BIOCHEMICAL AND GENETIC STUDIES OF QUEBEC PLATELET DISORDER

BIOCHEMICAL AND GENETIC STUDIES

OF

QUEBEC PLATELET DISORDER

By

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ABSTRACT

Inherited bleeding disorders can be caused by mutations affecting platelet, coagulation, or fibrinolytic proteins. Quebec platelet disorder (QPD) is a rare, autosomal dominant disorder associated with increased expression of the fibrinolytic enzyme urokinase plasminogen activator (uPA) in platelets. Individuals with QPD experience delayed-onset bleeding after hemostatic challenges that is attenuated with fibrinolytic inhibitor therapy. The aims of this thesis were to: 1) determine if increased platelet uPA contributes to QPD clot lysis in vitro; 2) investigate whether OPD individuals have increased urinary uPA. as some individuals experience hematuria; and 3) map the genetic locus of QPD, and look for the putative mutation. Studies of clot lysis indicated that OPD platelets induce a gain-of-function defect in fibrinolysis when platelets are incorporated into clots. This suggests that accelerated fibrinolysis may contribute to QPD bleeding. Studies of urinary uPA in QPD showed that uPA is not increased, indicating that hematuria in QPD is likely a consequence of increased platelet uPA. This finding also suggests that uPA overexpression in OPD may be megakaryocyte-specific. Linkage studies showed that QPD is strongly linked to a 2 megabase region on chromosome 10 that harbors the uPA gene, PLAU. No mutations in *PLAU* or its regulatory regions were identified; however, a common haplotype for a 32.5 kilobase region around PLAU, including inheritance of a rare, linked polymorphism, suggests this is the most likely locus for QPD. mRNA studies in OPD platelets showed that OPD selectively increases expression of the linked *PLAU* allele, without similar increases in megakaryocyte progenitors or in saliva. These findings implicate a *cis*-mutation near *PLAU* as the cause of OPD. This thesis provides novel insights on the fibrinolytic abnormality in QPD blood, and on the OPD genetic locus, which will be important for identifying the precise mutation that converts normally prohemostatic platelets to profibrinolytic cells.

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LIST OF SYMBOLS AND ABBREVIATIONS

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

transcription start site
chromosome conformation capture
alpha
beta
gamma
delta
α_2 -plasmin inhibitor
adenosine diphosphate
<i>trans-p</i> -aminomethyl-cyclohexane carboxylic acid; tranexamic acid
activated protein C
apolipoprotein E
AU-rich element
arginine
aspartic acid
amino terminal fragment
adenosine triphosphate
base pair
calcium
calcium/calmodulin-dependent protein kinase $II\gamma$
cyclic adenosine monophosphate
chromatin immunoprecipitation
cooperation mediator
cAMP- responsive element
diacylglycerol
ε-aminocaproic acid
extracellular matrix

EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
ELT	euglobulin clot lysis time
F	(coagulation) factor
FDP	fibrin(ogen) degradation product
FISH	fluorescence in situ hybridization
FPA	fibrinopeptide A
FPB	fibrinopeptide B
GGCX	γ-glutamyl carboxylase
Glu	glutamic acid
GP	glycoprotein
His	histidine
HIV	human immunodeficiency virus
HMW	high molecular weight
Ile	isoleucine
IP ₃	inositol triphosphate
kb	kilobase
LBS	lysine binding site
LMAN1	lectin-mannose-binding 1
LMW	low molecular weight
LOD	logarithm of odds
LRP	lipoprotein receptor-related protein
Lys	lysine
МАРК	mitogen activated protein kinase
Mb	megabase
MCFD2	multiple coagulation factor deficiency 2
МК	megakaryocyte
MMP	matrix metalloprotease
mRNA	messenger RNA
mRNABp	mRNA binding protein
orf	open reading frame

PAI-1, -2, -3	plasminogen activator inhibitor-1, -2, -3
PAP	plasmin- α_2 -plasmin inhibitor complex
PAR	protease activated receptor
PG	prostaglandin
PGI ₂	prostacyclin
Phe	phenylalanine
PIP ₂	phosphatidylinositol bisphosphate
РКС	protein kinase C
PLAU	plasminogen activator, urokinase (gene)
PLC	phospholipase C
poly-A	poly-adenylation
PSD	primary secretion defect
QPD	Quebec platelet disorder
RACE	rapid amplification of cDNA ends
RUNX-AML-1	runt-related transcription factor/acute myeloid leukemia-1
sc	single chain
SCCS	surface-connected canalicular system
Ser	serine
SNP	single nucleotide polymorphism
SPD	storage pool disorder
TAFI	thrombin activatable fibrinolysis inhibitor
tc	two chain
TEG	thromboelastography
TF	tissue factor
TFPI	tissue factor pathway inhibitor
Thr	threonine
ТМ	thrombomodulin
TNF-α	tumor necrosis factor- α
tPA	tissue plasminogen activator
ТРО	thrombopoietin
TxA_2	thromboxane A ₂

UEF	upstream enhancer factor
uPA	urokinase plasminogen activator
uPAR	uPA receptor
UTR	untranslated region
Val	valine
VCL	vinculin
VKORC1	vitamin K epoxide reductase complex subunit 1
VWD	von Willebrand disease
VWF	von Willebrand factor
Xase	tenase

INTRODUCTION

The hemostatic system is essential for preventing excessive blood loss at sites of vessel injury and for maintaining normal blood fluidity. In the process of hemostasis, platelets rapidly form a hemostatic plug to stop blood loss from injured vessels, which is reinforced by the coagulation system that generates a fibrin clot. The fibrinolytic system removes fibrin to reestablish normal blood flow. Normally, these systems are in a homeostatic balance, due to regulatory mechanisms that prevent excessive bleeding and thrombosis. Excessive bleeding can occur with inherited disorders that impair platelet adhesive function or fibrin clot formation, or that impair the control of fibrinolysis. This thesis investigated a rare, autosomal dominant fibrinolytic disorder, of unknown genetic cause, called Ouebec platelet disorder (OPD). OPD is characterized by increased expression of urokinase plasminogen activator (uPA) in platelets and delayed-onset bleeding after hemostatic challenges. My goals were to: 1) investigate the contribution of platelet uPA to the fibrinolytic abnormalities in OPD blood by studies of in vitro clot lysis; 2) determine if QPD is associated with increased uPA in urine as individuals with QPD are at risk for experiencing hematuria; and 3) gain insights on the genetic defect that causes QPD. The following sections introduce the concept of hemostasis, including the functions of platelets, the coagulation system and fibrinolytic cascade. Also introduced are the inherited disorders of hemostasis and fibrinolysis that cause bleeding. This is followed by a section on the biology of uPA, and a detailed review of the current state of knowledge on QPD.

