60)	0.23 (0.03 - 2.0)	0.26
Hearing problems	24 (7/29)	25 (15/60)	0.95 (0.3 - 2.7)	1.00
Kidney problems	10 (3/29)	8 (5/60)	1.3 (0.28 - 5.7)	0.71

Figure 18. Responses to questions about general health and family history for all affected subjects compared to general population controls. The proportions of affected family members (Affected) and general population controls (Controls) that reported each symptom/problem is summarized, along with the risk estimates for affected individuals, presented as odds ratios (ORs) and 95% confidence intervals (CIs).

Symptom / Problem	Affecteds % (#/n)	Controls % (#/n)	OR	(95% CI)	p
Excessive bleeding or bruising	86 (25/29)	10 (6/60)	56	(15 - 220)	< 0.01
Onset of bleeding symptoms <18 years	31 (9/29)	0 (0/60)	260	(15 - 4700)	< 0.01
Bleeding that led to a lifestyle change	41 (12/29)	3 (2/60)	20	(4.2 - 100)	< 0.01
Anemia or iron deficiency	52 (15/29)	15 (9/60)	6	(2.2 - 17)	< 0.01
Recommended or given medication to prevent or control a bleed	76 (22/29)	0 (0/60)	360	(20 - 6600)	< 0.01
Duration of minor wound bleeding					
>10 minutes	55 (16/29)	2 (1/60)	73	(8.8 - 600)	< 0.01
>1 hour	31 (9/29)	0 (0/60)	56	(3.1 - 1000)	< 0.01
Bruising					
For no reason	69 (20/29)	7 (4/60)	24	(7.3 - 82)	< 0.01
Disproportionate to trauma	48 (14/29)	3 (2/60)	27	(5.5- 130)	< 0.01
Lumpy	34 (10/29)	2 (1/60)	31	(3.7 - 260)	< 0.01
Leaving permanent marks	21 (6/29)	2 (1/60)	15	(1.8 - 140)	< 0.01
Spread lower	24 (7/29)	0 (0/60)	40	(2.2 - 740)	< 0.01
Size ≥ orange	34 (10/29)	0 (0/60)	65	(3.7 -1200)	< 0.01
Average number of bruises at one time					
>1	72 (21/29)	20 (12/60)	11	(3.7 - 30)	< 0.01
>2	66 (19/29)	5 (3/60)	36	(9.0 - 150)	< 0.01
>3	48(14/29)	3(2/60)	27	(5.5 - 130)	< 0.01
Nosebleeds					
>15 minutes	55 (16/29)	5 (3/60)	23	(5.9 - 92)	< 0.01
Requiring treatment	38 (11/29)	2 (1/60)	36	(4.4 - 300)	< 0.01
Medical attention	24 (7/29)	2 (1/60)	40	(1.5 - 520)	< 0.01
Nasal pack	21 (6/29)	0 (0/60)	33	(1.8 - 620)	< 0.01
Cautery	17 (5/29)	0 (0/60)	27	(1.5 - 510)	< 0.01
Admission to hospital	7 (2/29)	0 (0/60)	11	(0.5 - 237)	0.10
Muscle bleeds	3 (1/29)	5 (3/60)	0.68	(0.07 - 6.8)	1.00
Joint bleeds	3 (1/29)	0 (0/60)	6.4	(0.3 - 160)	0.33
Bleeding from stomach or bowels	34 (10/29)	12 (7/60)	4	(1.3 - 12)	0.02
Hematuria	0 (0/29)	0 (0/60)	-	-	-
CNS bleeds	10 (3/29)	2 (1/60)	6.8	0.68 - 69	0.10

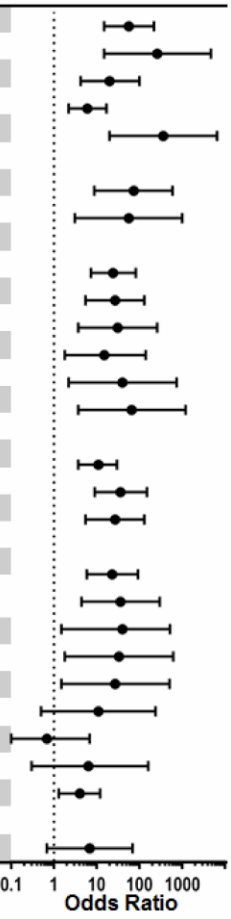


Figure 19. Bleeding symptoms experienced by all affected subjects compared to general population controls. The proportions of all affected (Affecteds) and general population controls (Controls) that reported each symptom/problem is summarized, along with the risk estimates for affected individuals, presented as odds ratios (ORs) and 95% confidence intervals (CIs).

Symptom / Problem	Affecteds % (#/n)	Controls % (#/n)	OR	(95% CI)	p
Serious accident or trauma, with excessive bleeding	19 (5/26)	2 (1/41)	9.5	(1.0 - 87)	0.03
Bleeding problems from an operation	52 (13/25)	6 (3/49)	17	(4.1 - 68)	< 0.01
Excessive oral or dental bleeding	46 (13/28)	2 (1/52)	44	(5.3 - 370)	< 0.01
Transfusions for bleeding	46 (13/29)	0 (0/29)	99	(5.6 -1800)	< 0.01
Problems with healing from an injury, wound, dental procedure or surgery	41 (12/29)	5 (3/60)	13	(3.4 - 53)	< 0.01

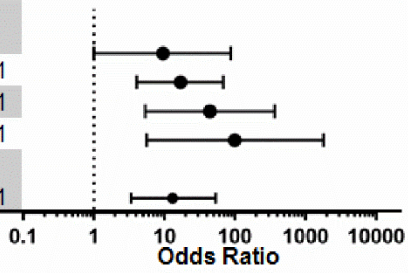


Figure 20. Challenge related bleeding symptoms. The proportions of all affected (Affecteds) and general population controls (Controls) that reported each symptom/problem is summarized, along with the risk estimates for affected individuals, presented as odds ratios (ORs) and 95% confidence intervals (CIs).

Symptom	All Affected % (#/n)	Control % (#/n)	OR	(95% CI)	p
Bleeding problems that interfere with sex life	29 (5/17)	6 (2/33)	6.5	(1.1 - 38)	0.04
Menstrual periods					
Length > 7 days	47 (9/19)	8 (3/40)	11	(2.5 - 49)	< 0.01
Heavy flow ≥3 days	80 (15/19)	30 (12/40)	8.8	(2.4 - 32)	0.03
Soaks through pads / tampons in ≤2 hours	89 (17/19)	40 (16/40)	13	(2.6 - 63)	< 0.01
Flooding or gushing accidents due to heavy bleed	53 (10/19)	23 (9/40)	3.8	(1.2 - 12)	0.04
Restricted lifestyle	42 (8/19)	30 (12/40)	2.3	(0.75 - 7.0)	0.15
Clots with menses	89 (17/19)	25 (16/40)	13	(2.6 - 63)	< 0.01
Required blood transfusions	11 (2/19)	0 (0/40)	12	(0.53 - 250)	0.10
Had fibroids	5 (1/19)	13 (5/40)	0.39	(0.04 - 3.6)	0.65
Had endometriosis	16 (3/19)	3 (1/40)	7.3	(0.71 - 76)	0.09
Treated for heavy periods	53 (10/19)	13 (5/40)	7.8	(2.1 - 29)	< 0.01
Childbirth related bleeding					
Been pregnant	76 (13/17)	70 (23/33)	1.4	(0.37 - 5.4)	0.75
Bleed excessively	62 (8/13)	9 (2/23)	17	(2.7 - 105)	< 0.01
Lochia >6 weeks after childbirth	23 (3/13)	13 (3/23)	2	(0.34 - 12)	0.65
Surgery required	46 (6/13)	0 (0/23)	41	(2.0 - 810)	< 0.01
Stay longer in hospital	23 (3/13)	9 (2/23)	3.2	(0.45 - 22)	0.33
Readmission to hospital	8 (1/13)	0 (0/23)	5.6	(0.21 - 150)	0.36
Intensive care treatment	8 (1/13)	0 (0/23)	5.6	(0.21 - 150)	0.36
Drug treatments	15 (2/13)	4 (1/23)	4.0	(0.33 - 49)	0.54
Transfusions	23 (3/13)	0 (0/23)	16	(0.74 - 330)	0.40
Treatment for bleeding with subsequent deliveries	0 (0/13)	0 (0/23)	-	-	-

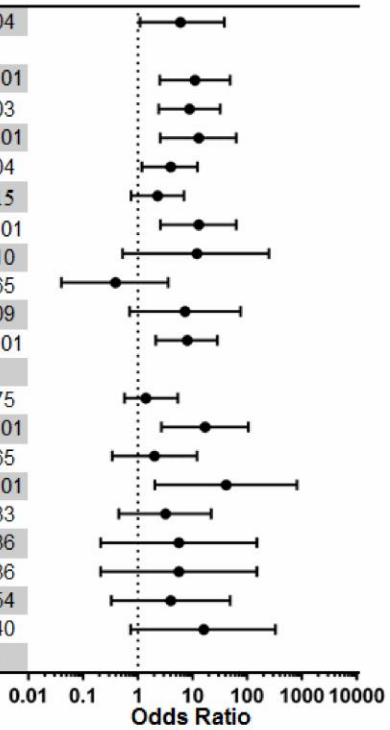


Figure 21. Sex related bleeding symptoms. The proportions of all affected (Affecteds; n=19) and general population controls (Controls; n=40) that reported each symptom/problem is summarized, along with the risk estimates for affected individuals, reported as odds ratios (ORs) and 95% confidence intervals (CIs).

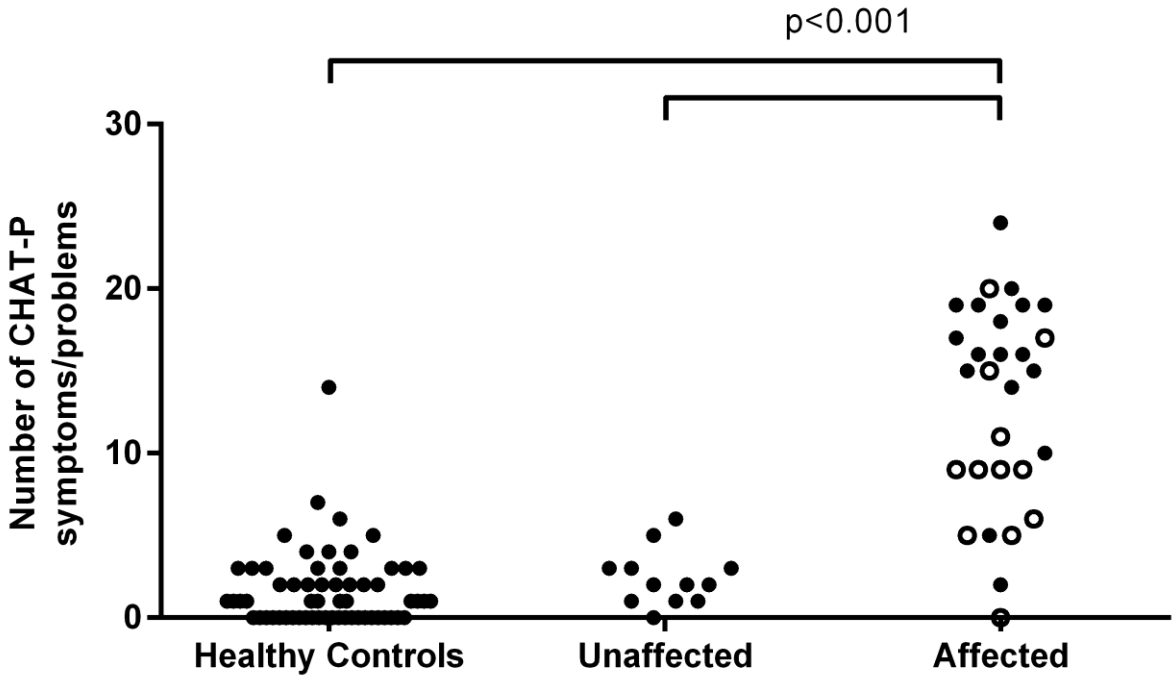


Figure 22. Number of CHAT-P symptoms/problems reported by affected individuals compared to unaffected family members and general population controls. Scores represent the number of CHAT-P symptoms/problems that had an OR that did not cross 1, reported by each subject, after exclusion of data for family history, whether they had been told that they had a bleeding disorder or had received treatment or treatment recommendations for bleeding. Affected (n=29) and unaffected (n=12) family members and general population controls (n=60) are denoted by solid circles. Open circles denote non-index case affected subjects. * indicate significantly different results (p values= <0.01)

3.2.8. CHAT-P bleeding risks for the family with the *RUNX1* mutations

The CHAT-P findings for affected family members with the *RUNX1* mutations are summarized in Figures 23-24 and Table A1.

