ANALYSIS OF PATIENTS WITH PLATELET FUNCTION DISORDERS

PHENOTYPIC ANALYSIS OF PLATELETS IN PATIENTS WITH PLATELET FUNCTION DISORDERS

By:

JUSTIN BRUNET, BHSC (HON)

A Thesis Submitted to the School of Graduate Studies

in Partial Fulfillment of the Requirements

for the Degree of

Master of Science

McMaster University

Masters 2018

Medical Sciences

McMaster University

Hamilton, Ontario

TITLE: Phenotypic analysis of platelets in patients with platelet function disorders

AUTHOR: Justin Brunet, BHSc (Hon) (McMaster University)

SUPERVISOR: Dr. Catherine P.M. Hayward

NUMBER OF PAGES: xii, 125

LAY ABSTRACT

Platelets are blood cells that are important to limit bleeding as they stick at sites of injury and help blood to clot. The medical term for conditions where platelets don't function properly is platelet function disorders (PFD). Laboratory tests are important to diagnose PFD. We evaluated our experiences with some tests for diagnosing PFD, including whole mount electron microscopy (EM), a test that looks for a type of PFD called platelet dense granule deficiency. We also evaluated other diagnostic tests, including a test for a platelet protein called MYH10. We also evaluated how platelets support blood clotting - an aspect of platelet function that hasn't been tested much in PFD. We found that whole mount EM is helpful to diagnose PFD, unlike tests for MYH10. We also found defects in how platelets support blood clotting in some but not all types of PFD. The findings help clarify what tests are useful to assess PFD.

ABSTRACT

Many rare and severe forms of platelet function disorders (PFD) are now well characterized, however, information is only emerging about the phenotype and causes of many common types of PFD. This thesis aimed to extend the knowledge on uncharacterized PFD that manifest with impaired platelet aggregation function with multiple agonists, and/or platelet dense granule (DG) deficiency (DGD) by addressing some unanswered questions about laboratory findings for these PFD and whether some PFD have defects in platelet procoagulant function in assays using platelet rich plasma. First, we assessed the test characteristics and diagnostic usefulness of testing for DGD by whole mount electron microscopy (EM). We found that this test has acceptable performance characteristics, with a within-subject coefficient of variation (CV) of 12% for samples with normal DG counts. Confirmed DGD by whole mount EM showed significant association with increased bleeding symptoms and the clinical diagnosis of a bleeding disorder (Odds Ratio: 97, 95% CI: 5.7-1700). Light transmission aggregometry, and DG adenosine triphosphate release (estimated by lumi-aggregometry), were not sufficiently sensitive for detecting DGD as their respective sensitivities for detecting platelet function abnormalities due to DGD were only ~52% and 70%. Next, we evaluated for aberrant persistence of platelet non-muscle myosin IIB (MYH10) in a cohort with PFD, including those caused by mutations in transcription factors, such as *RUNX1*. While some participants with *RUNX1* mutations showed the expected abnormal MYH10 findings, one had consistently normal findings. In another family with a PFD of unknown cause that impaired aggregation with multiple agonists, MYH10 findings varied from normal to abnormal among the affecteds. Lastly, we evaluated platelet procoagulant function in PFD using thrombin generation assays, and found that platelet-dependent thrombin generation was normal in many PFD with impaired aggregation responses and/or DGD, with abnormalities detected in the subgroup with *RUNX1* mutations. Platelet-dependent thrombin

generation was also impaired in Quebec platelet disorder (QPD). Unlike the other PFD subjects studied, QPD participants had platelet Factor V (FV) deficiency. In QPD, but not other participants, platelet FV showed a significant association to endogenous thrombin potential (R^2=0.81) and peak thrombin concentration endpoints (R^2=0.88) for PRP samples, suggesting platelet FV is important for thrombin generation. The findings from this thesis help clarify the phenotypic abnormalities associated with PFD, with implications for diagnostic testing. The studies also illustrated that the functional defects in some PFD extend to the ability of platelets to support thrombin generation.

ACKNOWLEDGMENTS

I would like to thank the numerous individuals who allowed me to succeed in my graduate project and the writing of this thesis. I'd like to first thank my supervisor, Dr. Catherine Hayward, for her support and mentorship over the past two years. I have gained valuable skills from working with her, which has allowed me to grow as a student and researcher. I thank her for all the opportunities she has given me to travel to present my work at international conferences, and I am grateful for her time, guidance and patience during my graduate studies. I would also like to thank my committee members, Drs. Peter Gross and Guillame Paré, who provided me with valuable insight and further guidance for my project. I thank Karen Moffat and the staff at the special coagulation laboratory, as well as Ernie Spitzer and Marnie Timleck, at McMaster University for their help with the electron microscopy studies in my thesis. I thank Dr. Georges-Étienne Rivard for his collaboration and recruitment of additional persons with Quebec platelet disorder for my studies of thrombin generation in platelet disorders. I would also like to thank the staff at the Boris Clinic at McMaster Children's Hospital for their help with collecting research samples from our study participants. Most importantly, I'd like to thank our study participants, for without your donations, this project would not be possible. I would also like to acknowledge my colleagues from the Hayward lab: Dr. Subia Tasneem, Alex Leatherdale, and Dr. Janaki Iyer. They have all provided me with endless guidance and support, and made my graduate work that much more enjoyable. Finally, I extend since gratitude and love to my family, who have supported me throughout my life and helped me stay positive through the tough times. Thank you everyone.

TABLE OF CONTENTS

LIST OF FIGURES AND TABLES

CHAPTER 1

CHAPTER 3

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:

α	Alpha
β	Beta
μ	Micro
α2M	Alpha-2-macroglobulin
a	Activated
ACD	Acid citrate dextrose
ADP	Adenosine diphosphate
AMC	7-amino-4-methylcoumarin
APC	Activated protein C
APTT	Activated partial thromboplastin time
ATP	Adenosine triphosphate
BAT	Bleeding assessment tool
BSS	Bernard-Soulier syndrome
CAT	Calibrated automated thrombogram
cDNA	Complimentary deoxyribonucleic acid
CHAT	Clinical Health Assessment Tool
CI	Confidence interval
COX	Cyclooxygenase
CTI	Corn trypsin inhibitor
CV	Coefficient of variation
dPCR	Digital polymerase chain reaction
DG	Dense granule
DGD	Dense granule deficiency
DNA	Deoxyribonucleic acid
EDS	Energy dispersive spectrometry
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
EQA	External quality assurance
ETP	Endogenous thrombin potential
ETV6	E26-transformation specific variant 6
F	Factor
FLI1	Friend leukemia integration 1
FPD/AML	Familial platelet disorder with predisposition to acute myelogenous leukemia
GATA1	GATA-binding factor 1
GFI1B	Growth factor independent 1B
GP	Glycoprotein
GT	Glanzmann thrombasthenia
HiREB	Hamilton Integrated Research Ethics Board
HPS	Hermansky-Pudlak syndrome
HPD	Hamilton platelet disorder
HRLMP	Hamilton Regional Laboratory Medicine Program
ISTH	International Society for Thrombosis and Haemostasis
JAK2	Janus kinase 2

LTA	Light transmission aggregometry
MA	Maximal aggregation
M/B	MYH10/β-actin
MK	Megakaryocyte
MPL	Myeloproliferative leukemia protein
MPV	Mean platelet volume
MVB	Multivesicular bodies
MYH	Myosin heavy chain
NASCOLA	North American Specialized Coagulation Laboratory Association
NSAID	Nonsteroidal anti-inflammatory drug
OR	Odds ratio
P2Y	Purinergic P2 receptor
PAI-1	Plasminogen activator inhibitor-1
PAR	Protease activated receptor
PE	Phosphatidylethanolamine
PFD	Platelet function disorder
PPP	Platelet-poor plasma
PRP	Platelet-rich plasma
PS	Phosphatidylserine
PT	Prothrombin time
PTS	Paris-Trousseau syndrome
QPD	Quebec platelet disorder
RI	Reference interval
RNA	Ribonucleic acid
RT	Reverse transcription
RUNX1	Runt-related transcription factor 1
SC	Sporadic case
SDS	Sodium dodecyl sulphate
SRM	Super-resolution light microscopy
TAFI	Thrombin-activatable fibrinolysis inhibitor
TCAG	The Center for Applied Genomics
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TGA	Thrombin generation assay
TM	Thrombomodulin
tPA	Tissue plasminogen activator
TPO	Thrombopoietin
TxA_2	Thromboxane A_2
uPA	Urokinase plasminogen activator
VWD	von Willebrand disease
VWF	von Willebrand factor

CHAPTER 1

INTRODUCTION

1.1 OVERVIEW OF HEMOSTASIS

1.1.1 Hemostatic plug formation:

Hemostasis is the process that controls and prevents bleeding, and involves both blood and blood vessel responses to hemostatic challenges. (1-3) Platelets are small, anucleate cells that have an important role in hemostasis. Platelets circulate within the blood, and when they are activated and recruited to sites of injury, they help to form a hemostatic plug by adhering, aggregating and supporting blood coagulation. (1, 2) Hemostasis involves three phases: 1) platelet plug formation; 2) the generation of thrombin and a fibrin mesh that attaches to the platelet plug; and 3) the dissolution of fibrin during wound healing, a process called fibrinolysis. (2)

When platelets participate in hemostasis, they adhere at the site of injury, become activated, stick to one another and release the contents of their granules through exocytosis. (4) The formation of a platelet plug involves three stages: 1) platelets attach to proteins exposed at the site of injury including exposed collagen (at the severed edge of the blood vessel and in the extravascular space that is not normally exposed to blood) and to von Willebrand factor (VWF) bound to collagen; 2) next, platelets are activated by collagen and other agonists; and 3) this is followed by platelet aggregation (the interactions of platelets with other activated platelets) to help seal the wound. (1, 2) In the first step, platelets adhere to collagen and VWF via $\alpha 2\beta 1$ and the glycoprotein (GP) Ib-IX-V complex. (1, 2) These interactions and collagen interactions with GPVI also

activate platelets, which induces platelet shape change due to cytoskeletal rearrangement that maximizes platelet surface contact at the site of injury. (1, 2) Platelet activation also leads to changes in phospholipids found on the platelet surface: it increases the exposure of negatively-charged procoagulant phospholipids, such as phosphatidylserine (PS), on the exterior surface of the activated platelet membrane that are required for the assembly of the tenase and prothrombinase complexes that lead to accelerated thrombin generation. (5) These coagulation reactions generate thrombin, a serine protease that converts the abundant plasma GP fibrinogen to fibrin monomers that then polymerize to form insoluble fibrin, which creates a clot. (3) The αIIbβ3 receptor on the platelet surface undergoes a conformational change that leads to binding of fibrinogen, fibrin and VWF to platelets. (1-3) The activation of platelets also: triggers the release of stored platelet granule contents via exocytosis; and the generation of thromboxane A_2 (TxA_2) and release of stored adenosine diphosphate (ADP) from granules to promote further platelet activation and recruitment. Interactions of platelet with fibrinogen, fibrin and VWF help form a stable platelet aggregate and hemostatic plug that limits further blood loss. (1-3, 6)

Platelet activation:

Platelet activation is stimulated by a variety of soluble agonists, which are subclassified into strong or weak agonists. Strong agonists are those that directly and simultaneously induce platelet aggregation, TxA_2 synthesis, and platelet granule secretion, whereas weak agonists are those that directly induce platelet aggregation without triggering secretion. (7) At low concentrations, strong agonists can act as weak

agonists. (7) Thrombin is a strong platelet agonist that binds to protease-activated receptor (PAR)-1 and PAR4 on human platelets. (8) Arachidonic acid is an agonist that is formed when platelets are activated and phospholipase A_2 cleaves membrane phospholipids. (8) Next, through multiple enzymatic steps, arachidonic acid is then converted into TxA_2, which further activates platelets via the thromboxane receptor. (8) ADP is a weak platelet agonist that is stored in platelet dense granules (DG), and upon platelet activation, ADP is released and binds to platelet $P2Y_1$ and $P2Y_{12}$ receptors to further activate platelets. (9) Once activated, platelets aggregate through αIIbβ3 binding to fibrinogen, fibrin, and VWF and form a hemostatic plug that acts as a barrier to limit further blood loss. (1, 2)

Platelet granules:

Platelet granules are formed early in megakaryocyte (MK) maturation (see 1.1.2 for platelet formation), when protein trafficking through the trans-golgi network leads to formation of small vesicles that then form multivesicular bodies (MVB). (10) These MVB further mature into storage granules upon receiving newly synthesized specific transmembrane proteins through multiple vesicular trafficking pathways. (11, 12) Platelets contain 2 types of granules: alpha and dense granules (DG). α-granules are the most abundant platelet granule (about 50-80 per platelet) (13), and store adhesive proteins (e.g. fibrinogen and VWF), proteins involved in coagulation (e.g. factor [F] V), fibrinolytic proteins (e.g. plasminogen activator inhibitor I [PAI-1]), growth factors, and immune modulators. (14) DG are less abundant (approximately 4-10/platelet) and store

electron dense molecules such as calcium and magnesium, in addition to polyphosphates, adenine nucleotides (e.g. adenosine triphosphate, ATP and ADP), polyphosphate and bioactive amines (e.g. serotonin and histamine). (14, 15) The release of granule contents upon platelet activation is a crucial step that promotes platelet aggregation (through the release of ADP) and hemostatic plug formation. (16)

1.1.2 Platelet formation:

Platelets are derived from MK, which are located in the bone marrow. (17) Megakaryopoiesis, the development of mature MK from their progenitor cells, takes approximately 9-12 days and yields cells with proplatelet extensions that then release platelets. (18) Recently, there is evidence that some platelet release occurs by MK in the lungs. (19) Megakaryopoiesis is supported by thrombopoietin (TPO) binding to the c-mpl receptor on hematopoietic progenitor cells, inducing a Janus kinase-2 (JAK2) signaling cascade that stimulates cell differentiation into MK. (17, 20, 21) MK undergo polyploidization and endomitosis, which are repeated cell cycles with a termination of mitosis at the later phase of cytokinesis. (20) During megakaryopoiesis, platelet-specific proteins are synthesized and cytoplasmic maturation occurs, with the packaging of granules and the development of an open canalicular membrane system that is found in mature human platelets. (22) Finally, MK develop branched extensions called proplatelets that protrude into bone marrow sinusoids, allowing its terminal buds to be released into circulation as platelets. (21, 22)

Each MK can produce approximately 5000-10,000 platelets during their lifespan. (4, 17) About two thirds of platelets are released from the bone marrow and circulate within the blood at a concentration of 150-400 x 10^9/L, with additional platelets sequestered in the spleen. (13, 14) The platelet lifespan varies between 5-9 days and platelets are eventually cleared from circulation by the liver and spleen via phagocytosis. (4, 13)

1.1.3 Blood coagulation:

Coagulation proteins are also involved in hemostasis. (5, 23, 24) Traditionally, coagulation was divided into the extrinsic, intrinsic and common pathways, but more contemporary descriptions describe coagulation as an overlapping, 3-phase process: initiation, amplification, and propagation. (23) With an injury, blood is exposed to cells outside the vasculature that express tissue factor (TF) on their surface, allowing FVII to bind to TF to form a TF-FVIIa complex, which can associate with and activate small amounts of FX. (5, 23-25) FXa (a, activated) activates FV and associates with FVa on the phospholipid membrane of activated platelets, or other cells, to form the prothrombinase complex and convert small amounts of prothrombin to thrombin. (5, 23, 24) Amplification of coagulation then occurs, which involves positive feedback loops that promote thrombin generation, including the proteolytic activation of FVIII, FV, and FXI by thrombin. (5, 23, 24) Upon activation of platelets by thrombin, platelet intracellular calcium levels rise, causing a loss of phospholipid asymmetry in the platelet plasma membrane. (5, 23, 24) PS and phosphotidylethanolamine (PE), which are normally found

on the internal platelet membrane, move to the external surface, which favours the assembly of the intrinsic tenase and prothrombinase complexes. Additionally, partially activated FV is secreted from platelet α-granules onto the platelet surface (see below for more information on FV and FVa) to enhance prothrombinase assembly. (5, 23, 24) This culminates in the propagation of coagulation, by the intrinsic tenase and prothrombinase complexes and increased thrombin generation due to the activation of factors V, VIII and XI. (5, 23, 24) FVIIIa participates in FXa generation by forming the intrinsic tenase complex with factor IXa, the substrate FX, phospholipids and calcium. (5, 23, 24) FVa participates in thrombin generation by forming the prothrombinase complex, which includes FXa, FVa, the substrate prothrombin, phospholipids and calcium. (5, 23, 24) *Ex vivo*, the intrinsic pathway is initiated when contact with negatively charged surfaces activates FXII, in the presence of high molecular weight kininogen and prekallikrein, which leads to the subsequent activation of FXI and FIX. (5, 23, 24) Thrombin converts fibrinogen to fibrin and it also simultaneously activates FXIII, stabilizing the fibrin clot by crosslinking fibrin monomers to each other. (5, 23, 24)

Factor V:

FV is a coagulation cofactor produced by the liver as a single-chain GP (330 kDa) consisting of six domains: A1-A2-A3-B-C1-C2. (26) The majority of FV (~80%) circulates in plasma as an inactive cofactor, while in humans, ~20% is stored in platelets following FV endocytosis from plasma. (27) In platelets, FV is stored in a partially activated state in platelet α-granules. (27, 28) FVa is generated upon proteolytic cleavage

of plasma or platelet FV by thrombin or FXa (29, 30) to liberate the B domain, leaving two non-covalently bound chains that comprise factor Va, which consists of: a heavy chain of A1 and A2 domains, and a light chain of A3, C1, and C2 domains. (26) To ensure thrombin generation is fast and localized, the prothrombinase complex assembly requires the exposure of negatively charged phospholipids, PS and PE, onto the platelet surface. (23, 26) The prothrombinase complex consists of: an enzyme (FXa); a cofactor (FVa); a protein substrate (prothrombin); negatively charged phospholipids; and calcium ions. (26) The conversion of prothrombin to thrombin is approximately 100,000 times more efficient when the prothrombinase complex is assembled compared to cleavage of prothrombin by FXa alone. (26) FVa is downregulated through its inactivation by proteolytic cleavage by activated protein C (APC). (31)

Regulation of coagulation:

There are numerous regulatory components that control coagulation. First, TF is not a normal component of blood. Second, TF pathway inhibitor (TFPI) associates with FXa and inhibits the TF-FVIIa complex, limiting the activation of FX. (32) TFPI also inhibits prothrombinase activity in early stages of coagulation by binding to the acidic region on the FV B domain. (33) In addition, once thrombin is generated, it can bind to thrombomodulin (TM) on endothelial cells to activate protein C to APC. (30) APC and its cofactor, protein S, inhibit membrane-bound FVIIIa and FVa before they form complexes with FIXa and FXa, respectively. (30) APC also proteolytically inactivates FVa, thereby downregulating the activity of the prothrombinase complex. (34) Lastly,

antithrombin is a serine protease inhibitor that acts at numerous steps in the coagulation pathway as it inhibits thrombin, FIXa, FXa, FXIa, and FXIIa. (35)

1.1.4 Fibrinolysis:

Thrombus dissolution, or fibrinolysis, is normally a highly localized process that contributes to wound healing and the reestablishment of blood flow after clotting. (36) Fibrinolysis is initiated by the conversion of plasminogen into plasmin by tissue plasminogen activator (tPA) or urokinase plasminogen activator (uPA). (37) Plasmin (which is a broad specificity protease) can then hydrolyze cross-linked fibrin into fibrin degradation products, dissolving the clot. (36)

There are several important molecules that inhibit fibrinolysis. The plasminogen activators uPA and tPA are both inhibited by PAI-1, which is released by endothelial cells and by activated platelets. (36) α2-plasmin inhibitor and α2-macroglobulin (α2M**)** are serine protease inhibitors that inhibit plasmin and help to limit fibrinolysis. (38) Lastly, plasmin activates thrombin-activatable fibrinolysis inhibitor (TAFI) to its activated form (TAFIa) that removes C-terminal lysine and arginine residues from partially degraded fibrin to further limit fibrinolysis. (39)

1.2 DEFECTS IN PLATELET FUNCTION

Defects in platelet function can occur due to acquired or inherited disorders, and can affect a variety of mechanisms. Acquired platelet function disorders (PFD) are a result of platelet dysfunction often due to drugs and bone marrow disorders. (40, 41) Substances that inhibit platelet function include: cyclooxygenase (COX)-1 inhibitors (e.g. Aspirin), nonsteroidal anti-inflammatory drugs (NSAIDs), thienopyridine drugs, proton pump inhibitors, alcohol, caffeine, and ginkgo-based herbal supplements. (40, 42, 43)

Inherited PFD are a group of bleeding disorders with diverse molecular causes. (44) Clinical laboratories that offer testing for PFD report diagnosing PFD as frequently as von Willebrand disease (VWD), which has a prevalence of approximately 1:1000. (45-47) The features and causes of rare and syndromic inherited PFD are largely well-characterized. However, the causes of more common types of inherited PFD, such as those that cause non-syndromic DG deficiency (DGD) and/or aggregation defects with multiple agonists are largely unknown. (48) There are thousands of proteins in platelets, which means that are many potential mechanisms for PFD and for the normal observed variability in platelet function. (49)

1.2.1 Assessment of inherited platelet function disorders:

Initial assessment:

The first step in evaluating for a suspected inherited PFD is a thorough assessment of the patient's medical history to assess the type, severity and frequency of bleeding. (50, 51) A family history is also important in a clinical evaluation as bleeding

symptoms shared by multiple members within a family suggest an inherited PFD and provide important information on the potential mode of inheritance. Knowledge of the range and type of symptoms experienced by affected members of the family is often helpful in understanding the disorder and its severity. (50) A complete physical examination is recommended to assess for bleeding manifestations and for syndromic features that are associated with some inherited PFD, such as: albinism; kidney disease; hearing loss; dysmorphic faces; defects in the heart or bones; ocular abnormalities; and mental retardation. (50)

Bleeding symptoms:

Some inherited PFD, such as Glanzmann thrombasthenia (GT) and Bernard-Soulier Syndrome (BSS), have more severe bleeding symptoms. (52-56) Mild and severe inherited PFD can present with symptoms of: epistaxis, heavy menstrual bleeding, extensive and/or unexplained bruising, heavy or prolonged bleeding with childbirth, and excessive challenge-related bleeding with surgical and dental procedures. (13, 54, 55, 57) As some bleeding symptoms are also common in healthy population controls (e.g. epistaxis, easy bruising, heavy menstrual bleeding), this needs to be factored in the assessment of bleeding from PFD. (55)

Bleeding assessment tools:

Bleeding history assessment tools (BAT) have been designed to help document and quantify symptoms associated with bleeding disorders and allows for a more

standardization assessment of bleeding histories and symptoms. (49) Additional background on BAT is discussed below as I used data from BAT for part of my analyses.

The International Society on Thrombosis and Haemostasis (ISTH) BAT is the currently recommended tool for evaluating bleeding histories for persons with PFD for research studies. This BAT was designed to be completed by a physician or other trained health professionals to quantify the overall burden of bleeding symptoms and categorize the type of bleeding. (51) There are 14 bleeding symptoms assessed by this BAT: epistaxis, bruising, bleeding from minor wounds, oral cavity, gastrointestinal bleeding, hematuria, tooth extraction, surgery, menorrhagia, post-partum hemorrhage, muscle hematomas, hemarthrosis, central nervous system bleeding, and other types of bleeding. (51) For each category, bleeding is scored from 0 (no/trivial bleeding) to 4 (severe bleeding needing treatment). (51) Only bleeding events and symptoms that occurred before or at the time of diagnosis are scored by this tool. (51) A total score across all categories is calculated to estimate the overall burden of bleeding symptoms experienced by the patient. (51) The ISTH BAT has been used in numerous cohorts, including cohorts of normal healthy controls (58), persons with type 1 VWD (59) and persons with inherited PFD. (57, 60-62) The normal range of ISTH BAT scores range from 0-4 for adult males, 0-6 for adult females, and 0-3 for children. (58) However, removal of gender-specific symptoms creates similar normal ranges for males and females (0-4). (58) The ISTH BAT has some drawbacks. The ISTH BAT scores each category based solely on the single worst bleeding episode in that category and does not take into account the overall frequency of mild or worse bleeding events. (63) Additionally, all studies report

overlap in BAT scores between individuals with and without a bleeding disorder which raises questions about the potential diagnostic usefulness of the tool. (64)

The Clinical Health Assessment Tool (CHAT) was the first BAT used to collect standardized information on bleeding to estimate bleeding risks for an inherited disorder as odds ratios (OR) for different bleeding symptoms and problems by comparing data for persons with Quebec Platelet Disorder (QPD) to their unaffected family members. (65) A different version of CHAT was designed to evaluate platelet disorders (CHAT-P) including the estimation of bleeding risks for a family with a PFD due to a *RUNX1* haploinsufficiency mutation (with six affected family members), by comparison to general population controls. (57) Estimating bleeding risks as OR allows each specific bleeding symptom (including those that indicate more severe bleeding problems) to be compared for affected and unaffected individuals. The OR approach takes into consideration that some persons without bleeding problems experience some mild bleeding symptoms and it also takes into account that not everyone has exposures to certain hemostatic challenges, such as surgery. The data generated by this tool can be used to communicate information on the magnitude of bleeding risks to healthcare provides and patients, to promote evidence-based care.

Laboratory investigations:

Laboratory tests of platelet function are an integral part of the diagnostic work up of PFD. (50) Some inherited PFD are associated with thrombocytopenia, reduced storage

granule numbers, impaired release of platelet DG contents and/or abnormal platelet aggregation responses to a panel of agonists. (50)

A complete blood count is routinely done to assess for thrombocytopenia (defined as a platelet count below 150 x 10^9/L) as part of the workup of a PFD as some disorders affect platelet numbers with or without altering platelet function. Persons undergoing investigations for bleeding are often investigated for other defects at the same time or before testing for a PFD. The commonly performed tests for other bleeding problems include an evaluation of citrated platelet-poor plasma (PPP) for: a prothrombin time (PT, assesses the extrinsic pathway of coagulation), activated partial thromboplastin time (APTT, assesses intrinsic pathway of coagulation), and a VWF screen (which includes an estimation of VWF antigen, VWF activity by ristocetin or mutant GPIb binding assays of VWF activity, and assessment of FVIII coagulant activity). (50) If a PFD is suspected in a person with thrombocytopenia, further testing is required to assess for accompanying defects in platelet function as some disorders reduce platelet numbers and cause characteristic impairments in platelet function (e.g., absent ristocetin-induced platelet agglutination in BSS; reduced or absent aggregation with other agonists in variant GT). (50)

Light transmission aggregometry (LTA) is the most common platelet function test performed by diagnostic laboratories and it is currently considered a gold standard test for diagnosing inherited PFD. (56, 66, 67) LTA evaluates platelet-platelet interactions in platelet-rich plasma (PRP) by monitoring the change in light transmission upon addition of a platelet agonist, while stirring the sample at low shear force. (66, 67) The typical

agonist panel used to assess platelet function contains: ADP, collagen, epinephrine, arachidonic acid, and ristocetin. (56, 66) Additional agonists (e.g., TxA_2 analogue U46619) are also useful for detecting and categorizing the type of platelet function abnormality. (56, 66, 68) A result is considered abnormal if maximal aggregation (MA) falls outside of the laboratory's established reference interval (RI). Impaired platelet aggregation with two or more agonists is suggestive of a PFD (47, 50, 68), although not all PFD impair aggregation responses and some rare disorders impair aggregation responses to single agonists (e.g., collagen in GPVI defects, ristocetin in BSS and some forms of VWD).