1.1 OVERVIEW OF HEMOSTASIS

Platelets are derived from a rare population of bone marrow cells, called megakaryocytes (MKs), which originate from hematopoietic stem cells [1]. Approximately 10^{11} platelets are produced each day through a process called thrombopoiesis (see section 1.3). Platelets circulate in blood at concentrations of 150-400 x 10^{9} /L, where they are available for rapid recruitment to sites of blood vessel injury [2]. Platelets are necessary for the formation of a hemostatic plug to limit bleeding, and when activated, they enhance blood coagulation by providing a procoagulant phospholipid membrane surface for the enzymatic reactions that generate thrombin [3]. The end result is the formation of an insoluble fibrin clot. As fibrin forms, the fibrinolytic system is activated, and this generates plasmin, a broad specificity protease that breaks down fibrin and prevents pathologic thrombosis [4].

1.1.1 Hemostatic plug formation

With vessel injury, subendothelial collagen is exposed and this leads to von Willebrand factor (VWF) binding, platelet adhesion and subsequent recruitment of more platelets that form a platelet-rich plug to limit bleeding [3.5]. Binding of VWF to collagen promotes platelet adhesion to VWF by the platelet receptor glycoprotein (GP) Iba (part of the GPIb/IX/V receptor complex) [6-8]. Platelet adhesion to the vessel wall is also mediated by the collagen receptor. integrin $\alpha_2\beta_1$ and it is enhanced by GPVI, a receptor important for collagenmediated signaling that activates platelets [6,7,9-11]. The process of platelet activation leads to downstream activation of phospholipase C (PLC), which cleaves membrane phosphatidylinositol bisphosphate (PIP₂) to form the second messengers inositol trisphosphate (IP₃) and diacylglycerol (DAG) that contribute to further platelet activation [12-15]. Platelet activation causes de novo production and release of thromboxane A_2 (TxA₂) and secretion of adenosine diphosphate (ADP) from platelet dense (δ) granules (see section 1.3) [16,17]. Thrombin (generated locally by the coagulation cascade; see section 1.1.2), and secreted ADP and TxA_2 bind to their respective receptors on the platelet surface and initiate G protein-mediated signaling, producing additional DAG and IP₃ [16-18]. The IP₃ generated leads to an increase in cytosolic calcium (Ca^{2+}), through the release of sequestered Ca^{2+} from the dense tubular system or by Ca^{2+} influx across the plasma membrane [18,19]. Increased cytosolic Ca²⁺ causes platelet shape change by rearrangement of the cytoskeleton, and stimulates secretion of platelet granule contents and activation of phospholipase A2, which produces additional TxA₂ [20-22]. The DAG generated activates protein kinase C (PKC), which is involved in both platelet granule release, as well as activation of the integrin $\alpha_{IIb}\beta_3$ - a receptor for fibringen that has a key role in platelet aggregation [22-24].

ADP and thrombin stimulation of human platelets leads to inhibition of adenylate cyclase, mediated by the binding of these ligands to their G proteincoupled cell surface receptors P2Y₁₂ (ADP) and protease activated receptors (PAR) PAR1 and PAR4 (thrombin) [25-27]. This inhibition may be potentiated by epinephrine-mediated stimulation of the α_2 -adrenergic receptor [27]. The result of adenylate cyclase inhibition is a reduction in cyclic adenosine monophosphate (cAMP), which normally blocks platelet signaling and limits platelet activation [25-27]. This culminates in the recruitment and activation of additional platelets to form a large, stable aggregate. Platelet aggregation at low shear is mediated by fibrinogen, which binds to $\alpha_{IIb}\beta_3$ integrins on adjacent activated platelets and after it forms cross-bridges between platelets, clot retraction occurs [28]. At high shear, platelet adhesion to the vessel wall is mediated mainly by VWF, through its binding to GPIb/IX/V and $\alpha_{IIb}\beta_3$ [7,29]. Other adhesive proteins, including fibronectin (binds to α_5b_1 and $\alpha_{IIb}\beta_3$), laminin (binds to $\alpha_6\beta_1$) and thrombospondin-1 (binds to GPIV, GPIb and fibrin) have a secondary role in supporting plateletsubendothelium interactions [30-34] or inhibiting platelet-platelet interactions [35] at sites of injury.

Platelet aggregation can be attenuated by inhibitory agonists such as nitric oxide and prostaglandins (PG), including prostacyclin (PGI₂) and PGE₁ derived from the vessel wall, and PGD₂ synthesized by platelets [36]. G protein-coupled receptors on the platelet surface bind their respective agonist, and activate adenylate cyclase, leading to the formation of cAMP, which inhibits platelet activation through the induction of protein kinase A [37]. The drug aspirin can also effectively block platelet activation by its irreversible inhibition of cyclooxygenase 1-dependent TxA_2 formation [38]. Clopidogrel, another antiplatelet drug, selectively inhibits platelet aggregation induced by ADP [39].

Activated platelets are important for the enhancement of thrombin generation and fibrin clot formation by a number of mechanisms. With platelet phospholipids (phosphatidylserine activation. negatively charged and phosphatidylethanolamine) are repositioned from the inside to the outside layer of the platelet membrane [40]. This provides the necessary catalytic surface for assembly of the tenase (Xase) and prothrombinase complexes on platelets, which respectively activate coagulation factor (F) X and prothrombin (FII) [41] (see below). Furthermore, platelets secrete the contents of their α -granules and δ granules into clots, which alters local concentrations of coagulation factors, adhesion molecules, receptors, chemokines, and other agonists (see section 1.3). Platelets also release large amounts of the serpin (serine protease inhibitor) plasminogen activator inhibitor-1 (PAI-1), that inhibits both uPA and tissue plasminogen activator (tPA) and contributes to the resistance of platelet-rich thrombi to fibrinolysis [3].

1.1.2 Blood coagulation

The role of the coagulation system is to form an insoluble fibrin clot to stop bleeding, which allows for vessel repair to take place. Coagulation consists of a cascade of reactions involving a number of vitamin K-dependent serine proteases that circulate as zymogens, and non-enzymatic cofactor proteins that are assembled on negatively charged membrane surfaces, such as the activated platelet phospholipid surface, in a Ca²⁺-dependent manner (reviewed in [3,41,42]) (figure 1.1). For *in vitro* testing of coagulation defects, it has been useful to divide the coagulation cascade into the extrinsic (tissue factor; TF) and intrinsic (contact) pathways [3]. Both pathways lead to FX activation and converge on the common pathway, the activation of prothrombin to thrombin that generates a fibrin clot, activates platelets and FXIII, and further accelerates coagulation by activating cofactors V and VIII [3]. *In vivo*, the exposure of blood to TF is the principal mechanism for initiation of coagulation. Proteins of the contact pathway (high

molecular weight kininogen, prekallikrein and FXII) are not thought to be important for *in vivo* initiation of coagulation, as individuals with severe deficiencies of these factors do not have bleeding problems [41,43].