Comparisons of the responses for affected individuals in this family to general population controls indicated no significant differences in their reported general health related problems, including cancer, and no family members reported heart problems (e.g., heart attack or angina) or thrombosis (DVT and stroke) (Figure 23). However, affected individuals (whose CHAT-P questionnaires were completed before genetic testing or discussions about a possible *RUNX1* defect) had a higher likelihood of reporting a family history of bleeding problems including: first degree relatives with bleeding problems (OR=304; 95 % CI, 13-7050; P<0.0001; family history of bleeding problems causing death or serious complications: OR=29; 95 % CI, 3.4-240; P=0.0041), thrombocytopenia (OR=440; 95 % CI, 16-12000; P<0.0001) and leukemia and other bone marrow problems (OR=29; 95 % CI, 3.4-240; P=0.0041) (Figure 23).

Affected individuals reported more bleeding symptoms than general population controls (OR estimates: 11-440) (Figure 24), including excessive bleeding that necessitated lifestyle changes (OR 15; 95 % CI, 1.6-130; P=0.04; Figure 24). Most (5/6) affected individuals experienced abnormal bleeding before the age of 18 OR=444; 95 % CI 16-12000; P<0.001) and half experienced abnormal bleeding before the age of 5 (Figure 24).

Proportionately more affected family members than general population controls reported prolonged bleeds from cuts and minor wounds that lasted longer than 10 minutes (OR=300; 95% CI, 15-5500; P<0.001) or an hour (OR 120; 95% CI, 5.2 - 2800; P=0.0004). None of the affected individuals required treatment for minor wounds (Figure 24).

Proportionately more affected family members reported abnormal bruising, including bruises that appeared without reason, were very large, disproportionate to trauma, lumpy and/or left permanent marks (OR 11-67) (Figure 24). They also reported having more bruises (>2 or 3 bruises at one time, OR 95 and 58 respectively) (Figure 24). No subjects reported bruises that tracked downward (Figure 24).

Proportionately more affected family members reported prolonged nosebleeds (>15 minutes) than general population controls (OR 19), with some requiring packing or cauterization (OR 121) but not transfusion, and uncommonly, admission to hospital (Figure 24).

Most affected subjects (4/6) reported wound healing problems after injuries, wounds, surgery or dental procedure (OR 38; 95% CI 4.9-300; P=<0.001), which was reported by only a few general population controls (3/60) (Figure 14). Proportionately more affected family members were given or recommended medications to prevent bleeding (OR 220; 95 % CI 9-5300; P=<0.001) (Figure 24). Four affected family members received desmopressin, and three reported that it worked whereas the other was not sure.

Proportionately more affected family members reported excessive oral or dental bleeding than general population controls (OR 77; 95% CI 5.3-1100; P=001) (Figure 24). The affected individuals who underwent prior dental procedures (n=3) reported excessive oral or dental bleeding that occurred with every dental procedure (2/3), lasted longer than a day (2/3) and/or resulted in extensive bruising (2/3). One affected individuals who received prophylactic treatment with desmopressin and tranexamic acid for a subsequent dental extraction reported no abnormal bleeding with that procedure.

Among subjects who underwent operations, proportionately more affected family members experienced excessive bleeding than general population controls (OR 15; 95 % CI 1.6-150; P=0.04) (Figure 24). Both affected individuals who reported excessive bleeding with surgery did not receive prophylactic treatment to prevent bleeding with surgery whereas the two with no abnormal surgical bleeding received prophylaxis with desmopressin or platelet transfusions. The affected individual who received prophylactic platelet transfusions was subsequently managed with prophylactic desmopressin, with good results.

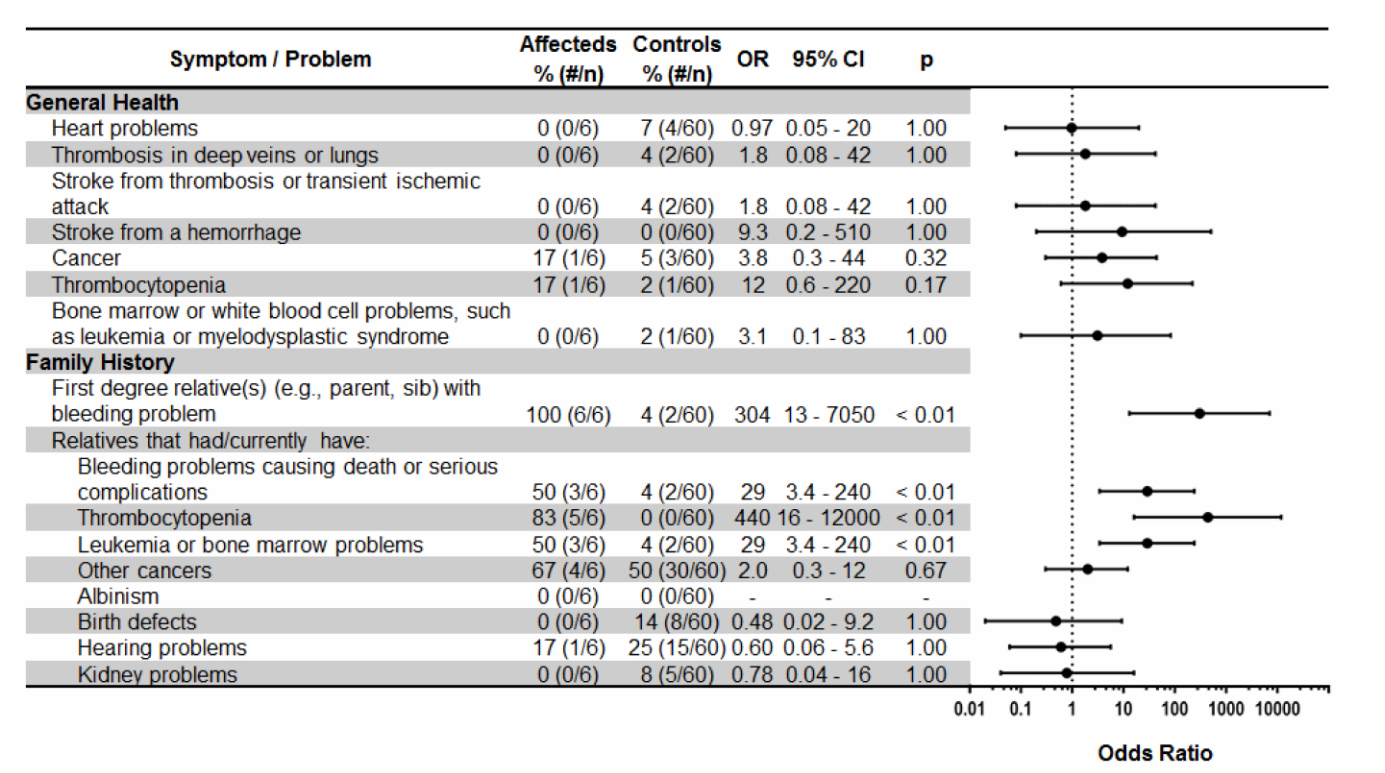
Excessive bleeding from trauma was uncommon among all subjects, and only one subject (a general population control) was admitted to hospital after a trauma.

Gastrointestinal bleeds were reported by few subjects (2/6 affected, 7/60 general population controls; OR not significant) (Figure 24), due to conditions such as hemorrhoids or anal fissures. One affected family

member reported a severe bleed from an ulcer that required hospital admission and platelet transfusions. None of the affected family members reported spontaneous hematuria, joint bleeds or muscle bleeds (Figure 24). One affected family member reported bleeding into the brain (Figure 24), which had been a subdural hematoma at birth.

OR estimates for female specific bleeding problems were limited by having data for only two affected female family members, although their risks for heavy menses, that limited lifestyle with most periods, were increased (OR 32; 95 % CI 1.4-770; P=0.02) (Figure 24). The proband had received oral contraceptive therapy for menorrhagia, and later, an endometrial ablation with Mirena IUD insertion at the end of the procedure, with good results. She experienced a post-partum hemorrhage after one of her two uncomplicated vaginal deliveries.

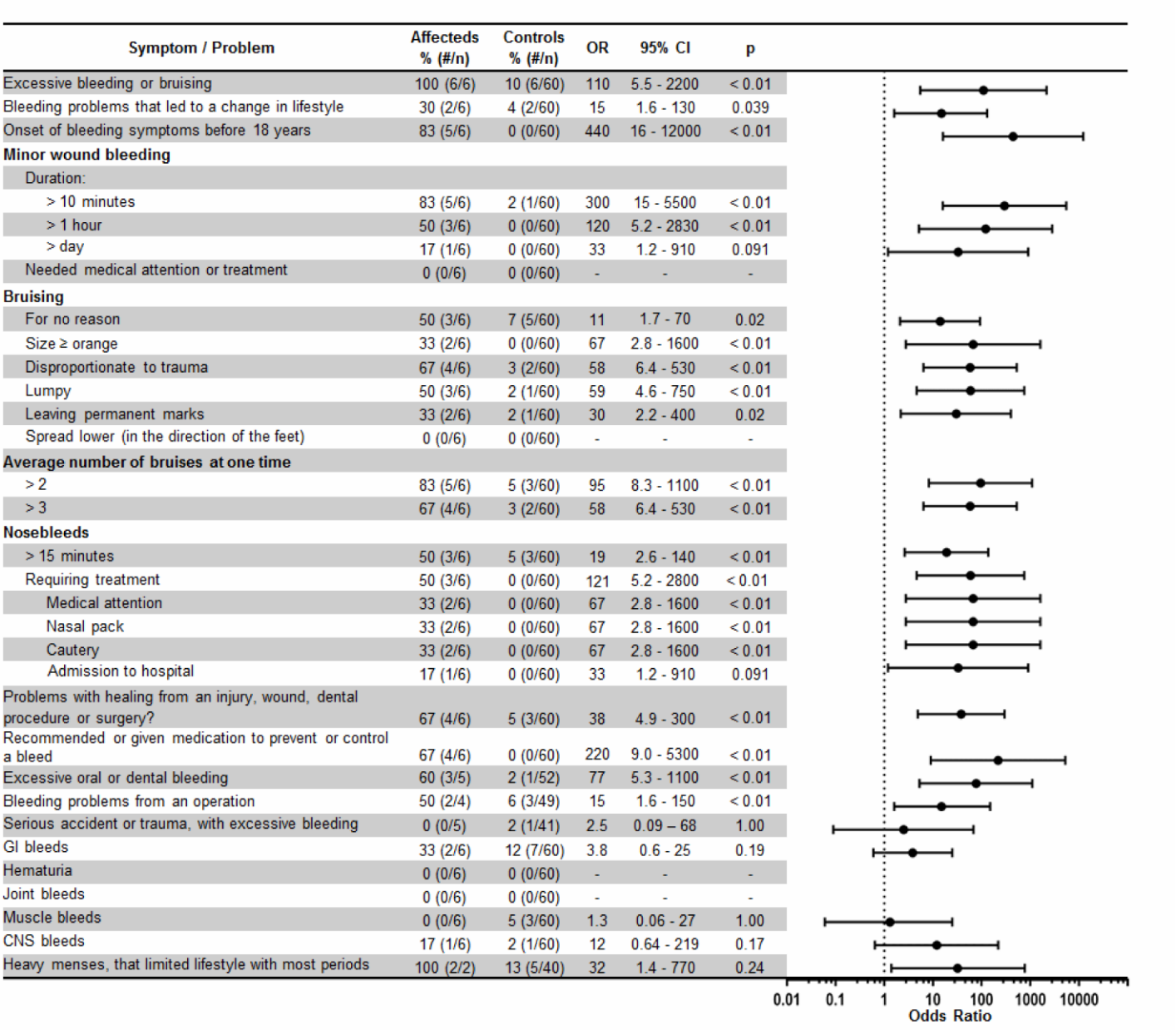
The number CHAT-P symptoms/problems reported by individual subjects (maximums, males: 18, females: 19) was significantly higher for affected family members (5-15, median 11) compared to unaffected family members (0-7, median 4) and general population control subjects (0-8, median 0) ($p=<0.01$) (Figure 25). The number of symptoms/problems were similar for affected family members with or without thrombocytopenia (median: 9 vs 11; $p= 0.6$), dense granule deficiency (median: 9 vs 12; $p= 0.5$) or prior surgery (median: 11 vs. 10; $p=0.9$); and showed no association to age ($R^2= 0.092$).