Some laboratories also perform platelet DG release assays as part of the evaluation of inherited PFD. (48, 56) When platelets are activated by agonists in PRP, DG release both ADP and ATP into the plasma and it is possible to quantify DG release by measuring the released ATP by lumiaggregometry. This is the most commonly-used technique assess DG release, which uses a luciferin/luciferase reagent and an ATP standard to quantify the amount of ATP released in response to an agonist. (48, 56) However, the assessment of DG ATP release has questionable diagnostic usefulness as the findings show considerable variability, and even if ATP release is consistently impaired, the finding does not show a significant relationship to increased bleeding scores or the diagnosis of a bleeding disorder. (60)

The ISTH has recommended that assays for platelet DGD be performed as a part of the diagnostic workup of a PFD. (50) An estimate of platelet DG numbers by whole mount electron microscopy (EM) is the most commonly used method to diagnose platelet

DGD. (48) The calcium and phosphorus content of DG make these structures electron dense, allowing them to be visualized and quantified by EM without fixation or staining. (15, 69) DGD is associated with mucocutaneous bleeding problems, and although it represents only one type of PFD, DGD is fairly common, with a similar prevalence to VWD. (45, 70) An assessment for DGD is helpful for diagnosing inherited PFD since some patients with DGD have normal MA and DG ATP release findings. (45, 70) External quality assurance (EQA) exercises on whole mount EM tests shown good agreement between laboratories on which electron-dense structures should be counted as a DG, and which samples show normal or abnormal findings. (71, 72) Although the finding of DGD has been associated with a bleeding disorder (68), the variability of the test findings have not been reported and it would be useful to determine the diagnostic usefulness of whole mount EM for detecting DGD in a larger study.

Common clinical tests, such as PT and APTT (which measure the time to clot formation), do not test the role of platelets in thrombin generation as these tests are performed with PPP. (73, 74) Since fibrin formation occurs when only approximately 5% of the total amount of thrombin has been generated, measuring thrombin generation over time has some advantages over the PT and APTT when testing for potential defects in the hemostatic pathway. (73, 74) In the method designed and described by H.C. Hemker, thrombin generation is measured using a fluorogenic substrate (Z-Gly-Gly-Arg) bound to 7-amino-4-methylcoumarin (AMC), which is cleaved by thrombin. (75, 76) In the calibrated automated thrombogram (CAT) method, the cleavage of the fluorogenic substrate is compared to a sample of the same plasma in which no thrombin generation is

triggered. (77, 78) Typically, α2M is used as a calibrator, where α2M binds thrombin, protecting thrombin from its inactivators in plasma but leaving its ability to split the fluorogenic substrate intact. (76) The use of α2M as a calibrator also normalizes individual differences between subjects, since α2M levels are higher in females and youth. (79, 80) Thrombin generation can be measured in citrate anticoagulated PRP and PPP, which is typically done by adding back calcium and relipidated TF to simulate vessel damage. (76, 77, 81, 82) In PPP, phospholipids are added to act as a surface for thrombin generation whereas activated platelets provide the phospholipid surface for thrombin generation assays with PRP. (76, 77, 81, 82) Measuring platelet-independent and -dependent thrombin generation by CAT is not routinely performed in the evaluation of PFD due to a lack information on findings for the test in many PFD. Many pre-analytical and analytical variables exist that may cause variability within and between laboratories in CAT assays, which were discussed in recently published ISTH guidelines that provide recommendations for measuring thrombin generation by CAT. (83) One source of inter-laboratory variation is the source and concentration of TF. Some laboratories have used lower concentrations of TF (i.e. 0.5 pM) for PPP samples to detect hypocoagulability induced by FVIII or FIX deficiencies (84, 85), and higher concentrations of TF for studying other conditions. The ISTH guidelines recommend the use of standardized, commercially-available reagents for measuring thrombin in PPP and PRP samples. (83) The use of corn trypsin inhibitor (CTI), a FXIIa inhibitor, to ensure that thrombin generation proceeds via the TF pathway has also been debated. (86-89) Some studies have reported that blood sampling directly into tubes containing CTI

reduces imprecision in CAT for both PPP and PRP samples. (87) However, others have found that addition of CTI is not needed when triggering at higher TF concentrations (>1 pM). (89)

Mutations in transcription factors are being implicated as the cause of some PFD, including some autosomal dominant forms (see 1.2.2 for further information). (90) Whole exome sequencing is an upcoming technique to identify mutations in many different PFD, such as those caused by mutations in transcription factors. (50, 91) However, this technique can be expensive and time-consuming for many laboratories. Accordingly, recent ISTH guidelines have recommended a biomarker test, that is an assessment for the aberrant persistence of platelet non-muscle myosin heavy chain IIB (MYH10), to help identify some forms of PFD caused by pathogenic transcription factor mutations in the genes for *RUNX1* and *FLI1* (see 1.2.2 for further information on disorders caused by mutations in transcription factors). (50, 90, 92) Normally, MYH10 is downregulated by RUNX1 and FLI1, and undetectable in platelets; however, if present during late stages of megakaryopoiesis, it leads to impaired MK polyploidization (18), which may cause reductions in platelet counts and intracellular contents. (17) Before laboratories consider implementing this biomarker test into a routine diagnostic workup of PFD, an assessment of typical findings in individuals with findings suggestive of a PFD deserve further investigation.

1.2.2 Summary of inherited platelet function disorders:

Diagnosis of an inherited PFD is often based on abnormalities detected during the diagnostic workup that are suspicious of a bleeding problem or particular type of platelet disorder. (93) Table 1 provides a summary of inherited PFD organized by the type of defect, the defective gene(s), and key features of the disorder.

Table 1. Summary of inherited platelet function disorders. Disorders are categorized by the type of defect, defective gene(s) and key features. Abbreviations: ADP (adenosine diphosphate), ATP (adenosine triphosphate), DGD (dense granule deficiency), GI (gastrointestinal), MK (megakaryocyte), PE (phosphatidylethanolamine), PS (phosphatidylserine), TPO (thrombopoietin), TxA_2 (thromboxane A_2).

Type of defect	Name of disorder	Defective gene(s)	Key features
Activation	ADP receptor ($P2Y_1$ and $P2Y_{12}$) defects	*P2RY12, P2RX1*	Impaired platelet activation by ADP; normal platelet count. (93, 94)
Activation	Collagen receptor (GPVI) defects	*GP6*	Impaired platelet activation by collagen; normal platelet count; mild to severe bleeding phenotype. (95, 96)
Activation	Thromboxane receptor defects	*TBXA2R*	Impaired TxA_2-dependent aggregation and secretion by thromboxane; normal platelet count. (97)
Activation & signaling	Thromboxane synthase defects	*TBXAS1*	Impaired production of TxA_2; moderate to severe bleeding phenotype. (93)
Adhesion	Bernard-Soulier Syndrome	*GP1BA, GP1BB, GP9*	Deficiency or absence of GPIb-V-IX complex; macrothrombocytopenia; absent ristocetin-induced agglutination; bleeding severity can range from mild to severe. (98, 99)
Adhesion	Platelet-type von Willebrand disease	*GP1BA*	Gain-of-function defect in platelet GPIbα, leading to increased binding of

			platelets to VWF and increased platelet clearance; prolonged bleeding time. (100, 101)
Adhesion	α2β1 defect	*ITGA2*	Thrombocytopenia associated with deficiency of the platelet integrin receptor for collagen; mild bleeding phenotype. (102)
Aggregation	Glanzmann thrombasthenia, including variant forms	*ITGA2B, ITGB3*	<5% (type I) or 5-20% (type II) of αIIbβ3; >20% of αIIbβ3 with dysfunctional properties (variant); normal platelet counts except in some variant forms with activating mutations of the receptor; absent or markedly reduced platelet aggregation with all agonists but normal agglutination in response to ristocetin; bleeding usually severe. (103, 104)
Aggregation	Leukocyte adhesion deficiency type III	*FERMT3*	Defective integrin activation involving platelets and leukocytes because of defects in kindlin 3; impaired platelet aggregation with various agonists. (105)
Cytoskeletal	Wiskott-Aldrich syndrome	*WAS*	Mild to moderate thrombocytopenia; small platelets with DGD; often present with immunodeficiency; common bleeding symptoms include GI bleeding and petechiae. (93)
Cytoskeletal	MYH9-related platelet disorder	*MYH9*	Macrothrombocytopenia; Dohlë-like inclusions in neutrophils; potential for nephritis and hearing loss; bleeding tendency related to platelet count. (106)
Fibrinolysis & procoagulant function	Quebec platelet disorder	*PLAU*	Gain-of-function defect in fibrinolysis with abnormal proteolysis of platelet FV and other stored platelet proteins due to increased

			platelet urokinase plasminogen activator (uPA) that triggers intraplatelet plasmin generation; delayed bleeding; normal to reduced platelet counts. (65, 107-109)
Platelet granules	Hermansky-Pudlak syndrome	*HPS* (6 genes), *AP3B1, DTNBP1, PLDN, AP3D1*	DGD associated with defects of lysosomes and melanosomes, affecting skin and hair pigmentation; normal platelet count; reduced or absent ATP release by lumiaggregometry; can result in pulmonary fibrosis; mild to severe bleeding severity. (110)
Platelet granules	Chediak-Higashi syndrome	*LYST*	Normal platelet counts with DGD; skin and hair hypopigmentation; immunodeficiency; mild to severe bleeding severity. (111)
Platelet granules	Gray platelet syndrome	*NBEAL2*	Deficiency of platelet α-granules; mild thrombocytopenia; large platelets; prolonged bleeding time. (112)
Platelet granules	Non-syndromic *δ*-storage pool disease	Unknown	Variable aggregation responses; prolonged bleeding time; decreased amounts of DG; increased ratio of total platelet ATP to ADP. (45)
Platelet granules	Arthrogryposis, renal dysfunction and cholestasis syndrome	*VSP33B, VIPAS39*	Arthrogryposis, renal dysfunction, and cholestasis associated with platelet α-granule deficiency; severe bleeding phenotype. (113)
Platelet granules	Griscelli syndrome	*MYO5A, RAB27A, MLPH*	DGD associated with immunological and central nervous system defects, lymphohistiocytosis, and hypopigmentation. (114, 115)

Platelet granules	Medich platelet syndrome	Unknown	Macrothrombocytopenia with markedly decreased α-granules; platelets contain membranous cigar-shaped inclusions. (116)
Platelet number	Congenital amegakaryocytic thrombocytopenia	*MPL*	Severe thrombocytopenia; absent MK in bone marrow; increased plasma TPO levels; easy bleeding. (117)
Platelet number	Congenital amegakaryocytic thrombocytopenia with radioulnar synostosis	*HOXA11*	Severe thrombocytopenia; absent MK in bone marrow; proximal radioulnar synostosis. (118)
Platelet number	Thrombocytopenia (THC2-linked)	*MASTL, ACBD5, ANKRD26*	Thrombocytopenia; mild bleeding phenotype; *ANKRD26* mutations associated with predisposition to leukemia (119, 120)
Platelet number	Macrothrombocytopenia with platelet expression of glycophorin A	Unknown	Macrothrombocytopenia and platelets expressing surface glycophorin A; late-onset hearing loss; mild bleeding phenotype. (121)
Platelet number	Thrombocytopenia associated with absent radii syndrome	*RMB8A*	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. (122, 123)
Platelet number & cytoskeletal	Thrombocytopenia and giant platelets	*TUBB1*	Thrombocytopenia with giant platelets; absent to mild bleeding phenotype. (124)
Platelet number & cytoseketal	Filaminopathy (thrombocytopenia with or without syndromic features)	*FLNA*	Defects in filamin A resulting in thrombocytopenia with or without periventricular nodular heteropenia or otopalatodigital syndromes. (125)
Procoagulant function	Scott syndrome	*TMEM16F (ANO6)*	Impaired ability of platelets to support coagulation due to impaired PS and PE expression on activated

			platelets; normal platelet aggregation. (126)
Procoagulant function	Stomorken syndrome	*STIM1*	Enhanced procoagulant activity of resting platelets; reduced platelet aggregation and secretion with all agonists except collagen. (127)
Procoagulant function	Platelet Factor V-New York	Unknown	Impaired platelet FV prothrombinase activity; normal prothrombinase function with added FVa; no FV proteolysis or multimerin deficiency. (128)
Signaling	Cytoplasmic phospholipase A2 deficiency	*PLA2G4A*	Impaired arachidonic acid production due to defects in phospholipase A2 deficiency; severe bleeding phenotype. (129)
Signaling	Cyclooxygenase deficiency	*PTGS1*	Impaired ability to convert arachidonic acid to thromboxane; mild bleeding tendency. (130)
Signaling	Signaling defects involving G-protein pathways	*GNAS1, GNAQ*	Defective G-protein coupled signaling; reduced platelet aggregation and secretion; mild bleeding phenotype. (131)
Signaling	York platelet syndrome	*STIM1*	Thrombocytopenia associated with a gain of function mutation in the calcium sensor, STIM1; large ER-derived inclusion bodies in platelets; immune deficiency and non-progressive myopathy. (132)
Transcription factor	Familial platelet disorder with propensity to acute myeloid leukemia and myelodysplastic syndrome	*RUNX1*	Mild thrombocytopenia, some with normal platelet counts; abnormal aggregation in response to multiple agonists; some with mild DGD; mild to moderate bleeding tendency. (57, 80, 90)
Transcription factor	Paris-Trousseau-Jacobsen syndrome	*FLI1*	Macrothrombocytopenia; developmental delay and facial abnormalities; mild

			bleeding phenotype. (90, 133)
Transcription factor	Macrothrombocytopenia with dyserythropoiesis/anemia/beta-thalassemia	*GATA1*	Macrothrombocytopenia associated with thalassemia, neutropenia and megakaryoblastic leukemia; reduced number of AG. (134)
Transcription factor	Macrothrombocytopenia with dyserythropoiesis	*GFI1B*	Moderate macrothrombocytopenia; reduction in AG contents; variable bleeding tendencies. (135)
Transcription factor	ETV-related thrombocytopenia	*ETV6*	Thrombocytopenia with predisposition to hematologic malignancy; reduced MK maturation. (135, 136)

Platelet disorders that impair activation:

Defects in a variety of platelet receptors that are important for platelet activation have been identified to cause some inherited PFD. (54) There have been reports of quantitative and qualitative defects in the receptors for: TxA_2 (TP) (97), ADP ($P2Y_1$ and $P2Y_{12}$) (94), and collagen (GPVI). (95, 96) In addition, there have been reports of defects in thromboxane synthase, which is involved inTxA_2 generation. (137)

Platelet disorders that impair adhesion:

Defects in platelet adhesion can impair interactions between platelets and the vessel wall, and the ability of platelets to bind to subendothelial proteins. (93) BSS is a rare disorder that is caused by defects in the GPIb-IX-V complex, resulting in impaired platelet adhesion to VWF, macrothrombocytopenia, and absent platelet agglutination in response to ristocetin. (93, 98) Individuals with BSS also have impaired platelet-dependent thrombin generation (138), since there are abnormalities in the binding of thrombin and fibrin to platelets. Platelets can bind fibrin through an interaction between fibrin-bound VWF and GPIb (139), and the platelet-bound fibrin enhances TF-induced platelet procoagulant activity. (140, 141) Thus, defects in fibrin-VWF-platelet binding in BSS may contribute to impaired platelet-dependent thrombin generation. Individuals with BSS have mild to life-threatening bleeding symptoms, which can include epistaxis, extensive bruising, and gastrointestinal bleeding. (98) Platelet-type VWD is an inherited disorder that is caused by a gain-of-function defect in GPIbα, allowing large VWF multimers to bind to resting platelets and be cleared from the system. (100, 101)

Platelet disorders that impair aggregation:

Defects in platelet aggregation impair platelet-platelet interactions. (93) GT is an autosomal recessive bleeding disorder that is caused by a severe deficiency or absence of $\alpha_{IIb}\beta_3$, the platelet receptor that binds fibrinogen and VWF when platelets are activated at low and high shear, respectively. (103, 104) Individuals with GT have impaired platelet aggregation responses to all agonists and ristocetin-induced platelet agglutination is present, but not followed by aggregation. (93) There have been reports of impaired platelet-dependent thrombin generation in some individuals with GT (138, 142), but there is variability in findings due to methodical differences when measuring thrombin generation. In variant GT, platelets express non-functional $\alpha_{IIb}\beta_3$, and typically present with moderate macrothrombocytopenia. (104) Other causes of platelet aggregation defects exist, including severe plasma fibrinogen deficiency (93) and leukocyte adhesion deficiency-III. (105)

Platelet disorders that impair the cytoskeleton:

Defects in the platelet cytoskeleton can impair megakaryopoiesis and platelet production. (44) MYH9-related platelet disorder is an autosomal dominant bleeding disorder that is caused by a mutation in the *MYH9* gene. (106) Individuals with this disorder have macrothrombocytopenia since MYH9 is important for normal megakaryopoiesis. (106) These individuals can also present with Dohlë-like inclusions in their neutrophils, and may be at risk for nephritis and hearing loss. (44, 106) Wiskott-Aldrich syndrome, is an X-linked disorder caused by a mutation in the *WAS* gene, causes

macrothrombocytopenia and often neutropenia associated with eczema and immune dysfunction. (93) Defects in other platelet cytoskeletal proteins, such as filamin (125) and tubulin (124), have also been reported, which cause macrothrombocytopenia.

Platelet disorders that impair fibrinolysis:

Accelerated fibrinolysis from premature dissolution of a fibrin clot can result in bleeding complications. (143) QPD is a gain-of-function PFD that affects fibrinolysis due to an increase in platelet stores of uPA (107-109) caused by a tandem duplication mutation in *PLAU*. (143) Individuals with QPD have: mildly reduced to normal platelet counts (65); normal numbers of α-granules and DG (107, 144); and typically have absent platelet aggregation in response to epinephrine. (107, 144) QPD α-granule proteins (e.g. FV, VWF, thrombospondin-1, fibrinogen) show partial degradation due to intraplatelet but not systemic plasmin generation. (107, 144-146) Platelet FV activity is also reduced and prothrombinase activity of QPD pre-activated, washed platelets is impaired, unless purified FVa is added. (146) The function of QPD platelets in supporting coagulation in the presence of plasma FV has not been tested by CAT. CHAT has been used to assess bleeding risks for QPD, and compared to their unaffected relatives, individuals with QPD have a higher likelihood of reporting abnormal bleeding, including delayed bleeding after dental and surgical procedures unless they receive treatment with fibrinolytic inhibitor drugs. (65)

Platelet disorders that affect storage granules:

Defects in platelet storage granules can affect DG and/or α-granules. (93) There are several PFD that result in DGD. First, Hermansky-Pudlak syndrome (HPS) is a disorder that is caused by a disruption in DG formation. (110) Individuals with HPS present with oculocutaneous albinism, mild to moderate bleeding symptoms, absent or impaired secondary platelet aggregation with weak agonists, and may also develop pulmonary fibrosis. (110) Another DG disorder, known as Chediak-Higashi syndrome, presents with similar features as HPS but possesses severe immunologic and neurological defects. (111) Griscelli syndrome is associated with lymphohistiocytosis and immunological and central nervous system defects. (114, 115) Lastly, non-syndromic DGD are a much more common form of PFD with individuals presenting with variable aggregation responses and an increased ratio of total platelet ATP to ADP. (45)

There are also many PFD that result in a reduction of AG numbers. Gray platelet syndrome is a disorder that is characterized by absent or decreased numbers of α-granules, and mild thrombocytopenia. (112) Other forms of α-granule deficiency have been reported, including arthrogryposis, renal dysfunction and cholestasis syndrome (113) and Medich platelet syndrome. (114, 115)

Platelet disorders that influence platelet number:

There are many inherited PFD that cause thrombocytopenia in conjunction with impairments in platelet function and/or morphology (i.e., altered platelet size). Congenital amegakaryocytic thrombocytopenia with or without radioulnar synostosis are rare disorders that result in severe thrombocytopenia and absent MK in bone marrow. (117,

118) Individuals with thrombocytopenia associated with absent radii syndrome also present with low platelet counts, and have an increased risk for bone defects, cardiac, or renal problems. (122, 123) Lastly, individuals with mutations in *ANKRD26* are associated with thrombocytopenia 2, and present with reduced α-granule numbers, mild bleeding, and have an increased risk of developing leukemia. (119)

Platelet disorders that impair procoagulant function:

Impaired procoagulant function presents as an inability of platelets to support the enzymatic reactions in the coagulation system. (126) Scott syndrome is a rare, inherited disorder that is associated with a decreased exposure of anionic phospholipids on the platelet surface, resulting in reduced thrombin generation. (126) Mutations in *TMEM16F* (*ANO6*) have been associated with Scott syndrome. (126) Conversely, Stomorken syndrome is characterized by enhanced procoagulant activity due to a mutation in *STIM1*. (127) In addition to accelerating fibrinolysis, QPD is a PFD that also impairs procoagulant function due to increased proteolysis of platelet FV that impairs prothrombinase complex assembly. Platelet FV-New York is an additional disorder that impairs platelet FV prothrombinase activity, but distinct from QPD as there is no proteolysis of platelet FV or other α-granule proteins. (128)

Platelet disorders that impair signaling:

Platelet signaling defects represent a heterogeneous group of disorders characterized by impairment in signal amplification or other processes involved in

intracellular messages. They may be caused by qualitative or quantitative defects in intracellular signalling molecules (e.g. phospholipase A_2), GTP-binding proteins (α, β and γ subunits) or enzymes involved in TxA_2 production (e.g. COX-1 or thromboxane synthetase). (54, 93)

Platelet disorders caused by transcription factor mutations:

Hematopoietic transcription factors are known to be important in the development and maturation of MK and platelets. (147) Mutations in transcription factors, such as Runt-related transcription factor 1 (RUNX1), Friend leukemia integration 1 (FLI1), GATA-binding factor 1 (GATA1), growth factor independent 1B (GFI1B), and E26-transformation specific variant 6 (ETV6) have been shown to result in MK and platelet dysfunction, thrombocytopenia, and variable bleeding symptoms in patients. (21, 134, 135, 147)

RUNX1 plays a fundamental role in megakaryopoiesis, and knockout gene studies show a decrease in MK polyploidization and cytoplasmic development. (147-149) Patients with a *RUNX1* mutation carry a high risk of developing familial platelet disorder with predisposition to acute myelogenous leukemia (FPD/AML). (21) FPD/AML is an autosomal dominant disorder, characterized by normal platelet morphology and size, and mild to moderate thrombocytopenia. (21, 57, 90, 147) Despite the platelet dysfunctions, patients with FPD/AML usually report a mild to moderate bleeding history. (147) Some affected individuals even have normal platelet counts and lack a bleeding history, making it challenging to identify all affected individuals within an affected family. (57)

Numerous platelet abnormalities have been reported in patients with *RUNX1* mutations, such as storage pool deficiencies (57, 150), impaired maximal aggregation and DG secretion (57, 90), aberrant persistence of platelet MYH10 (57, 92), and decreased activation of αIIbβ3. (151, 152)

FLI1 plays a major role in megakaryopoiesis, since a mutation in the transcription factor or a partial deletion of the chromosomal region that includes *FLI1*, results in macrothrombocytopenia, dysmorphic facial features, giant α-granules, and dysmegakaryopoiesis in the bone marrow. (21, 135, 147) There is also impairment in the release of platelet α-granules contents upon activation by thrombin (21, 147), a decrease in the number of platelet DG (133), and abnormal persistence of MYH10 in platelets. (90, 92, 133) Disorders associated with FLI1 include Paris-Trousseau syndrome (PTS) and Jacobsen syndrome. The two disorders differ in severity, with PTS being characterized primarily by thrombocytopenia, while Jacobsen syndrome is characterized by intellectual disabilities and dysmorphic features. (135, 153) The differences in clinical expression are likely to reflect the size and location of the chromosomal breakpoint. (135)

GATA1 is mainly expressed in erythroid cells, mast cells, and MK. (21, 44) Mutations in *GATA1* create an X-linked thrombocytopenia and affects the number and maturation of MK. (21) These MK have decreased polyploidization and immature cytoplasms. (21) Patients with a *GATA1* mutation produce fewer, abnormally-sized platelets and have fewer α-granules. (21)

GPI1b is crucial for normal hematopoiesis and an essential transcriptional regulator of erythroid and megakaryocytic development. (154) Individuals with mutations

in *GPI1b* have moderate macrothrombocytopenia, red cell anisopoikilocytosis, variable aggregation responses, and reduced platelet α-granules contents. (135, 155)

ETV6 is widely expressed in all tissues and essential for definitive hematopoiesis in the bone marrow. (135) It also modulates the activity of other transcription factors, such as FLI1. (156) Individuals with mutations in ETV6 have thrombocytopenia and a predisposition to hematologic malignancies; however, no defects in platelet function have been reported. (136)

1.3 IMPORTANT UNANSWERED QUESTIONS

1.3.1 Aim 1 – Diagnostic usefulness of whole mount electron microscopy for diagnosing platelet dense granule deficiency:

As uncharacterized PFD are commonly found among patients referred to a hematologist for abnormal bleeding, this has raised questions about how much testing, and which testing, is required to diagnose or exclude a PFD. (46) Recent ISTH guidelines have recommended that assays for platelet DGD be performed as a part of the diagnostic workup of a PFD. (50) Confirmed DGD by whole mount EM has been associated with a bleeding disorder (68), but this study did not evaluate if the findings were reproducible over time. Since whole mount EM is the most commonly used method to diagnose platelet DGD (48), there is a need to study a larger cohort to assess the diagnostic usefulness of the assay for evaluating PFD due to DGD.

Based on this knowledge, the following hypothesis was generated:
Quantification of DG numbers by whole mount EM is a useful diagnostic assay for the evaluation of a suspected PFD due to DGD.

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

1. Analyze prospectively collected data on diagnostic EM tests for DGD to evaluate the within-subject variability of DG counts in referred patients and general population controls who had multiple tests performed.

2. Evaluate sensitivities of LTA and DG ATP release tests for detecting PFD due to DGD.
3. Determine if confirmed DGD shows relationships to increased bleeding and the clinical diagnosis of a bleeding disorder.

1.3.2 Aim 2 – Analysis of platelet MYH10 as a biomarker for impaired megakaryopoiesis in uncharacterized platelet function disorders:

Transcription factor defects are implicated as an important cause of some PFD, and one study reported finding *RUNX1* or *FLI1* mutations in 6/13 (46%) of patients with a suspected inherited PFD. (90) The recent ISTH guidelines have recommended an assessment of platelet MYH10 as a potential biomarker for PFD cases that result from a mutation in a transcription factor such as *RUNX1* or *FLI1*. (50) Although previous studies have found that there is an aberrant persistence of platelet MYH10 in individuals with *RUNX1* and *FLI1* mutations (57, 90, 92, 133), further analyses are needed to assess the usefulness of evaluating for platelet MYH10, particularly for typical cohorts with an uncharacterized inherited PFD that could be due to a pathogenic mutation in *RUNX1* or *FLI1*. These analyses will provide further insights on the use of platelet MYH10 as a biomarker test for cases suspected to have mutations in *RUNX1*, *FLI1*, and/or potentially other hematopoietic transcription factors.