Coagulation in vivo consists of two phases: initiation and propagation [41]. The initiation phase occurs when the subendothelial protein TF is exposed to blood after damage to the vessel wall, or when TF is expressed on the surface of activated monocytes or microparticles [44-47]. Small amounts of the circulating serine protease FVIIa ("a" denotes activated form) bind to TF and the resulting "extrinsic Xase" complex then associates with the zymogens FIX and FX to produce the serine proteases FIXa and FXa [48,49] (figure 1.1). The FVIIa-TF complex, FIXa and FXa then activate additional FVII in blood [3]. Small amounts of FXa, formed by extrinsic Xase, assemble into the prothrombinase complex with cofactor FVa and FII to generate picomolar concentrations of thrombin (FIIa) [50] (figure 1.1). The limited amount of thrombin that is generated has several effects including: 1) partially activating platelets; 2) activating FV to FVa; and 3) activating FVIII to FVIIIa [41,51]. FVIIIa associates with FIXa and FX on membrane surfaces to form the "intrinsic Xase" complex, which activates FX at a 50-100-fold higher rate than the FVIIa-TF complex [52]. As more thrombin is produced, it serves to amplify its own production by completing the activation of platelets and activating additional FV and FVIII [51], and also by activating FXI of the intrinsic pathway (the propagation phase of coagulation) [41,53] (figure 1.1). 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 subsequent activation of FXI and FIX (figure 1.1). At the beginning of the propagation phase, thrombin also cleaves fibrinogen to fibrin and, in the presence of fibrin, activates FXIII to FXIIIa, a transglutaminase that cross-links fibrin monomers, which makes fibrin insoluble and more resistant to fibrinolysis [41,54-56] (figure 1.1) (see section 1.2).

There are a number of important regulatory factors of coagulation. First, tissue factor pathway inhibitor (TFPI) limits TF-mediated activation of coagulation as it associates with FXa and inhibits the TF-VIIa complex, preventing further activation of FIX and FX by the extrinsic pathway that limits thrombin generation [57]. Additionally, when thrombin is generated, it binds to its cofactor thrombomodulin (TM) on endothelial cells and proteolytically activates plasma protein C to activated protein C (APC), an anticoagulant, serine protease. APC, and its cofactor, protein S are involved in downregulating the procoagulant response by APC-mediated inactivation of membrane-bound cofactors VIIIa and Va before they form a complex with the serine proteases FIXa and FXa, respectively [58-60]. Coagulation is also downregulated at a number of steps by the plasma serpin antithrombin, whose inhibitory effects are potentiated by its cofactors heparin and heparan sulfate [61]. Antithrombin directly blocks the

action of fibrin-unbound thrombin, as well as inhibiting soluble factors IXa, Xa, XIa, and XIIa [62,63].

The fibrin clot, once formed, is susceptible to lysis by the fibrinolytic enzyme plasmin [4]. Release of PAI-1 from platelets can protect fibrin from premature lysis by attenuating plasmin generation [64]. Furthermore, the physiologic plasmin inhibitor α_2 -plasmin inhibitor (α_2 -PI) circulates in plasma at high concentrations (1 μ M) and it directly blocks the actions of plasmin on fibrin [65]. In addition, thrombin, in complex with TM, acts on thrombin-activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase that cleaves exposed C-terminal lysine residues on fibrin strands [66]. Exposed lysines on fibrin are recognized by lysine-binding sites (LBS) on plasminogen, whose binding to fibrin is important for fibrinolysis [4]. These processes are described in detail in the next section.



Figure 1.1: The coagulation cascade: intrinsic and extrinsic pathways (reviewed in [3.41]). Coagulation involves a cascade of reactions between the zymogen forms of serine proteases and non-enzymatic cofactor proteins. Complexes between zymogens and cofactors are assembled on phospholipid surfaces (+PL) in a calcium-dependent (+ Ca^{2+}) manner. In the extrinsic pathway, exposure of the subendothelial protein tissue factor leads to binding of small amounts of circulating factor VIIa ("a" denotes activated form), and this complex activates factor IX and factor X in blood. In the intrinsic pathway, contact with negatively charged surfaces ex vivo activates soluble factor XII, which leads to activation of factor XI, and then factor IX. Assembly of factor IXa with its cofactor factor VIIIa results in large amounts of factor Xa generation. Factor Xa assembles into the prothrombinase complex with its cofactor Va to generate thrombin (factor IIa) from prothrombin (factor II). Thrombin then converts soluble fibrinogen into insoluble fibrin, and activates factor XIII, which crosslinks (XL) fibrin strands in the clot. The generation of thrombin causes a positive feedback mechanism that amplifies further thrombin production by activating additional factor V, factor VIII, and factor XI. (Figure modified from: [3]).

1.1.3 Fibrinolysis

Fibrinolysis is the process of thrombus dissolution, which is necessary for the reestablishment of normal blood flow after clotting [4]. It is a highly localized process, and the fibrin generated by clot formation has enhancing effects on the activation of fibrinolysis [4,64]. The central zymogen of the fibrinolytic system is plasminogen, the precursor of the serine protease plasmin. Plasminogen is incorporated into forming clots by binding to exposed lysine residues on fibrin via its LBS [4]. Plasminogen may also associate with other cells in the thrombus (eg. platelets, endothelial cells, monocytes, neutrophils), through the interactions of its LBS with lysine residues on cell-surface proteins, which may potentiate local plasmin generation [67-69].