Symptom / Problem	Affecteds % (#/n)	Controls % (#/n)	OR	95% CI	p
General Health					
Heart problems	0 (0/6)	7 (4/60)	0.97	0.05 - 20	1.00
Thrombosis in deep veins or lungs	0 (0/6)	4 (2/60)	1.8	0.08 - 42	1.00
Stroke from thrombosis or transient ischemic attack	0 (0/6)	4 (2/60)	1.8	0.08 - 42	1.00
Stroke from a hemorrhage	0 (0/6)	0 (0/60)	9.3	0.2 - 510	1.00
Cancer	17 (1/6)	5 (3/60)	3.8	0.3 - 44	0.32
Thrombocytopenia	17 (1/6)	2 (1/60)	12	0.6 - 220	0.17
Bone marrow or white blood cell problems, such as leukemia or myelodysplastic syndrome	0 (0/6)	2 (1/60)	3.1	0.1 - 83	1.00
Family History					
First degree relative(s) (e.g., parent, sib) with bleeding problem	100 (6/6)	4 (2/60)	304	13 - 7050	< 0.01
Relatives that had/currently have:					
Bleeding problems causing death or serious complications	50 (3/6)	4 (2/60)	29	3.4 - 240	< 0.01
Thrombocytopenia	83 (5/6)	0 (0/60)	440	16 - 12000	< 0.01
Leukemia or bone marrow problems	50 (3/6)	4 (2/60)	29	3.4 - 240	< 0.01
Other cancers	67 (4/6)	50 (30/60)	2.0	0.3 - 12	0.67
Albinism	0 (0/6)	0 (0/60)	-	-	-
Birth defects	0 (0/6)	14 (8/60)	0.48	0.02 - 9.2	1.00
Hearing problems	17 (1/6)	25 (15/60)	0.60	0.06 - 5.6	1.00
Kidney problems	0 (0/6)	8 (5/60)	0.78	0.04 - 16	1.00

Figure 23. Information on general and familial health for the family with the *RUNX1* mutations.

Responses to questions about general health and family history for individuals that tested positive for the *RUNX1* mutations (Affecteds) were compared to general population controls (Controls). The proportions that reported each symptom/problem is summarized, along with the risk estimates, presented as odds ratios (ORs) and 95% confidence intervals (CIs).



Symptom / Problem	Affecteds % (#/n)	Controls % (#/n)	OR	95% CI	p
Excessive bleeding or bruising	100 (6/6)	10 (6/60)	110	5.5 - 2200	< 0.01
Bleeding problems that led to a change in lifestyle	30 (2/6)	4 (2/60)	15	1.6 - 130	0.039
Onset of bleeding symptoms before 18 years	83 (5/6)	0 (0/60)	440	16 - 12000	< 0.01
Minor wound bleeding					
Duration:					
> 10 minutes	83 (5/6)	2 (1/60)	300	15 - 5500	< 0.01
> 1 hour	50 (3/6)	0 (0/60)	120	5.2 - 2830	< 0.01
> day	17 (1/6)	0 (0/60)	33	1.2 - 910	0.091
Needed medical attention or treatment	0 (0/6)	0 (0/60)	-	-	-
Bruising					
For no reason	50 (3/6)	7 (5/60)	11	1.7 - 70	0.02
Size ≥ orange	33 (2/6)	0 (0/60)	67	2.8 - 1600	< 0.01
Disproportionate to trauma	67 (4/6)	3 (2/60)	58	6.4 - 530	< 0.01
Lumpy	50 (3/6)	2 (1/60)	59	4.6 - 750	< 0.01
Leaving permanent marks	33 (2/6)	2 (1/60)	30	2.2 - 400	0.02
Spread lower (in the direction of the feet)	0 (0/6)	0 (0/60)	-	-	-
Average number of bruises at one time					
> 2	83 (5/6)	5 (3/60)	95	8.3 - 1100	< 0.01
> 3	67 (4/6)	3 (2/60)	58	6.4 - 530	< 0.01
Nosebleeds					
> 15 minutes	50 (3/6)	5 (3/60)	19	2.6 - 140	< 0.01
Requiring treatment	50 (3/6)	0 (0/60)	121	5.2 - 2800	< 0.01
Medical attention	33 (2/6)	0 (0/60)	67	2.8 - 1600	< 0.01
Nasal pack	33 (2/6)	0 (0/60)	67	2.8 - 1600	< 0.01
Cautery	33 (2/6)	0 (0/60)	67	2.8 - 1600	< 0.01
Admission to hospital	17 (1/6)	0 (0/60)	33	1.2 - 910	0.091
Problems with healing from an injury, wound, dental procedure or surgery?	67 (4/6)	5 (3/60)	38	4.9 - 300	< 0.01
Recommended or given medication to prevent or control a bleed	67 (4/6)	0 (0/60)	220	9.0 - 5300	< 0.01
Excessive oral or dental bleeding	60 (3/5)	2 (1/52)	77	5.3 - 1100	< 0.01
Bleeding problems from an operation	50 (2/4)	6 (3/49)	15	1.6 - 150	< 0.01
Serious accident or trauma, with excessive bleeding	0 (0/5)	2 (1/41)	2.5	0.09 – 68	1.00
GI bleeds	33 (2/6)	12 (7/60)	3.8	0.6 - 25	0.19
Hematuria	0 (0/6)	0 (0/60)	-	-	-
Joint bleeds	0 (0/6)	0 (0/60)	-	-	-
Muscle bleeds	0 (0/6)	5 (3/60)	1.3	0.06 - 27	1.00
CNS bleeds	17 (1/6)	2 (1/60)	12	0.64 - 219	0.17
Heavy menses, that limited lifestyle with most periods	100 (2/2)	13 (5/40)	32	1.4 - 770	0.24

Figure 24. Bleeding risks for the family with the *RUNX1* mutations. Bleeding symptoms experienced by individuals with the RUNX1 mutations (Affecteds) compared to general population controls (Controls). The proportions that reported each symptom/problem is summarized, along with the risk estimates, presented as odds ratios (ORs) and 95% confidence intervals (CIs).

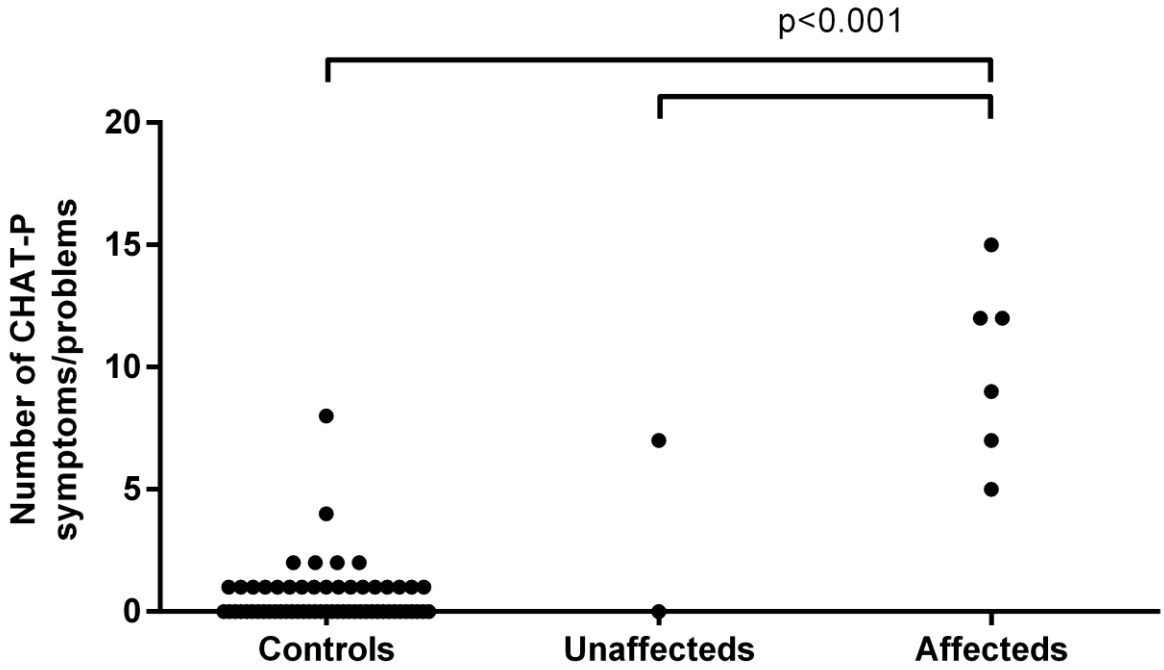


Figure 25. Number of CHAT-P symptoms/problems experienced by members of the family with the *RUNX1* mutations compared to general population controls. Scores represent the number of bleeding symptoms with OR that did not cross 1 for each subject, after exclusion of data for family history, being told that they had a bleeding disorder, and having received treatment or treatment recommendations for bleeding. Data is shown for both affected (who tested positive for the RUNX1 mutation; n=6) and unaffected (n=2) family members and general population controls (N=60). * indicate significantly different results (p values= <0.01).

CHAPTER 4

DISCUSSION

Bleeding disorders that impair platelet function due to unknown causes represent a common type of bleeding problem that is not well characterized. The present studies were undertaken to study the phenotype of inherited platelet function disorders of unknown cause, including a family that we discovered had an inherited platelet disorder due to a *RUNX1* mutation. First, I assessed if dense granule ATP release using lumi-aggregometry would be helpful in determining who has a platelet function disorder as such testing has been recommended for routine assessment of inherited platelet function disorders (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). We observed that the quantity of platelet dense granule ATP release with all agonists tested varied significantly between tests for individuals (Figures 2-3). Normal findings were often confirmed whereas abnormalities with multiple agonists were frequently not confirmed (Figure 4), even though the testing was done in parallel with a control sample to verify test performance was appropriate. Importantly, no association was detected between the presence of confirmed abnormal ATP release with multiple agonists and the bleeding score, or between the presence of confirmed abnormal ATP release with multiple agonists and the diagnosis of a definite bleeding disorder (Figure 5). As lumi-aggregometry was not able to reliably predict for subjects with an bleeding disorder it was not considered part of the case definition for determining who has, or does not have a platelet function disorder.

Second, I analyzed the laboratory and bleeding phenotype of the entire cohort in the Hamilton study on inherited platelet disorders of unknown cause that had confirmed platelet aggregation abnormalities with two or more agonists, and/or dense granule deficiency. Among the 29 affected individuals recruited to the Hamilton study that met the revised case definition criteria (Figure 9-10), 9 had dense granule deficiency and only five had thrombocytopenia (Figure 8). Aside from impaired platelet function, the majority of the cohort also demonstrated a mild bleeding phenotype indicated by elevated ISTH BAT scores (Figure 14). Interestingly, 4

subjects in which the family members of subjects recruited to the study had bleeding scores below 4. In some cases, the low bleeding score could be attributed to their relatively young age.