Based on this knowledge, the following hypothesis was generated:

Evaluation of the aberrant persistence of platelet MYH10 is a useful diagnostic assay to detect PFD resulting from mutations in transcription factors that are implicated to cause some PFD.

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

1. Evaluate platelet MYH10 protein levels by immunoblotting and densitometry, for individuals with uncharacterized PFD, in a cohort that included persons found to have a pathogenic *RUNX1* mutation.
2. Evaluate platelet *MYH10* transcript levels by digital polymerase chain reaction (dPCR) for individuals in a cohort with uncharacterized inherited PFD, including persons found to have a pathogenic *RUNX1* mutation.

1.3.3 Aim 3 – Evaluation of platelet procoagulant capacities in uncharacterized platelet function disorders and Quebec platelet disorder:

Some rarer forms of PFD, like Scott's syndrome, are known to affect platelet procoagulant activity. However, it is possible that other PFD (including those presenting with uncharacterized PFD that impair aggregation responses and/or cause DGD) may also impair the ability of platelets to support thrombin generation, given that some are caused by mutations in transcription factors, such as *RUNX1*, that impair multiple aspects of platelet function. (57, 90, 157) QPD is known to affect prothrombinase activity due to increased proteolysis of platelet FV (107, 146), but the disorder has not been studied by CAT where thrombin generation can be assessed using PRP. Since ISTH recommends the

assessment of platelet procoagulant activity in the diagnostic workup of some inherited PFD (50), we undertook a cohort study of the findings for participants with uncharacterized PFD (including some with mutations in transcription factors) and also studied platelet procoagulant activity in QPD by CAT, to better understand the pathogenesis of these disorders.

Based on this knowledge, the following hypothesis was generated:
Individuals with QPD, and some individuals with uncharacterized PFD, have impairments in the ability of their platelets to support thrombin generation.

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

1. Assess platelet procoagulant defects in persons with uncharacterized PFD and QPD by evaluating:
    a. Platelet-independent and -dependent thrombin generation by CAT.
    b. FV antigen levels in plasma and platelets by ELISA.
    c. Relationships between plasma and platelet FV levels and thrombin generation endpoints.
2. Re-evaluate platelet uPA levels in QPD to assess associations between platelet uPA, platelet FV, and thrombin generation endpoints.

CHAPTER 2

METHODS

2.1 ETHICS APPROVAL AND PARTICIPANT CONSENT

The study was conducted in accordance with the requirements of the revised Helsinki accord, the Hamilton Integrated Research Ethics Board (HiREB) and the Hamilton Regional Laboratory Medicine Program (HRLMP). Written informed consent of participants was obtained for the second and third aims only as the HRLMP and HiREB did not require informed consent to be obtained to evaluate HRLMP data for quality improvement purposes (aim 1). Samples from QPD participants (aim 3) were collected with the approval of the HiREB and the Centre Hospitalier Universitaire Sainte Justine Research Ethics Board. All participant identities were anonymized prior to sample preparation and data entry.

2.2 PARTICIPANTS

2.2.1 Participants in the Hamilton study cohort on phenotype and molecular causes of uncharacterized platelet function disorders:

Participants in Hamilton that were recruited for a study on the phenotype and molecular causes of uncharacterized PFD (referred to subsequently as the "HPD study cohort"), led by Dr. Catherine Hayward, donated samples for aims 1-3. Persons in the HPD cohort were classified as "affected" by a PFD if they met the following inclusion criteria:

1. Confirmed bleeding problems, based on the recorded opinion of the patient's hematologist (obtained by medical record review).
2. One or more of the following platelet function abnormalities of unknown cause:
    a. Impaired MA responses to ≥2 agonists by LTA, not due to a well-characterized disorder (e.g. GT, BSS), and confirmed by repeat testing if DG numbers were normal.
    b. Confirmed DGD (lower limit of RI: 4.9 average DG/platelet), estimated by whole mount EM.

Affected and unaffected relatives of participants had their bleeding histories assessed, using the ISTH BAT and CHAT-P tools, as previously described. (57, 60) Medical records were reviewed to gather HRLMP laboratory findings. The goals of my thesis did not include determining the molecular defects in study participants, although results from sequence analyses were used to sub-categorize the subjects tested.

As of August 2018, the HPD study cohort included: 9 individuals with a PFD and a confirmed *RUNX1* haploinsufficiency mutation (from 3 families, one of which was previously reported (57)); 28 persons with a PFD of unknown cause (n=27 without a causative mutation identified by exome sequencing, n=1 whose exome sequencing was not completed), which included 18 affected individuals from 10 families and 10 affected single ("sporadic") cases; and 23 unaffected relatives. General population controls with "no known problem with bleeding" were also evaluated (n=60 for bleeding history determination; n=20 and n=18 respectively for aims 2 and 3). Each participant was given an anonymous study code prior to sample collection and data entry.

For HPD study cohort participants, the data that was gathered included: sex; age at time of testing; whether the participant had a bleeding disorder (based on the hematologist's opinion, as described (47)); ISTH BAT scores (51, 57, 60); all evaluations of mean DG numbers per platelet by whole mount EM (71, 72); and the most recent HRLMP platelet count, mean platelet volume (MPV), DG ATP release findings (evaluated by lumi-aggregometry as described (60)), using an agonist panel that included: 1 U/ml thrombin, 5.0 μg/ml Horm collagen, 6 μM epinephrine, 1 μM thromboxane analogue U46619 and 1.6 mM arachidonic acid; and MA responses (evaluated by LTA as described (47, 57, 68)), with the agonists: 1.25 and 5.0 μg/ml Horm collagen, 2.5 and 5.0 μM ADP, 6 μM epinephrine, 1 μM thromboxane analogue U46619, 1.6 mM arachidonic acid, and 0.5 and 1.25 mg/ml ristocetin.

For aims 2 and 3, participants in the HPD study cohort that consented to donate additional research samples were evaluated (n=26 for aim 2, n=18 for aim 3) (see 3.2.1 and 3.3.1 for further information on participant demographics).

2.2.2 Other participants:

For aim 1 (evaluation of diagnostic usefulness of quantifying DG numbers by whole mount EM), participants were organized into two cohorts: 1) all referred patients and simultaneously evaluated controls that were tested by the HRLMP for DGD by whole mount EM over a 12-year period from January 1st 2006 to December 4th 2017 (referred to as Cohort I); and 2) all HPD study cohort participants that had DG investigations completed by December 4th 2017 (referred to as Cohort II). There was overlap between Cohorts I and II, as 24/29 persons in Cohort II were a part of Cohort I. Five persons with QPD from Hamilton and Montreal provided samples for aim 3.

2.3 METHODS FOR AIM 1 STUDIES

2.3.1 Quantification of platelet dense granule numbers:

HRLMP data was gathered on platelet DG numbers for clinical and control test samples evaluated between January 1st 2006 and December 4th 2017 (Cohort I) by the whole mount EM procedure that was originally described by Dr. James White. (15, 69). The DG counts had been quantified by one of two staff members in the EM facility, using PRP (from blood anticoagulated with 0.105 M sodium citrate) prepared by the HRLMP Special Coagulation Laboratory, as published. (68) A general population control sample was drawn and tested on each day the patient tests were performed. Briefly, small drops of PRP were placed on Formvar grids, with blotting of excess liquid, followed by a quick rinse with distilled water before air-drying of the sample. Samples were first examined by transmission EM at ~100x magnification to assess the overall DG distribution in platelets before moving to a higher magnification (~4000x) to quantify DG numbers. Platelets were examined by methodically moving the grid from one side to the next. DG were not quantified in platelets that overlapped, showed bubble artifacts, or shape change. Structures that were counted as DG included round DG and DG with tails or "purse-like" shapes. Structures that were not counted as DG included faint, tiny or chain-like granules. Structures that were counted as DG were typical of the electron dense structures that the majority of North American Specialized Coagulation Laboratory Association (NASCOLA) laboratories classified as DG. (71, 72) As recommended by Dr. White when the assay at McMaster University was first set up, DG were counted in 30 platelets and

the average was calculated. Grids from 10 random samples were reexamined to compare DG counts for 30 and 100 platelets.

DG counts for HRLMP samples prepared for NASCOLA EQA exercises on quantifying DG numbers were exclusively used to evaluate within-subject variability. To assess within-subject variability in DG counts for participants with DGD, older EM records (from January 1999 to December 2005) were searched for additional data on Cohort I patients with ≥1 test showing reduced DG numbers/platelet.

2.3.2 Reference interval determination:

Differences in male and female control data were evaluated before determining the lower limit of the RI using stepwise logistic regression to determine the lower limit of the 95% confidence intervals (CI) for all unique Cohort I general population controls. (158) The method of Taylor et al. (159) was used to estimate RI limits as this method allows for the inclusion of repeated measures on subjects and takes into account within-subject variability, with an adjustment in weightings for repeated measures so that each participant's data contributes equally to the RI.

2.3.3 Statistical analyses:

Student's t-test was used to assess differences in average number of DG/platelet for Cohort I: pediatric versus adult patients; female versus male patients; female versus male controls; and patients versus controls. Coefficients of variation (CV) (160) were

used to evaluate the within-subject variability in average DG counts/platelet for all participants (Cohort I and II) that had ≥3 unique samples assessed. Linear regression was used to assess relationships between: the average number of DG/platelet and MPV; CVs and mean DG counts for participants with ≥3 assessments; and the average number of DG/platelet and age for Cohort I participants <18 years old on their first test, since a recent study reported that young children have lower DG numbers. (161) Chi-square test was used to assess the proportions of: Cohort I patient versus control samples with DG numbers below the RI; and Cohort II participants with PFD, with or without DGD, that had reduced MA with ≥2 agonists by LTA. Mann-Whitney U tests were used to evaluate the relationships between confirmed DGD and ISTH BAT scores. OR and 95% CI were used to assess relationships between confirmed DGD and the diagnosis of a bleeding disorder.

2.4 METHODS FOR AIM 2 STUDIES

2.4.1 Blood sample collection:

Thirty milliliters of blood was collected from general population controls and affected participants in the HPD study cohort that consented to provide additional samples. Blood was obtained by venipuncture and syringe, and transferred to tubes containing acid citrate dextrose (ACD, 1:6 volume/volume). PRP was collected after a 170 x *g* spin, followed by CD45-depletion (STEMCELL^{TM} Technologies, Vancouver, BC, Canada) to reduce leukocyte contamination.

2.4.2 Sample preparation for *MYH10* transcript analysis:

For dPCR analyses, platelets were isolated, washed, and dissolved in TRIzol® (Life Technologies Inc., Burlington, ON, Canada) for platelet RNA isolation, as described. (162) Briefly, chloroform was added to samples, followed by a spin at 12000 x *g*. RNA was precipitated after addition of isopropanol and GlycoBlue (Thermo Fisher Scientific Inc., Waltham, MA, USA), and then washed in 75% ethanol at 7500 x *g*. Pellets were dissolved in RNase-free water, followed by evaluation of RNA concentration and quality by a NanoDrop 2000c (Thermo Fisher Scientific Inc.) and by the Mobix Lab (McMaster University, ON, Canada), respectively. Reverse transcription (RT) was performed, as described (162), using High-Capacity RNA-to-cDNA kits (Applied Biosystems, ThermoFisher Scientific Inc.), by incubating RNA in a Thermocycler (37°C, 1 hour, 20 µl reaction volume) with the recommended RT Kit

components and 20 units of RNasin® Plus RNase Inhibitor (Promega Corp., Madison, WI). Concentrations of cDNA ranged from 50-200 ng/μl.

2.4.3 Quantification of *MYH10* transcripts:

cDNA samples for dPCR were submitted to the Center for Applied Genomics (TCAG, The Hospital for Sick Children, Toronto, ON, Canada). Estimation of *MYH10* transcript expression was performed in triplicate at TCAG using: QX200 Droplet Digital PCR system (Bio-Rad Laboratories, Inc., Hercules, CA, USA), *MYH10* Taqman probe Hs00413181_m1 (Life Technologies), and *PPIA* probe Hs99999904_m1 as an endogenous control (92), in a duplex reaction mode. The 20 μl gene expression reaction mix consisted of: 10 μl of 2x dPCR SuperMix for Probes (Cat No. 1863023, Bio-Rad Laboratories); 1 μl of the target assay (labeled with FAM); 1 μl of the endogenous control assay (labeled with VIC); 5.5 μl nuclease free water; and 2.5 μl of cDNA. The gene expression assays were previously validated by temperature gradients to ensure optimal separation of target and control droplets. Cycling conditions for the reaction were: 95°C for 10 minutes; 45 cycles of 94°C for 30 seconds and 58°C for 1 minute; 98°C for 10 minutes; and finally a 10°C hold on a Life Technologies Veriti thermal cycler. Data was analyzed using QuantaSoft v1.7.4 (Bio-Rad Laboratories). No template, no RT and Human Universal RNA controls were run in parallel with the study samples. Since *MYH10* transcript levels were very low relative to *PPIA* transcript levels, ratios were multiplied by 1000.

2.4.4 Immunoblot analysis of MYH10:

Platelets were isolated from PRP after a 1300 x *g* spin, washed, and solubilized at 10^{10} platelets/ml in lysing buffer containing: 2 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, St. Louis, MO, USA); 1% Halt protease inhibitor cocktail (Thermo Fisher Scientific Inc.); 1% Triton X-100 (Sigma-Aldrich); and 2% sodium dodecyl sulphate (SDS, Bio-Rad Laboratories), as described. (57) DC protein assays (Bio-Rad Laboratories) were completed, as recommended by the manufacturer, to estimate total protein concentration in the platelet lysate samples. Most samples were tested by loading 30 μg of platelet protein/lane on 6% SDS-polyacrylamide gels, as previously described. (92) Participants with unexpectedly normal findings, based on findings for other affected family members or the presence of a *RUNX1* haploinsufficiency mutation, were further evaluated by loading 30, 60 and 90 μg total platelet protein/lane for two samples (each collected on a different day). After transfer of the proteins onto nitrocellulose membranes, membranes were cut between 100 and 75 kDa markers to probe the upper half for MYH10 (230 kDa) and the lower half for β-actin (45 kDa, loading control), as previously described. (57) Positive and negative controls on each gel included pooled platelet lysate prepared from 5 individuals with a known *RUNX1* haploinsufficiency mutation (57) and normal pooled platelet lysate prepared from 20 donors, respectively. Densitometry was used to assess the ratio of the MYH10/β-actin (M/B) band intensities with subtraction of background using ImageJ.

2.4.5 Statistical analyses:

Two-tailed Mann-Whitney tests, with Bonferroni correction for multiple comparisons, were used to assess differences in *MYH10*/*PPIA* transcript ratios. Individual participants' densitometry data were compared to the densitometry data of pooled platelet lysate prepared from the individuals with a known *RUNX1* haploinsufficiency mutation. (57)

2.5 METHODS FOR AIM 3 STUDIES

2.5.1 Blood sample collection and sample preparation:

For this aim, participants were required to complete an HRLMP drug questionnaire, with deferral of those who had taken NSAIDs or thienopyridines in the past 7 days to limit potential drug interference. Twenty milliliters of blood was collected for thrombin generation assays (TGA) from affected participants in the HPD study cohort, persons with QPD, and age- and gender-matched general population controls. Blood was collected by venipuncture and syringe, and transferred to tubes containing 0.109M sodium citrate (1:9 volume/volume) for isolation of PPP and PRP. PRP was collected after a 170 x *g* spin. Some PRP was spun at 1300 x *g* to collect PPP, followed by an additional spin to remove platelet debris. Additional citrated PPP was stored at -80°C for plasma FV antigen quantification (see Section 2.5.3). Platelet count was adjusted to 150 x 10^9/L in PRP with autologous PPP.

2.5.2 Thrombin generation assays:

Thrombin generation was assessed by CAT on a Fluoroskan (Thermo Fisher Scientific AG, Reinach, Switzerland) using Thrombinoscope software (Thrombinoscope, Synapse BV, Maastricht, The Netherlands). (76) Experiments were completed within 3-4 hours of blood draw, and were tested according to ISTH recommendations on measuring thrombin generation. (83) Briefly, thrombin generation was measured in triplicate with PPP or PRP adjusted to 150 x 10^9/L. As per the Hemker method, each PPP test well contained: 80 µl PPP and 20 µl PPP reagent (containing 4 µM phospholipids and 5 pM

TF, from Thrombinoscope BV). (76) Each PRP test well contained: 80 µl adjusted PRP and 20 µl PRP reagent (containing 0.5 pM TF, from Thrombinoscope BV). (76) To start the test, 20 µl of FluCa reagent (Thrombinoscope BV) was dispensed into each well and thrombin generation was measured in 20 second intervals for 60 minutes at 37°C. (76) Endpoints measured included: endogenous thrombin potential (ETP, nM·min), peak thrombin concentration (nM), time to peak (min), and lag time (min). (76, 82) If thrombin generation curves did not return to baseline during the data recording, the area under the curve was manually calculated, as recommended by recent ISTH guidelines. (83)

2.5.3 Enzyme-linked immunosorbent assays for factor V antigen and urokinase plasminogen activator levels:

Double-spun citrated PPP (prepared for TGA, see 2.4.2) and platelet lysate samples from selected subjects were prepared to evaluate for relationships between TGA endpoints and FV antigen and/or uPA levels. Briefly, platelet lysate was prepared and total platelet protein was quantified (as outlined in Section 2.4.4) except that for these analyses, platelets were lysed with 1% Triton X-100 (Sigma-Aldrich) at a final count of 10^{10} platelets/ml in lysing buffer containing 2 mM EDTA (Sigma-Aldrich) and 1% Halt protease inhibitor cocktail (Thermo Fisher Scientific Inc., USA). Protein levels were described as amount/mg of total cellular protein. FV antigen was quantified by ELISA as described (107, 128) and platelet uPA was quantified by the commercial R&D Systems uPA ELISA kit (Minneapolis, MN, USA).

2.5.4 Statistical analyses:

Two-tailed Mann-Whitney tests, with Bonferroni correction for multiple comparisons, were used to assess differences in: 1) TGA endpoints; 2) platelet FV; 3) plasma FV; and 4) and uPA levels. Linear regression was used to assess relationships between: 1) plasma FV and TGA endpoints in PPP and PRP; and 2) platelet FV and TGA endpoints in PRP. For QPD participants only, multiple regression was used to assess relationships between platelet FV or platelet uPA with TGA endpoints in PRP.

CHAPTER 3

RESULTS

3.1 EVALUATION OF DENSE GRANULE DEFICIENCY BY WHOLE MOUNT ELECTRON MICROSCOPY

For this section of the thesis, I evaluated: HRLMP's experiences with whole mount EM for diagnosing platelet DGD; the within-subject variability of DG counts for individuals with multiple assessments; the proportions of DGD participants that had reduced MA or DG ATP release with ≥2 agonists; and the relationships between confirmed DGD and ISTH BAT scores. Quantification of DG numbers over the 12-year period was completed by Ernie Spitzer and Marnie Timleck. Laboratory data from the HRLMP was collected with the assistance of Catherine Hayward and Karen Moffat. The findings from this aim were presented at the 2017 meeting of the International Society for Laboratory Hematology and at the 2018 Thrombosis and Hemostasis Societies of North America Summit, and were published in the International Journal of Laboratory Hematology. The figures and content presented in this aim are reproduced from the published article with permission from Wiley Company.

3.1.1 Demographics of participants evaluated for dense granule deficiency by electron microscopy:

Cohort I consisted of 1115 unique patients (ages in years: median 37, range 0.25-89; females: 77%) and 126 unique controls (ages in years, if recorded: 18-64; females: 52%) who had platelet whole mount preparations evaluated by HRLMP between January

1st 2006 and December 4th 2017. Some participants in Cohort I were tested on multiple occasions (number of unique samples/participant, as medians [ranges]: patients: 1 [1-6]; controls: 1 [1-63]). Means were used for comparisons when there were multiple determinations for a subject, since the DG count data for individuals with the largest number of determinations were normally distributed.

Cohort II consisted of 26 females and 16 males (median age in years, range: 47, 7-80). In this cohort, 29/42 had a PFD whereas the remaining 13 were unaffected relatives who had normal DG counts and LTA findings. In the subgroup with PFD, 34% (10/29) had confirmed DGD. There was overlap between Cohorts I and II, where 24/29 persons with PFD in Cohort II were a part of Cohort I. The remaining 5 persons from Cohort II either had DG counts completed before 2006 or did not have them completed. Platelet DG numbers showed no relationship to MPV ($R^2 = 0.06$, $p = 0.15$).

3.1.2 Analysis of dense granule counts for cohort I participants:

DG counts were similar for evaluated pediatric (<18 years old) and adult patients (respective means [range]: 6.6 [2.7-11.6] vs. 6.7 [0.8-15.5] (p=0.28) (Figure 1). One adult patient with a PFD, thrombocytopenia and an elevated MPV (MPV: 12.1, upper limit of RI: 10.4 fL) had the highest DG counts (2 determinations, averages: 14.5 and 15.8 DG/platelet) of all study participants. There was no relationship between age and DG counts among participants <18 years of age (R^2=0.005, p=0.35). There were no significant differences in mean DG counts for female vs. male patients (6.7 vs. 6.7, p=0.32); however, among the controls, the mean DG counts were slightly higher for

females than males (6.9 vs. 6.5, p=0.03). Thus, the lower limit of the RI for DG counts were determined separately for males and females using all Cohort I control data for unique samples. The lower RI was estimated to be 4.9 average DG/platelet for both males and females (respective 95% CIs: 4.9-8.2 and 4.9-8.8), which was identical to HRLMP's previous estimate.

Although the mean DG counts for patients and controls in Cohort I were identical (respective means: 6.7 vs. 6.7, p=0.84), proportionately more patient samples had reduced DG counts (% <4.9 DG/platelet for patients vs. controls: 6.5% vs. 0.3%, p<0.01). DG counts determined by examining 30 or 100 platelets were comparable (respective: means [ranges]: 5.2 [1.8-7.6] vs. 4.9 [1.7-6.8]; standard deviations: 1.6 vs. 1.3; p=0.60).

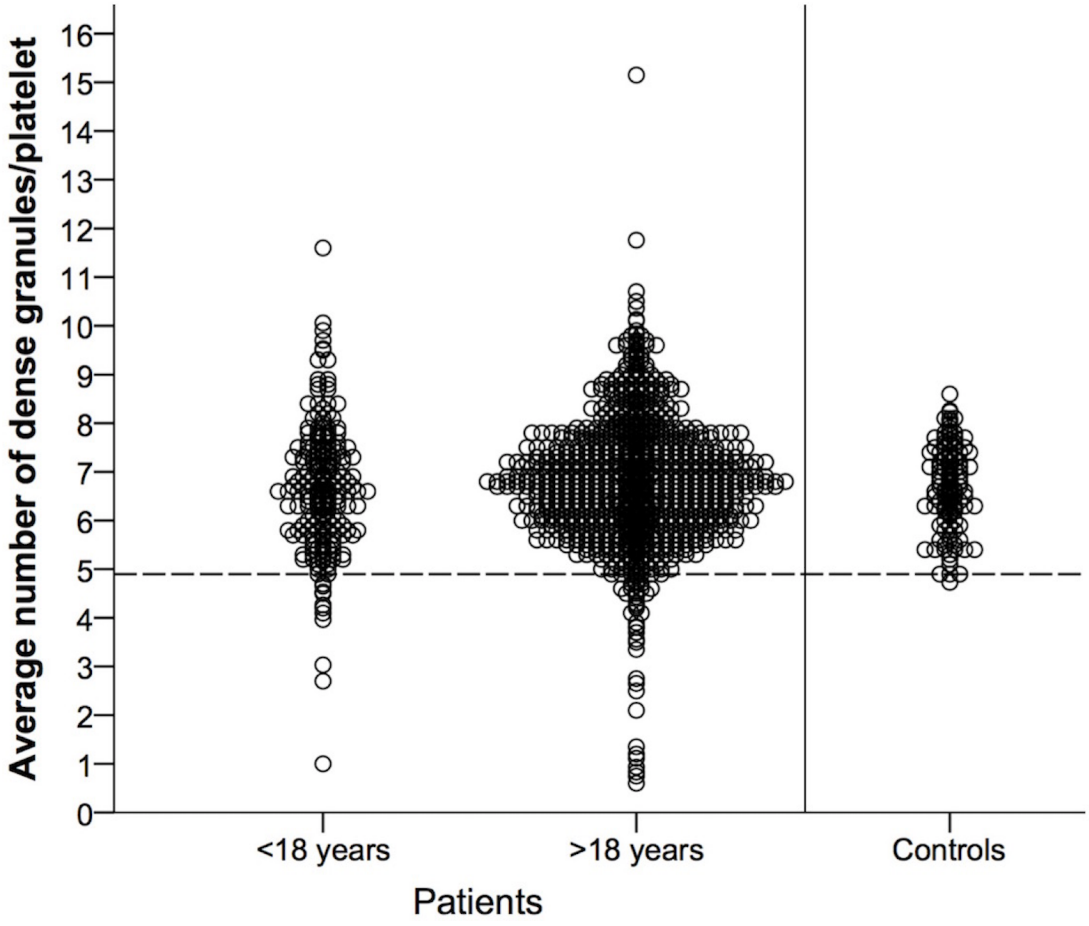

Figure 1. Average number of dense granules/platelet in whole mount electron microscopy assessment of dense granule numbers for patients and controls evaluated over a twelve-year period. Results for Cohort I participants compare the DG counts for 126 unique adult controls and 1115 unique patients. Mean values are shown for persons with multiple determinations. Patient data were grouped according to whether the individual was <18 years old (n=215) or ≥18 years old (n=900) at the time of their last test. The horizontal dashed line indicates the estimated lower limit of 4.9 DG/platelet, which was identical for male and female controls.

3.1.3 Within-subject variability in dense granule counts for participants with multiple assessments:

Figure 2 shows data for participants from Cohorts I and II (n=56 controls, n=64 patients) that had DG counts determined for multiple unique samples, each drawn on a different occasion, including samples drawn for NASCOLA EQA exercises. Almost all controls with repeat tests (55/56) had DG counts ≥ the lower RI limit on all tests. Sixteen controls had ≥10 determinations because they volunteered to donate a sample on many different occasions (Figure 2A). Among patients with multiple tests, the median time between first and second tests was 63 days (range: 7-2882 days). Many patients that were retested had confirmed normal findings on each test (41%, 26/64), whereas others had confirmed DGD on each test (30%, 19/64) or discordant results (30%, 19/64) (Figure 2B). Among patients with discordant results, the majority (17/19, 89%) had a mild reduction in DG numbers (i.e., 4.0-4.8 DG/platelet) on the first test only (Figure 2B).

Among participants who had their platelet DG counts assessed ≥3 times, the within-subject CV for average DG counts/platelet showed an inverse relationship to the subject's mean DG count ($R^2 = 0.65$, $p<0.01$), where non-DGD participants had a lower within-subject CV than DGD subjects (respective median [ranges] for within-subject CV for normal vs. DGD participants: 12% [5-24%] vs. 28% [18-46%]) (Figure 3). Nonetheless, DGD was evident on each sample for all participants with <4.0 DG/platelet.