Plasminogen is a single-chain (sc) GP (92 kDa; 791 amino acids) that circulates at high concentrations in plasma (2 µM), and some is also stored in platelet α -granules [4]. Native plasminogen has a glutamic acid residue (Glu) at its N-terminus, and thus it is also known as Glu-plasminogen [4]. Another form, lysine (Lys)-plasminogen, also exists that is missing the first N-terminal 77 amino acids and it has a shorter half-life in the circulation [70]. The two main physiological activators of plasminogen are uPA and tPA, which cleave at a single peptide bond to generate a two-chain (tc) plasmin molecule [71] (see below). SctPA is synthesized and secreted by endothelial cells, and is activated to the more active form, tctPA, by plasma kallikrein, FXa, or plasmin [4]. ScuPA, which is produced by many types of cells and circulates in the plasma, is converted to tcuPA by plasma kallikrein, FXIIa, or plasmin [4] (see section 1.5). The activation of plasminogen can be mediated by tcuPA or tctPA, but the end result is the same: Glu- plasminogen is converted to plasmin (Lys-plasmin) by cleavage at the Arg⁵⁶¹-Val⁵⁶² bond [72] (figure 1.2). Lys-plasmin has a greater affinity for binding to fibrin than Glu-plasmin and a greater reactivity with plasminogen activators, thus promoting further plasmin generation [73,74]. Of the heavy chain (A) and light chain (B) of plasmin, the A-chain is important for fibrin binding and interactions with cell surfaces and inhibitors, while the B-chain contains the enzymatic serine protease site [75]. Cells associated with the thrombus, such as endothelial cells and leukocytes, which bind plasminogen, may also release and bind plasminogen activators (either uPA, tPA, or both), and thus plasmin generation can be facilitated on cell surfaces, in addition to fibrin [76-79]. Localized fibrinolytic activity requires the formation of a ternary complex of plasminogen activator-plasminogen-fibrin [80,81]. Although tPA can directly bind to fibrin, uPA comes in close contact with fibrin by binding to its cell surface receptor, uPA receptor (uPAR), expressed on several cell types associated with the thrombus (see section 1.5).

Once plasmin is generated, it hydrolyzes susceptible lysyl and arginyl bonds of fibrin to expose new lysine residues with increased affinity for plasminogen and tPA [4,82]. Furthermore, plasmin converts more Glu- to Lys-

plasminogen, to induce a positive feedback effect on fibrinolysis. Cleavage of cross-linked fibrin produces unique fibrin degradation products (FDPs) of variable sizes, the smallest of which is the D-dimer [83] (see section 1.2). This process is much slower than for plasmic degradation of non-cross-linked fibrin or fibrinogen, due to the relative insolubility of cross-linked clots. Other coagulation and platelet proteins are also substrates for plasmin, including: FV [84], FVIII [85], FX [86], TFPI [87], fibronectin [88], thrombospondin-1 [88], VWF [88,89], multimerin 1 [90], GPIb [91], and $\alpha_{IIb}\beta_3$ [92]. In addition to having a key role in fibrinolysis, plasmin has additional roles in extracellular matrix (ECM) degradation, tissue and vascular remodeling, cell migration, angiogenesis and tumor biology (reviewed in [93,94]).

Fibrinolysis is regulated by several proteins, including TAFI, PAI-1, α_2 -PI, and α_2 -macroglobulin (figure 1.3) [4,95]. During coagulation, TAFI is activated by the thrombin/TM complex (see section 1.1.2), but it may also be activated by plasmin(ogen) [96]. The removal of C-terminal Arg and Lys residues on fibrin by TAFIa eliminates the positive feedback steps in plasminogen activation by reducing the availability of the substrate for the reaction [96]. The serpin PAI-1, which can exist in both active and latent forms, is released primarily from platelets and endothelial cells [97-100]. It irreversibly inhibits tPA and uPA by interacting with their active site to form a 1:1 stoichiometric complex (see section 1.5) [101]. In plasma, PAI-1 circulates bound to vitronectin, which maintains its active conformation and enhances plasminogen activator inhibition [102,103]. In platelets, PAI-1 exists in both forms, although less than 5% appears to be active [97,104]. It can be activated in vitro by negatively charged phospholipids, suggesting that it may also be converted to the active form by activated cells during hemostasis [105]. PAI-1 may also exert local activity on fibrin by binding directly to fibrin strands and suppressing plasminogen activator activity by competitive binding [106]. α_2 -PI, the primary inhibitor of plasmin, circulates in blood, but is also released from platelets and acts in several ways: 1) it inhibits plasmin(ogen) irreversibly by binding to the catalytic site in the light chain, forming the plasmin- α_2 -PI (PAP) complex; and 2) it blocks LBS on plasmin(ogen), thereby inhibiting plasminogen interactions with fibrin [65,107-109]. Furthermore, FXIIIa can crosslink α_2 -PI to fibrin, rendering thrombi more resistant to fibrinolysis [110]. Finally, α_2 -macroglobulin is another plasma inhibitor of plasmin that exerts its role when the capacity of α_2 -PI is exceeded by plasmin, and it also inhibits tPA and uPA [111].

Although these inhibitors are effective in attenuating fibrinolysis, their effects are reduced when plasminogen activators or plasmin(ogen) are already associated with fibrin or with their cell-surface receptors during fibrin formation and fibrinolysis [4]. This protects plasmin(ogen), uPA and tPA from inactivation, and promotes plasmin generation at the fibrin surface [4]. Because fibrin is a

necessary substrate for fibrinolysis to take place, the degradation of fibrin limits further activation of plasminogen, which eventually attenuates fibrinolysis [112].



Figure 1.2: Plasminogen activation [4,95]. The interaction of plasminogen activators (uPA or tPA) with Glu (G)-plasminogen (left side) results in the generation of trace amounts of plasmin (G-plasmin) by cleavage of the Arg (R)561-Val (V)562 bond. This is followed by plasmic cleavage of the N-terminus of G-plasmin to generate Lys (K)-plasmin, which is missing 77 amino-terminal residues. Plasmin is also capable of cleaving the N-terminus of G-plasminogen (right side) to produce a more reactive form, K-plasminogen. This is then activated by uPA or tPA to produce K-plasmin, which is the most active form of the enzyme. The two chains (heavy chain, HC; light chain, LC) of plasmin are held together by two disulfide (cysteine) bonds (C548-C666, and C558-C566) (Figure modified from: [95]).



Figure 1.3: Fibrinolysis and its regulation (reviewed in [4,95]). Fibrinolysis is regulated by several different mechanisms, some of which are activated during coagulation. The principal zymogen of the fibrinolytic system, plasminogen, is activated to plasmin by urokinase- and tissue-type plasminogen activators (uPA and tPA). The action of uPA and tPA on plasminogen is inhibited by plasminogen activator inhibitor-1 (PAI-1), released from endothelial cells (ECs) and platelets upon thrombin stimulation. Plasmin hydrolyzes cross-linked (XL) fibrin into unique fibrin degradation products (FDPs), the smallest of which is the D-dimer. This process is inhibited by α_2 -plasmin inhibitor (α_2 -PI) and α_2 -macroglobulin $(\alpha_2$ -MG). Plasmin also activates thrombin activatable fibrinolysis inhibitor (TAFI), a carboxypeptidase that cleaves exposed C-terminal lysine residues on fibrin to limit plasmin(ogen) binding via lysine-binding sites (LBS). TAFI's principal activator is thrombin bound to thrombomodulin (TM). Generation of thrombin during coagulation is attenuated by antithrombin (AT) and by activated protein C (APC), which inactivates factors VIIIa and Va, the cofactors in Xase and prothrombinase complex generation.