I also evaluated the features of an inherited platelet disorder in one of the families recruited to the study that was identified to have an inherited mutation in the gene encoding the hematopoietic transcription factor, *RUNX1*. The index case for this family was the only one out of the first twelve index cases that had NGS exome sequencing performed, that had a *RUNX1* mutation. Through follow up Sanger Sequencing, I verified that this individual and their affected family members, had a novel single base pair duplication in exon 6 of the *RUNX1* (c. 583dup) (Figure 7), which introduced a frameshift and truncation of *RUNX1* at a site where other frameshifts have been demonstrated to cause haploinsufficiency (Nakao et al., 2004). The affected family members with *RUNX1* mutations had similar, clear cut platelet function abnormalities in LTA and dense granule ATP release assays (Figure 12-13), even though only a few were dense granule deficient or thrombocytopenic (Figure 11).

In addition, a new questionnaire was piloted to identify general health and bleeding related risks for individuals with inherited platelet function disorders. Affected individuals within the study cohort had higher risks for bleeding (OR 3 to 360; Figures 18-21) from minor cuts/wounds, abnormal bruising, prolonged nosebleeds and those requiring treatment, wound healing problems, excessive bleeding from injuries/trauma, oral/dental challenges and surgery than general population controls. Affected females also reported increased risk of bleeding that interferes with their sex life, lengthened menses with clots and flooding, and excessive bleeding during childbirth sometimes requiring surgical intervention compared to general population controls. These symptoms are consistent with bleeding symptoms for mild platelet function disorders. Within the family with *RUNX1* mutations, there were similar increases the likelihood for experiencing wound healing problems and clinically significant bleeding, including challenge-related bleeding (OR 14 to >400; Figure 23-24). Very few of the affected subjects in our study had required hospitalization or interventions for challenges and

bleeding episodes, other than desmopressin or platelet transfusions (Figure 24) suggesting a milder form of platelet dysfunction.

4.1. IMPLICATIONS FOR STUDIES USING LUMI-AGGREGOMETRY ABNORMALITIES AS A CASE DEFINITION

Outlined in the recent recommendations from the ISTH committee, lumi-aggregometry may be used to assess dense granule release in the diagnostic workup of a platelet function disorder (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Despite these recent recommendations, our study (which was the first to assess reproducibility of findings for subjects tested for bleeding disorders) suggests lumi-aggregometry is not a reliable diagnostic tool for diagnosing individuals with a platelet function disorder. A previous study had noted a higher degree of variability in the test findings for general population controls (Pai et al., 2011). Our current study identified that lumi-aggregometry is not reliable to identify subjects with platelet function disorders even among individuals with a high pre-test probability or those individuals who are likely to be seen in the workup for a bleeding disorder. Accordingly, we recommended that measuring dense granule ATP release using lumi-aggregometry should not be used for diagnostic purposes (Badin et al., 2016).

4.1.1. Overstating disease incidence within a population with a high pre-test probability

Studies that have used lumi-aggregometry alone to establish the diagnosis of a platelet function disorder may be overstating the reported incidence in populations with a high pre-test probability such as individuals assessed for a suspected bleeding disorder. A recent article out of the UK-GAAP study group, stated that the prevalence of a platelet defect as identified by an abnormal response to lumi-aggregometry, was around 52% in participants with bleeding symptoms (Lowe et al., 2013) and a study from Milano, Italy reported a much lower value (18.8%). In both studies. the finding of an abnormality with one or more agonists was used to establish the diagnosis of a platelet function disorder (Lotta et al., 2013) and the findings for follow-up tests was not

reported. This may be problematic because our study indicates that a large proportion of referred individuals have inconsistent findings in lumiaggregometry assessments of ATP release.

4.1.2. Impact on the Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders

Previously, the case definition for our study on uncharacterized platelet function disorder included all subjects with reproducible aggregation defects, dense granule deficiency and/or impaired dense granule ATP release by lumi-aggregometry. However, we found that lumi-aggregometry is not reliable for the diagnosis of platelet function disorder when the test is performed for individuals with a high pre-test probability of a platelet function disorder. Consequentially, 3 index cases, and their affected and unaffected family members, were excluded from our study and the analysis of lab findings and their bleeding symptoms.

There is still a need to find a sensitive, specific and reproducible method for evaluating platelet secretion as it seems current diagnostic methods are either not reliable or not widely utilized (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015).

4.1.3. Value of results from lumi-aggregometry where other reproducible indications of platelet dysfunction are present

While an analysis of ATP release findings for families with inherited platelet secretion defects was not the focus of the first aim of this thesis, it was observed that the test findings are very similar amongst affected members of families with some inherited platelet function disorders. This is particularly evident when the findings are well below the lower limit of the RI amongst affected individuals. In gathering the data for cohort II, we observed that repeat ATP release testing was often not performed on individuals who were diagnosed with a familial platelet function disorder when their observed abnormal ATP release findings resembled other affected family members and/or less variable tests had confirmed the subject's platelet function abnormalities (i.e., aggregation tests and assessments of platelet dense granule numbers by EM). The practice of using the diagnostic tests with greater reproducibility to confirm platelet abnormalities seems reasonable given that

aggregation tests and dense granule EM are also more predictive of a bleeding disorder (Hayward et al., 2008; Castilloux et al., 2011).

We observed that some large families in our subsequent study had very abnormal dense granule ATP release findings among those affected by a platelet function disorder. For example, in the family that was identified to have a *RUNX1* mutation (Figure 13), the affected individuals had reduced dense granule ATP release with all agonists tested: their dense granule ATP release was markedly decreased with strong agonists and it was completely absent with weak agonists (Figure 10). This pattern of abnormalities was shared by all family members that tested positive for the *RUNX1* mutations and it was absent in those that tested negative the *RUNX1* mutations. These findings highlight the potential usefulness of lumi-aggregometry to further phenotype platelet function abnormalities large families that demonstrate consistent patterns in abnormal agonists and could aid researchers attempting to identify their molecular causes.

4.2. INVESTIGATION OF *RUNX1* MUTATIONS IN A FRENCH-CANADIAN FAMILY WITH REPRODUCIBLE PLATELET FUNCTION ABNORMALITIES

Within the cohort of subjects in Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders study, a family an inherited mutation in *RUNX1* was found to have a distinct laboratory phenotype.

Preliminary investigations identified a striking difference in both platelet count and dense granule count per platelet between the affected and unaffected family members (Figure 11). While not all affected family members would be classified as thrombocytopenic (2/6), there is a clear shift in their platelet counts which ranged from mildly thrombocytopenic to results in the lower end of the normal range (range: 125–169 x 10^9 platelets/L) compared to the reference interval and platelet counts of unaffected family members, which were normal. The mild reduction in platelet counts in this family's disorder suggests that other factors are important determinants of their bleeding, such as impairments in platelet aggregation function and dense granule release.

4.2.1. Reproducible LTA and Lumi-aggregometry results within the family

Indeed, all affected family members displayed impaired MA with low and high concentrations of collagen, thromboxane analogue and most affected family members also had reduced MA with arachidonic acid (Figure 9). These findings illustrate the usefulness of these agonists for an evaluation of *RUNX1*-related platelet function disorders as well as in excluding aspirin-like defects. Alternatively, all affected family members demonstrated normal responses to ADP while only two had absent secondary aggregation with epinephrine and one had reduced MA with ristocetin (Figure 9), suggesting these agonists may not be as informative for detecting *RUNX1* related platelet function. With the exception of epinephrine, these results are consistent with the abnormal aggregation response for agonists identified in recent study of nine unrelated families with *RUNX1* mutations (Latger-Cannard et al., 2016). Similar to the results of the family with the *RUNX1* mutations, the majority of affected subjects in our entire study had impaired MA with thromboxane analogue U46619, the low concentration of collagen and arachidonic acid in addition to predominantly displaying a normal response to ADP and epinephrine (Figure 17). This further emphasizes the usefulness of these agonists in detecting abnormal platelet function in individuals assessed for a bleeding disorder.

One of the most striking laboratory findings was absent dense granule secretion in response to all weak agonists and severely impaired response to strong agonists (Figure 13). When comparing across the whole cohort of affected subjects, 7 other subjects also displayed impaired platelet secretion to all agonists tested and another 5 affected subjects had impaired dense granule release in response to all weak agonists (Figure 10). All affected subjects in the study displayed impaired dense granule ATP release to at least one agonist, and the majority (25/29) of subjects displayed an impaired dense granule ATP release to at least two agonists. While lumi-aggregometry may not be particularly useful for determining which subjects have a platelet function disorder amongst individuals seen for an assessment of a bleeding disorder, impaired dense granule ATP release is common among subjects with other evidence of a platelet disorder such as impaired LTA, dense granule deficiency or a family history of platelet dysfunction.

4.2.2. The relationship of test platelet function tests to RUNX1 mutations

It is unsurprising to find that affected family members with *RUNX1* mutations would have impaired platelet function as transcription factor mutations have been identified as a cause for platelet function disorders (Cattaneo, 2013b; Stockley et al., 2013; Songdej and Rao, 2015; Bianchi et al., 2016). The current analysis was focused on the phenotype of a family with two autosomal dominantly inherited mutations in *RUNX1*. As a hematopoietic transcription factor, *RUNX1* influences platelet physiology through binding to the promoter regions of downstream genes (Bunimov et al., 2013; Stockley et al., 2013; Songdej and Rao, 2015; Bianchi et al., 2016). Consequentially, mutations in *RUNX1* often result in abnormal expression of downstream genes and may ultimately manifest as abnormalities of both platelet number and function (Stockley et al., 2013; Latger-Cannard et al., 2016). Most transcripts harboring premature termination codons are subjected to degradation by nonsense-mediated decay (Holbrook et al., 2004). The *RUNX1* mutations identified in the family under investigation are predicted to result in haploinsufficiency, similar to other frameshift mutations identified at the reported start site (Nakao et al., 2004). Mutations in *RUNX1* have also been associated with low mpl receptor expression (Heller et al., 2005). As the mpl receptor and its agonist thrombopoetin are important regulators of megakaryopoesis, a *RUNX1* mutation that impairs the production of mpl could account for lower levels of circulating platelets observed in the affected family members (Heller et al., 2005); although corroborating protein expression data would need to be obtained to provide reasonable evidence. In addition, this overall impairment also negatively affects the platelets ability to aggregate and to secrete dense granules in response to agonist stimulation (Figures 9 and 10).

At the molecular level, in addition to the reduction in platelet production, the bleeding phenotype could be due to a variety of factors including a reduced number of platelet receptors present on the platelet surface, or a qualitative defect in the receptors or signalling pathway leading to platelet activation (Songdej and Rao, 2015). Platelet transcript profiling in individuals with *RUNX1* haploinsufficiency revealed multiple pathways with prominent roles in platelet structure and function to be downregulated (Sun et al., 2007; Jalagadugula et al., 2011; Tanaka et al., 2012; Umansky, Feldmesser and Groner, 2015). Previous studies have also implicated

RUNX1 in regulating genes involved with cytoskeletal dynamics (Sun et al., 2007; Rao, 2013b). Australian researchers found that an overexpression of *RUNX1* resulted in an enrichment of microtubule and cytoskeleton related molecules (Michaud et al., 2008). They also found an increase in polymerized microtubules in cells cultured from FPD-AML affected cells compared to controls (Michaud et al., 2008). As microtubules are important in processes such as cell migration, cell division, cellular transport and intracellular signal transduction (Pearson and Bloom, 2004; Hartwig, 2013; White, 2013), impairment in *RUNX1* could contribute to the reduction in platelet counts and the defects in platelet function observed in the family under investigation.