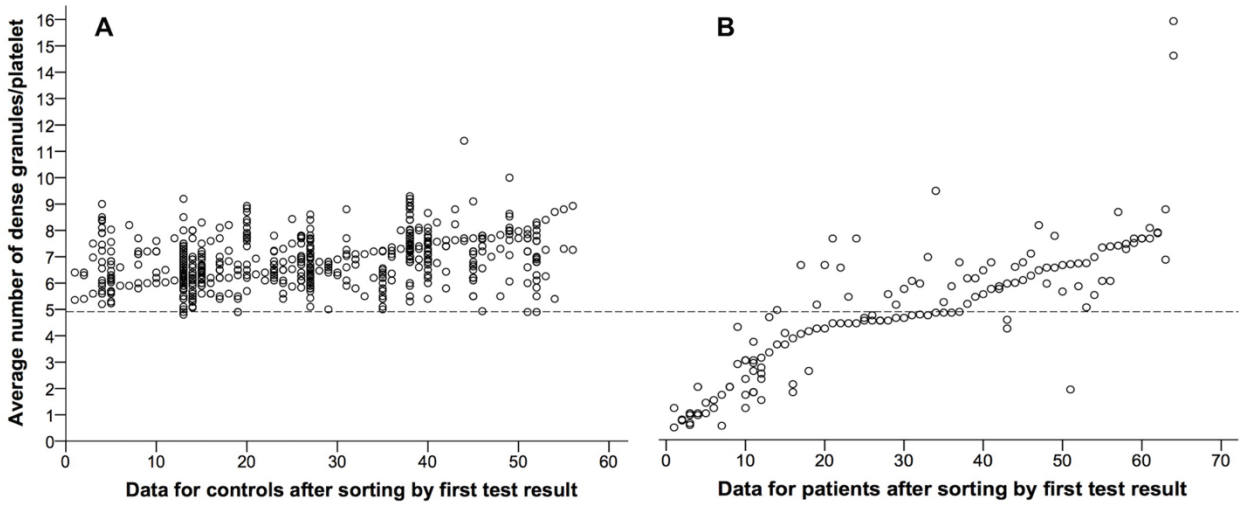

Figure 2. Observations on platelet dense granule counts for individuals who had multiple unique samples tested, each on a different occasion. The figure shows data for multiple tests performed on 56 different controls (panel A) and 64 different patients (panel B), sorted in ascending order of first test results. The dashed line indicates the lower limit of the RI (4.9 DG/platelet).

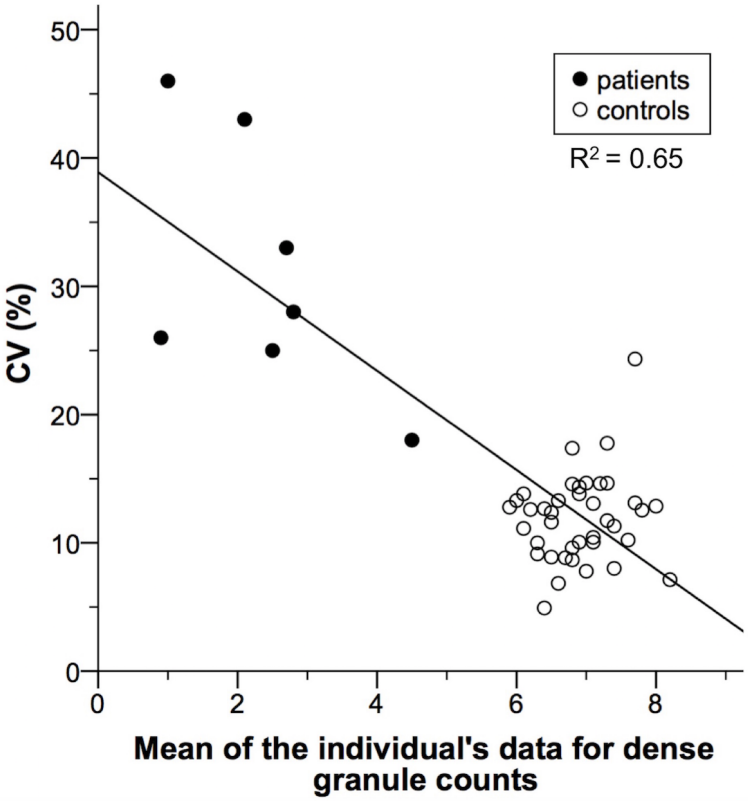

Figure 3. Intra-individual coefficient of variation for the average number of dense granules/platelet. Data for individuals with three or more independent tests (each on a unique sample; n=7 patients, n=39 controls) is shown and compares the estimated intra-individual CV to the mean of the participant's DG count estimations (R^2 shown).

3.1.4 Aggregation and dense granule secretion findings for dense granule deficient participants:

The data for the most recent platelet function tests for the 19 participants with confirmed DGD (from Cohorts I and II) are displayed in Figure 4. LTA findings with 0.5 mg/ml ristocetin are not shown as data was normal for all participants. While none of the unaffected relatives in Cohort II (n=13) had abnormal LTA findings (not shown), 52% of the DGD participants (10/19) had reduced MA with ≥2 agonists (Figure 4A). Comparisons of aggregation data for Cohort II participants that had a PFD, with or without DGD (two studied with limited numbers of agonists due to their young age), indicated that proportionately less with DGD had impaired MA with: 2.5 μM ADP (0/19 vs. 6/18; $p<0.01$); 5 μM ADP (0/19 vs. 5/18; $p=0.01$); 1.6 mM arachidonic acid (7/18 vs. 14/18; $p=0.02$); and 1 μM thromboxane analogue U46619 (9/18 vs. 17/18; $p<0.01$).

Figure 4B summarizes the lumi-aggregometry data for DG ATP release, which was available for 52% (10/19) of participants with confirmed DGD (median [range] of average DG counts/platelet: 2.2 [0.7-4.7]). While most individuals with DGD had reduced ATP release with at least two agonists (70%, 7/10), only half showed reduced release with all agonists (5/10) and others (30%, 3/10) had non-diagnostic findings. The sensitivities of LTA and DG ATP release, for detecting platelet function abnormalities due to DGD, were ~52% and 70% respectively (based on detection of impaired responses to ≥2 agonists).

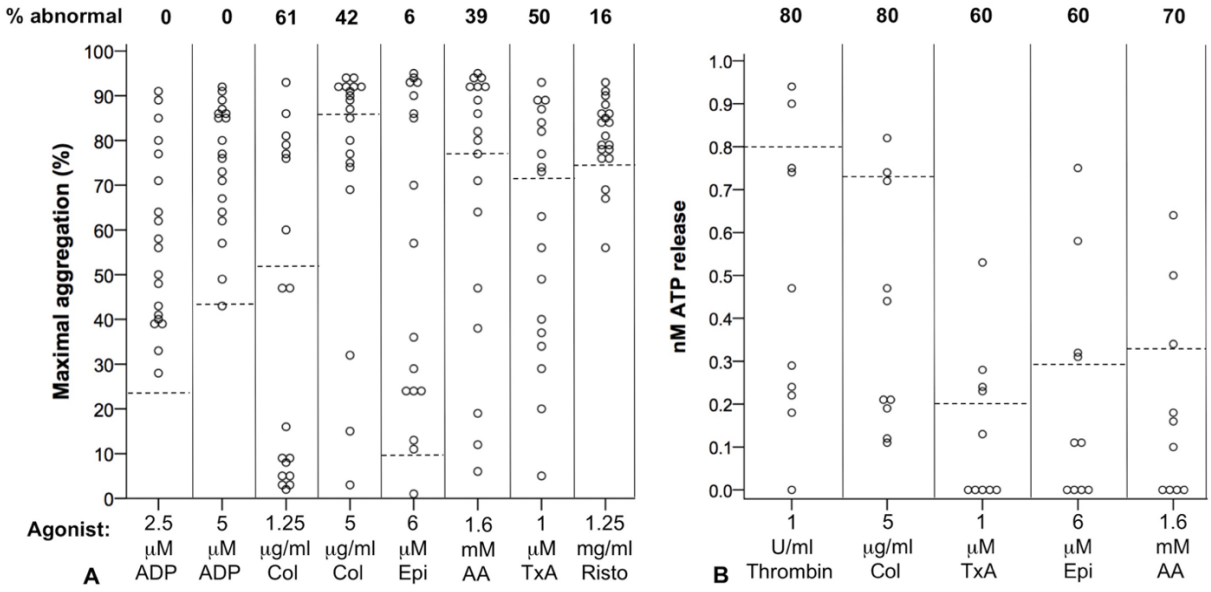

Figure 4. Maximal platelet aggregation responses and lumi-aggregometry dense granule ATP release findings with different agonists for individuals with confirmed dense granule deficiency. A) Percent maximal aggregation responses are shown for DGD participants, evaluated with the following agonists: 2.5 and 5.0 μM adenosine diphosphate (ADP), 1.25 and 5.0 μg/ml collagen (Col), 6 μM epinephrine (Epi), 1.6 mM arachidonic acid (AA), 1 μM thromboxane analogue (TxA) (U46619), and 1.25 mg/ml ristocetin (Risto). B) Lumi-aggregometry estimates of dense granule ATP release for DGD participants, evaluated with the following agonists: 1 U/ml thrombin, 5.0 μg/ml collagen (Col), 1 μM thromboxane analogue (TxA) (U46619), 6 μM epinephrine (Epi), and 1.6 mM arachidonic acid (AA). A&B). Dotted lines show the lower limit of the RI for each agonist. The percentage of DGD patients that were abnormal with each agonist is indicated.

3.1.5 Relationship between dense granule deficiency and bleeding:

Cohort II participants with confirmed DGD had higher ISTH BAT bleeding scores (median: 9.5, range: 4-17) than unaffected relatives (median: 1, range: 0-8, $p<0.01$) and general population controls (median: 0, range: 0-6, $p<0.01$) (Figure 5). However, ISTH BAT scores were not significantly different for the subgroups with a PFD, that had or did not have DGD (respective data: medians [ranges] = 8 [0-17] vs. 9.5 [4-17], $p=0.20$). The finding of confirmed DGD by EM was associated with a bleeding disorder (OR = 97, 95% CI = 5.4-1700, $p<0.01$).

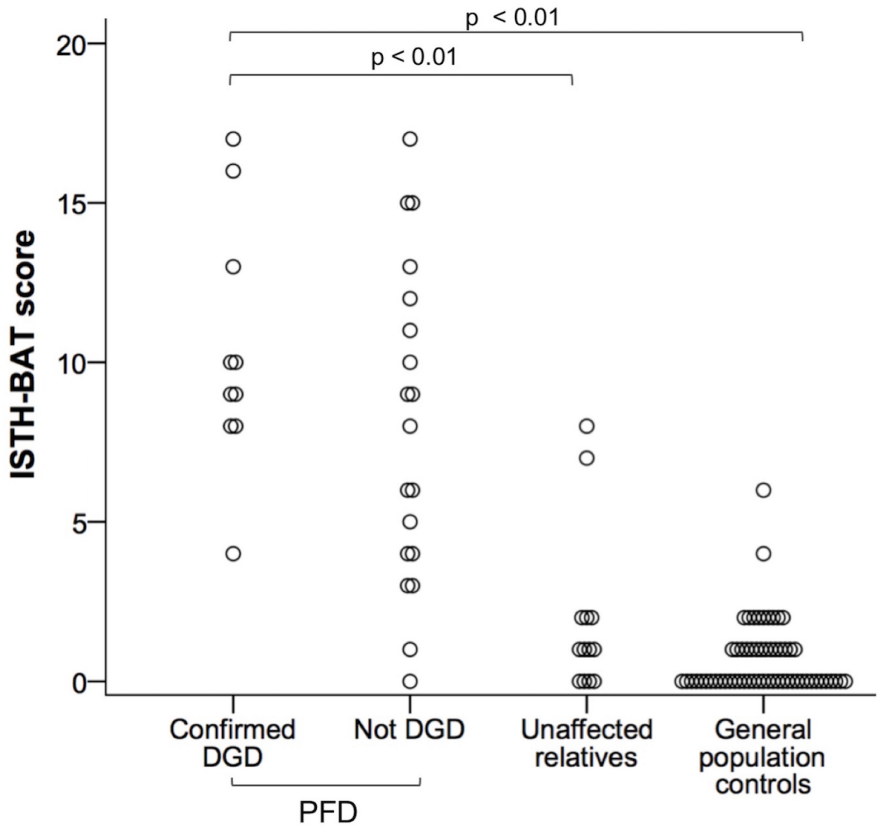

Figure 5. Bleeding scores for cohort II participants. ISTH BAT scores are compared for participants with: PFD and confirmed DGD (n=10), PFD without DGD (n=19, indicated as Not DGD), unaffected relatives (n=13) and general population controls (n=60). p values indicate significant differences.

3.2 ABERRANT PERSISTENCE OF PLATELET MYH10 IN PARTICIPANTS WITH UNCHARACTERIZED PLATELET FUNCTION DISORDERS

For this section of the thesis, I assessed the diagnostic usefulness of evaluating for the aberrant persistence of platelet MYH10 transcript and protein in participants from the HPD study cohort, who had an uncharacterized PFD due to impaired MA with ≥2 agonists and/or DGD, which included a subgroup that were found to have a pathogenic *RUNX1* mutation. The additional research sample donations obtained from PFD participants and controls were organized by myself and Janaki Iyer. I also: 1) prepared platelet RNA, cDNA and lysates; 2) performed protein quantification and immunoblotting assays; and 3) analyzed protein data. Transcript quantification by dPCR and subsequent analyses were performed at TCAG (The Hospital for Sick Children, Toronto, ON, Canada).

3.2.1 Demographics of participants evaluated for platelet MYH10 transcript and protein levels:

Additional research samples for MYH10 protein analyses were provided by 26 affected participants from the HPD study cohort (17 index cases; 5 single cases, 21 from 12 families with ≥2 participants, including 7 persons from 3 families with a pathogenic *RUNX1* mutation; number with PFD/family as median, range: 1, 1-5; 69% females; age in years: median 42, range 17-77). The majority of affected participants who provided samples for these analyses had impaired MA only (15/26; 58%), while some had DGD but normal MA findings (5/26; 19%) or DGD with impaired MA (6/26; 23%).

21/26 affected participants (81%) who provided samples for protein analyses had dPCR tests completed to quantify *MYH10* transcripts (15 index cases; 5 single cases, 16 from 10 families ≥2 participants, which included 7 from 3 families with a pathogenic *RUNX1* mutation; number with PFD/family as median, range: 1, 1-5; 79% females; age in years: median 46, range 17-77). The majority of affected participants that provided samples for dPCR tests had impaired MA only (11/21; 52%), DGD but normal MA findings (4/21; 19%) or DGD with impaired MA (6/21; 29%).

General population controls (n=20, 70% females) provided samples for both molecular and protein analyses.

3.2.2 MYH10 immunoblot findings in participants with an uncharacterized platelet function disorder:

Figures 6-10 depict MYH10 Western blot findings for n=26 affected participants from the HPD study cohort and n=20 general population controls. Participants are designated by identifiers that indicate the unique participants by family (F), single case (SC) or control (C) numbers. MYH10 protein data for F1 were previously published but reassessed using the recommended protocol (30 μg platelet protein/lane) from the Antony-Debré article cited in the ISTH guidelines. (50, 92)

The analysis of 30 μg of platelet protein/lane revealed increased MYH10 in 8 participants, that included: 6/7 (86%) with a pathogenic *RUNX1* mutation (5 from F1, Figure 6A; and SC1, Figure 6B; data for the other case described later) and 2/4 affected members of F4 (a family with a PFD of unknown molecular cause after exome

sequencing). Densitometry analysis indicated that SC1 had a similar M/B ratio (100%) to the abnormal pooled platelet lysate prepared from the samples of 5 affected members of the F1 family (Figure 6B).

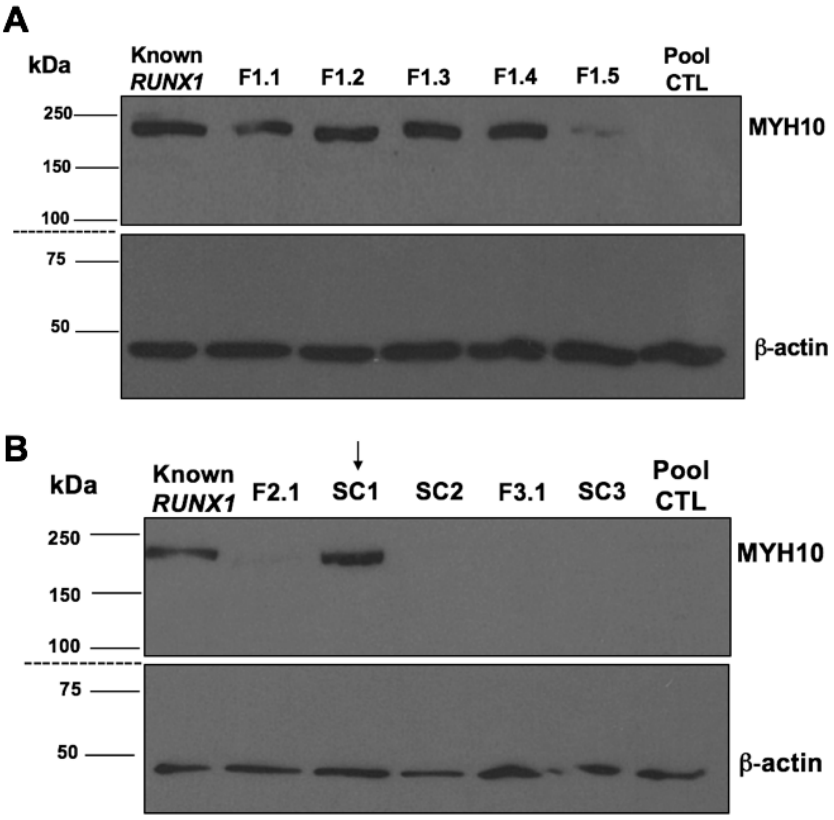

Figure 6. Western blot findings for platelet MYH10 in participants with uncharacterized platelet function disorders. Panels show data for: A) 5 participants from family 1 (F1) with a pathogenic *RUNX1* mutation; and B) 5 participants (4 with PFD of unknown molecular cause, and a single case [SC1, indicated with arrow] with a different *RUNX1* mutation). Each panel shows data for an abnormal control, which was pooled platelet lysates prepared from n=5 participants from F1 who have a *RUNX1* haploinsufficiency mutation (Known *RUNX1*), and normal pooled platelet lysate, prepared from n=20 general population controls (Pool CTL). The lower half of the membrane was probed for β-actin as a loading control.

In family 4 (F4.1-4.4), who have an uncharacterized PFD of unknown molecular cause, two of the four affecteds had similar M/B densitometric ratios (F4.2: 110%, F4.4: 110%) to the abnormal pool, while the remaining two (F4.1 and F4.3) had no detectable MYH10 by densitometry (Figure 7A), even when 60 and 90 μg of platelet protein/lane was evaluated (Figure 7B-C). The negative findings for F4.1 and F4.3 were confirmed by analysis of additional samples from these participants (Figure 7D-E).

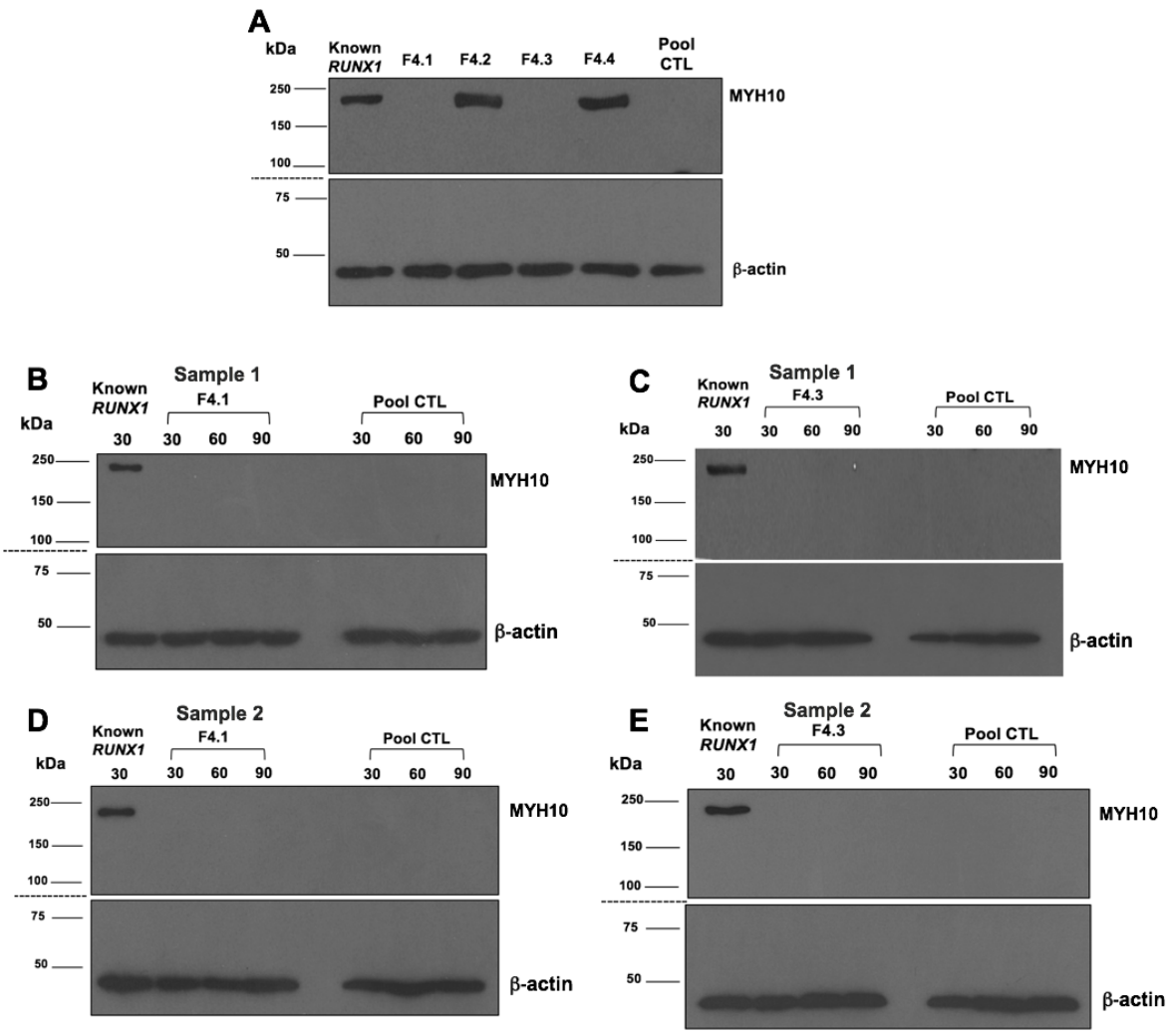

Figure 7. Western blot findings for platelet MYH10 for relatives from family 4 with an uncharacterized platelet function disorder of unknown molecular cause. Panels show data for 4 affected participants from a family (F4) with an uncharacterized platelet function disorder (PFD) of unknown molecular cause compared to positive and negative controls. A) Western blot analyses of 30 μg of platelet protein/lane. B-E) MYH10 findings for F4.1 and F4.3 were further evaluated by analyzing more platelet protein/lane (amount in μg indicated) using several independent samples (indicated by sample number). In all panels, the pooled platelet lysates from n=5 participants from family 1 (F1) with a *RUNX1* haploinsufficiency mutation (Known *RUNX1*) and n=20 general population controls (Pool CTL), were used as positive and negative controls, respectively. The lower half of the membrane was probed for β-actin as a loading control.

The affected index case of family 5 (F5.1), who had a different *RUNX1* haploinsufficiency mutation from F1, had no detectable MYH10 in Western blots prepared with 30 μg of platelet protein/lane, and analyses of larger amounts of platelet protein (i.e., 60 or 90 μg of total platelet protein/lane) revealed only faint bands that were similar to those detected in analysis of the same amount of total protein/lane for control pooled lysate (Figure 8A). The negative findings for F5.1 in Western blots of MYH10 were confirmed by testing a second sample donated by this participant (Figure 8B). Her affected son declined to donate samples for MYH10 analyses.

MYH10 was undetectable by densitometry in the remaining participants with uncharacterized PFD of unknown molecular cause (Figures 6B & 9A-B) and 20 general population controls (Figure 10A-D).

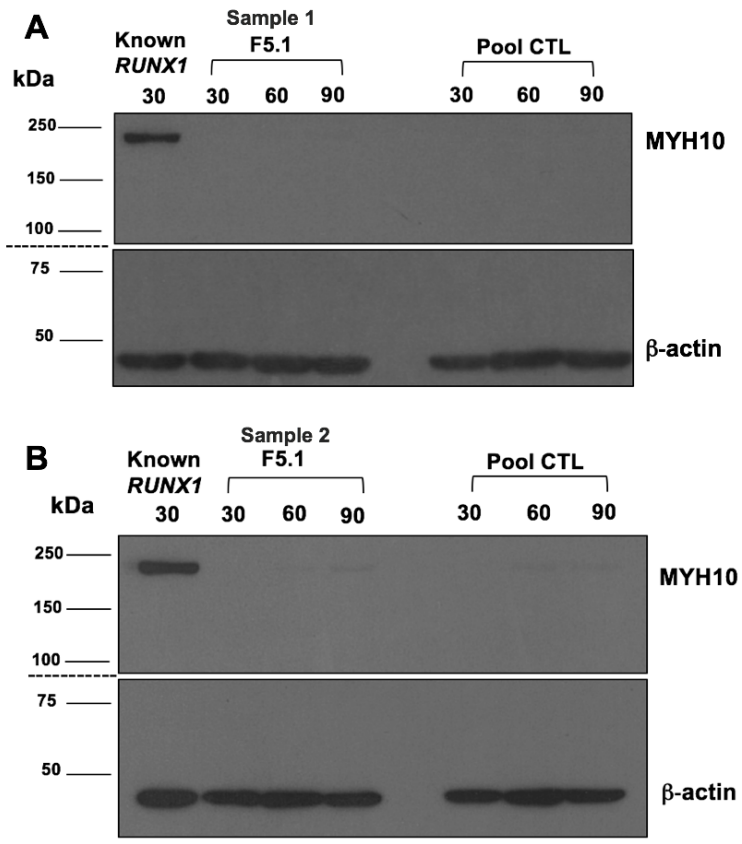

Figure 8. Western blot findings for platelet MYH10 in participant 5.1 with a pathogenic *RUNX1* mutation. A&B) Western blot findings are shown for the index case from a family (F5.1) with a platelet function disorder (PFD) due to a *RUNX1* haploinsufficiency mutation; the amount of platelet protein/lane (in μg) is indicated for two unique samples from F5.1 (indicated by sample number). In both panels, the pooled platelet lysate from n=5 participants from family 1 (F1) who have a known *RUNX1* haploinsufficiency mutation (Known *RUNX1*) and n=20 general population controls (Pool CTL) were used as positive and negative controls, respectively. The lower half of the membrane was probed for β-actin as a loading control.