1.1.4 Fibrinolytic inhibitor drugs

Several synthetic and naturally-occurring inhibitors have been used to attenuate fibrinolysis in individuals with bleeding problems [113,114]. Lysine analogues are a class of synthetic antifibrinolytic compounds that include ε -aminocaproic acid (EACA) and *trans-p*-aminomethyl-cyclohexane carboxylic acid (tranexamic acid; AMCA). These drugs act by inhibiting the interactions of plasmin(ogen) with fibrin, through competitive binding to LBS on plasmin(ogen) [115]. *In vitro*, lysine analogues cause a conformational change in plasminogen upon binding, which paradoxically accelerates plasminogen activation, but plasmin is still inhibited from interacting with the fibrin substrate because the LBS are occupied [115,116]. It is not known if this accelerated activation occurs *in vivo* [115,116].

Another drug that has been used to attenuate fibrinolysis is the broadspecificity serpin of the Kunitz family aprotinin [117]. Aprotinin is derived from bovine lung tissue and it has a high affinity for plasmin [118,119]. It acts by binding to the catalytic site Asp residue of plasmin, to form a stable complex [119]. Aprotinin is a very effective plasmin inhibitor in the presence of a fibrin clot and maintains its inhibitory capacity even when binding sites for α_2 -PI and α_2 -macroglobulin on plasmin are occupied [113]. The inhibition is further potentiated by the fact that plasmin cannot hydrolyze aprotinin upon binding [113]. Recently, aprotinin was identified to increase thrombotic risks when given in certain clinical situations (e.g. cardiopulmonary bypass surgery) [120].

1.2 FIBRIN DEGRADATION BY PLASMIN: EFFECTS ON CLOT ARCHITECTURE

Fibrin is one of the important substrates for plasmin and its formation also provides a stimulus for plasminogen activation [112]. The rate and extent of fibrinolysis is dependent on the structural features of the fibrin clot that is generated, the availability of different activators and inhibitors of the system, and the interactions of these components with fibrin.

1.2.1 Fibrin production, polymerization and cross-linking

Fibrinogen is a soluble protein that is cleaved by thrombin to form fibrin, the main protein component of clots. Fibrinogen is a dimeric protein, whose monomers are made up of three pairs of disulfide-bonded polypeptide chains, designated A α , B β , and γ , which assemble into a symmetrical hexamer with three

globular domains [121]. The two peripheral domains of fibrinogen are the D domains, and these are connected to the central E domain by α -helical coiled-coils [121]. Fibrinogen circulates in blood as a non-covalent complex with FXIII, which following activation, forms FXIIIa, a transglutaminase enzyme that is localized to the fibrin clot [122].

Thrombin hydrolyzes fibrinogen to fibrin by cleavages at specific Arg-Gly bonds, located at the N-terminal ends of the A α and B β chains, to release two short fibrinopeptides, fibrinopeptide A (FPA) and fibrinopeptide B (FPB), respectively from the central E domain [121]. Cleavage of FPA occurs first, to produce an intermediate molecule called a fibrin monomer, which can spontaneously polymerize into a two-stranded, half-staggered structure known as the protofibril. Protofibrils are stabilized by non-covalent interactions between a region on the central E-domain of one fibrin monomer and the outer D-domain of another [123,124]. The release of FPA is followed by FPB release, which allows for several protofibrils to undergo lateral aggregation and twist around each other to make up a thicker fibrin fiber [125]. The three-dimensional network of the clot is a result of fiber branching, the most important characteristic of clots in terms of their biophysical and fibrinolytic properties [126]. Fiber branching makes clots more dense, less porous, less susceptible to lysis, and contributes to fibrin elasticity [126].

The release of FPA enhances FXIII activation by thrombin [127]. The final step in clot formation is cross-linking by FXIIIa, which creates covalent isopeptide bonds between adjacent fibrin molecules in the same strand of a protofibril [128]. The unique bonds formed in cross-linked fibrin are responsible for the liberation of specific degradation products during plasmin proteolysis [129]. The smallest unique degradation product, the D-dimer, consists of fragment D moieties of two adjacent covalently bound fibrin monomers [83]. Fragment E and noncovalent complexes of D-D with E have also been identified, as well as larger fragments, but these are eventually degraded to the smallest sizes [83,130].

1.2.2 Structural features of fibrin clots affecting fibrinolysis

Fibrin clots can vary in their size, density, and viscoelastic properties which are dependent on the structure of the fibrin strands, their degree of crosslinking and association with other cells in the thrombus. All of these properties contribute to clot stability, which is the resistance of a clot to mechanical stress and fibrinolytic dissolution [126,131,132].

Several factors contribute to the mechanical properties of fibrin strands, including fibrinogen concentrations, fiber branching, fiber diameter and FXIIIainduced cross-linking. High fibrinogen concentrations result in the production of dense, highly branched fibrin fibers [133]. Fiber branching is a key architectural detail because it is associated with thinner fibers and more elastic clots [125]. Fibrin clots composed of thin fibers have been shown to dissolve more slowly than those made from thicker fibers, as the conversion of plasminogen to plasmin by tPA or uPA occurs more slowly in the presence of thin fibers [131,132]. Finally, cross-linking by FXIIIa enhances the elasticity and mechanical strength of fibrin clots, and increases their resistance to lysis by stabilizing interactions between preassembled protofibrils [134].

The incorporation of platelets contributes unique architectural qualities to forming clots and confers increased resistance of thrombi to fibrinolysis [126,135-140]. One factor that reduces the speed of lysis of platelet-rich compared to platelet-poor clots, is the release of large amounts of PAI-1 from activated platelets [139,141,142]. In addition, the integrin receptor for fibrinogen, $\alpha_{IIb}\beta_3$, contributes to platelet aggregation and clot retraction [135,136,143], and the interactions between platelets and fibrin via $\alpha_{IIb}\beta_3$ are a key contributor to resistance of platelet-rich thrombi to lysis [137,138,140]. This could be because: 1) fibrin binding to its receptor creates local areas of high fibrin concentration, which may limit the diffusion of fibrinolytic proteins through the clot [126,138,144]; or 2) compression of the clot may extrude fluid containing any plasminogen or plasminogen activator molecules that are not tightly bound to the thrombus [138,145]. Finally, fibrin(ogen) can also bind to other platelet-associated clot components like thrombospondin-1 and VWF, further enhancing platelet-fibrin and platelet-platelet contacts [142,143,146,147].