At present, the risks for developing leukemia or myelodysplasia in individuals presenting with bleeding from a *RUNX1* mutation have not been estimated. However, *RUNX1* dosage has been linked to the likelihood of developing hematological malignancies (Song et al., 1999), with mutations causing dysregulation of megakaryocyte maturation, an expansion of progenitor cells and an increased potential for malignant transformation (Bluteau et al., 2012; Toya et al., 2013). Dominant negative mutations that preserve the ability of *RUNX1* to bind its co-factor CBFβ, and result in CBFβ sequestration (Langlois et al., 2015), may have a higher risk of hematologic malignancy than haploinsufficiency mutations (Michaud et al., 2002; Owen et al., 2008; Latger-Cannard et al., 2016). In the family under investigation, the oldest affected individual's aunt (whom we could not test as she was deceased) was known to have developed leukemia; a history consistent with rates of hematological malignancies tracked for other *RUNX1* haploinsufficiency mutations (Stockley et al., 2013; Latger-Cannard et al., 2016). Nonetheless, the discovery of this family's *RUNX1* mutation led to increased surveillance for more serious blood disorders as this family's defect would warrant early consideration of bone marrow transplantation.

4.3. EVALUATION OF BLEEDING PHENOTYPES AND BLEEDING ASSESSMENT TOOLS

Bleeding assessment tools have been used to capture information about the general health and bleeding symptoms of individuals with bleeding disorders in order to characterize and occasionally quantify the risks for further incidences of bleeding and other general health related issues. Subsequently, I investigated the bleeding

phenotype of subjects within study cohort using both the established bleeding assessment tool (ISTH BAT) as well as a new tool designed specifically to assess symptoms and problems that can reflect platelet dysfunction.

4.3.1. Bleeding phenotype in the cohort of subjects in Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders study

Within the cohort of individuals in the present study, elevated ISTH-BAT scores (median: 11, range: 6-18; Figure 19) were found for all affected subjects in the study. Previous studies assessing the utility of the ISTH BAT for predicting the diagnosis of platelet function defects report similar ISTH BAT scores (median: 12, range 8-16) (Lowe et al., 2013). A recent study from Pakistan on ISTH BAT bleeding scores in individuals with clinical evidence of bleeding and suspected inherited platelet function disorder reported much lower bleeding scores (median: 4, range 0-15), however the researchers indicated that this was likely due to the young age of study participants (median: 11, range 1-67) (Rashid et al., 2016). It is likely that these subjects would not have had sufficient time to experience sufficient hemostatic challenges such as tonsillectomy, pregnancy or dental work that would likely raise the median bleeding score (Rashid et al., 2016).

Within the family investigated with a *RUNX1* mutation, elevated ISTH-BAT scores (median: 10.5, range 4-20 compared to 0-1 for unaffected relatives and 0-6 for healthy controls; Figure 16) is consistent with the bleeding we have observed in other *RUNX1* related platelet function disorders, but it is inconsistent with a recent report that most individuals with *RUNX1* mutations have normal ISTH-BAT scores (Latger-Cannard et al., 2016). The disparities could reflect differences in molecular causes (e.g., haploinsufficiency vs. dominant negative mutations) and/or exposures to hemostatic challenges (Badin et al., in press). Unlike the recent French study (Latger-Cannard et al., 2016), we were able to compare subject findings as platelet counts, dense granule quantification, platelet aggregation and secretion responses which were all evaluated by standardized validated assays performed at the same center.

4.3.2. Insights and pitfalls of the ISTH BAT for the characterization of inherited platelet disorder bleeding phenotypes

While BAT do provide some information about mild bleeding disorders, these tools have limitations. For example, researchers from the UK GAAP study, noted that in a cohort with suspected inherited platelet function disorders, the ISTH BAT is unable to discriminate between individuals with a platelet function disorder detected by lumiaggregometry from those who had no platelet function disorders detected (Lowe et al., 2013). It should be noted that the bleeding symptoms detected by the ISTH BAT are not necessarily indicative of a platelet defect as bleeding symptoms may also reflect defects in VWF, coagulation factors or vessel walls (Watson et al., 2010).. In addition to VWD and platelet disorders, the ISTH BAT has also recently been used to assess bleeding in hemophilia: in a study of 168 hemophilia carriers, the median BS was 4 (range: 0–35) (James et al., 2016) and the tool was not recommended for use as a screen for hemophilia carriers. Our findings showed that the ISTH BAT scores do not show a significant relationship to the findings of platelet secretion tests (Figure 5A). As consistently abnormal release by lumi-aggregometry did not show a significant relationship to whether a subject has or does not have a bleeding disorder (Badin et al., 2016), the test results should be interpreted with caution.

Like other studies, we found overlap in ISTH BAT bleeding scores between subjects with a bleeding disorder and healthy controls (Figure14, 16 and (Lowe et al., 2013). Some healthy control individuals may experience a wide variety of bleeding symptoms; however, this alone does not necessarily indicate a true bleeding disorder is present. For example, menorrhagia is known to be common among the general population (reported by approximately 44% of women (Tosetto et al., 2013)) and many females require treatment for menorrhagia including birth control which was reported commonly (15%) by our female, general population controls. This symptom alone is not necessarily predictive of a platelet function disorder however, that subject would receive a bleeding score of 3 for that category alone. Alternatively, some affected family members report few bleeding symptoms, as demonstrated by at least 4 of the subjects presenting with a bleeding score of less

than 4. This is particularly true when assessing the bleeding history of children who have had limited exposure to hemostatic challenges (Watson et al., 2010).

One way clinicians and researchers could compare the bleeding phenotype among related disorders is to compare categories of bleeding symptoms are elevated among the populations (as performed in Figures 15 and 17). In the current study, the whole cohort (including the family with the *RUNX1* mutations) demonstrated elevated scores in the categories of epistaxis, cutaneous bleeding, minor wound bleeding, menorrhagia and bleeding from hemostatic challenges such as tooth extraction, surgeries and post-partum hemorrhage. As demonstrated, severity scores do assist in determining which categories of symptoms are relatively more severe (Figures 15 and 17). However, grouping related symptoms into a single score limits determining the specific symptoms experienced by the subjects. For example, a subject with a bleeding score of 1 for menorrhagia could have experienced the need to change pads more frequently than every 2 hours during menses, or may have had clotting or flooding with menses (Rodeghiero et al., 2010). Further, the scores do not distinguish singular from multiple incidences of a symptom or take into account whether the subject was exposed to any major hemostatic challenges.

4.3.3. Bleeding risks associated with uncharacterized platelet function disorders

We found that the CHAT-P tool was also useful for gathering standardized, detailed information on subjects' medical histories including the details of their bleeding symptoms and problems, including if they had bleed excessively before and after diagnosis and treatment.

There are many benefits to using odds ratios for analyzing clinical symptoms. Odds ratios compare the relative incidence of symptoms between groups rather than the absolute number of cases, which allows researchers to make comparisons between different populations and it allows for meaningful comparisons (e.g. symptoms experienced by those with a disease vs. general population). In comparing the bleeding risks for those to have experienced bleeding challenges (such as surgery or dental extractions), odds ratios analyses exclude the data for subjects who did not have the exposure. Odds ratio finings are relatively easy to explain to health care providers and individuals in the general population. Whereas a bleeding score may give clinicians with a background in hematology an indication of a individual's overall burden of bleeding symptoms, the score can be difficult for individuals with bleeding to understand. An estimate of the likelihood of experiencing specific bleeding symptoms for someone with a condition, compared to general population controls, is relatively easy to communicate to an individual. For example, communicating: "that compared to the general population, an affected individual in your family, that has the *RUNX1* mutations, is about 44 times more likely to experience excessive oral or dental bleeding", may provide more meaningful information to individuals about their bleeding problems than a score of their bleeding symptoms estimated by the ISTH BAT.

Using the recently developed CHAT-P, it was identified that this family's disorder increases the likelihood of experiencing wound healing problems and clinically significant bleeding, including challenge-related bleeding (OR 14 to >400; Figure 14). Their symptoms indicate a mild rather than a severe bleeding disorder, as few required interventions for challenges and bleeding episodes, other than desmopressin or platelet transfusions (Figure 24).

The cohort of all affected individuals identified in our study to have a platelet function disorder demonstrated a wide variety of bleeding symptoms common to other documented causes of mild bleeding disorders. There were no syndromic features or general health related problems unique to our cohort (Figure 18 and 23), which excluded some rare and well characterized disorders such as oculocutaneous albinism in HPS or CHS (Hayward, 2011; Cattaneo, 2013b; Ray et al., 2013). This is not surprising as other studies have reported that individuals with uncharacterized mild bleeding disorders are a heterogenous group of subjects with increased bleeding symptoms (Mezzano, Quiroga and Pereira, 2009; Pai and Hayward, 2009; Hayward, 2011; Carubbi et al., 2014; Westbury et al., 2015; Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015; Leo et al., 2015; Yoshimi et al., 2016).

Lowe and colleagues from the GAAP study recently performed an investigation assessing the ISTH bleeding scores of individuals with suspected inherited platelet function disorders (n=79) (Lowe et al., 2013). They found that these individuals typically presented with epistaxis, cutaneous bleeding, post-operative bleeding and menorrhagia (Lowe et al., 2013). A larger study (n=707) from Westbury and colleagues from the BRIDGE consortium, recently published their findings (Westbury et al., 2015) on the genetic analysis of individuals with disorders of platelet number, volume, morphology or function, or with pathological bleeding that cannot be explained by laboratory tests (Westbury et al., 2015). In addition to the symptoms identified in the previous analysis, they also identified oral cavity, muscle, joint, CNS and GI bleeds. The most common bleeding symptoms were cutaneous bleeds, minor wound bleeds and epistaxis (Westbury et al., 2015). Similarly, the bleeding symptoms in common platelet function disorders identified in our study were epistaxis, menorrhagia, bleeding from dental extractions, surgery, cutaneous bleeding and bleeding from minor wounds as relatively typical (reported in >40% of subjects). GI bleeds were also relatively common (34% of affected subjects), however they were often associated with an underlying cause such as a fissure or hemorrhoids (Figure 19-20, 24). Taken together, our study provides additional information on the symptoms commonly seen in individuals that have mild bleeding disorders due to platelet function abnormalities.

The bleeding symptoms of some platelet disorders may be quite severe. Symptoms such as prior traumas with excessive bleeding, abnormal bleeding lasting longer than 24 hours after dental extraction, hematuria and joint bleeds are reported more common reported by individuals with QPD than by subjects in our current study (outlined in the Table A2). It will be interesting to compare specific bleeding symptoms for platelet function disorders using an estimation of odds ratios in order to determine if there are distinct phenotypes influencing risks for different bleeding symptoms.

Interestingly, affected subjects reported proportionately more healing problems from an injury, wound, dental procedure or surgery than healthy controls. This could, in part, be due to the delay in bleeding cessation which prevents the initiation of wound healing (Nurden et al., 2008; Golebiewska and Poole, 2015).Alternatively, platelets contribute to wound healing through the secretion of various mitogenic and pro-angiogenic factors including platelet derived growth factor (PGDF) (Li et al., 2011), platelet derived stromal cell-derived factor 1 α (SDF-1α) (Massberg et al., 2006), vascular endothelial growth factor (VGEF) and endostatin (Nurden et al., 2008; Golebiewska and Poole, 2015). An inherited molecular defect that impairs platelet function could potentially impair delivery and/or release of these pro-healing factors and subsequently impair overall wound healing (Gawaz and Vogel, 2013). The family with the *RUNX1* mutation had an increased proportion of subjects with wound healing problems relative to the other affected subjects in the study (67% vs 35%) however, this was not significantly different ($p= 0.2$). The finding of delayed wound healing should, however, be interpreted with caution as it was identified through self-reporting and thus is inherently subjective.

This study has illustrated that bleeding risks can be estimated for typical families with an inherited platelet function disorder. Moving forward, it will be important to quantify the bleeding risks for other types of platelet function disorders as done in this study for a single family as well as multiple families and individuals with platelet function defects. The information gleaned from the analysis may be informative for individuals

with bleeding problems, and for researchers and healthcare providers who are interested in diagnosing and treating bleeding disorders.