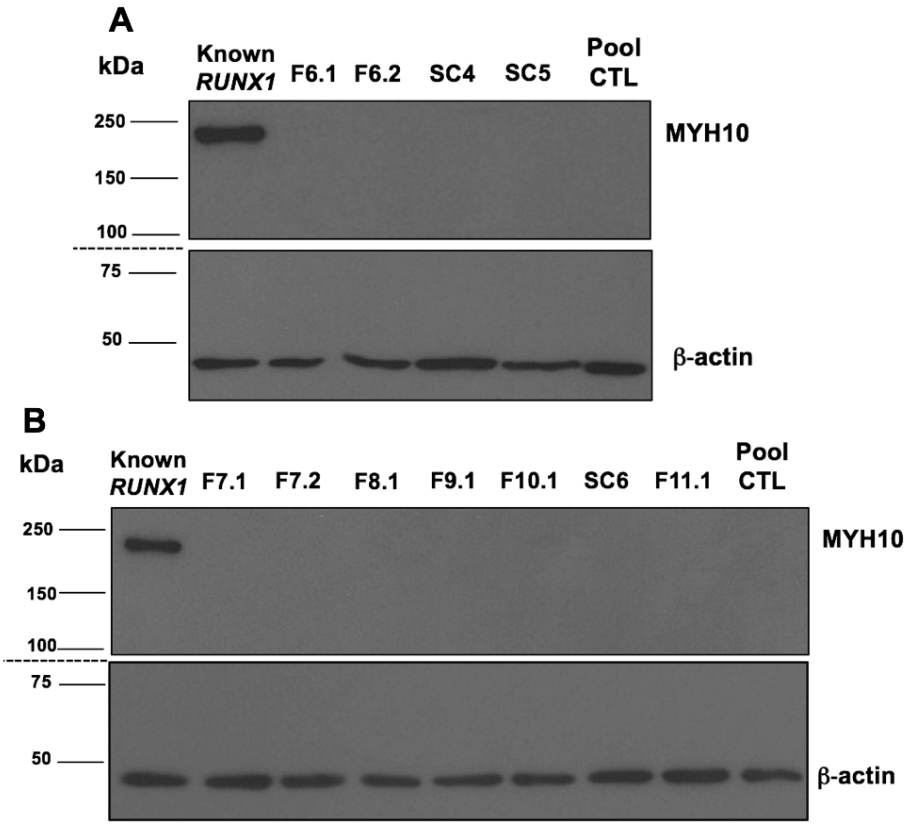

Figure 9. Western blot findings for platelet MYH10 in participants with uncharacterized platelet function disorder of unknown molecular cause. A&B) Western blot findings are shown for platelet MYH10 in n=11 participants with uncharacterized platelet function disorders (PFD) of unknown molecular cause after exome sequencing. In both panels, the pooled platelet lysate from n=5 participants from family 1 (F1) who have a known *RUNX1* haploinsufficiency mutation (Known *RUNX1*) and n=20 general population controls (Pool CTL) were used as positive and negative controls, respectively. β-actin was probed in the lower half of the gel as a loading control.

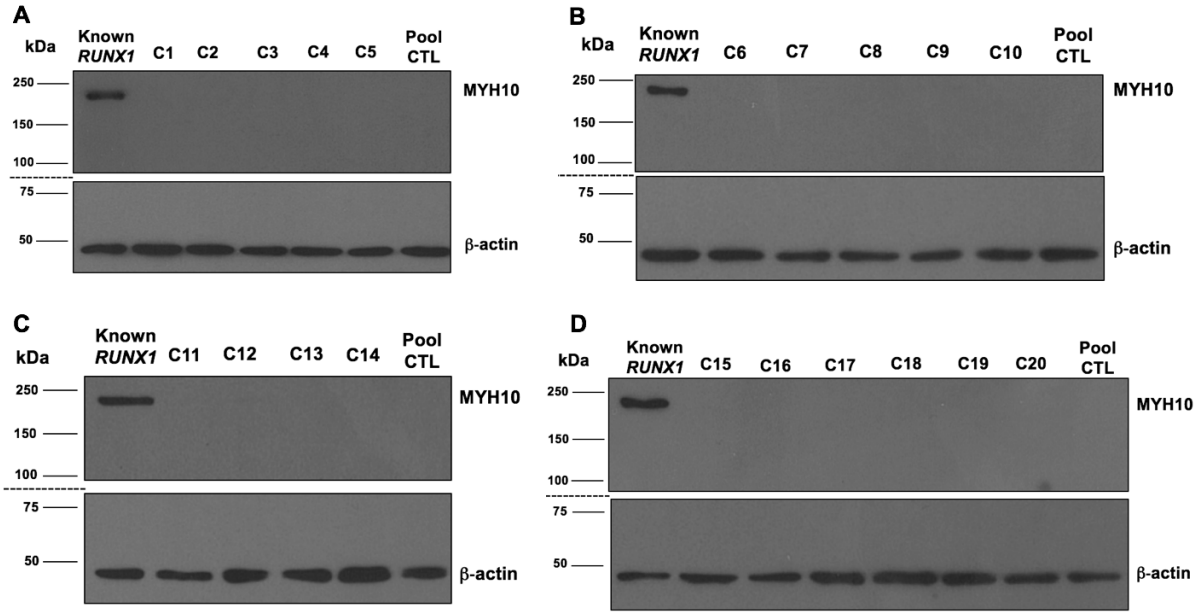

Figure 10. Western blot findings for platelet MYH10 in general population controls. A-D) Western blot findings are shown for platelet MYH10 in n=20 general population controls. In both panels, the pooled platelet lysate from n=5 participants from family 1 (F1) who have a known *RUNX1* haploinsufficiency mutation (Known *RUNX1*) and pooled platelet lysate, prepared from n=20 general population controls (Pool CTL), were used as positive and negative controls, respectively. β-actin was probed in the lower half of the gel as a loading control.

3.2.3 *MYH10* transcript levels in participants with uncharacterized platelet function disorders:

Compared to general population controls, *MYH10*/*PPIA* transcript ratios were significantly higher for PFD participants with a pathogenic *RUNX1* mutation unless the outlier with with the highest value was excluded (reported as median [range] ratio multiplied by 1000: controls: 0.16 [0.04-1.1]; *RUNX1*: 0.62 [0.13-4.3], p=0.008; *RUNX1* with outlier excluded: 0.45 [0.13-1.9], p=0.10). The findings for the other PFD were not significantly different from the controls (data as median [range] ratio multiplied by 1000: 0.30 [0.01-1.2], p=0.22) (Figure 11).

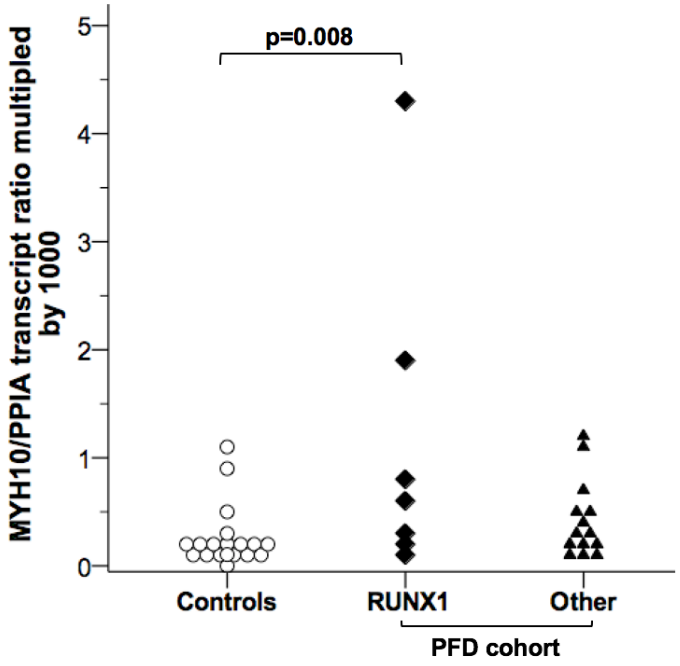

Figure 11. *MYH10* transcript findings for participants with an uncharacterized platelet function disorder. Transcript ratios (described as concentration of *MYH10* transcripts divided by *PPIA* transcripts multiplied by 1000) evaluated by digital PCR are compared for participants with: PFD and *RUNX1* mutation (n=7, closed diamonds), PFD of unknown molecular cause (n=14, closed triangles), and general population controls (n=20, open circles). p-values indicate significant differences without exclusion of the outlier in the *RUNX1* subgroup.

3.3 PLATELET PROCOAGULANT FUNCTION IN PARTICIPANTS WITH PLATELET FUNCTION DISORDERS

For this section of the thesis, I evaluated: 1) platelet-independent and -dependent thrombin generation by CAT, and 2) FV antigen levels (in platelets and plasma) by ELISA for general population controls, persons with QPD, and participants from the HPD study cohort, which included a subgroup with pathogenic *RUNX1* mutations. I also measured platelet uPA by ELISA for the participants with QPD to evaluate for potential associations between their platelet FV and uPA levels, and between their platelet FV levels and TGA endpoints. I procured additional research sample donations from controls and PFD participants (including QPD participants from Montreal) with the help of Catherine Hayward, Georges Rivard, and Subia Tasneem. I prepared platelet lysates and plasmas for testing, performed TGA and ELISA, and analyzed the data. The findings from this aim were presented at the 2018 American Society of Hematology Annual Meeting.

3.3.1 Demographics of participants evaluated for impaired thrombin generation and factor V levels:

Samples for TGA and platelet lysates were provided by 18 affected participants from the HPD study cohort (11 index cases; 4 single cases, 14 from 9 families with ≥2 participants, which included 5 from 2 families with a pathogenic *RUNX1* mutation; number with PFD/family evaluated as median, range: 1, 1-4; 61% females). Their median age (range) was 44 (20-72) years. The majority of affected PFD participants had impaired

MA without DGD (10/18, 56%), whereas the others had DGD and impaired MA (6/18, 33%) or only DGD (2/18, 11%).

Eighteen age- and gender-matched general population controls (median age in years, range: 42, 22-68; female: 61%) were tested, along with 5 QPD participants from Hamilton and Montreal (median ages in years, range: 57, 37-78; all available donors were male).

3.3.2 Thrombin generation findings in platelet-poor plasma:

There were no significant differences in the TGA endpoints for PPP samples from participants from the HPD study cohort (with or without a pathogenic *RUNX1* mutation) or QPD compared to controls (ETP: p-values ≥ 0.3; peak thrombin concentration: p-values ≥ 0.10; time-to-peak: p-values ≥ 0.10; lag time: p-values ≥ 0.15) (Figure 12A-D and Table 2). There were no significant associations between age and TGA endpoints for the controls ($R^2 \leq 0.06$, p values ≥ 0.16), participants from the HPD study cohort ($R^2 \leq 0.03$, p values ≥ 0.50), or QPD participants ($R^2 \leq 0.04$, p values ≥ 0.25). Figure 13 depicts a representative thrombin generation curve for PPP for a general population control.

Table 2. Thrombin generation endpoints when tested in platelet-poor plasma. Data are expressed as: median, [range], p-value when compared to controls.

	Controls	**PFD with *RUNX1* mutation**	**PFD with no causative mutation**	**QPD**
ETP (nM·min)	2020 [1530-2830]	1590 [1160-2420] p=0.30	1850 [1120-2720] p=0.40	2080 [1620-2160] p=0.82
Peak thrombin concentration (nM)	317 [213-370]	267 [186-315] p=0.10	267 [149-404] p=0.10	287 [264-334] p=0.39
Time-to-peak (min)	7 [6-12]	7 [6-8] p=0.84	8 [6-11] p=0.10	7 [7-10] p=0.52
Lag time (min)	4 [3-9]	4 [3-4] p=0.71	4 [3-6] p=0.15	4 [3-6] p=0.58

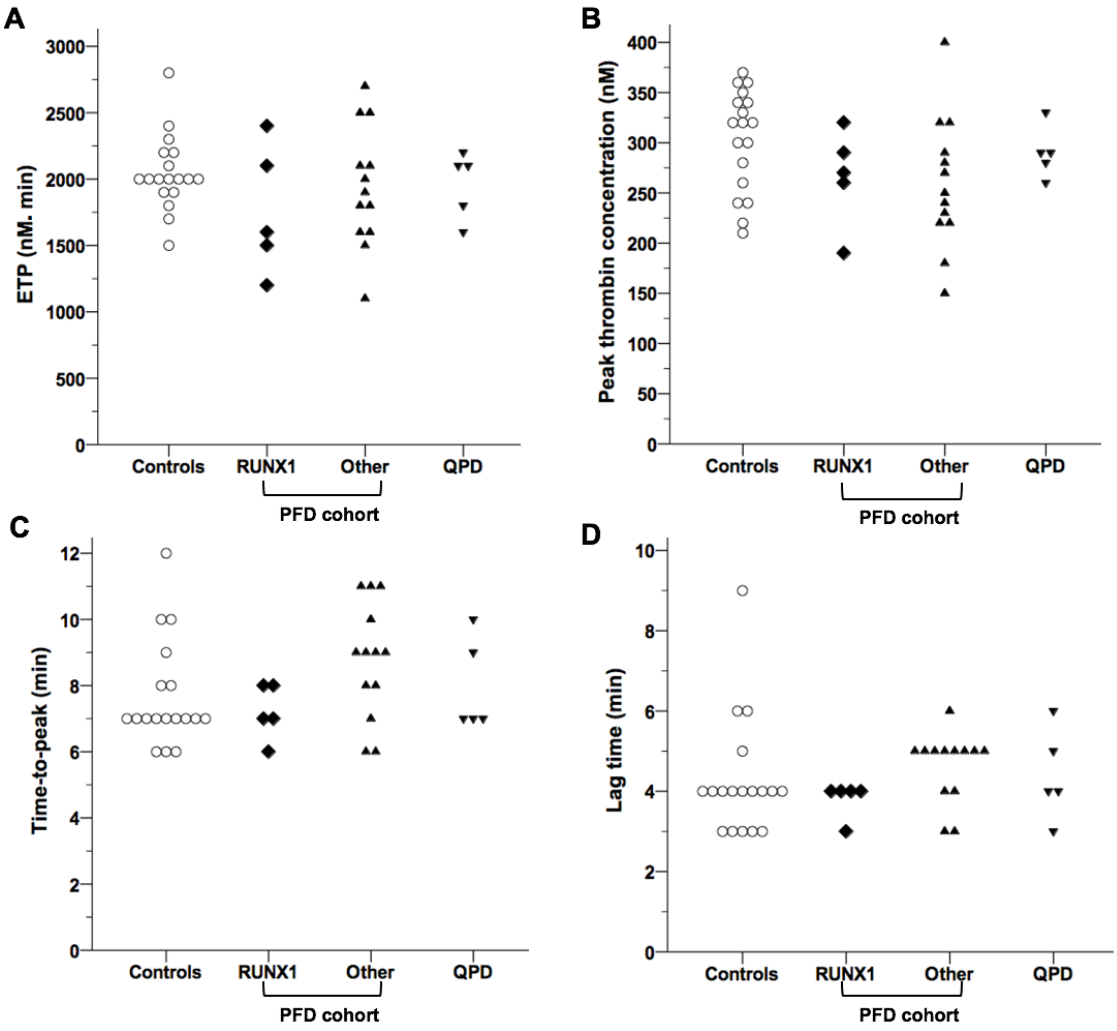

Figure 12. Thrombin generation endpoints when tested in platelet-poor plasma for participants with platelet function disorders. (A) Endogenous thrombin potential (ETP, nM·min), (B) peak thrombin concentration (nM), (C) time-to-peak (min), and (D) lag time (min) are compared in platelet-poor plasma (PPP) for: general population controls (n=18, open circles); participants from the Hamilton PFD cohort with either a pathogenic *RUNX1* (n=5, closed diamonds) or an unknown causative mutation (n=13, closed upright triangles); and participants with QPD (n=5, closed inverted triangles).

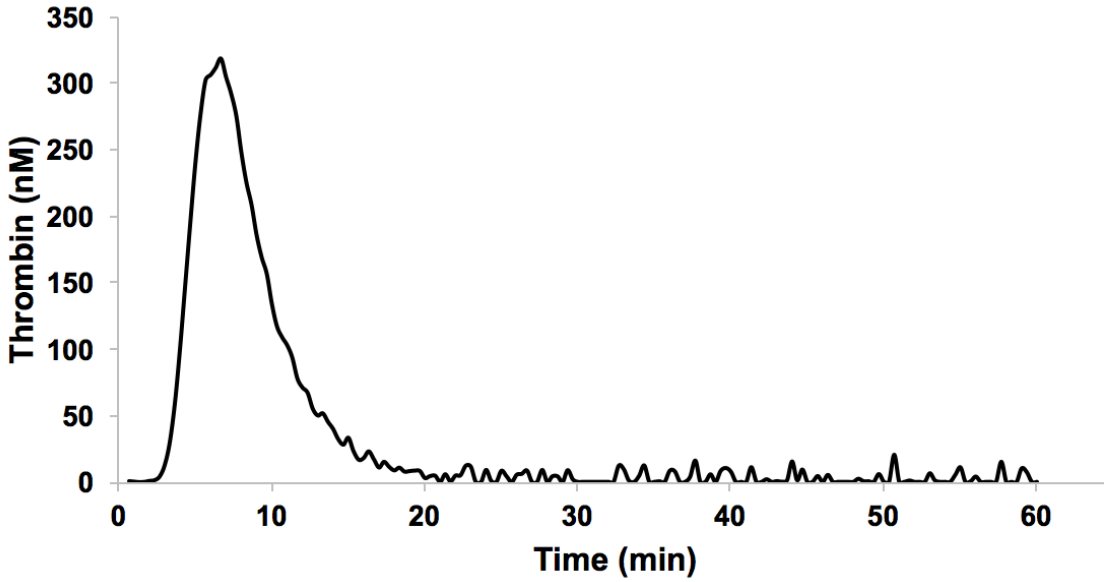

Figure 13. Representative thrombin generation curve in platelet-poor plasma. A curve from a representative general population control depicting the generation of thrombin over time in platelet-poor plasma (PPP) when triggered with 5 pM tissue factor and 4 μM phospholipids.

3.3.3 Thrombin generation findings for platelet-rich plasma:

Participants from the HPD study cohort without a known causative mutation had similar TGA endpoints compared to controls when tested in PRP (ETP: p=0.72; peak thrombin concentration: p=0.04, insignificant after Bonferroni correction; time-to-peak: p=0.11; lag time: p=0.76). However, participants from the HPD study cohort with a pathogenic *RUNX1* mutation or with QPD had reduced ETP and peak thrombin concentration, but similar time-to-peak and lag times compared to the controls (data as median [range]: ETP: controls: 1860 [1530-2630]; *RUNX1* mutation subgroup: 1520 [805-1640], p=0.004; QPD: 1370 [981-2010], p=0.01; peak thrombin concentration: controls: 111 [65-152]; *RUNX1* mutation subgroup: 66 [32-93], p=0.006; QPD: 59 [41-91], p=0.005; time-to-peak: p-values $\geq$ 0.17; lag time: p-values $\geq$ 0.23) (Figure 14 A-D and Table 3). Figure 15 depicts representative thrombin generation curves when tested in PRP for a general population control, PFD participant with a pathogenic *RUNX1* mutation, and participant with QPD. All curves showed single peaks.

Table 3. Thrombin generation endpoints when tested in platelet-rich plasma. Data is expressed as: median, [range], p-value when compared to controls. All PRP samples tested were adjusted to a platelet count of 150 X 10^9/L with autologous PPP.

	Controls	**PFD with *RUNX1* mutation**	**PFD with no causative mutation**	**QPD**
ETP (nM·min)	1860 [1530-2630]	1520 [805-1640] p=0.004	1630 [1400-2540] p=0.72	1370 [981-2010] p=0.01
Peak thrombin concentration (nM)	111 [65-152]	66 [32-93] p=0.006	75 [57-169] p=0.04	59 [41-91] p=0.005
Time-to-peak (min)	25 [18-34]	19 [16-36] p=0.28	28 [25-40] p=0.11	32 [23-34] p=0.17
Lag time (min)	12 [7-21]	10 [9-17] p=0.23	10 [10-23] p=0.76	15 [10-17] p=0.27

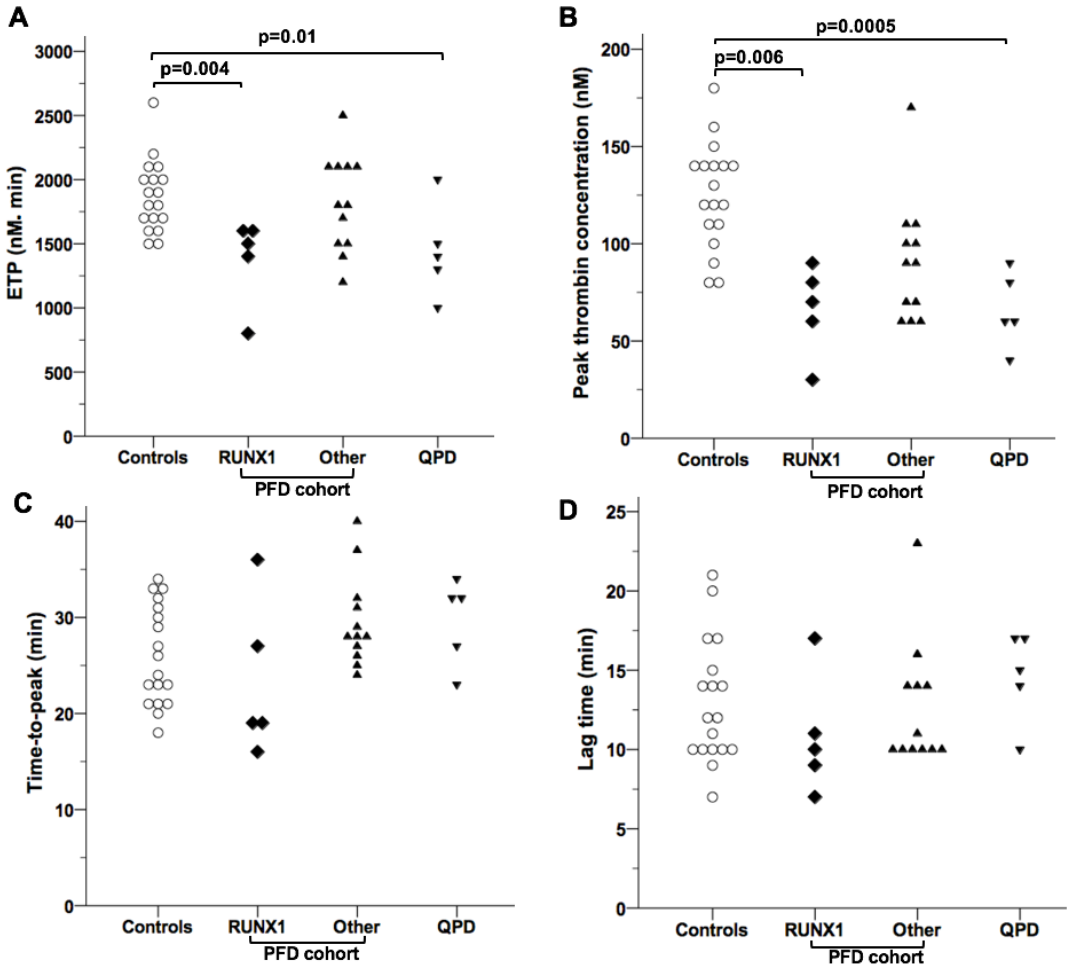

Figure 14. Thrombin generation endpoints when tested in platelet-rich plasma for participants with platelet function disorders. All PRP samples tested were adjusted to a platelet count of 150 X 10^9/L with PPP. (A) Endogenous thrombin potential (ETP, nM·min), (B) peak thrombin concentration (nM), (C) time-to-peak (min), and (D) lag time (min) are compared in PRP (platelet count: 150 x 10^9/L) for: general population controls (n=18, open circles); participants from the Hamilton PFD cohort with either a pathogenic *RUNX1* (n=5, closed diamonds) or an unknown causative mutation (n=13, closed upright triangles); and participants with QPD (n=5, closed inverted triangles). Significant p-values after Bonferroni correction are indicated.

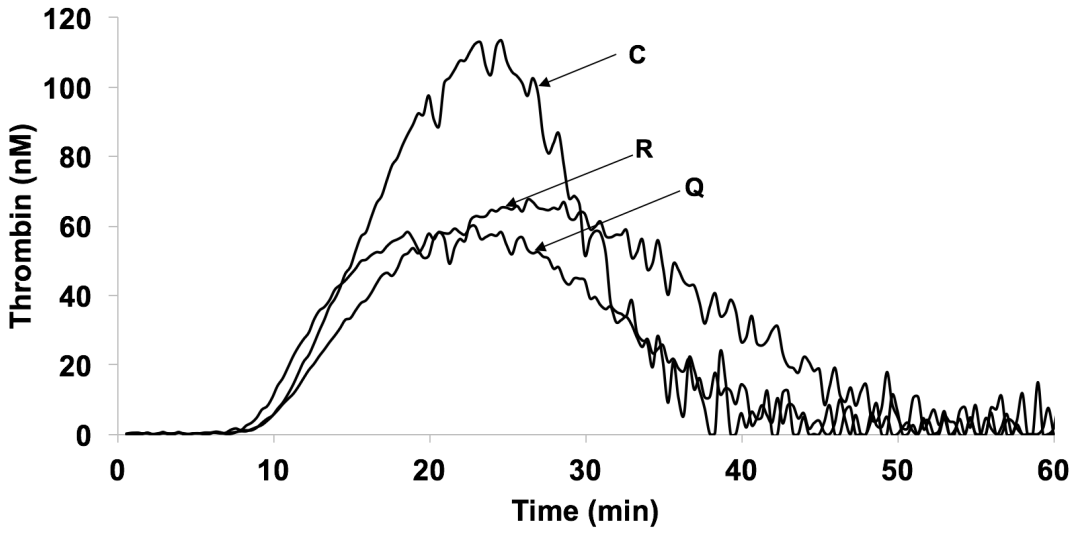

Figure 15. Representative thrombin generation curves in platelet-rich plasma. Representative curves depicting the generation of thrombin over time when tested in platelet-rich plasma (PRP, platelet count: 150 x 10^9/L) triggered with 0.5 pM tissue factor, from a general population control (C), PFD participant with a pathogenic *RUNX1* mutation (R), and QPD participant (Q).

3.3.4 Factor V antigen levels for participants with uncharacterized platelet function disorders and Quebec platelet disorder:

Plasma FV antigen levels were similar in all participants (μg/ml PPP, median [range]: controls: 7.7 [6.3-10.7]; QPD: 8.4 [7.0-10.2], p=0.33; *RUNX1* mutation subgroup: 7.8 [6.5-8.1], p=0.83; other PFD: 7.5 [5.4-12], p=0.74) (Figure 16A) and showed no associations to TGA endpoints for PPP or PRP samples (p values ≥ 0.14). Platelet FV antigen levels were reduced in QPD participants, but were normal in all other participants (μg FV/mg platelet protein, median [range]: controls: 0.89 [0.63-1.54]; QPD: 0.35 [0.18-0.46], p<0.001; other PFD: 0.83 [0.47-1.75], p=0.48; *RUNX1* mutation subgroup: 0.73 [0.50-1.53], p=0.41) (Figure 16B). In QPD, but not other participants, platelet FV antigen levels showed significant associations to the ETP (R^2=0.81, p=0.04) and peak thrombin concentrations (R^2=0.88, p=0.01) for PRP samples (Figure 17A-B).