1.2.3 Effects of plasmin on clot morphology

An examination, by scanning electron microscopy, of the surfaces of clots being digested by plasmin, shows that fibrinolysis creates many free fiber ends and gaps in the continuity of fibers within clots [148]. These changes are associated with the large complex fragments, with portions of individual fibers/protofibrils, in clot supernatants [148]. The very large pieces liberated suggests that the mechanism of fibrin degradation is that of transection, rather than surface erosion of fibers [126]. Progressive transection of fibers eventually leads to clot collapse [149].

In clots containing platelets, fibrinolysis is visibly heterogeneous, as lysis proceeds faster in platelet-poor regions compared to platelet-rich regions, with platelet-rich regions being left largely undegraded [137]. The protective effects of platelets on platelet-rich clots are attenuated by platelet activation or platelet aggregation ($\alpha_{IIb}\beta_3$) inhibitors, such as aspirin or abciximab [137,140,150,151]. These drugs prevent platelet-mediated organization of the fibrin network (via $\alpha_{IIb}\beta_3$), and attenuate release of PAI-1, thus increasing fibrinolysis rates. The incorporation of other cells into a thrombus, including leukocytes, may also have

an effect on the rate and efficiency of fibrinolysis as leukocytes bind to fibrin and platelets and are capable of releasing plasminogen activators [76,152-154].

1.2.4 Laboratory assays of fibrinolysis

Biochemical assays of fibrinolysis include measuring plasma levels of fibrinolytic proteins, and assays of clot lysis times, plasmin generation and the degradation of crosslinked fibrin by plasmin [155-158]. The euglobulin clot lysis time (ELT) is an assay that uses the euglobulin fraction (acid insoluble proteins) from plasma (contains fibrinogen, plasminogen activators and fibrinolytic inhibitors) to assess fibrinolysis [159,160]. The euglobulin fraction is precipitated, dissolved, clotted in a borate buffer by adding Ca^{2+} at 37°C, and then the clot lysis time is measured, with short lysis times indicating accelerated fibrinolysis. ELT is influenced by concentrations of plasminogen activators and PAI-1, so short ELT can indicate both increased plasminogen activator activity or reduced inhibitory capacity [159]. Quantitative assays for various components of the fibrinolytic system in vivo include enzyme-linked immunosorbent assays (ELISA) of plasminogen activators, plasminogen, α_2 -PI, plasmin generation (measurement of PAP complexes in blood), and D-dimer (measurement of degraded cross-linked fibrin) [156,157,161]. D-dimer levels can also be evaluated using semiquantitative, latex bead agglutination assays. In this type of assay, latex beads are coated with antibodies against unique epitopes on crosslinked fibrin, and agglutination occurs in the presence of D-dimers [162]. Other quantitative assays of fibrinolysis include: chromogenic assays, which can be used to measure levels of plasminogen activators, plasmin generation, or inhibitors of fibrinolysis based on their cleavage of specific chromogenic substrates [163,164]; turbidimetric assays, which measure changes in optical density as a fibrin clot is lyzed in vitro [165-167]; and radioisotope assays, which can be used to measure release of soluble, radiolabeled fibrin(ogen) from clots with lysis [168]. These clinical assays of fibrinolysis typically use blood plasma for measurement of fibrinolytic markers, but other types of samples, including serum and the supernatants of fibrin clots can also be used to measure changes in fibrinolytic protein levels. Fibrinolysis may also be evaluated using in vitro flow chamber assays, in which surfaces that are pre-coated with fibrin are perfused with blood, and the effects of different blood components (platelets, fibrinolytic proteins, inhibitors) on the fibrin substrate is assessed by microscopy or by measuring FDPs in the collected fluid after it flows through the chamber ([169] and see chapter 2). Finally, zymography and fibrin substrate gels have been used to evaluate the effects of solutions of fibrinolytic proteins on fibrin breakdown [170].

Thromboelastography (TEG) is a point-of-care method used to measure the viscoelastic changes that occur during clot formation in whole blood *ex vivo* [171-173]. The predominant clinical use of TEG has been to assess clot formation

and lysis in the settings of liver transplantation and cardiopulmonary bypass surgery [174,175]. TEG has been investigated for potential uses in evaluating other aspects of platelet function, coagulation and fibrinolysis, including the effects of different hemostatic treatments [173,176-182]. TEG is typically performed by mixing citrated whole blood with added Ca²⁺ and thrombin or TF to initiate coagulation [171]. Clot formation, strength and lysis are assessed at 37°C by changes in tortion measured by a pin in a cup for up to 3 hours after initiation. The measured parameters include: the time for clot formation; the strength of the clot (maximum amplitude); and the efficacy of fibrinolysis (reduction in amplitude) [171]. Figure 1.4 shows typical TEG traces for normal, hypercoagulable and hyperfibrinolytic states.



Figure 1.4 Schematic representations of thromboelastography (TEG) traces. TEG is used to measure the viscoelastic changes in whole blood when it is clotted *ex vivo*. The measured parameters include: 1) r time, the time to clot formation; 2) maximum amplitude (MA), an indicator of clot strength; and 3) reduction in amplitude (occurs after MA), indicating the efficacy of fibrinolysis. Panels A, B and C show typical traces for normal, hyperfibrinolytic, or hypercoagulable states, respectively. (Figure modified from: [171]).

1.3 PLATELETS: ORIGINS AND FUNCTION

1.3.1 Megakaryocyte differentiation

Platelets are derived from hematopoietic stem cells in the bone marrow, which first differentiate to a common myeloid progenitor that further differentiates into MK and other myeloid lineages [183]. There are three stages of MK differentiation: proliferation, endoreplication, and maturation [184]. Normal megakaryopoiesis is regulated by the cytokine thrombopoietin (TPO), which binds to the *c-mpl* receptor to stimulate MK differentiation [185]. In the endoreplication phase of megakaryopoiesis, repeated cycles of DNA replication occur without cell division and this generates polyploid MK (2N up to 128N), with an expanded cytoplasmic mass that is thought to be important for platelet production [186]. TPO is the signal that stimulates MKs to increase in cell size and ploidy, and to form proplatelet processes that fragment into single platelets which are released into the circulation [185] (see below). The process also involves transcription factors, which are discussed in chapter 5.