4.4. ADVANTAGES AND LIMITATIONS OF THE CURRENT INVESTIGATIONS

4.4.1. Study size and bleeding score composition in the assessment of Lumi-aggregometry

One of the strengths of the assessment of Lumi-aggregometry was that it studied a large cohort of individuals with multiple dense granule ATP tests. Studies assessing platelet diagnostic endpoints have used similar subject numbers to power their analysis (Hayward et al., 2009b; Pai et al., 2011; Castilloux et al., 2011). Having a large study population allows the comparison within subgroups (for example, those classified as having a definite, probable and no bleeding disorder). The large number of subjects enabled valuable comparisons of variation among individual agonists between tests.

Another important strength of our current study is that we investigated individuals with a range of bleeding scores, not just those with high bleeding scores. In a study out of Milano, Italy estimating the prevalence of platelet secretion defects, the researchers only recruited individuals that had a bleeding score of over 4 (Lotta et al., 2013). Consequently, individuals that were referred to the testing centre and assessed for a bleeding disorder but did not reach that cut-off were excluded from the analysis. This exclusion is particular problem for younger individuals as they are less likely to have experienced bleeding challenges such as surgeries or dental extractions (Pai and Hayward, 2009; Rydz and James, 2012). As a result, the data are biased toward those with a high burden of symptoms and cannot claim a “typical” population of individuals seen for the diagnostic work-up of a bleeding disorder. In our study, subjects were retrospectively identified from all individuals referred for the diagnostic work-up of a suspected platelet function disorder, and did not exclude the responses of individuals based on the ISTH BAT as it was performed retrospectively and independent of their diagnostic work-up. Consequentially, it is reasonable to state the population is representative of what is typically seen for the evaluation of bleeding disorder.

4.4.2. Limitations in subject selection and assay comparisons in the study of Lumi-aggregometry

Despite the advantages of the assessment of lumi-aggregometry, some limitations that must be considered. These include a possible bias based on the rationale for repeat testing, limited numbers with ISTH BAT scores and testing on >2 occasions and no comparison to other methods of dense granule ATP release.

While the reason for repeat testing was rarely recorded, it was a common practice of the local hematologists to repeat tests on many individuals as the consistency of findings has not been established. As a consequence, the majority of the subjects retested had abnormal findings on their first test (78% of subjects had at least one abnormal agonist in cohort I), which could have introduced some bias. However, among the individuals with normal findings, these results were confirmed upon repeat testing (Figure 4) which iterates previous studies with healthy controls (Pai et al., 2011).

Another limitation was that few subjects were administered the ISTH BAT for comparison. In cohort I, all subjects were identified retrospectively, and thus were not given the questionnaire. In the second cohort, while every study subject was administered the ISTH BAT questionnaire the analysis was alternatively limited by the number of subjects (both affected and unaffected) that had multiple dense granule ATP release tests performed. Often, affected family members were recruited based upon similar test findings to their already recruited family members and did not require a subsequent test to confirm their diagnosis. The absence of a statistically significant relationship between the burden of clinically relevant bleeding symptoms (suggested by the bleeding score) and the presence of consistent multiple abnormal dense granule ATP responses may be, in part due to a limited sample size. However, despite a significant association between ISTH BAT scores and clinical diagnosis (Figure 1), no trend between ISTH BAT scores and ATP release findings is immediately apparent (Figure 5a).

Additionally, there were minimal subjects tested on more than 2 occasions. The reproducibility across multiple tests with the same subject and get an accurate representation of the intra-individual variability of certain agonists across varying lengths of time could not be assessed. It is unlikely that this there will be a

correlation between the amount of time between testes and the agonist variation, rather there may be a trend to decrease with age as studies have shown. It would be interesting to assess in a cohort that is likely to be seen for a work up of their bleeding disorder, similar to the cohort currently studied.

Conclusions for the diagnostic use of platelet dense granule release testing in general could not be drawn as only one endpoint for secretion (lumi-aggregometry and the detection of light from the luciferin/luciferase) was assessed. There is limited information on other assays of platelet secretion. The measurement of radioactive serotonin (5-HT) release is used to assess platelet secretion in some laboratories (Quiroga et al., 2009; Quiroga and Mezzano, 2012) and indeed has been identified as reproducible and concordant with LTA in some populations (Quiroga et al., 2009). Presently, radioactive serotonin (5-HT) release assays are not in widespread use as clinical laboratories have largely abandoned the use of radioisotopes (Bolton-Maggs et al., 2006). In addition, we did not assessed if the affected subjects had reduced total or releasable platelet adenine nucleotides because the diagnostic utility of such assays is uncertain and these assays are rarely performed (Gresele et al., 2014).

4.4.3. Advantages of a large study cohort and specific study criteria in the investigation of inherited platelet function disorder phenotypes

The cohort in the Hamilton study of the genotype, phenotype relationships in mild bleeding disorders related to abnormal platelet function has many advantages over similar study cohorts as it included large families and employed focused and validated study criteria.

Large families are helpful when determining the genetic cause of an inherited platelet disorder as this allows for an assessment of mutations that co-segregate with the disease phenotype (MacArthur et al., 2014). Unaffected family members allows researchers to disregard mutations present in both affected and unaffected family members as they are less likely to contribute to the bleeding phenotype (MacArthur et al., 2014).

We also excluded well documented cases of platelet function abnormalities (e.g., QPD) to focus on identifying the bleeding risks of common but largely uncharacterized disorders affecting platelet function.

4.4.4. Limitations in family enrollment and potential for index case bias

While the study cohort provided many advantages, it was limited by various aspects of recruitment. Typical of human research studies, subject enrollment can limit the ability to adequately power the analysis of outcomes. In the case of the family with the *RUNX1* mutation, there were limited numbers of available affected females and as a result we were unable to adequately assess risks for some menorrhagia symptoms and childbirth related bleeding, unlike our previous study of a larger family (McKay et al., 2004). Despite this, we did obtain other useful information on their bleeding risks. Recruitment of additional family members to the study might allow for a reassessment of the family's bleeding risks in the future.

Traditionally, index cases have more severe bleeding symptoms as they are more likely to be referred for an assessment and thus are more likely to be invited to participate in a study. One problem that could arise if the study population is skewed towards index cases is that average severity scores could be overstated. In our study, there was a high incidence of index cases compared to non-index cases (17 vs 12). This may be partially attributed to the high incidence of sporadic cases where only a single member of a family was affected. In other

cases, there was a lack of locally available family members for recruitment. However, when comparing both the ISTH BAT bleeding score and the overall CHAT-P bleeding scores in our study population, there does not seem to be a significant difference between index cases and their subsequently recruited affected family members. The inability to detect a statistically significant difference between the study groups allows their results to be aggregated.

While we were unable to detect a difference in bleeding scores between index and non-index cases, we do acknowledge that the majority of non-index case affected family members were from two of the large families in our study (9/11 from 2 families). For certain analyses, this may have skewed the phenotype to the individual family profile. Where possible, a secondary analysis was performed by excluding each of the families to test consistency. With further recruitment of study subjects including affected and unaffected family members, the overrepresentation by large families will be minimized and a more accurate representation of the bleeding symptoms can be assessed.

4.5. CONCLUSION AND FUTURE DIRECTIONS

In the present studies, it was determined that lumi-aggregometry should not be recommended as a diagnostic test for inherited platelet function disorders. Additionally, the cohort recruited to the Hamilton study on the phenotype and molecular cause of uncharacterized platelet function disorders were individuals with problems that are consistent with a mild, mucocutaneous bleeding disorder. Within that cohort, a family with a heritable mutation in *RUNX1*, and reproducible platelet aggregation and secretion abnormalities, also reported symptoms and problems typical of a mild bleeding disorder. Finally, CHAT-P is a useful tool for documenting and characterizing bleeding symptoms and problems associated with platelet disorders.

4.5.1. Phenotyping additional subjects with platelet function disorders

At present, we have assessed bleeding risks for a cohort with aggregation defects and/or dense granule deficiency, and for a single family with an inherited defect in *RUNX1*. Investigations of other large families would give insight into their family specific bleeding risks. Compiling additional bleeding risks from with other families with platelet function disorders will subsequently permit comparisons between different platelet disorders.

As a determination of risks (using odds ratios) requires a large number of subjects, identifying bleeding risks may not be feasible for all families. These analyses require that a sufficient number of affected subjects are available (e.g., 5-6 individuals, as a minimum).

Overall, the methods for determining bleeding risks using the CHAT-P are relatively simple and easy to use given a sufficient number of subjects with a definite bleeding phenotype, a control group of general population controls for comparison, and a large database to store and analyse the collected data. I would encourage researchers studying similar platelet type bleeding disorders to utilize the tool to permit comparisons of bleeding phenotypes across a diverse range of disorders and populations.

4.5.2. Exome sequencing for the identification of genes suspected to cause platelet dysfunction

Another interesting aspect of the current study is the implication of genetic analysis to give us insight into the preponderance of experiencing bleeding problems. As demonstrated by ours and other studies, identifying individuals that have pathologic inherited mutations in genes that influence platelet function, who may be more likely to bleed in the future (Cattaneo, 2013b; Westbury et al., 2015; Leo et al., 2015). Some factors hampering the diagnostic utility of first round genetic testing in the diagnosis of mild platelet bleeding disorders include the cost of investigations, variable penetrance of bleeding symptoms among some families with inherited disorders, the absence of a gold-standard clinical assay to test for abnormal platelet function and the prevalence of some bleeding symptoms, such as easy bruising and menorrhagia, within the general population (Watson et al., 2010).

Current guidelines for the diagnosis of platelet function disorders have included the possibility of first-line exome sequencing as an alternative to platelet function testing (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). However, the guidelines acknowledge that it is difficult to definitively prove that a specific sequence variation is causative for the inherited platelet function disorder in the absence of corroborating phenotypic evidence (Gresele and Subcommittee on Platelet Physiology of the International Society on Thrombosis and Hemostasis, 2015). Many genes affecting various aspects of hemostasis can contribute to the overall presentation of clinically relevant bleeding (Bunimov et al., 2013; Cattaneo, 2013b; Westbury et al., 2015; Leo et al., 2015). In order to exclude benign mutations and focus on those that are more influential on their overall bleeding phenotype, it is therefore important to study large families comprised of other similarly affected subjects as well as unaffected, but closely related family members.

Aside from the potential to screen for individuals with platelet dysfunction, identifying mutations that result in platelet dysfunction is also beneficial for identifying potential targets for future gene-therapy (Songdej

and Rao, 2015). For example, Connelly and colleagues (Connelly et al., 2014) demonstrated that pluripotent stem cells from individuals with FPD/AML harboring *RUNX1* mutations could recover from MK defects through targeted gene correction. The corrected clones resulted in approximately 40-60% more $CD41^+$ and $CD42^+$ megakaryocytes and an upregulation of megakaryocyte genes resulting in a rescue of phenotypic features of abnormal megakaryocyte differentiation (Connelly et al., 2014). While this is preliminary work, studies like these provide great potential to treat congenital defects such as the *RUNX1* mutations identified in our current study.

A DNA based diagnosis is also much less invasive than a blood draw. This is of particular interest for the pediatric population where extensive diagnostic tests of platelet function may be difficult (Watson et al., 2010; Lentaigne et al., 2016). Indeed, some of the young subjects in our investigation were unable to give a large enough sample for full platelet function testing. Despite this, we did have platelet function and genetic information that identified this child possessed the *RUNX1* mutation found in the affected members of the family.

As the body of knowledge expands on genes known to cause platelet dysfunction, a detailed phenotypic analysis remains useful to determine the molecular causes as well as identify the risk for bleeding in families with those genetic mutations. Through continued use of Next Generation sequencing technology, we are able to broaden our knowledge on the relationships between genetic variation and its impact on human health. Work towards creating a standardized method for characterizing these disorders and identifying the bleeding risk for these individuals are important steps in establishing a basis of knowledge for the impact of the identified mutations on human health.