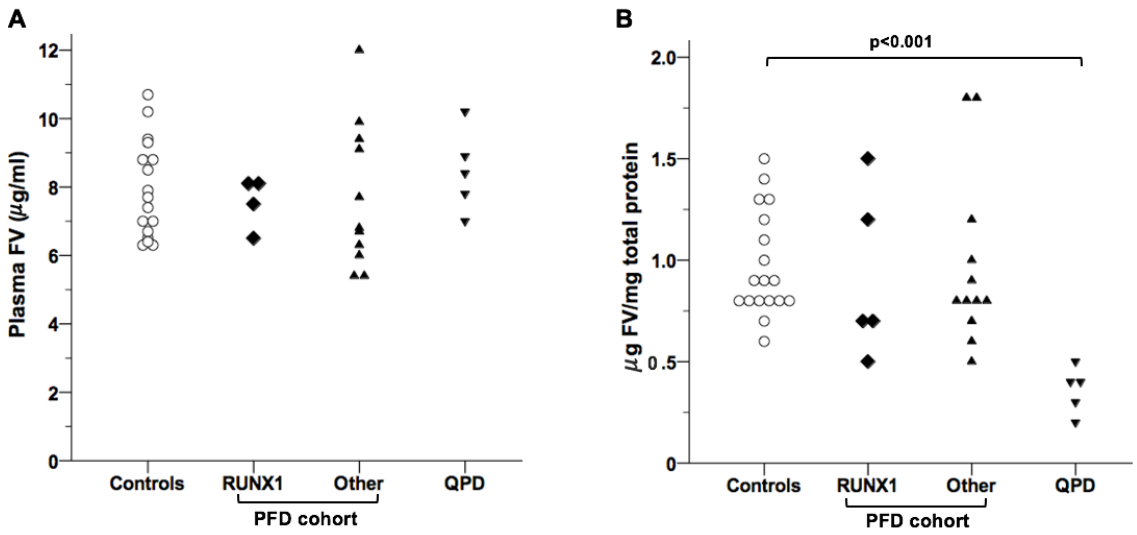

Figure 16. Factor V antigen levels in participants with platelet function disorders. A) Plasma FV (µg/ml) and B) platelet FV (µg FV/mg total protein) antigen levels are compared for: general population controls (n=18, open circles); participants from the Hamilton PFD cohort with either a pathogenic *RUNX1* (n=5, closed diamonds) or an unknown causative mutation (n=13, closed upright triangles); and participants with QPD (n=5, closed inverted triangles). Significant p-values after Bonferroni correction are indicated.

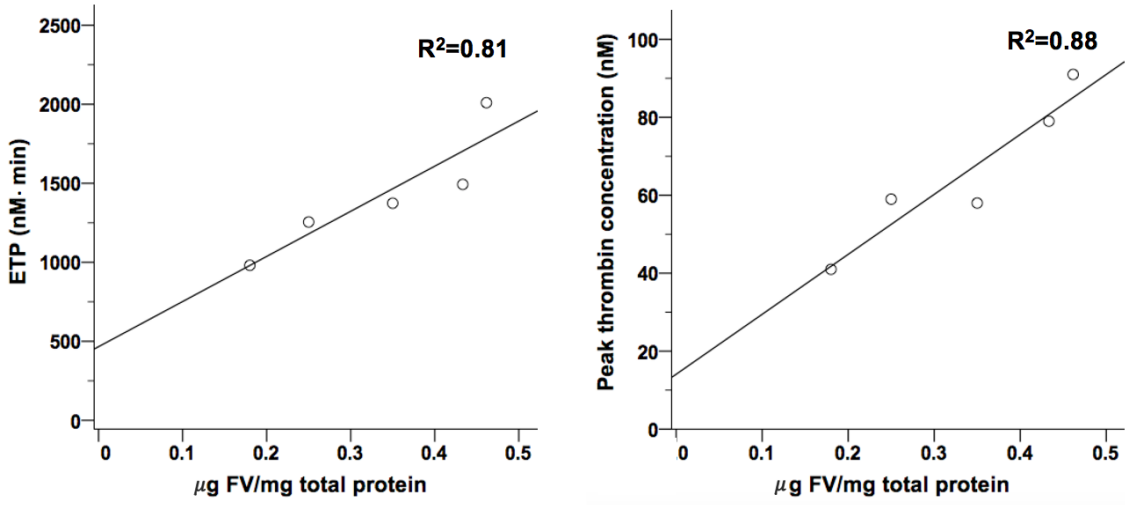

Figure 17. Relationships between platelet factor V antigen levels and thrombin generation endpoints when tested in platelet-rich plasma in participants with Quebec platelet disorder. Data for QPD participants (n=5) is shown and compares platelet FV antigen levels (μg FV/mg total protein) to (A) endogenous thrombin potential (ETP, nM·min) and (B) peak thrombin concentration (nM) when tested in platelet-rich plasma (PRP) (R^2 shown).

3.3.5 Platelet urokinase plasminogen activator levels for participants with Quebec platelet disorder:

Platelets from QPD participants contained >100 fold more uPA than control platelets (median ng uPA/mg total platelet protein [range]: QPD: 57 [24-110]; controls, n=6 tested: 0.15 [0.12-0.16], $p<0.0001$; one control that contained <0.01 ng uPA/mg cellular protein, and their lysate contained less uPA than the lowest ELISA standard [0.03 ng/ml]. In QPD participants, neither platelet FV antigen levels or TGA endpoints in PRP showed a significant association to platelet uPA levels (p values ≥ 0.11).

CHAPTER 4

DISCUSSION

4.1 SUMMARY OF THESIS WORK

This thesis aimed to extend the current knowledge on uncharacterized PFD by addressing some unanswered questions about the diagnostic assays used for evaluating some forms of PFD and by evaluating platelet procoagulant function in these disorders. We found that whole mount EM is a useful test with acceptable within-subject variability for evaluating PFD due to DGD. As LTA and ATP release tests did not have high adequate sensitivity for detecting impaired platelet function due to DGD, and DGD has a similar prevalence to VWD, we suggest that testing for DGD should be considered regardless of LTA and ATP release findings. Additionally, we found that evaluating MYH10 transcript and protein levels, to help identify PFD due to transcription factor mutations, did not always yield the expected findings since we observed consistently normal MYH10 protein findings for a participant with PFD due to a *RUNX1* haploinsufficiency mutation and noted variable findings amongst affected members of a family with an undefined PFD and consistently abnormal LTA findings. Lastly, we found impaired platelet-dependent thrombin generation in several forms of PFD, namely QPD and PFD associated with pathogenic *RUNX1* mutations. We observed that the platelet FV stores helped to distinguish between these disorders as platelet FV levels were only reduced in QPD participants. The next sections discuss the key findings and implications for future work with this cohort of individuals with uncharacterized PFD.

4.2 EVALUATION OF DENSE GRANULE DEFICIENCY BY WHOLE MOUNT ELECTRON MICROSCOPY

4.2.1 Summary of key findings:

The main goal of the aim of this thesis was to evaluate HRLMP's experiences with using platelet whole mount EM to diagnose DGD over a twelve-year period, including the findings for an overlapping cohort whose bleeding symptoms, DG counts, platelet function and MPV were assessed for a study on uncharacterized PFD. We evaluated typical findings, including: the lower limit of the assay RI by gender; age differences in findings; within-subject variability in DG numbers/platelet; and relationships between confirmed DGD, bleeding symptoms, and the presence of a bleeding disorder.

Analysis of Cohort I data indicated that there were no significant age differences in the average numbers of DG/platelet. The lower limit of the RI was 4.9 DG/platelet for both genders. DGD was present in 6.5% of patient samples tested between 2006-2017 (excluding donors exclusively used for NASCOLA EQA samples), and in a higher proportion (34%) of individuals from the Hamilton PFD study that had confirmed DGD as an inclusion criterion. Like previous studies (47, 60, 68), we observed that the majority of patients referred for PFD testing were female, likely due to their increased burden of hemostatic challenges from menstruation and childbirth. Importantly, the within-subject CV was acceptable at 12% for control samples with normal DG counts. The respective sensitivities of LTA and DG ATP release (estimated by lumi-aggregometry), for detecting abnormalities due to DGD, were only ~52% and 70%, which is inadequate to

screen for DGD. Although impaired DG ATP release with multiple agonists was present in most participants with DGD, some had normal findings. Additionally, the diagnostic usefulness of the assessment of DG ATP release has been questioned as the findings show considerable variability, and even if ATP release is consistently impaired, the finding does not show a significant relationship to elevated bleeding scores or the clinical diagnosis of a bleeding disorder. (60) Participants with DGD by whole mount EM had a high likelihood of having a bleeding disorder (OR=97) and increased bleeding, reflected by higher ISTH BAT scores than unaffected relatives and general population controls. Given our major findings, we recommend that specific testing for DGD, by a validated method (such as whole mount EM) that has an acceptable, within-subject variability, should be considered when testing for a suspected PFD, even if LTA and ATP release findings are non-diagnostic or inconsistent.

4.2.2 Variability of whole mount electron microscopy test for dense granule deficiency:

The variability of the whole mount EM test for DGD was formally evaluated since tests with high CV (such as DG ATP release) perform poorly for diagnostic applications. (60, 160) Since the optimal number of platelets to assess DG counts is uncertain, and some laboratories assess more than 30 platelets (which is the number assessed by the HRLMP laboratory) (161), we re-evaluated 10 random samples to compare DG counts using 30 or 100 platelets and observed very similar means, ranges, and standard deviations. This suggests that counting DG in 30 platelets is an appropriate

amount to calculate the average number of DG/platelet. Additionally, an EM assessment for DGD using whole mount preparations had an acceptable within-subject CV (12% for samples with normal DG counts). Although the within-subject CV was higher (28%) for samples with significantly reduced DG counts, DGD was confirmed in all samples from persons with an initial count that was <4.0 DG/platelet. The observation that many mild reductions in DG numbers (i.e., 4.0-4.8 DG/platelet) were not confirmed when another sample was collected and tested, illustrates that it is important to verify reduced DG counts with another sample. Some of the mild reductions in DG counts that were observed could reflect false positives and also the biology of some PFD. For example, familial PFD due to *RUNX1* haploinsufficiency reduces the DG content of platelets, however, only ~50% of the affected family members in a family that our group studied had DG counts below the RI. (57) Our observations on the test reproducibility and within-subject CV are important, as whole mount EM is the most commonly used method for diagnosing platelet DGD in North America. (71) This aim reports an expert center's experience as two expert EM technologists performed the test during the period of data collection, using PRP prepared by the Special Coagulation Laboratory. While many centers that use EM to evaluate platelet DG cannot perform energy dispersive spectrometry (EDS) (72), our site had the technical capability during the initial assay validation, which was helpful to verify that the platelet structures counted as DG did indeed contain calcium and phosphorus. Nonetheless, EQA exercises have shown excellent agreement between laboratories on what electron dense structures in platelets should be counted as a DG. (71, 72)

4.2.3 Reference interval validation for diagnosing platelet dense granule deficiency:

An important analysis from this section of the thesis was the validation of the RI for DG counts used by the HRLMP. Differences in RI between laboratories have been noted for this test. It is possible that pre-analytical and analytical differences, and differences in the number of control samples used to determine RI, contribute to differences in RI between laboratories for whole mount EM DG counts. For example, a recent study that reported a lower limit cutoff (based on estimating the 95% CI) of 1.96 DG/platelet used single measures for 40 control samples from participants with a median age of 10 (range: 2 months to 20 years) (161) whereas we used data for all 615 samples from 126 unique adult controls and the method described by Taylor et al. that allows inclusion of all data when some subjects have multiple determinations and takes within-subject variability into account in the RI determination. (159) Unlike some centers, the HRLMP does not accept shipped samples for whole mount EM tests and grids are prepared using subsamples of PRP used for aggregation testing (completed within a few hours of sample collection) to limit the possibility of platelet activation and DG release *ex vivo*. The estimate of the average DG count/platelet calculated in this thesis is closer to the RI reported by several other groups, which were 4-6 and 4-8 DG/platelet. (45, 116) There were no differences in DG numbers/platelet between pediatric and adult Cohort I samples, nor was there any significant correlation between the participant age and DG numbers for the participants under 18 years of age. This suggests that the lower limit of the RI that was validated for adult samples (i.e., 4.9 DG/platelet for both males and females) is suitable for samples from children. Nonetheless, we would recommend to

retest all individuals with abnormal counts and to retest all younger children with abnormal DG counts at an older age. It is also recommended that each laboratory determine their own RI using samples from control participants, to control for differences in sample preparation and DG quantification between laboratories.

4.2.4 Other methods used to quantify platelet dense granules:

While there is no generally accepted "gold standard" for diagnosing DGD, this aim of the thesis illustrates that whole mount EM provides estimates of platelet DG counts with limited within-subject variability, with low values having important clinical associations. One limitation of whole mount EM (which is fairly simple to perform) is that it requires an expensive piece of equipment: a transmission electron microscope. Recently, super-resolution light microscopy (SRM) assessment of CD63-stained platelets was proposed as an alternative way to detect DGD, based on observations for known DGD individuals versus control subjects. (163, 164) It was found that SRM could successfully resolve CD63-positive structures in fixed platelets, and that this method was effective and rapid in objectively differentiating between patients with a platelet disorder and healthy controls. (163) A prospective study comparing SRM to whole mount EM for diagnosing PFD due to DGD, amongst patients referred for diagnostic PFD testing, would be the ideal way to establish if SRM is non-inferior or superior to whole mount EM for diagnosing DGD. However, this would be impractical as such a study would require multicenter recruitment to achieve sufficient power given how infrequently new cases of DGD are diagnosed, even at tertiary referral centers, like HRLMP.

4.2.5 Sensitivities of light transmission aggregometry and dense granule ATP release for detecting dense granule deficiency:

We analyzed LTA and lumi aggregometry findings for DGD participants from Cohorts I and II. Similar to previous studies (45, 165), we found that only 52% of DGD participants had reduced MA. Interestingly, all DGD participants had normal MA with both 2.5 and 5.0 μM ADP, suggesting that this agonist is not informative for detecting DGD. This finding is similar to reports by Weiss et al., where DGD patients had similar ADP-dose response curves to normal controls (166), and that ADP addition after platelet stimulation corrected intracellular calcium levels and prothrombinase activity in HPS platelets. (167) This suggests that addition of ADP may compensate for the decreased amounts of ADP stored in DG in DGD patients, resulting in normal aggregation findings in response to the agonist at both concentrations. In addition, only half of the DGD participants showed reduced ATP release with all agonists, while 30% had non-diagnostic findings. This is counter-intuitive as reduced DG numbers should be associated with reduced release of DG contents. Although impaired MA to multiple agonists is predictive of a bleeding disorder (47, 68), it has been found that the assessment of DG ATP release does not show a significant relationship to elevated bleeding scores or the clinical diagnosis of a bleeding disorder. (60) Accordingly, it is recommended to assess for DGD by whole mount EM when evaluating patients for a PFD, even if LTA and ATP release findings are non-diagnostic or inconsistent.

4.2.6 Dense granules and their relationship to platelet function disorders:

As outlined previously, DG are important for normal platelet function, as they store and secrete ADP, ATP, calcium, magnesium, polyphosphates and bioactive amines (14, 15), with DGD leading to a PFD. DGD can occur as a part of a syndrome, such as HPS, or in isolation (like the participants in Cohort II). (12) The causes of syndromic DGD are more clear-cut, where numerous genes have been identified, like the *HPS* group, that encode for proteins involved in DG biogenesis and maturation. (12, 110) However, there are patients with non-syndromic DGD who do not present with HPS or other related disorders, like those found in Cohort II. Some of the DGD participants in HPD study cohort have a pathogenic mutation in *RUNX1*, which affects multiple aspects of platelet function, including reduced DG numbers and DG ATP release. (57, 90, 133) Interestingly, one study has found that RUNX1 is a direct transcriptional target of *PLDN,* which encodes for pallidin, an important protein involved in DG biogenesis. (150) Whole exome sequencing has been completed by the Pare laboratory for the remaining participants with DGD in the HPD study cohort but has not identified any potential molecular causes of PFD. However, it is possible that some participants have mutations in other non-explored genes that encode for transcription factors or other elements that regulate DG biogenesis.

4.3. EVALUATING FOR ABERRANT PERSISTENCE OF PLATELET MYH10 IN PARTICIPANTS WITH UNCHARACTERIZED PLATELET FUNCTION DISORDERS

4.3.1 Summary of key findings:

The main goal of the second aim of this thesis was to evaluate the aberrant persistence of platelet MYH10 transcript and protein by dPCR and Western blotting, respectively, in participants from the HPD study cohort with an uncharacterized PFD due to impaired MA with ≥2 agonists and/or DGD, including a subgroup with pathogenic *RUNX1* mutations.

As recommended by a recent study (92) which was included in recent ISTH guidelines for diagnosing PFD (50), we performed Western blot analyses using 30 μg of platelet protein/lane from 26 participants from the HPD study cohort. This revealed increased MYH10 protein in 6/7 evaluated participants with pathogenic *RUNX1* mutations, which included five from F1 with a *RUNX1* haploinsufficiency mutation whose data was previously published (57), and a single case (SC1) with a recently identified *RUNX1* missense mutation. We also found increased MYH10 protein in two of the four affected members from F4 who have a PFD of unknown molecular cause, which indicates that the findings are not always consistent within a family with a PFD that impairs platelet aggregation responses to multiple agonists. A concerning observation of our study was that the MYH10 protein levels were normal for: an index case (F5.1) with PFD due to a *RUNX1* haploinsufficiency mutation. The normal findings in the remaining 15 participants with PFD of unknown molecular cause and in all general population

control participants were anticipated. dPCR analyses of platelet *MYH10* transcript levels were largely non-informative, as 5/7 evaluated participants with a *RUNX1* haploinsufficiency mutation had transcript levels within the range seen for general population controls. These observations indicate that caution is warranted before applying Western blot or molecular analysis of platelet MYH10 to the routine diagnostic workup of PFD. We suggest that the test should only be used to describe the phenotype of an individual or family with a PFD given that it was not abnormal in all participants with a PFD in association with a pathogenic *RUNX1* mutation.

4.3.2 Normal MYH10 protein levels in a participant with a pathogenic *RUNX1* mutation:

MYH10 is a cytoskeletal protein that is normally downregulated during megakaryopoiesis by transcription factors, like *RUNX1* and *FLI1*, and has been suggested as a potential biomarker for PFD associated with these mutations. (18, 92, 168) However, the total number of cases reported in the literature who were evaluated for MYH10 levels by Western blotting, and quantitative analysis of platelet mRNA, is limited. By Western blotting, we found increased platelet MYH10 protein in affected members from F1 with a *RUNX1* haploinsufficiency mutation and in a single case (SC1) with a *RUNX1* missense mutation. However, we found consistently negative findings in F5.1, who has a PFD due to a pathogenic *RUNX1* mutation (predictive of haploinsufficiency), which contradicts previously published conclusions by Antony-Debré et al. (92) In that study, MYH10 was present in platelets of all patients with *RUNX1* mutations. (92) While there are no other

studies that have reported any cases with *RUNX1* mutations without increased platelet MYH10, the total number of cases reported is small and insufficient to estimate the test sensitivity. Since 30 μg of platelet protein may not have been sensitive enough to detect increased MYH10 in F5.1, we also used increasing amounts of platelet protein loaded per lane by two- and three-fold but, again, noted that F5.1 had MYH10 levels very similar to the pooled platelet lysate from 20 general population controls. The assay was also performed on platelet protein from an additional, unique sample from F5.1 to exclude a preanalytical error and we, again, noted that F5.1 had MYH10 levels similar to the normal pool. It would be interesting to assess MYH10 protein levels in F5.1's affected son (who has the same *RUNX1* frameshift mutation but declined to provide additional samples). Overall, these findings suggest that caution is warranted when evaluating platelet MYH10 by Western blot as it may not have sufficient sensitivity to detect all pathogenic *RUNX1* mutations.

4.3.3 Inconsistent MYH10 findings in a family with a platelet function disorder of unknown molecular cause:

While the majority of affected participants with a PFD of unknown molecular cause had negative platelet MYH10 findings, we found increased MYH10 levels in two of the four affected members from F4, who have a PFD with impaired MA and normal DG numbers in whom the Pare laboratory did not find a molecular cause by whole exome sequencing. The negative MYH10 findings for F4.1 and F4.3 were confirmed by testing larger amounts of platelet protein, and additional, unique samples to exclude a

preanalytical or analytical error. Antony-Debré et al. found increased platelet MYH10 in some patients without *RUNX1* or *FLI1* mutations (92), which would be consistent with our findings for F4.2 and F4.4, however, no families have been reported with a PFD and variable MYH10 findings by Western blot analysis of affecteds. The inconsistent findings amongst affected members of F4 raises questions about the usefulness of evaluating MYH10 protein levels in platelets as a diagnostic test or phenotypic criteria, since all affecteds of this family, who have a similar phenotype by aggregation and DG ATP release tests, did not show the same MYH10 protein findings. The sensitivity and specificity of the test deserves further evaluation. Caution is warranted in using this test as part of the diagnostic evaluation of a PFD.

4.4.4 Molecular findings for platelet MYH10 in participants with a platelet function disorder:

We also measured platelet *MYH10* transcript levels by dPCR, a technique that is able to detect genes with relatively low expression. (169) dPCR analyses found low *MYH10* expression in all participants relative to the endogenous control, and 5/7 evaluated participants with *RUNX1* haploinsufficiency had transcript levels within the range seen for general population control participants. This large overlap in molecular findings between *RUNX1* participants and controls suggests that dPCR would not be a useful test for diagnosing PFD associated with pathogenic *RUNX1* mutations.

Our molecular data contradicts our MYH10 protein findings, since 6/7 participants with *RUNX1* mutations showed increased protein levels, but only 2/7 had

increased transcript levels. mRNA are synthesized, processed and translated into amino acid chains, and subsequently folded into functional proteins, with mRNA and protein having different half lives. Most reports comparing mRNA and protein levels find weak correlations. (170) There are many biological factors that may have created discrepancies in the molecular and protein findings, such as RNA structure, the presence of regulatory proteins, and protein stability. (170) We were not able to evaluate associations between mRNA transcript and protein levels in our findings since Western blotting only provided semi-quantitative results. A more quantitative approach for measuring protein levels, like ELISA, is ideal. However, MYH10 is found in the platelet cytoskeleton and the protein can only be made accessible to antibody binding by SDS solubilization. Since SDS denatures proteins and ELISA best measures antigens in their non-denatured state, measuring platelet MYH10 protein levels using SDS-PAGE and Western blotting with densitometry, and comparisons to pooled platelet lysates, is currently the best quantitative approach. (171)

4.4.5 Effect of aberrant persistence of platelet MYH10 on platelet function:

In FPD/AML patients with *RUNX1* mutations, there are defects in MK differentiation and polyploidization, and proplatelet formation. (168) During MK polyploidization, MYH10 expression is repressed by RUNX1. (18) MYH10 down-regulation is necessary for the switch from mitosis to endomitosis, since re-expression decreases MK ploidy. (18) The exact mechanism of the role MYH10 plays in this switch, and how increased MYH10 expression is linked with impaired platelet function, is still

unknown. It is possible that impairments in proplatelet formation, and MK maturation and polyploidization, lead to reduced platelet numbers and platelet intracellular contents. However, not all participants from the HPD study cohort with increased MYH10 protein levels have similar platelet phenotypes (3/8 with thrombocytopenia and 2/8 with confirmed DGD). It is possible that the participants with increased MYH10 protein levels have similar phenotypes in other aspects of MK polyploidization that were not assessed in this thesis. Regardless, the inconsistencies found in this section of the thesis suggests that the role of increased MYH10 in impaired platelet function requires further study.

4.4 EVALUATING PLATELET PROCOAGULANT CAPACITIES IN PARTICIPANTS WITH PLATELET FUNCTION DISORDERS

4.4.1 Summary of key findings:

The main goals of the third aim of this thesis were to evaluate: platelet-independent and -dependent thrombin generation by CAT, with accompanying analyses of FV antigen levels in platelets and plasma by ELISA, for participants from the HPD study cohort with uncharacterized PFD with impaired MA with ≥2 agonists and/or DGD (including a subgroup with a pathogenic *RUNX1* mutation), and for QPD. As anticipated, there were no significant differences in the TGA endpoints for PPP samples from participants with QPD or an uncharacterized PFD (with or without a pathogenic *RUNX1* mutation) compared to controls. In tests with PRP, samples from participants with PFD of unknown molecular cause showed normal thrombin generation whereas those with a pathogenic *RUNX1* mutation or QPD showed a reduction in both ETP and peak thrombin concentration compared to the controls. Plasma FV antigen levels were normal in all participants and, as previously reported (107), platelet FV antigen levels were reduced in QPD participants. All other PFD participants had normal platelet FV antigen levels. We also evaluated for associations between platelet and plasma FV antigen levels and TGA endpoints for PPP and PRP. In QPD, platelet FV antigen levels, but not uPA levels, correlated with ETP and peak thrombin concentration in PRP TGA, even though their plasma FV antigen levels were normal. In QPD platelets, uPA levels were >100 fold higher than controls, as previously reported. (65, 109)

4.4.2 Normal platelet-dependent thrombin generation in participants with uncharacterized platelet function disorders of unknown molecular cause:

There were no significant differences between controls and PFD participants from the HPD study cohort without *RUNX1* mutations or QPD in any TGA endpoint for PPP samples, which was expected since all participants in the HPD study cohort had normal findings on coagulation screening tests. This finding is similar to reports from two previous studies which assessed thrombin generation for PPP samples in: individuals with bleeding symptoms of unknown origin (172) and individuals with platelet aggregation and/or secretion defects. (173) Interestingly, we found no significant differences in TGA endpoints for PRP samples from PFD participants in the HPD study cohort without *RUNX1* mutations or QPD, compared to controls. While we grouped participants with uncharacterized PFD of unknown molecular cause together, we recognize that there is heterogeneity in these disorders. We also recognize that some of the analyses for PRP samples may be underpowered, such as peak thrombin concentration (p-value = 0.04 before Bonferroni correction). It is also possible that other subgroups of PFD have mild impairments in platelet procoagulant function. The recruitment and analysis of additional participants with PFD of unknown molecular cause is needed, as it may detect a significant decrease in peak thrombin concentration in some subjects compared to controls.

Recent ISTH guidelines suggest the assessment of platelet procoagulant defects to detect some forms of PFD, such as Scott's syndrome, a rare disorder caused by a defect in PS exposure on activated platelets. (50, 126) Thrombin generation in PRP has not been

evaluated by CAT, and compared to controls, in individuals with phenotypes similar to those in the HPD study cohort. Thrombin generation for PRP samples has been evaluated by CAT in other rarer forms of PFD, with normal findings in patients with MYH9-related platelet disorders (174), and impairments in GT (142) and BSS. (138) There have also been reports of impaired prothrombinase activity in response to collagen and thrombin in HPS, but insignificant impairments in non-HPS patients with DGD, who are similar to the participants with DGD in the HPD study cohort. (167) The authors attribute this platelet procoagulant defect to decreased secretion of DG adenine nucleotides that play a role in maintaining the elevated intracellular calcium levels required for the optimum development of prothrombinase. (167) Prothrombinase activity was also impaired in QPD, and in this disorder, it showed a unique relationship to reduced platelet FV antigen levels (146) (see 4.4.4 for further discussion). It would be helpful to study a larger cohort using many different kinds of PFD and newer, standardized CAT protocols to further evaluate how platelet procoagulant function is affected by PFD.