MKs at different stages of differentiation can be distinguished by cellsurface markers [184]. CD34 (a marker on hematopoietic stem cells and early MKs) expression begins to disappear as CD41 (the $\alpha_{IIb}\beta_3$ integrin that is expressed only by MKs) expression emerges. CD41 expression is followed by CD42 (the GPIb/IX/V complex), which marks later differentiation steps and correlates with increased expression of mpl, GPVI, $\alpha_2\beta_1$, and with detection of α -granule proteins (eg. VWF). β 1-tubulin, which composes microtubules, is expressed at the stage of proplatelet formation [184].

1.3.2 Platelet release into the circulation

Each MK is capable of giving rise to 1000-5000 platelets, a process which involves fragmentation of pseudopodial projections, called proplatelets, to release platelets into the circulation [187]. During the proplatelet phase, organelles and granules are delivered to the ends of proplatelets along microtubules [188]. The ends of proplatelets are also the site for the assembly of the final platelet cytoskeleton. Microtubule bundles extend through the length of the proplatelets and continue into the nascent platelet buds, where they loop back to re-enter the proplatelet shaft and twist into a coil, which releases the platelet [188,189]. Platelets contain organelles, granules, soluble macromolecules, messenger RNA (mRNA) and pre-mRNA (mRNA that has not yet undergone post-transcriptional processing), but they do not have nuclei or contain DNA [190,191]. However, platelets are capable of some *de novo* protein synthesis (they contain ribosomes), and this has been demonstrated for several proteins [192-194]. Following release

into blood, platelets survive in the circulation for about 8-9 days. MKs can respond to changes in platelet requirements (eg. in conditions of thrombocytopenia) by increasing platelet production more than 10-fold [195]. A constant supply of functioning platelets is physiologically important as platelets support hemostasis, and are thought to have roles in inflammation, innate immunity, wound healing and neoangiogenesis [196].

1.3.3 Platelet granules

Platelet granules are formed early in MK maturation as protein trafficking through the trans-golgi network leads to formation of small vesicles that transit through multivesicular bodies, then increasing in density until they reach their mature granule state [197]. The largest and most abundant platelet granules are the α -granules (40-80 per platelet), which contain diverse MK-synthesized and plasma-derived proteins [190,196,198] (see table 1.1). MK- synthesized proteins include: adhesive proteins, coagulation proteins, inhibitors, and other mediator proteins. Many MK-synthesized proteins are found in low or undetectable levels in plasma. Exceptions include VWF and PAI-1 (also made by endothelial cells) and fibronectin, which are found in high concentrations in plasma [190,198]. Some proteins stored in platelets are derived from plasma by receptor-mediated endocytosis (eg. fibrinogen via $\alpha_{IIb}\beta_3$), or by fluid-phase pinocytosis (eg. albumin) of plasma [198-201]. The origin of platelet plasminogen has not yet been determined, but recent evidence suggests it is probably plasma-derived (see Appendix I). The α -granule membrane contains platelet adhesion receptors, such as $\alpha_{IIb}\beta_3$, and activation molecules, such as P-selectin (table 1.1). These α -granule membrane proteins are translocated to the plasma membrane upon platelet activation and contribute to additional cell recruitment (platelets, leukocytes) to sites of injury. This increases the numbers of adhesive receptors on the outer surface of platelets by about one third [190].

Platelet δ -granules store ADP, adenoside triphosphate (ATP), Ca²⁺, magnesium, serotonin and pyrophosphate and platelets contain much smaller numbers of these granules (2-7 per platelet) than α -granules [196]. δ -granule membranes also contain receptors, such as $\alpha_{IIb}\beta_3$, and P-selectin. Platelets also have lysosomes, similar to other cells [196].

Table 1.1 MK-synthesized and plasma-derived α-granule proteins.

Data compiled from reviews [190,196,198]. *indicates platelet-specific proteins; *indicates proteins also present on the plasma membrane; [†]indicates unknown origin, although some evidence supports plasma origin (see Appendix I).
1.3.4 Proteome and transcriptome studies of megakaryocytes/platelets

The vast array of proteins and mRNA molecules present in platelets/MKs has been elucidated by studies of the platelet proteome and transcriptome [202-204]. Platelets contain an estimated 3000-6000 transcripts, despite their limited amounts of total RNA [205]. These studies have identified some differences in the transcripts expressed by platelets from healthy individuals and those with platelet/MK-related disorders [206-209]. Furthermore, proteomic studies comparing resting and activated platelets have revealed some previously unknown proteins that are released from platelets upon their stimulation [210,211].

1.3.5 Platelet activation

Circulating platelets have a compact discoid shape in their inactive state, but are capable of increasing their surface area upon their activation. Normally, the resting platelet shape is maintained by microtubules and the cytoskeleton, and during activation, the microtubule coils constrict, allowing platelets to become spherical and extend filopodia [212]. With platelet activation, secretory granules are directed toward the cell center, where they can fuse with the surface-connected canalicular system (SCCS), a network of invaginated channels through the interior of the platelet that is connected to the plasma membrane [196]. When platelet granules fuse with the SCCS, they release their contents into the lumen [213] and the α -granule membrane proteins are incorporated into the SCCS membrane, where they can redistribute onto the plasma membrane [196,214].

Human platelet activation by thrombin, through PAR1 and PAR4, activates signaling pathways that lead to a conformational change in $\alpha_{IIb}\beta_3$ that initiates fibrinogen binding and further signal transduction [215,216]. This process leads to polymerization of submembranous actin, causing platelet shape change [217,218]. In the final steps of activation, platelets also shed procoagulant microparticles, which have several functions similar to platelets including: 1) support of coagulation, by formation of Xase and prothrombinase complexes; 2) participation in recruitment of more platelets to the site of injury (via $\alpha_{IIb}\beta_3$); and 3) participation in recruitment of monocytes and neutrophils (via P-selectin) [219]. Platelet activation is marked by the presence of P-selectin on the platelet surface, as well as the release of soluble platelet-specific α -granule proteins, including β -thromboglobulin and platelet factor 4 [220].

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1.4 INHERITED BLEEDING DISORDERS

The important role of soluble coagulation factors, platelets and proteins of the fibrinolytic system in hemostasis is highlighted by the abnormal bleeding that occurs in genetic disorders that impair the production or functions of coagulation factors, platelets, or fibrinolytic inhibitors. Some bleeding disorders are due to inherited abnormalities in the vessel wall and connective tissue [221,222].