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APPENDIX

Table A1. CHAT-P bleeding risks for all affected family members versus general population controls.

Bleeding risks were calculated as likelihood or odds ratios (OR), with 95% confidence intervals (CI), and p values. Blue shaded cells indicate symptoms or problems with significant OR. * indicate items with significant OR that were not included in the CHAT-P score. ‡ indicate bleeding symptoms/problems were no longer proportionally more frequent for affected subjects compared to general population controls when index cases (n=17s) were removed.

Symptom	Affected #/n	Control #/n	OR	95% CI	p-value
A. General Questions					
1. Heart problems (e.g. heart attack, angina) :	1/29	4/60	0.5	0.053 - 4.7	0.50
2. Thrombosis (blood clots) in deep veins (DVT) or lungs (pulmonary embolism or PE)	1/29	2/60	1.0	0.090 - 12	1.00
3. Stroke from thrombosis (blockage of a blood vessel inside the brain) or transient ischemic attack (TIA)	1/29	2/60	1.0	0.090 - 12	1.00
4. Stroke from a hemorrhage (bleed inside your brain)	0/29	0/60			
5. Cancer	2/29	3/60	1.4	0.22 - 8.9	0.6593
6. Thrombocytopenia (low platelet count)	2/29	1/60	4.4	0.38 - 50	0.2467
7. Bone marrow or white blood cell problems, such as leukemia or myelodysplastic syndrome	1/29	1/60	2.1	0.13 - 35	0.5480
B. Family History					
1. Who has (or had) bleeding problems in your natural family (pick all that apply) i. grandparent(s)*	9/29	2/60	13	2.6 - 66	0.0005
ii. mother*	10/29	1/60	31	3.7 - 259	< 0.0001
iii. father*	5/29	1/60	12	1.4 - 111	0.0131
iv. brothers*	4/29	0/60	21	1.1 - 412	0.0097
v. sisters*	7/29	0/60	40	2.2 - 736	0.0002
vi. uncles	3/29	0/60	16	0.80 - 321	0.322
vii. aunts	2/29	0/60	11	0.51 - 237	0.1037
viii. other relatives	1/29	0/60	0.27	0.032 - 2.3	0.2656
ix. first degree relative(s)(e.g., parent, sib), based on responses above*	22/29	2/60	44	12 - 165	< 0.0001
2. Bleeding problems causing death or serious complications*	9/29	2/60	13	2.6 - 66	0.0005
3. Low platelet counts*	6/29	0/60	33	1.8 - 618	0.0008
4. Leukemia (blood cancer) or bone marrow problems*	6/29	2/60	7.6	1.4 - 40	0.0133

5. Other cancers	21/29	30/60	2.6	1.0 - 6.8	0.668
6. Albinism	1/29	0/60	6.4	0.25 - 161	0.3258
7. Birth defects	1/29	8/60	0.23	0.028 - 2.0	0.2614
8. Hearing problems	7/29	15/60	0.95	0.34 - 2.7	1.00
9. Kidney problems	3/29	5/60	1.3	0.28 - 5.7	0.7122
C. Questions About Your Bleeding Problems					
1. Do you experience excessive bleeding or bruising (e.g. longer or more bleeding, bigger bruises, more frequent bruises than most people)?	25/29	6/60	56	15 - 217	< 0.0001
2. How old were you when bleeding problems began (pick the best answer)?					
i. newborn	2/29	0/60	11	0.51 - 237	0.1037
ii. less than 1 year old*	4/29	0/60	21	1.1 - 412	0.0097
iii. 1-5 years old*	7/29	0/60	40	2.2 - 736	0.0002
iv. 6-18 years old*	7/29	0/60	40	2.2 - 736	0.0002
v. adult (older than 18 years)* ‡	9/29	2/60	13	2.6 - 66	0.0005
vi. Onset of bleeding symptoms before 18 years of age, based on above responses*	20/29	0/60	261	15 - 4690	< 0.0001
3. Have bleeding problems caused you to change your lifestyle or pay extra attention when doing certain things or activities?	12/29	2/60	20	4.2 - 101	< 0.0001
4. Has a doctor ever told you that you have a bleeding problem?*	24/29	0/60	539	29 - 10130	< 0.0001
5. Have you had transfusions for bleeding (that is, to prevent, control or treat bleeding)? ‡	13/29	0/60	99	5.6 - 1756	< 0.0001
6. How many times have you been transfused					
i. once	6/29	N/A			
ii. twice	5/29	N/A			
iii. three times	0/29	N/A			
iv. four	2/29	N/A			
v. more than once (based on above)*	13/29	0/60	99	5.6 - 1756	< 0.0001
b) What did you receive?					
i. red cells	1/13	N/A			
ii. platelets	5/13	N/A			
iii. plasma	2/13	N/A			
iv. other	2/13	N/A			
7. Has a doctor ever suggested or given you a medication to prevent or control a bleed?*	22/29	0/60	363	20 - 6624	< 0.0001
b) What medication was given or suggested to prevent or control a bleed:					
i. DDAVP or desmopressin	16/22	N/A			
ii. fibrinolytic inhibitor drug (e.g. amicar, tranexamic acid, Cyklokapron)	5/22	N/A			
iii. other type of medication	1/22	N/A			
Did it work?					
i. yes	13/22	N/A			
ii. no	0/22	N/A			

iii. not sure	9/22	N/A			
8. Have you had bruises that (pick all that apply):					
i. show up for no reason (without trauma)	20/29	5/60	11	1.7 - 70	0.0204
ii. are disproportionate to trauma (e.g. bruise appears from a touch without force)	14/29	2/60	27	5.5 - 132	< 0.0001
iii. form a lump lasting days	10/29	1/60	31	3.7 - 259	< 0.0001
iv. leave a permanent mark on my skin ‡	6/29	1/60	15	1.8 - 135	0.0044
v. spread lower (in the direction of the feet) ‡	7/29	0/60	40	2 - 736	0.0002
vi. are the size of an orange, or larger	10/29	0/60	65	4 - 1164	< 0.0001
9. Average number of bruises at one time:					
i. > 2	19/29	3/60	36	9 - 145	< 0.0001
ii. > 3	14/29	2/60	27	5.5 - 132	< 0.0001
10. When you had a little cut (a paper cut, a razor gash, a needle prick…) has your bleeding					
i. lasted longer than 10 minutes but less than an hour	16/29	1/60	73	8.8 - 598	< 0.0001
ii. lasted more than an hour but less than a day	9/29	0/60	56	3 - 1007	< 0.0001
iii. lasted longer than a day	3/29	0/60	16	0.80 - 32	0.0322
iv. needed medical attention or treatment	1/29	0/60	6.4	0.25 - 161	0.3258
11. Have you ever had problems with healing from an injury, wound, dental procedure or surgery?	12/29	3/60	13	3.4 - 53	< 0.0001
12. Have you ever had anemia, iron deficiency or treatment with iron (tablets, injections or intravenous iron)?	15/29	9/60	6	2 - 17	0.0006
13. Have you had nose bleeds that last longer than 15 minutes?	16/29	3/60	23	6 - 92	< 0.0001
14. Have you had nosebleeds that needed (pick all that apply):					
i. treatment*	7/29	0/60	40	2 - 736	0.0002
ii. medical attention.	6/29	0/60	33	2 - 618	0.0008
iii. nasal pack ‡	5/29	0/60	27	1.4 - 511	0.0029
iv. cautery	5/29	0/60	27	1.4 - 511	0.0029
v. admission to hospital	2/29	0/60	11	0.50 - 237	0.1037
vi. drug treatments	1/29	0/60	6.4	0.25 - 161	0.3258
vii. transfusion of red cells	0/29	0/60	-	-	-
viii. transfusion of platelets	0/29	0/60	-	-	-
ix. other blood products	0/29	0/60	-	-	-
x. surgery	0/29	0/60	-	-	-
xi. other treatments	0/29	0/60	-	-	-
15. Have you ever had a serious accident or trauma, with excessive bleeding (e.g. doctor said there was more bleeding than expected)? ‡	5/26	1/41	9.5	1.0 - 87	0.0293
b) If you had a serious accident or trauma with excessive bleeding, did you need					
i. admission to hospital	1/5	1/1	0.11	0.0028 - 4.5	0.333
ii. drug treatments	2/5	0/1	2.1	0.059 - 78	1.00
iii. transfusion of red cells	0/5	0/1	-	-	-
iv. other blood products	1/5	0/1	1.0	0.025 - 40	1.00

v. surgery	2/5	0/1	2.1	0.059 - 78	1.00
vi. other treatments	1/5	0/1	1.0	0.025 - 40	1.00
16. Have you ever had bleeding problems from an operation (including large bruises around the incision or bleeding causing a large blood collection or hematoma)	13/25	3/49	17	4.1 - 68	< 0.0001
17. If you had excessive bleeding from operations, did this happen :					
i. with every operations	5/13	0/3	4.5	0.19 to 106	0.5089
ii. only with some operations	4/13	3/3	0.068	0.0028 - 1.6	0.0625
iii. only when operations were done without treatments to prevent bleeding	4/13	0/3	3.3	0.14 - 79	0.528
iv. other	0/13	0/3	-	-	-
b) If you had excessive bleeding from operations, did bleeding problems begin					
i. on the day of the operation*	11/13	0/3	32	1.2 - 843	0.0179
ii. the next day	1/13	0/3	0.84	0.028 - 26	1.00
iii. two or more days after the operation	0/13	1/3	0.062	0.0019 - 2.0	0.1875
iv. other	3/13	0/3	2.3	0.095 - 57	1.00
c. If you had excessive bleeding from operations, please indicate stayed in hospital longer because of bleeding					
i. stayed longer in hospital because of bleeding	6/13	0/3	6.1	0.26 - 141	0.25
ii. admission to hospital because of bleeding	2/13	0/3	1.5	0.26 - 141	1.00
iii. drug treatments for bleeding	3/13	0/3	2.3	0.095 - 57	1.00
iv. platelet transfusion	5/13	0/3	4.5	0.19 - 106	0.5089
v. transfusion of red cells	2/13	0/3	1.5	0.26 - 141	1.00
vi. other blood products	3/13	0/3	2.3	0.095 - 57	1.00
vii. another operation to control bleeding	1/13	0/3	0.84	0.028 - 26	1.00
viii. other treatments	3/13	0/3	2.3	0.095 - 57	1.00
19. If you had operations without excessive bleeding, were you given treatments to prevent bleeding?					
i. Yes*	7/25	0/49	40	2.2 - 739	0.0003
ii. Yes, but not for every operation without bleeding	1/25	1/49	2.0	0.12 - 33	1.00
20. If you had treatments to prevent bleeding with operations, what did you receive (pick all that apply)					
i. DDAVP or desmopressin	5/8	0/1	4.7	0.15 - 152	0.4444
ii. fibrinolytic inhibitor drugs (tranexamic acid, Cyklokapron, amicar)	2/8	0/1	1.2	0.034 - 39	1.00
iii. platelets	2/8	0/1	1.2	0.034 - 39	1.00
iv. other	1/8	0/1	0.6	0.016 - 23	1.00
22. Have you ever had excessive oral or dental bleeding (more than expected or longer bleeding) during or after tooth extractions, other dental procedures, or after biting your tongue or cheek?	13/28	1/52	44	5.3 - 366	< 0.0001
b) If you had excessive oral or dental bleeding, did it happen					
i. with every dental procedure	4/13	0/1	1.4	0.048 - 42	1.00
ii. only with some dental procedures	5/13	1/1	0.22	0.0074 - 6.3	0.4286