4.4.3 Impaired platelet-dependent thrombin generation but normal platelet factor V levels in participants with *RUNX1* mutations:

We evaluated platelet-dependent thrombin generation for the subgroup in the HPD study cohort that had pathogenic *RUNX1* mutations. This group showed a reduction in ETP and peak thrombin concentrations in TGA with PRP, but the time-to-peak and lag times were similar to controls. Platelet and plasma FV antigen levels were similar to control participants (similar to control levels in other studies (175, 176)), suggesting an

alternate mechanism for their impaired platelet-dependent thrombin generation, possibly from impaired activation required for optimal expression of platelet procoagulant activity. There have been reports of decreased expression of numerous genes that are downstream targets of RUNX1 in these disorders, including a number of genes that influence platelet activation, such as *ALOX12*, *MYL9, PKCθ* and *PCTP*. (177-180) Accordingly, the defect in platelet-dependent thrombin generation in PFD due to *RUNX1* mutations may arise from multiple steps in the platelet activation pathway, such as defects in agonist-receptor binding, downstream intracellular protein interactions, the release of partially activated platelet FV onto the platelet surface, and/or the assembly of the prothrombinase complex, which requires movement of PS to the platelet's external surface. One study, by Glembotsky et al., evaluated platelet activation in a family with a *RUNX1* frameshift mutation and found reduced PAC-1 and P-selectin expression upon activation compared to controls. (157) To better understand the findings seen in this thesis, it would be beneficial to assess platelet activation in those participants with a *RUNX1* mutation, including if there is reduced annexin V binding upon agonist stimulation. It would also be interesting to evaluate expression of the gene implicated in Scott's syndrome, *TMEM16F* (also termed *ANO6*), (181, 182) and to evaluate for defects in microparticle formation in the PFD participants with pathogenic *RUNX1* mutations.

4.4.4 Impaired platelet-dependent thrombin generation and reduced platelet factor V levels in participants with Quebec platelet disorder:

Our study evaluated platelet-dependent thrombin generation in 5 QPD participants and demonstrated reduced ETP and peak thrombin concentrations but similar times-to-peak and lag times to controls for PRP samples. These data indicate an impairment in the total amount of thrombin formed in QPD PRP, despite the normal rate of thrombin generation. Like previous studies (107, 146), we found reduced platelet FV, but normal plasma FV, antigen levels in QPD participants. A past study by Tracy et al. (146) evaluated platelet FV activity in Factor V Quebec (now called QPD) and found reduced activity compared to controls. Their study also found that prothrombinase activity of pre-activated, washed QPD platelets was also impaired, unless purified FVa was added. (146) Due to the reduced activity and amount of platelet FV in QPD, there is an impairment in prothrombinase complex assembly for the normal burst of thrombin generation, leading to reductions in ETP and peak thrombin concentrations. We also found that platelet FV antigen levels showed significant associations to the ETP and peak thrombin concentrations for PRP samples, suggesting that the normal levels of plasma FV in their samples are insufficient to completely compensate for the platelet procoagulant abnormalities in QPD. This makes sense since, unlike plasma FV, platelet FV is stored in its partially-activated state in α-granules (28, 183, 184) and it is more resistant to proteolytic cleavage by APC. (28) In addition, one study evaluated the role of platelet FV in thrombin generation and found that plasma FV was not essential for thrombin generation when platelets were present. (185) They also evaluated thrombin generation in

patients with congenital FV deficiency and found that thrombin generation was absent in PPP but present (although reduced) in PRP, and in the presence of platelet activators, thrombin generation in PRP was enhanced to levels close to control PRP without activators. (185) This suggests that their platelet FV levels, although reduced, could partially correct for their bleeding defect. This conclusion is also supported by animal models. (186, 187)

Further analyses are needed to assess if the impaired proteolysis of other α-granule proteins, such as the FV binding protein multimerin-1, show an association to the TGA defect in QPD PRP. Multimerin-1 antigen is a polymeric FV binding protein that is reduced in QPD platelets, which overlaps with the C1 and C2 domain membrane binding sites that are essential for FVa procoagulant function. (107, 188, 189) Like previous studies (65, 109), we found increased uPA levels in QPD platelets compared to controls; however, platelet FV antigen levels and TGA endpoints in PRP did not show a significant association to platelet uPA levels. This is not surprising since uPA triggers the generation of plasmin, a broad-specificity protease that could potentially modulate numerous proteins involved in supporting coagulation on platelets, not just FV.

4.5 STRENGTHS OF THE THESIS

This thesis aimed to better characterize platelet phenotypes of uncharacterized PFD that manifest with impaired aggregation with multiple agonists and/or DGD. One of the strengths of the aim 1 analyses was that it studied a large, prospective cohort of real-life patients and controls over an extensive period of time (12 years). Having a large study population allowed for comparisons within subgroups, such as pediatric vs. adult and male vs. female, and robust estimates of the RI for diagnosing DGD at our laboratory. Additionally, DG quantification for samples from Cohort I participants were performed by one of two expert EM technologists, limiting sources of inter-rater variability. Despite the low prevalence of DGD, we also had access to multiple samples from DGD participants and controls which allowed for helpful CV estimates when determining the reproducibility of the assay. By incorporating DGD participants from the HPD study cohort into this aim, we were also able to assess clinically important associations between DGD and the presence of a bleeding disorder, an important finding for hematologists who routinely evaluate for DGD in the diagnostic workup of a PFD. In aims 2 and 3, we studied participants from the HPD cohort who have an uncharacterized PFD, which are common types of PFD. We were able to study participants from numerous families (12 for aim 2, 9 for aim 3), which included some participants from families with pathogenic *RUNX1* mutations. In aim 2, we performed similar Western blotting experiments to a study cited by ISTH guidelines for diagnosing PFD (50, 92), with additional comparisons of participant findings to abnormal and normal controls, and quantification of bands using densitometry. We complemented our semi-quantitative

protein findings with molecular findings using dPCR, a technique that is beneficial when quantifying genes with relatively low expression, like *MYH10*. (169) In aim 3, we followed recently published ISTH guidelines when measuring thrombin generation in PPP and PRP (83), which included comparisons to age- and gender-matched controls, since one study showed that TGA parameters, particularly ETP and peak thrombin concentration, increased with age in PPP samples. (190) We found similar TGA endpoints for PPP (86, 191, 192) and PRP samples (192-195) in our control participants compared to reports from other laboratories that used commercially-available reagents and the protocol recommended in the ISTH guidelines. (83) We also evaluated thrombin generation in other forms of PFD, like QPD, and were able to perform additional protein analyses to better understand the mechanisms leading to impaired thrombin generation. Overall, this thesis possesses many strengths that allow the findings to be applicable to other laboratories that evaluate for PFD associated with impaired aggregation responses and/or DGD.

4.6 LIMITATIONS OF THE THESIS

Despite the strengths of this thesis, there are some limitations that must be considered. In aim 1, there were a minimal number of Cohort I participants with DGD that were tested on ≥3 occasions (n=7). We estimated the median CV for DGD participants to evaluate within-subject variability of the assay, but CV estimates would have been benefited from having more observations. Additionally, we did not evaluate for associations between confirmed DGD and CHAT-P symptoms in Cohort II participants, as it would have been interesting to assess if DGD participants had a higher risk of certain bleeding symptoms. In aim 2, we evaluated MYH10 transcript and protein levels for a minimal number of participants with pathogenic *RUNX1* mutations (n=7 from 3 families), which might not represent the findings of all forms of *RUNX1* mutations. We also did not further evaluate the mechanism causing the inconsistent MYH10 protein findings in F4, which raises unanswered questions about the potential dysmegakaryopoeisis in some members of this family. In aim 3, we did not further evaluate the mechanism of the impaired platelet-dependent thrombin generation in PFD participants with a *RUNX1* mutation. Additional analyses that evaluate platelet activation and prothrombinase complex assembly is needed to fully understand the mechanism in this participant subgroup. Although we did find normal platelet FV antigen levels in the *RUNX1* subgroup, we did not directly assess platelet FV activity. Unlike aim 1, we were not able to robustly estimate the median CV for TGA endpoints in PPP and PRP samples, since only two controls donated research samples for TGA experiments on 3 occasions. Lastly, it is possible that TGA experiments in PPP and PRP were affected by contact

activation, recognizing that there is still some debate about the use of CTI in TGA. (86-89)

4.7 FUTURE DIRECTIONS

This current investigation into characterizing common inherited PFD is multifaceted; however, some aspects have not been addressed in the current analysis. These aspects include: a further evaluation of the mechanism causing impaired platelet-dependent thrombin generation in PFD participants with a *RUNX1* mutation; and increasing patient and control sample sizes for additional thrombin generation analyses.

4.7.1 Evaluation of the mechanism causing impaired platelet-dependent thrombin generation in participants with a platelet function disorder due to a pathogenic *RUNX1* mutation:

We found impaired platelet-dependent thrombin generation in PFD participants with a *RUNX1* mutation, but normal platelet and plasma FV antigen levels. However, there are additional mechanistic questions that have not been addressed in this thesis. For example, we did not assess for impaired platelet activation in the *RUNX1* subgroup. Individuals with *RUNX1* mutations are known to have impaired platelet activation, as one study found reduced PAC-1 and P-selectin expression upon activation compared to controls. (157) There have been no reports of impaired annexin V binding, a marker that identifies procoagulant platelets by binding to the head group of PS on activated platelets. (196) Future studies should evaluate these three activation markers (PAC-1, P-selectin, and annexin V) by flow cytometry in the *RUNX1* subgroup to better understand the mechanism causing the procoagulant defect, which could reflect impaired platelet activation. Lastly, it would be interesting to evaluate the expression of the gene

implicated in Scott's syndrome, *ANO6*, given the reports that reduced expression is associated with impairments in calcium-dependent PS exposure. (182, 197) It is possible that *ANO6* expression is regulated by RUNX1, since RUNX1 interacts with numerous genes implicated in platelet function. It would also be interesting to assess the shedding of microparticles by flow cytometry in the HPD participants with a *RUNX1* mutation, as defective microparticle shedding is an additional phenotypic abnormality that is characteristic of Scott's syndrome. (198)

4.7.2 Increasing sample size of participants with platelet function disorders to detect differences in thrombin generation assay endpoints:

We found no significant differences in TGA endpoints for PRP samples between controls and patients with PFD of unknown molecular cause. Particularly, we found an insignificant difference after Bonferroni correction when comparing median peak thrombin concentrations (p-value = 0.04 before Bonferroni correction). It is possible that the analyses for PRP samples performed in this thesis were underpowered to determine if some others with PFD have impaired platelet-dependent thrombin generation. This thesis evaluated thrombin generation in 13 PFD participants with PFD of unknown molecular cause; therefore, performing TGA on the remaining affecteds in the HPD study cohort, in addition to new affecteds that are continuing to be recruited to the HPD study, are needed to adequately power the PRP analyses. Increasing sample size may uncover a significant difference in peak thrombin concentration for PRP samples in this group of PFD participants.

4.7.3 Evaluation of variability in thrombin generation assay endpoints:

We compared TGA endpoints for PPP and PRP samples from PFD participants to eighteen age- and gender-matched controls. This thesis did not evaluate the reproducibility or variability of TGA for PPP and PRP samples, since only 2 controls donated research samples on multiple occasions. Concerns have been raised about using tests with a within-subject CV above 20% for diagnostic purposes. (160) Thus, multiple samples from both controls and PFD participants are needed to assess within-subject variability in TGA endpoints for PPP and PRP samples before questioning the usefulness of CAT as a diagnostic tool. Nonetheless, previous studies by other groups, using the recommended methods, have reported within-subject CVs between 4-8% and 7-10% for TGA endpoints in PPP and PRP samples, respectively. (76, 195, 199)

References

1. Marder V. Hemostasis and Thrombosis. 1st ed. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins Health; 2013.
2. Michelson AD. Platelets. 3rd ed. St. Louis, MO: Elsevier; 2013.
3. Gross P, Murray R, Weill P, Rand M. Harper's Illustrated Biochemistry. New York, NY: McGraw-Hill Education; 2015.
4. Josefsson E, Dowling M, Lebois M, BT K. Platelets 2013.
5. Triplett DA. Coagulation and bleeding disorders: review and update. Clin Chem. 2000;46(8 Pt 2):1260-9.
6. Brass L, Newman D, Wannermacher K, Zhu L, TJ S. Platelets 2013.
7. Cattaneo M. Light transmission aggregometry and ATP release for the diagnostic assessment of platelet function. Semin Thromb Hemost. 2009;35(2):158-67.
8. Clemetson K, Clemetson J. Platelets 2013.
9. Cattaneo M. Platelets 2013.
10. Heijnen HF, Debili N, Vainchencker W, Breton-Gorius J, Geuze HJ, Sixma JJ. Multivesicular bodies are an intermediate stage in the formation of platelet alpha-granules. Blood. 1998;91(7):2313-25.
11. Blair P, Flaumenhaft R. Platelet alpha-granules: basic biology and clinical correlates. Blood Rev. 2009;23(4):177-89.
12. Ambrosio AL, Di Pietro SM. Storage pool diseases illuminate platelet dense granule biogenesis. Platelets. 2017;28(2):138-46.
13. Ghoshal K, Bhattacharyya M. Overview of platelet physiology: its hemostatic and nonhemostatic role in disease pathogenesis. ScientificWorldJournal. 2014;2014:781857.
14. White JG. Platelets2013.
15. White J. The dense bodies of human platelets: Inherent electron opacity of serotonin storage particles. Blood. 1969;33(4):606.
16. Flaumenhaft R. Platelets2013.
17. Bianchi E, Norfo R, Pennucci V, Zini R, Manfredini R. Genomic landscape of megakaryopoiesis and platelet function defects. Blood. 2016;127(10):1249-59.
18. Lordier L, Bluteau D, Jalil A, Legrand C, Pan J, Rameau P, et al. RUNX1-induced silencing of non-muscle myosin heavy chain IIB contributes to megakaryocyte polyploidization. Nat Commun. 2012;3:717.
19. Lefrancais E, Ortiz-Munoz G, Caudrillier A, Mallavia B, Liu F, Sayah DM, et al. The lung is a site of platelet biogenesis and a reservoir for haematopoietic progenitors. Nature. 2017;544(7648):105-9.
20. Mazzi S, Lordier L, Debili N, Raslova H, Vainchenker W. Megakaryocyte and polyploidization. Exp Hematol. 2017.
21. Tijssen MR, Ghevaert C. Transcription factors in late megakaryopoiesis and related platelet disorders. J Thromb Haemost. 2013;11(4):593-604.
22. Italiano J, Hartwig J. Platelets: Elsevier; 2013.
23. Hoffman M. Remodeling the blood coagulation cascade. J Thromb Thrombolysis. 2003;16(1-2):17-20.

24. Crawley JT, Zanardelli S, Chion CK, Lane DA. The central role of thrombin in hemostasis. J Thromb Haemost. 2007;5 Suppl 1:95-101.
25. Norledge BV, Petrovan RJ, Ruf W, Olson AJ. The tissue factor/factor VIIa/factor Xa complex: a model built by docking and site-directed mutagenesis. Proteins. 2003;53(3):640-8.
26. Stoilova-McPhie S, Parmenter CD, Segers K, Villoutreix BO, Nicolaes GA. Defining the structure of membrane-bound human blood coagulation factor Va. J Thromb Haemost. 2008;6(1):76-82.
27. Cripe LD, Moore KD, Kane WH. Structure of the gene for human coagulation factor V. Biochemistry. 1992;31(15):3777-85.
28. Gould WR, Silveira JR, Tracy PB. Unique in vivo modifications of coagulation factor V produce a physically and functionally distinct platelet-derived cofactor: characterization of purified platelet-derived factor V/Va. J Biol Chem. 2004;279(4):2383-93.
29. Foster WB, Nesheim ME, Mann KG. The factor Xa-catalyzed activation of factor V. J Biol Chem. 1983;258(22):13970-7.
30. Suzuki K, Dahlback B, Stenflo J. Thrombin-catalyzed activation of human coagulation factor V. J Biol Chem. 1982;257(11):6556-64.
31. Kalafatis M, Rand MD, Mann KG. The mechanism of inactivation of human factor V and human factor Va by activated protein C. J Biol Chem. 1994;269(50):31869-80.
32. Rapaport SI. The extrinsic pathway inhibitor: a regulator of tissue factor-dependent blood coagulation. Thromb Haemost. 1991;66(1):6-15.
33. Wood JP, Bunce MW, Maroney SA, Tracy PB, Camire RM, Mast AE. Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation. Proc Natl Acad Sci U S A. 2013;110(44):17838-43.
34. Kim PY, Nesheim ME. Down regulation of prothrombinase by activated protein C during prothrombin activation. Thromb Haemost. 2010;104(1):61-70.
35. Olson ST, Bjork I, Shore JD. Kinetic characterization of heparin-catalyzed and uncatalyzed inhibition of blood coagulation proteinases by antithrombin. Methods Enzymol. 1993;222:525-59.
36. Maron B, Loscalzo J. Platelets2013.
37. Robbins KC, Summaria L, Hsieh B, Shah RJ. The peptide chains of human plasmin. Mechanism of activation of human plasminogen to plasmin. J Biol Chem. 1967;242(10):2333-42.
38. Wiman B, Collen D. On the mechanism of the reaction between human alpha 2-antiplasmin and plasmin. J Biol Chem. 1979;254:9291-97.
39. Marx PF, Dawson PE, Bouma BN, Meijers JC. Plasmin-mediated activation and inactivation of thrombin-activatable fibrinolysis inhibitor. Biochemistry. 2002;41(21):6688-96.
40. Rao A. Platelets 2013.
41. Konkle BA. Acquired disorders of platelet function. Hematology Am Soc Hematol Educ Program. 2011;2011:391-6.

42. McEwen BJ. The influence of diet and nutrients on platelet function. Semin Thromb Hemost. 2014;40(2):214-26.
43. Yagmur E, Piatkowski A, Groger A, Pallua N, Gressner AM, Kiefer P. Bleeding complication under Gingko biloba medication. Am J Hematol. 2005;79(4):343-4.
44. Bolton-Maggs PH, Chalmers EA, Collins PW, Harrison P, Kitchen S, Liesner RJ, et al. A review of inherited platelet disorders with guidelines for their management on behalf of the UKHCDO. Br J Haematol. 2006;135(5):603-33.
45. Israels SJ, McNicol A, Robertson C, Gerrard JM. Platelet storage pool deficiency: diagnosis in patients with prolonged bleeding times and normal platelet aggregation. Br J Haematol. 1990;75(1):118-21.
46. Pai M, Wang G, Moffat KA, Liu Y, Seecharan J, Webert K, et al. Diagnostic usefulness of a lumi-aggregometer adenosine triphosphate release assay for the assessment of platelet function disorders. Am J Clin Pathol. 2011;136(3):350-8.
47. Castilloux JF, Moffat KA, Liu Y, Seecharan J, Pai M, Hayward CP. A prospective cohort study of light transmission platelet aggregometry for bleeding disorders: is testing native platelet-rich plasma non-inferior to testing platelet count adjusted samples? Thromb Haemost. 2011;106(4):675-82.
48. Hayward CP, Moffat KA, Plumhoff E, Van Cott EM. Approaches to investigating common bleeding disorders: an evaluation of North American coagulation laboratory practices. Am J Hematol. 2012;87 Suppl 1:S45-50.
49. Hayward CP. Diagnostic evaluation of platelet function disorders. Blood Rev. 2011;25(4):169-73.
50. Gresele P, Subcommittee on Platelet Physiology of the International Society on T, Hemostasis. Diagnosis of inherited platelet function disorders: guidance from the SSC of the ISTH. J Thromb Haemost. 2015;13(2):314-22.
51. Rodeghiero F, Tosetto A, Abshire T, Arnold DM, Coller B, James P, et al. ISTH/SSC bleeding assessment tool: a standardized questionnaire and a proposal for a new bleeding score for inherited bleeding disorders. J Thromb Haemost. 2010;8(9):2063-5.
52. Hayward CP, Rao AK, Cattaneo M. Congenital platelet disorders: overview of their mechanisms, diagnostic evaluation and treatment. Haemophilia. 2006;12 Suppl 3:128-36.
53. Israels SJ, El-Ekiaby M, Quiroga T, Mezzano D. Inherited disorders of platelet function and challenges to diagnosis of mucocutaneous bleeding. Haemophilia. 2010;16 Suppl 5:152-9.
54. Rao AK. Inherited platelet function disorders: overview and disorders of granules, secretion, and signal transduction. Hematol Oncol Clin North Am. 2013;27(3):585-611.
55. Watson SP, Lowe GC, Lordkipanidze M, Morgan NV, consortium G. Genotyping and phenotyping of platelet function disorders. J Thromb Haemost. 2013;11 Suppl 1:351-63.
56. Gresele P, Harrison P, Bury L, Falcinelli E, Gachet C, Hayward CP, et al. Diagnosis of suspected inherited platelet function disorders: results of a worldwide survey. J Thromb Haemost. 2014;12(9):1562-9.

57. Badin MS, Iyer JK, Chong M, Graf L, Rivard GE, Waye JS, et al. Molecular phenotype and bleeding risks of an inherited platelet disorder in a family with a RUNX1 frameshift mutation. Haemophilia. 2017;23(3):e204-e13.
58. Elbatarny M, Mollah S, Grabell J, Bae S, Deforest M, Tuttle A, et al. Normal range of bleeding scores for the ISTH-BAT: adult and pediatric data from the merging project. Haemophilia. 2014;20(6):831-5.
59. Tosetto A, Rodeghiero F, Castaman G, Goodeve A, Federici AB, Batlle J, et al. A quantitative analysis of bleeding symptoms in type 1 von Willebrand disease: results from a multicenter European study (MCMDM-1 VWD). J Thromb Haemost. 2006;4(4):766-73.
60. Badin MS, Graf L, Iyer JK, Moffat KA, Seecharan JL, Hayward CP. Variability in platelet dense granule adenosine triphosphate release findings amongst patients tested multiple times as part of an assessment for a bleeding disorder. Int J Lab Hematol. 2016;38(6):648-57.
61. Lowe GC, Lordkipanidze M, Watson SP, group UGs. Utility of the ISTH bleeding assessment tool in predicting platelet defects in participants with suspected inherited platelet function disorders. J Thromb Haemost. 2013;11(9):1663-8.
62. Rashid A, Moiz B, Karim F, Shaikh MS, Mansoori H, Raheem A. Use of ISTH bleeding assessment tool to predict inherited platelet dysfunction in resource constrained settings. Scand J Clin Lab Invest. 2016;76(5):373-8.
63. Rydz N, James PD. The evolution and value of bleeding assessment tools. J Thromb Haemost. 2012;10(11):2223-9.
64. Quiroga T, Mezzano D. Is my patient a bleeder? A diagnostic framework for mild bleeding disorders. Hematology Am Soc Hematol Educ Program. 2012;2012:466-74.
65. McKay H, Derome F, Haq MA, Whittaker S, Arnold E, Adam F, et al. Bleeding risks associated with inheritance of the Quebec platelet disorder. Blood. 2004;104(1):159-65.
66. Cattaneo M, Hayward CP, Moffat KA, Pugliano MT, Liu Y, Michelson AD. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: a report from the platelet physiology subcommittee of the SSC of the ISTH. J Thromb Haemost. 2009;7(6):1029.
67. Hayward C, Moffat KA. Platelet Aggregation2013.
68. Hayward CP, Pai M, Liu Y, Moffat KA, Seecharan J, Webert KE, et al. Diagnostic utility of light transmission platelet aggregometry: results from a prospective study of individuals referred for bleeding disorder assessments. J Thromb Haemost. 2009;7(4):676-84.
69. White J. Use of the electron microscope for diagnosis of platelet disorders. Seminars in Thrombosis and Hemostasis. 1998;24(2):163-8.
70. Nieuwenhuis HK, Akkerman JW, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on one hundred six patients. Blood. 1987;70(3):620-3.
71. Hayward CP, Moffat KA, Plumhoff E, Timleck M, Hoffman S, Spitzer E, et al. External quality assessment of platelet disorder investigations: results of international

surveys on diagnostic tests for dense granule deficiency and platelet aggregometry interpretation. Semin Thromb Hemost. 2012;38(6):622-31.
72. Hayward CP, Moffat KA, Spitzer E, Timleck M, Plumhoff E, Israels SJ, et al. Results of an external proficiency testing exercise on platelet dense-granule deficiency testing by whole mount electron microscopy. Am J Clin Pathol. 2009;131(5):671-5.
73. Young G, Sorensen B, Dargaud Y, Negrier C, Brummel-Ziedins K, Key NS. Thrombin generation and whole blood viscoelastic assays in the management of hemophilia: current state of art and future perspectives. Blood. 2013;121(11):1944-50.
74. Baglin T. The measurement and application of thrombin generation. Br J Haematol. 2005;130(5):653-61.
75. Lance MD. A general review of major global coagulation assays: thrombelastography, thrombin generation test and clot waveform analysis. Thromb J. 2015;13:1.
76. Hemker HC, Giesen P, Al Dieri R, Regnault V, de Smedt E, Wagenvoord R, et al. Calibrated Automated Thrombin Generation Measurement in Clotting Plasma. Pathophysiology of Haemostasis and Thrombosis. 2003;33(1):4-15.
77. Hemker HC, Giesen P, AlDieri R, Regnault V, de Smed E, Wagenvoord R, et al. The calibrated automated thrombogram (CAT): a universal routine test for hyper- and hypocoagulability. Pathophysiol Haemost Thromb. 2002;32(5-6):249-53.
78. Berntorp E, Salvagno GL. Standardization and clinical utility of thrombin-generation assays. Semin Thromb Hemost. 2008;34(7):670-82.
79. Ignjatovic V, Lai C, Summerhayes R, Mathesius U, Tawfilis S, Perugini MA, et al. Age-related differences in plasma proteins: how plasma proteins change from neonates to adults. PLoS One. 2011;6(2):e17213.
80. Schmit JM, Turner DJ, Hromas RA, Wingard JR, Brown RA, Li Y, et al. Two novel RUNX1 mutations in a patient with congenital thrombocytopenia that evolved into a high grade myelodysplastic syndrome. Leuk Res Rep. 2015;4(1):24-7.
81. Hemker HC, Al Dieri R, De Smedt E, Beguin S. Thrombin generation, a function test of the haemostatic-thrombotic system. Thromb Haemost. 2006;96(5):553-61.
82. Hemker HC, Giesen PL, Ramjee M, Wagenvoord R, Beguin S. The thrombogram: monitoring thrombin generation in platelet-rich plasma. Thromb Haemost. 2000;83(4):589-91.
83. Dargaud Y, Wolberg AS, Gray E, Negrier C, Hemker HC, Subcommittee on Factor Viii FIX, et al. Proposal for standardized preanalytical and analytical conditions for measuring thrombin generation in hemophilia: communication from the SSC of the ISTH. J Thromb Haemost. 2017;15(8):1704-7.
84. Dargaud Y, Beguin S, Lienhart A, Al Dieri R, Trzeciak C, Bordet JC, et al. Evaluation of thrombin generating capacity in plasma from patients with haemophilia A and B. Thromb Haemost. 2005;93(3):475-80.
85. van Veen JJ, Gatt A, Bowyer AE, Cooper PC, Kitchen S, Makris M. Calibrated automated thrombin generation and modified thromboelastometry in haemophilia A. Thromb Res. 2009;123(6):895-901.