iii. only when dental procedures were done without treatments to prevent bleeding	3/13	0/1	1.0	0.033 - 31	1.00
iv. other	1/13	0/1	0.36	0.0096 - 13	1.00
c) If you had excessive oral or dental bleeding, did it: i. last longer than one day	7/13	1/1	0.38	0.013 - 11	1.00
ii. cause extensive bruising	3/13	1/1	0.11	0.0036 - 3.4	0.2857
iii. cause swelling and problems with breathing or swallowing	1/13	0/1	0.36	0.0096 - 13	1.00
iv. need medical attention	2/13	0/1	0.65	0.020 - 21	1.00
v. need packing or resuturing	4/13	0/1	1.4	0.048 - 42	1.00
vi. need admission to hospital	0/13	0/1	0.11	0.001556 - 7.935	1.00
vii. need drug treatments	2/13	0/1	0.65	0.020 - 21	1.00
viii. need red cell transfusions	0/13	0/1	-	-	-
ix. need platelet transfusion	0/13	0/1	-	-	-
x. need other blood products	0/13	0/1	-	-	-
xi. need sutures or surgery	0/13	0/1	-	-	-
xii. need other treatments	0/13	0/1	-	-	-
23. Have you ever had bleeding into your muscles?	1/29	3/60	0.68	0.067 - 6.8	1.00
b) did it : i. occur spontaneously	1/1	0/3	21	0.27 - 1648	0.25
ii. occur after trauma	0/1	3/3	0.048	0.00061 - 3.7	0.25
iii. need treatments	0/1	0/3	2.3	0.030 - 183	1.00
24. Have you ever had bleeding into your joints?	1/29	0/60	6.4	0.25 - 161	0.3258
b. If yes, was the joint bleeding after trauma?	1/1	N/A			
25. Have you ever had bleeding from your stomach or bowels	10/29	7/60	4.0	1.3 - 12	0.0191
c) if this bleeding needed : i. urgent medical attention	4/10	0/7	10	0.47 - 232	0.1029
ii. admission to hospital	2/10	0/7	4.4	0.18 - 107	0.4853
iii. drug treatments	0/10	0/7	-	-	-
iv. transfusion of platelets	0/10	0/7	-	-	-
v. transfusion of red cells	0/10	0/7	-	-	-
vi. transfusion of other blood products	2/10	0/7	4.4	0.18 - 107	0.4853
vii. surgery	0/10	0/7	-	-	-
viii. other treatments	0/10	3/7	0.061	0.0026 - 1.4	0.0515
27. Have you ever had blood in your urine i. yes, happened spontaneously – for no reason	0/29	0/60	-	-	-
ii. yes, with a urinary tract / bladder infection	0/29	0/60	-	-	-
iii. yes, from stones or kidney disease	0/29	0/60	-	-	-
iv. yes, but for another reason	0/29	0/60	-	-	-
v. Hematuria any reason (based on above)	0/29	0/60			
28. Have you had bleeding in your brain? i. yes, happened spontaneously	1/29	1/60	2.1	0.13 to 35	0.5480
ii. yes, after trauma	0/29	0/60			

iii. yes, other reason	2/29	0/60	11	0.51 - 237	0.1037
iv. CNS bleeding any reason (based on above)	3/29	1/60	6.8	0.68 - 69	0.0995
E. Questions For Women Only					
1. Have you had menstrual periods? (Yes)	19/19	40/40	0.48	0.0092 - 25	1.00
2. On average, how many days, do/did your menstrual periods last? (longer than a week) ‡	9/19	3/40	11	2.5 - 49	0.0009
3. How many days have been a heavy flow : i. 3 days*‡	8/19	8/40	2.9	0.88 - 9.6	0.1161
ii. longer*‡	7/19	4/40	5.25	1.3 - 21	0.0277
iii. 3 days or longer	15/19	12/40	8.8	2.4 - 32	0.0006
b. On the heaviest days of a menstrual period, how long would it typically take for you to soak through pads or tampons? i. 1-2 hours*	11/19	15/40	2.3	0.75 - 7.0	0.1685
ii. less than an hour*	6/19	1/40	18	2.0 - 164	0.0033
iii. Less than 2 hours	17/19	16/40	13	2.6 - 63	0.0005
4. Have you been treated for heavy periods? Yes ‡	10/19	5/40	7.8	2.1 - 29	0.0027
5. If you have been treated for heavy periods, please indicate the average number of days that your periods lasted when you were NOT on treatments i. longer	5/10	2/40			
b. If you have been treated for heavy periods, please indicate the average numbers of days of heavy flow during your menstrual periods when you were NOT on treatment (if there is a range, give the typical number, e.g. 2 days): i. 3 days	2/8	2/40			
ii. longer	5/8	2/40			
iii. 3 days or longer	7/8	2/40			
c. If you have been treated for heavy periods, please indicate how long it would take you to soak through pads or tampons on NO treatments? i. in 1-2 hours	2/8	2/40			
ii. less than an hour	4/8	2/40			
iii. less than 3 hours	6/8	2/40			
6. Please indicate if you have had (pick all that apply) i. flooding, gushing accidents with menstrual periods, due to heavy bleeding ‡	10/19	9/40	3.8	1.2 - 12	0.0355
ii. bleeding from menstrual periods that restricted your lifestyle, with most periods	4/19	5/40	1.9	0.44 - 7.9	0.4503
iii. bleeding from menstrual periods that restricted your lifestyle, but less frequently	5/19	8/40	1.9	0.61 - 5.7	0.3880
iv. Lifestyle restriction	8/19	12/40	2.3	0.75 - 7.0	0.1514
v. menstrual bleeding with clots up to 1 cm in size*‡	5/19	7/40	10	1.7 - 58	0.0095

vi. menstrual bleeding with clots between 1-3 cm in size*‡	6/19	2/40	8.8	1.6 – 49	0.0105
vii. menstrual bleeding with clots bigger than 3 cm*	6/19	3/40	5.7	1.2 – 26	0.0247
viii. clots with menstrual bleeding	12/19	12/40	5.4	1.7 – 17	0.0041
ix. menstrual bleeding needing emergency treatment or hospital admission ‡	3/19	0/40	17	0.84 - 352	0.0298
x. menstrual bleeding needing blood transfusions	2/19	0/40	12	0.53 - 254	0.0999
xi. were told by a doctor that you had fibroids	1/19	5/40	0.39	0.042 - 3.6	0.6532
xii. were told by a doctor that you had endometriosis	3/19	1/40	7.3	0.71 - 76	0.0937
7. Have you ever been treated for heavy periods with: i. birth control pill*‡	8/19	6/40	4.1	0.71 - 76	0.0460
ii. fibrinolytic inhibitor drugs	2/19	0/40	12	0.53 - 254	0.0999
iii. Mirena IUD	0/19	0/40	-	-	-
iv. Depo Provera	2/19	0/40	12	0.53 - 254	0.0999
v. hysterectomy	4/19	2/40	5.1	0.84 - 31	0.078
vi. D&C	2/19	1/40	4.6	0.39 - 54	0.2402
vii. endometrial ablation	3/19	2/40	3.6	0.54 - 23	0.3157
viii. other	1/19	1/40	2.2	0.13 - 37	0.5441
8. Have you had bleeding problems that interfere with your sex life?	5/17	2/33	6.5	1.1 - 38	0.037
9. Have you ever been pregnant	13/17	23/33	1.4	0.37 - 5.4	0.7455
10. Did you have bleeding problems at the time of childbirth, a miscarriage or after delivery? ‡	8/13	2/23	17	2.7 - 105	0.0013
11. If you had bleeding problems at the time of childbirth, a miscarriage or after delivery, did you receive treatments to prevent bleeding with your other deliveries?* ‡	5/13	1/23	14	1.4 - 136	0.0161
12. Did you bleed for more than 6 weeks after childbirth?	3/13	3/23	2	0.34 - 12	0.6454
13. If you ever gave birth, or had a miscarriage, did you: i. stay longer in hospital because of bleeding	3/13	2/23	3.2	0.45 - 22	0.3282
ii. need readmission to hospital because of bleeding	1/13	0/23	5.6	0.21 - 149	0.3611
iii. need intensive care treatment for bleeding	1/13	0/23	5.6	0.21 - 149	0.3611
iv. receive drug treatments for controlling bleeding	2/13	1/23	4.0	0.33 - 49	0.5394
v. receive drug treatments for controlling bleeding with your next pregnancies	0/13	0/23			
vi. receive platelet transfusions	1/13	0/23	5.6	0.21 - 149	0.3611
vii. receive a red cell (blood) transfusions	2/13	0/23	10	0.45 - 231	0.1238
viii. receive other blood products	1/13	0/23	5.6	0.21 - 149	0.3611
ix. transfusions (based on answers above) ‡	3/13	0/23	16	0.74 - 331	0.0401
x. need an operation to control bleeding ‡	6/13	0/23	41	2.0 - 812	0.0009
xi. need other treatments for bleeding	1/13	0/23	5.6	0.21 - 149	0.3611

Table A2. Comparison of bleeding risks for affected individuals versus individuals QPD. Bleeding risks for QPD were adapted from data from a previous publication (McKay et al., 2004) and were calculated as likelihood or odds ratios (OR), with 95% confidence intervals (CI), and p values. Blue shaded cells indicate symptoms or problems with significant OR.

Symptom	Affected % (#/n)	QPD % (#/n)	OR	95% CI	p-value
Abundant/excessive bleeding or bruising	86 (25/29)	57(13/23)	4.8	1.3 - 18	0.027
Had bleeding problems that led to a change in lifestyle	41 (12/29)	60 (12/20)	0.53	0.16 - 1.7	0.38
History of transfusions	45 (13/29)	52 (12/23)	0.74	0.25 - 2.2	0.78
Transfusion following surgery	24 (7/29)	38 (5/13)	0.51	0.12 - 2.1	0.46
Other treatments recommended for bleeding	76 (22/29)	87 (20/23)	0.47	0.11 - 2.1	0.48
Problems healing from an injury	41 (12/29)	26 (6/23)	0.2	0.61 - 6.6	0.38
Bruising					
Without reason	69 (20/29)	55 (12/22)	1.9	0.59 - 5.9	0.38
Disproportionate to trauma	48 (14/29)	39 (9/23)	2.6	0.81 - 8.6	0.15
That spreads towards feet or size of an orange or larger	31 (9/29)	32 (7/23)	1.0	0.31 - 3.4	1.00
Nosebleeds					
Lasting longer than 15 minutes	55 (16/29)	77 (10/13)	0.37	0.084 - 1.6	0.30
Requiring nasal packing	17 (5/29)	15 (2/13)	1.1	0.19 - 6.9	1.00
Requiring cautery	17 (5/29)	24 (3/13)	0.69	0.14 - 3.5	0.69
Requiring transfusion	0 (0/29)	8 (1/13)	0.15	0.0058 - 4.0	0.33
Requiring hospitalization	7 (2/29)	17 (2/12)	0.37	0.046 - 3.0	0.57
Trauma or Serious Accident					
Bleeding excessively afterward	19 (5/26)	86 (12/14)	0.04	0.0066 - 0.24	0.0001
Required hospitalization	20 (1/5)	67 (10/15)	0.13	0.011 - 1.4	0.13
Required transfusion	20 (1/5)	31 (4/13)	0.81	0.069 - 9.5	1.00
Dental Extractions					
With abnormal bleeding	46 (13/28)	94 (16/17)	0.054	0.0063 - 0.47	0.0012
Bleeding lasting longer than 24 hours afterward	54 (7/13)	94 (16/17)	0.073	0.0073 - 0.72	0.025
Hematuria	0 (0/29)	50 (11/22)	0.017	0.00092 - 0.31	<0.0001
Joint bleeds	3 (1/29)	43 (10/23)	0.046	0.0054 - 0.40	0.0006
Sex Specific					
Menses lasting longer than 7 days	47 (9/19)	50 (3/6)	0.9	0.14 - 5.6	1.00
Hysterectomy to control menses	21 (4/19)	17 (1/6)	1.3	0.12 - 15	1.00
Transfused when gave birth	23 (3/13)	40 (2/5)	0.45	0.050 - 4.1	0.58