86. Luddington R, Baglin T. Clinical measurement of thrombin generation by calibrated automated thrombography requires contact factor inhibition. J Thromb Haemost. 2004;2(11):1954-9.
87. Dargaud Y, Luddington R, Baglin TP. Elimination of contact factor activation improves measurement of platelet-dependent thrombin generation by calibrated automated thrombography at low-concentration tissue factor. J Thromb Haemost. 2006;4(5):1160-1.
88. Spronk HM, Dielis AW, Panova-Noeva M, van Oerle R, Govers-Riemslag JW, Hamulyak K, et al. Monitoring thrombin generation: is addition of corn trypsin inhibitor needed? Thromb Haemost. 2009;101(6):1156-62.
89. van Veen JJ, Gatt A, Cooper PC, Kitchen S, Bowyer AE, Makris M. Corn trypsin inhibitor in fluorogenic thrombin-generation measurements is only necessary at low tissue factor concentrations and influences the relationship between factor VIII coagulant activity and thrombogram parameters. Blood Coagul Fibrinolysis. 2008;19(3):183-9.
90. Stockley J, Morgan NV, Bem D, Lowe GC, Lordkipanidze M, Dawood B, et al. Enrichment of FLI1 and RUNX1 mutations in families with excessive bleeding and platelet dense granule secretion defects. Blood. 2013;122(25):4090-3.
91. Romasko EJ, Devkota B, Biswas S, Jayaraman V, Rajagopalan R, Dulik MC, et al. Utility and limitations of exome sequencing in the molecular diagnosis of pediatric inherited platelet disorders. Am J Hematol. 2018;93(1):8-16.
92. Antony-Debre I, Bluteau D, Itzykson R, Baccini V, Renneville A, Boehlen F, et al. MYH10 protein expression in platelets as a biomarker of RUNX1 and FLI1 alterations. Blood. 2012;120(13):2719-22.
93. Cattaneo M. Inherited platelet-based bleeding disorders. J Thromb Haemost. 2003;1(7):1628-36.
94. Remijn JA, MJ IJ, Strunk AL, Abbes AP, Engel H, Dikkeschei B, et al. Novel molecular defect in the platelet ADP receptor P2Y12 of a patient with haemorrhagic diathesis. Clin Chem Lab Med. 2007;45(2):187-9.
95. Hermans C, Wittevrongel C, Thys C, Smethurst PA, Van Geet C, Freson K. A compound heterozygous mutation in glycoprotein VI in a patient with a bleeding disorder. J Thromb Haemost. 2009;7(8):1356-63.
96. Pollitt A. Platelets 2013.
97. Ushikubi F, Okuma M, Kanaji K, Sugiyama T, Ogorochi T, Narumiya S, et al. Hemorrhagic thrombocytopathy with platelet thromboxane A2 receptor abnormality: defective signal transduction with normal binding activity. Thromb Haemost. 1987;57(2):158-64.
98. Pham A, Wang J. Bernard-Soulier syndrome: an inherited platelet disorder. Arch Pathol Lab Med. 2007;131(12):1834-6.
99. Phillips DR, Jennings LK, Berndt MC. Studies of inherited bleeding disorders to identify platelet membrane glycoproteins involved in adhesion and aggregation. Prog Clin Biol Res. 1982;97:151-63.
100. Miller JL, Cunningham D, Lyle VA, Finch CN. Mutation in the gene encoding the alpha chain of platelet glycoprotein Ib in platelet-type von Willebrand disease. Proc Natl Acad Sci U S A. 1991;88(11):4761-5.

101. Othman M. Platelet-type Von Willebrand disease: three decades in the life of a rare bleeding disorder. Blood Rev. 2011;25(4):147-53.
102. Noris P, Guidetti GF, Conti V, Ceresa IF, Di Pumpo M, Pecci A, et al. Autosomal dominant thrombocytopenias with reduced expression of glycoprotein Ia. Thromb Haemost. 2006;95(3):483-9.
103. George JN, Caen JP, Nurden AT. Glanzmann's thrombasthenia: the spectrum of clinical disease. Blood. 1990;75(7):1383-95.
104. Nurden AT, Fiore M, Nurden P, Pillois X. Glanzmann thrombasthenia: a review of ITGA2B and ITGB3 defects with emphasis on variants, phenotypic variability, and mouse models. Blood. 2011;118(23):5996-6005.
105. Jurk K, Schulz AS, Kehrel BE, Rapple D, Schulze H, Mobest D, et al. Novel integrin-dependent platelet malfunction in siblings with leukocyte adhesion deficiency-III (LAD-III) caused by a point mutation in FERMT3. Thromb Haemost. 2010;103(5):1053-64.
106. Althaus K, Greinacher A. MYH9-related platelet disorders. Semin Thromb Hemost. 2009;35(2):189-203.
107. Hayward CP, Rivard GE, Kane WH, Drouin J, Zheng S, Moore JC, et al. An autosomal dominant, qualitative platelet disorder associated with multimerin deficiency, abnormalities in platelet factor V, thrombospondin, von Willebrand factor, and fibrinogen and an epinephrine aggregation defect. Blood. 1996;87(12):4967-78.
108. Diamandis M, Paterson A, Rommens J, Veljkovic D, Blavignac J, Bulman D, et al. Quebec platelet disorder is linked to the urokinase plasminogen activator gene (PLAU) and increases expression of the linked allele in megakaryocytes. Blood. 2009;113(7):1543-6.
109. Kahr WH, Zheng S, Sheth PM, Pai M, Cowie A, Bouchard M, et al. Platelets from patients with the Quebec platelet disorder contain and secrete abnormal amounts of urokinase-type plasminogen activator. Blood. 2001;98(2):257-65.
110. Ray A, Ray S, Matthew J. Case report : Hermansky Pudlak syndrome (Presenting as late onset heavy menstrual bleeding). Clinical and diagnostic laboratory immunology. 2013;9:1235-9.
111. Lozano ML, Rivera J, Sanchez-Guiu I, Vicente V. Towards the targeted management of Chediak-Higashi syndrome. Orphanet J Rare Dis. 2014;9:132.
112. Gunay-Aygun M, Zivony-Elboum Y, Gumruk F, Geiger D, Cetin M, Khayat M, et al. Gray platelet syndrome: natural history of a large patient cohort and locus assignment to chromosome 3p. Blood. 2010;116(23):4990-5001.
113. Abu-Sa'da O, Barbar M, Al-Harbi N, Taha D. Arthrogryposis, renal tubular acidosis and cholestasis (ARC) syndrome: two new cases and review. Clin Dysmorphol. 2005;14(4):191-6.
114. Sandrock K, Zieger B. Current Strategies in Diagnosis of Inherited Storage Pool Defects. Transfus Med Hemother. 2010;37(5):248-58.
115. Huizing M, Helip-Wooley A, Westbroek W, Gunay-Aygun M, Gahl WA. Disorders of lysosome-related organelle biogenesis: clinical and molecular genetics. Annu Rev Genomics Hum Genet. 2008;9:359-86.

116. Gunning WT, Calomeni EP. A brief review of transmission electron microscopy and applications in pathology. J Histotechnol. 2000;23(3):237-46.
117. Ballmaier M, Germeshausen M. Congenital amegakaryocytic thrombocytopenia: clinical presentation, diagnosis, and treatment. Semin Thromb Hemost. 2011;37(6):673-81.
118. Horvat-Switzer RD, Thompson AA. HOXA11 mutation in amegakaryocytic thrombocytopenia with radio-ulnar synostosis syndrome inhibits megakaryocytic differentiation in vitro. Blood Cells Mol Dis. 2006;37(1):55-63.
119. Noris P, Perrotta S, Seri M, Pecci A, Gnan C, Loffredo G, et al. Mutations in ANKRD26 are responsible for a frequent form of inherited thrombocytopenia: analysis of 78 patients from 21 families. Blood. 2011;117(24):6673-80.
120. Di Paola J, Johnson J. Thrombocytopenias due to gray platelet syndrome or THC2 mutations. Semin Thromb Hemost. 2011;37(6):690-7.
121. Gilman A, Sloand E, White J, Sacher R. A novel hereditary macrothrombocyotpenia. J Pediatr Hematol Oncol 1995;17(4):295-305.
122. Albers CA, Paul DS, Schulze H, Freson K, Stephens JC, Smethurst PA, et al. Compound inheritance of a low-frequency regulatory SNP and a rare null mutation in exon-junction complex subunit RBM8A causes TAR syndrome. Nat Genet. 2012;44(4):435-9, S1-2.
123. Toriello HV. Thrombocytopenia-absent radius syndrome. Semin Thromb Hemost. 2011;37(6):707-12.
124. Kunishima S, Kobayashi R, Itoh TJ, Hamaguchi M, Saito H. Mutation of the beta1-tubulin gene associated with congenital macrothrombocytopenia affecting microtubule assembly. Blood. 2009;113(2):458-61.
125. Nurden P, Debili N, Coupry I, Bryckaert M, Youlyouz-Marfak I, Sole G, et al. Thrombocytopenia resulting from mutations in filamin A can be expressed as an isolated syndrome. Blood. 2011;118(22):5928-37.
126. Weiss HJ. Impaired platelet procoagulant mechanisms in patients with bleeding disorders. Semin Thromb Hemost. 2009;35(2):233-41.
127. Misceo D, Holmgren A, Louch WE, Holme PA, Mizobuchi M, Morales RJ, et al. A dominant STIM1 mutation causes Stormorken syndrome. Hum Mutat. 2014;35(5):556-64.
128. Weiss HJ, Lages B, Zheng S, Hayward CP. Platelet factor V New York: a defect in factor V distinct from that in factor V Quebec resulting in impaired prothrombinase generation. Am J Hematol. 2001;66(2):130-9.
129. Adler DH, Cogan JD, Phillips JA, 3rd, Schnetz-Boutaud N, Milne GL, Iverson T, et al. Inherited human cPLA(2alpha) deficiency is associated with impaired eicosanoid biosynthesis, small intestinal ulceration, and platelet dysfunction. J Clin Invest. 2008;118(6):2121-31.
130. Dube JN, Drouin J, Aminian M, Plant MH, Laneuville O. Characterization of a partial prostaglandin endoperoxide H synthase-1 deficiency in a patient with a bleeding disorder. Br J Haematol. 2001;113(4):878-85.
131. Van Geet C, Izzi B, Labarque V, Freson K. Human platelet pathology related to defects in the G-protein signaling cascade. J Thromb Haemost. 2009;7 Suppl 1:282-6.

132. Markello T, Chen D, Kwan JY, Horkayne-Szakaly I, Morrison A, Simakova O, et al. York platelet syndrome is a CRAC channelopathy due to gain-of-function mutations in STIM1. Mol Genet Metab. 2015;114(3):474-82.
133. Saultier P, Vidal L, Canault M, Bernot D, Falaise C, Pouymayou C, et al. Macrothrombocytopenia and dense granule deficiency associated with FLI1 variants: ultrastructural and pathogenic features. Haematologica. 2017;102(6):1006-16.
134. Tijssen MR, Cvejic A, Joshi A, Hannah RL, Ferreira R, Forrai A, et al. Genome-wide analysis of simultaneous GATA1/2, RUNX1, FLI1, and SCL binding in megakaryocytes identifies hematopoietic regulators. Dev Cell. 2011;20(5):597-609.
135. Daly ME. Transcription factor defects causing platelet disorders. Blood Rev. 2017;31(1):1-10.
136. Zhang MY, Churpek JE, Keel SB, Walsh T, Lee MK, Loeb KR, et al. Germline ETV6 mutations in familial thrombocytopenia and hematologic malignancy. Nat Genet. 2015;47(2):180-5.
137. Defreyn G, Machin SJ, Carreras LO, Dauden MV, Chamone DA, Vermylen J. Familial bleeding tendency with partial platelet thromboxane synthetase deficiency: reorientation of cyclic endoperoxide metabolism. Br J Haematol. 1981;49(1):29-41.
138. Beguin S, Keularts IM, Al Dieri R, Bellucci S, Caen J, Hemker HC. Fibrin polymerization is crucial for thrombin generation in platelet-rich plasma in a VWF-GPIb-dependent process, defective in Bernard-Soulier syndrome. J Thromb Haemost. 2004;2(1):170-6.
139. Loscalzo J, Inbal A, Handin RI. von Willebrand protein facilitates platelet incorporation in polymerizing fibrin. J Clin Invest. 1986;78(4):1112-9.
140. Beguin S, Kumar R, Keularts I, Seligsohn U, Coller BS, Hemker HC. Fibrin-dependent platelet procoagulant activity requires GPIb receptors and von Willebrand factor. Blood. 1999;93(2):564-70.
141. Kumar R, Beguin S, Hemker HC. The effect of fibrin clots and clot-bound thrombin on the development of platelet procoagulant activity. Thromb Haemost. 1995;74(3):962-8.
142. Reverter JC, Beguin S, Kessels H, Kumar R, Hemker HC, Coller BS. Inhibition of platelet-mediated, tissue factor-induced thrombin generation by the mouse/human chimeric 7E3 antibody. Potential implications for the effect of c7E3 Fab treatment on acute thrombosis and "clinical restenosis". J Clin Invest. 1996;98(3):863-74.
143. Paterson AD, Rommens JM, Bharaj B, Blavignac J, Wong I, Diamandis M, et al. Persons with Quebec platelet disorder have a tandem duplication of PLAU, the urokinase plasminogen activator gene. Blood. 2010;115(6):1264-6.
144. Hayward CP, Cramer EM, Kane WH, Zheng S, Bouchard M, Masse JM, et al. Studies of a second family with the Quebec platelet disorder: evidence that the degradation of the alpha-granule membrane and its soluble contents are not secondary to a defect in targeting proteins to alpha-granules. Blood. 1997;89(4):1243-53.
145. Janeway CM, Rivard GE, Tracy PB, Mann KG. Factor V Quebec revisited. Blood. 1996;87(9):3571-8.

146. Tracy PB, Giles AR, Mann KG, Eide LL, Hoogendoorn H, Rivard GE. Factor V (Quebec): a bleeding diathesis associated with a qualitative platelet Factor V deficiency. J Clin Invest. 1984;74(4):1221-8.
147. Songdej N, Rao AK. Hematopoietic transcription factor mutations: important players in inherited platelet defects. Blood. 2017;129(21):2873-81.
148. Ichikawa M, Asai T, Saito T, Seo S, Yamazaki I, Yamagata T, et al. AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis. Nat Med. 2004;10(3):299-304.
149. Muntean AG, Pang L, Poncz M, Dowdy SF, Blobel GA, Crispino JD. Cyclin D-Cdk4 is regulated by GATA-1 and required for megakaryocyte growth and polyploidization. Blood. 2007;109(12):5199-207.
150. Mao GF, Goldfinger LE, Fan DC, Lambert MP, Jalagadugula G, Freishtat R, et al. Dysregulation of PLDN (pallidin) is a mechanism for platelet dense granule deficiency in RUNX1 haplodeficiency. J Thromb Haemost. 2017;15(4):792-801.
151. Sun L, Mao G, Rao AK. Association of CBFA2 mutation with decreased platelet PKC-theta and impaired receptor-mediated activation of GPIIb-IIIa and pleckstrin phosphorylation: proteins regulated by CBFA2 play a role in GPIIb-IIIa activation. Blood. 2004;103(3):948-54.
152. Gabbeta J, Yang X, Sun L, McLane MA, Niewiarowski S, Rao AK. Abnormal inside-out signal transduction-dependent activation of glycoprotein IIb-IIIa in a patient with impaired pleckstrin phosphorylation. Blood. 1996;87(4):1368-76.
153. Favier R, Akshoomoff N, Mattson S, Grossfeld P. Jacobsen syndrome: Advances in our knowledge of phenotype and genotype. Am J Med Genet C Semin Med Genet. 2015;169(3):239-50.
154. van der Meer LT, Jansen JH, van der Reijden BA. Gfi1 and Gfi1b: key regulators of hematopoiesis. Leukemia. 2010;24(11):1834-43.
155. Stevenson WS, Morel-Kopp MC, Chen Q, Liang HP, Bromhead CJ, Wright S, et al. GFI1B mutation causes a bleeding disorder with abnormal platelet function. J Thromb Haemost. 2013;11(11):2039-47.
156. Kwiatkowski BA, Bastian LS, Bauer TR, Jr., Tsai S, Zielinska-Kwiatkowska AG, Hickstein DD. The ets family member Tel binds to the Fli-1 oncoprotein and inhibits its transcriptional activity. J Biol Chem. 1998;273(28):17525-30.
157. Glembotsky AC, Bluteau D, Espasandin YR, Goette NP, Marta RF, Marin Oyarzun CP, et al. Mechanisms underlying platelet function defect in a pedigree with familial platelet disorder with a predisposition to acute myelogenous leukemia: potential role for candidate RUNX1 targets. J Thromb Haemost. 2014;12(5):761-72.
158. Horn PS, Pesce AJ. Reference intervals: an update. Clin Chim Acta. 2003;334(1-2):5-23.
159. Taylor JM, Cumberland WG, Meng X, Giorgi JV. Normal range estimation for repeated immunologic measures. Clin Diagn Lab Immunol. 1996;3(2):139-42.
160. Reed GF, Lynn F, Meade BD. Use of coefficient of variation in assessing variability of quantitative assays. Clin Diagn Lab Immunol. 2002;9:1235-9.

161. Sorokin V, Alkhoury R, Al-Rawabdeh S, Houston RH, Thornton D, Kerlin B, et al. Reference Range of Platelet Delta Granules in the Pediatric Age Group: An Ultrastructural Study of Platelet Whole Mount Preparations from Healthy Volunteers. Pediatr Dev Pathol. 2016;19(6):498-501.
162. Hayward CP, Liang M, Tasneem S, Soomro A, Waye JS, Paterson AD, et al. The duplication mutation of Quebec platelet disorder dysregulates PLAU, but not C10orf55, selectively increasing production of normal PLAU transcripts by megakaryocytes but not granulocytes. PLoS One. 2017;12(3):e0173991.
163. Westmoreland D, Shaw M, Grimes W, Metcalf DJ, Burden JJ, Gomez K, et al. Super-resolution microscopy as a potential approach to diagnosis of platelet granule disorders. J Thromb Haemost. 2016;14(4):839-49.
164. Knight AE, Gomez K, Cutler DF. Super-resolution microscopy in the diagnosis of platelet granule disorders. Expert Rev Hematol. 2017;10(5):375-81.
165. Israels SJ, Robertson C, McNicol A. Identification of patients with storage pool deficiency using ATP release and dense granule counts. Hematology. 1997;2(2):161-7.
166. Weiss HJ, Lages B. The response of platelets to epinephrine in storage pool deficiency--evidence pertaining to the role of adenosine diphosphate in mediating primary and secondary aggregation. Blood. 1988;72(5):1717-25.
167. Weiss HJ, Lages B. Platelet prothrombinase activity and intracellular calcium responses in patients with storage pool deficiency, glycoprotein IIb-IIIa deficiency, or impaired platelet coagulant activity--a comparison with Scott syndrome. Blood. 1997;89(5):1599-611.
168. Bluteau D, Glembotsky AC, Raimbault A, Balayn N, Gilles L, Rameau P, et al. Dysmegakaryopoiesis of FPD/AML pedigrees with constitutional RUNX1 mutations is linked to myosin II deregulated expression. Blood. 2012;120(13):2708-18.
169. Hindson CM, Chevillet JR, Briggs HA, Gallichotte EN, Ruf IK, Hindson BJ, et al. Absolute quantification by droplet digital PCR versus analog real-time PCR. Nat Methods. 2013;10(10):1003-5.
170. Maier T, Guell M, Serrano L. Correlation of mRNA and protein in complex biological samples. FEBS Lett. 2009;583(24):3966-73.
171. Geumann C, Gronborg M, Hellwig M, Martens H, Jahn R. A sandwich enzyme-linked immunosorbent assay for the quantification of insoluble membrane and scaffold proteins. Anal Biochem. 2010;402(2):161-9.
172. Ay C, Haselbock J, Laczkovics C, Koder S, Pabinger I. Thrombin generation in patients with a bleeding tendency of unknown origin. Ann Hematol. 2011;90(9):1099-104.
173. Quiroga T, Goycoolea M, Giesen P. Thrombin generation in platelet-poor plasma is normal in patients with hereditary mucocutaneous haemorrhages. Pathophysiol Haemost Thromb. 2003;33(1):30-5.
174. Zetterberg E, Carlsson Alle MS, Najm J, Greinacher A. Thrombin generation in two families with MYH9-related platelet disorder. Platelets. 2016;27(3):264-7.
175. Tracy PB, Eide LL, Bowie EJ, Mann KG. Radioimmunoassay of factor V in human plasma and platelets. Blood. 1982;60(1):59-63.

176. Veljkovic DK, Cramer EM, Alimardani G, Fichelson S, Masse JM, Hayward CP. Studies of alpha-granule proteins in cultured human megakaryocytes. Thromb Haemost. 2003;90(5):844-52.
177. Kaur G, Jalagadugula G, Mao G, Rao AK. RUNX1/core binding factor A2 regulates platelet 12-lipoxygenase gene (ALOX12): studies in human RUNX1 haplodeficiency. Blood. 2010;115(15):3128-35.
178. Jalagadugula G, Mao G, Kaur G, Goldfinger LE, Dhanasekaran DN, Rao AK. Regulation of platelet myosin light chain (MYL9) by RUNX1: implications for thrombocytopenia and platelet dysfunction in RUNX1 haplodeficiency. Blood. 2010;116(26):6037-45.
179. Jalagadugula G, Mao G, Kaur G, Dhanasekaran DN, Rao AK. Platelet protein kinase C-theta deficiency with human RUNX1 mutation: PRKCQ is a transcriptional target of RUNX1. Arterioscler Thromb Vasc Biol. 2011;31(4):921-7.
180. Mao G, Songdej N, Voora D, Goldfinger LE, Del Carpio-Cano FE, Myers RA, et al. Transcription Factor RUNX1 Regulates Platelet PCTP (Phosphatidylcholine Transfer Protein): Implications for Cardiovascular Events: Differential Effects of RUNX1 Variants. Circulation. 2017;136(10):927-39.
181. Baig AA, Haining EJ, Geuss E, Beck S, Swieringa F, Wanitchakool P, et al. TMEM16F-Mediated Platelet Membrane Phospholipid Scrambling Is Critical for Hemostasis and Thrombosis but not Thromboinflammation in Mice-Brief Report. Arterioscler Thromb Vasc Biol. 2016;36(11):2152-7.
182. Mattheij NJ, Braun A, van Kruchten R, Castoldi E, Pircher J, Baaten CC, et al. Survival protein anoctamin-6 controls multiple platelet responses including phospholipid scrambling, swelling, and protein cleavage. FASEB J. 2016;30(2):727-37.
183. Bouchard BA, Williams JL, Meisler NT, Long MW, Tracy PB. Endocytosis of plasma-derived factor V by megakaryocytes occurs via a clathrin-dependent, specific membrane binding event. J Thromb Haemost. 2005;3(3):541-51.
184. Monkovic DD, Tracy PB. Functional characterization of human platelet-released factor V and its activation by factor Xa and thrombin. J Biol Chem. 1990;265(28):17132-40.
185. Duckers C, Simioni P, Spiezia L, Radu C, Dabrilli P, Gavasso S, et al. Residual platelet factor V ensures thrombin generation in patients with severe congenital factor V deficiency and mild bleeding symptoms. Blood. 2010;115(4):879-86.
186. Yang TL, Pipe SW, Yang A, Ginsburg D. Biosynthetic origin and functional significance of murine platelet factor V. Blood. 2003;102(8):2851-5.
187. Sun H, Yang TL, Yang A, Wang X, Ginsburg D. The murine platelet and plasma factor V pools are biosynthetically distinct and sufficient for minimal hemostasis. Blood. 2003;102(8):2856-61.
188. Jeimy SB, Woram RA, Fuller N, Quinn-Allen MA, Nicolaes GA, Dahlback B, et al. Identification of the MMRN1 binding region within the C2 domain of human factor V. J Biol Chem. 2004;279(49):51466-71.
189. Jeimy SB, Quinn-Allen MA, Fuller N, Kane WH, Hayward CP. Location of the multimerin 1 binding site in coagulation factor V: an update. Thromb Res. 2008;123(2):352-4.

190. Haidl H, Cimenti C, Leschnik B, Zach D, Muntean W. Age-dependency of thrombin generation measured by means of calibrated automated thrombography (CAT). Thromb Haemost. 2006;95(5):772-5.
191. Dargaud Y, Wolberg AS, Luddington R, Regnault V, Spronk H, Baglin T, et al. Evaluation of a standardized protocol for thrombin generation measurement using the calibrated automated thrombogram: an international multicentre study. Thromb Res. 2012;130(6):929-34.
192. Vanschoonbeek K, Feijge MA, Van Kampen RJ, Kenis H, Hemker HC, Giesen PL, et al. Initiating and potentiating role of platelets in tissue factor-induced thrombin generation in the presence of plasma: subject-dependent variation in thrombogram characteristics. J Thromb Haemost. 2004;2(3):476-84.
193. Machlus KR, Colby EA, Wu JR, Koch GG, Key NS, Wolberg AS. Effects of tissue factor, thrombomodulin and elevated clotting factor levels on thrombin generation in the calibrated automated thrombogram. Thromb Haemost. 2009;102(5):936-44.
194. Panova-Noeva M, Marchetti M, Spronk HM, Russo L, Diani E, Finazzi G, et al. Platelet-induced thrombin generation by the calibrated automated thrombogram assay is increased in patients with essential thrombocythemia and polycythemia vera. Am J Hematol. 2011;86(4):337-42.
195. Gerotziafas GT, Depasse F, Busson J, Leflem L, Elalamy I, Samama MM. Towards a standardization of thrombin generation assessment: the influence of tissue factor, platelets and phospholipids concentration on the normal values of Thrombogram-Thrombinoscope assay. Thromb J. 2005;3:16.
196. Ramstrom S, O'Neill S, Dunne E, Kenny D. Annexin V binding to platelets is agonist, time and temperature dependent. Platelets. 2010;21(4):289-96.
197. Suzuki J, Umeda M, Sims PJ, Nagata S. Calcium-dependent phospholipid scrambling by TMEM16F. Nature. 2010;468(7325):834-8.
198. Sims PJ, Wiedmer T, Esmon CT, Weiss HJ, Shattil SJ. Assembly of the platelet prothrombinase complex is linked to vesiculation of the platelet plasma membrane. Studies in Scott syndrome: an isolated defect in platelet procoagulant activity. J Biol Chem. 1989;264(29):17049-57.
199. Brummel-Ziedins KE, Pouliot RL, Mann KG. Thrombin generation: phenotypic quantitation. J Thromb Haemost. 2004;2(2):281-8.
3.