1.4.1 Disorders of coagulation

Hemorrhagic disorders of coagulation factors can have mild, moderate and/or severe forms. The most common inherited coagulation disorders are hemophilia A and B, which have a prevalence in the general population of 1 in 10,000 and 1 in 50,000, respectively [223]. Hemophilia A (reductions in FVIII) and B (reductions in FIX) are X-linked disorders, and more commonly affect males [224]. Other coagulation factor disorders are mainly transmitted as autosomal recessive traits (see table 1.2), with homozygotes or compound heterozygotes (factor levels $\leq 10\%$) presenting with abnormal bleeding [223]. Although autosomal recessive bleeding disorders are generally rare, they can be prevalent in regions where consanguineous marriages are common, where their prevalence can exceed that of hemophilia B [223]. For some diseases, no known living individuals exist with complete deficiencies, suggesting that complete deficiency of some factors (e.g. FII, FX) is incompatible with life, as further suggested by data from knockout mouse models [223,225].

An important clinical manifestation of inherited coagulation factor deficiencies is excessive or prolonged bleeding with hemostatic challenges (eg. dental extractions, surgery) and during menses and childbirth in women [223]. The diagnosis is established by assessment of a patient's bleeding history, laboratory tests to determine the specific defect in coagulation, and genetic testing, if available (reviewed in [226]). The causative mutations of characterized coagulation factor disorders include: reduced or abnormal clotting protein production, deficient secretion, or reduced function [223,227]. In rare instances, the defect is in a gene that encodes a protein that is key for the processing or transport of coagulation factors into blood [226,228-230]. For example, combined FV and FVIII deficiency is caused by defects in the lectin-mannose-binding 1 (LMAN1) or multiple coagulation factor deficiency 2 (MCFD2) genes, which are involved in intracellular transport of factors V and VIII [223,229]. Combined, inherited deficiencies of vitamin K-dependent factors, are caused by defects in the genes encoding vitamin K epoxide reductase complex subunit 1 (*VKORC1*) or γ glutamyl carboxylase (GGCX), two enzymes involved in vitamin K metabolism and post-translational modifications of vitamin K dependent coagulation factors [223,230].

The types of DNA mutations responsible for inherited coagulation factor deficiencies are extremely heterogeneous, and range from single base-pair changes (missense or nonsense mutations, which are common and cause amino acid substitutions or premature stop codons, respectively), insertions, small deletions, frameshift, splicing and promoter mutations ([227]; reviewed in [223,224]). Large gene deletions are rare but have been described for several disorders (fibrinogen, FVIII, FX, and FXIII deficiencies) [231-234]. Inversions involving introns 1 or 22 of the FVIII gene are found in a large proportion of individuals with severe hemophilia A [234,235]. Gene duplications or amplifications have never been reported as causes of bleeding disorders [223,224].

X-linked	Autosomal Recessive	Autosomal Dominant
FVIII deficiency (hemophilia A) FIX deficiency (hemophilia B)	Afibrinogenemia Hypofibrinogenemia Dysfibrinogenemia FII (prothrombin) deficiency FV deficiency FXI deficiency FXII deficiency Combined FV and FVIII deficiency [†] Vitamin K-dependent factor deficiencies (FII, FVII, FIX, FX, Protein S, Protein C) [‡]	Hypofibrinogenemia Dysfibrinogenemia

Table 1.2. Types of inherited coagulation factor disorders.

Reviewed in [223,228]. These disorders are caused by mutations in the genes 1) encoding coagulation factors; 2) [†]lectin mannose-binding 1 (*LMAN1*) or multiple coagulation factor deficiency 2 (*MCFD2*); 3) [‡]vitamin K epoxide reductase complex subunit 1 (*VKORC1*) or γ -glutamyl carboxylase (*GGCX*). In inherited fibrinogen disorders, mutations have been described in the genes encoding the A α -, B β - or γ - chains.

1.4.2 Disorders of primary hemostasis

Disorders of primary hemostasis (hemostatic plug formation) include von Willebrand disease (VWD) and platelet disorders. These disorders are usually manifested by rapid onset, excessive or prolonged bleeding following surgery or trauma [228].

von Willebrand Disease, VWD

VWD is a common inherited bleeding disorder (prevalence is 1 in 1000 or more in the general population), caused by quantitative or qualitative deficiencies of VWF, and may also be associated with reductions of FVIII in blood [228,236]. Three subtypes of VWD exist, as follows: Type 1 (autosomal dominant) is a partial, quantitative deficiency of VWF (60-80% of cases), whereas Type 3 (autosomal recessive) is a virtual absence of VWF antigen (reviewed in [236]); Type 2 VWD is due to qualitative defects in VWF and is further subdivided into four types: Types 2A and 2B VWD (autosomal dominant) are associated with loss of high molecular weight VWF multimers, and the type 2B form is distinguished by an abnormally high affinity of VWF for GPIb; Type 2M (autosomal dominant) is characterized by reduced binding of VWF to GPIb without loss of high molecular weight multimers; and Type 2N (autosomal recessive) is associated with a defect in the FVIII binding properties of VWF, resulting in low FVIII levels [236]. Many forms of VWD result from mutations in the VWF genes [237,238].

Platelet disorders

Platelet disorders include common and rare, inherited and acquired types of bleeding disorders [239]. Mucocutaneous bleeding is a common feature of platelet disorders, and it can manifest by ecchymosis (bruising), epistaxis (nosebleeds), oral bleeding, and menorrhagia [228]. The diagnosis of a platelet disorder is often based on the bleeding history, platelet function testing (eg. aggregation responses to various agonists), microscopic evaluation of platelets, and genetic testing, if available [228]. Platelet disorders can be caused by qualitative or quantitative defects of: membrane glycoproteins that function as receptors for hemostatic and adhesive proteins; receptors for platelet agonists; platelet granules; signaling molecules; structural proteins; and in one case, a defect of phospholipid translocation across the plasma membrane that impairs platelet procoagulant activity (reviewed in [239-241]). Some platelet disorders, including several that affect α - and δ -granules, are also associated with thrombocytopenia [239,242]. QPD is a platelet α -granule disorder, associated with mild thrombocytopenia or low-normal platelet counts, and is also classified as a fibrinolytic disease due to the marked overexpression of uPA in platelets of individuals with this condition [243]. QPD is unique from other platelet disorders because the bleeding with hemostatic challenges is delayed in onset (12-24 hours after hemostatic challenge) (reviewed in section 1.6).

Primary secretion defects (PSD), conditions that impair intracellular signal transduction pathways, are the most common type of platelet disorder. These disorders are very heterogeneous and are generally associated with mild bleeding problems [244,245]. Receptor defects have been documented for TxA_2 , collagen, and ADP. PSD also include defects in G protein activation, phosphatidylinositol metabolism, and abnormalities in arachidonic acid pathways and TxA_2 synthesis (reviewed in [246]). Table 1.3 summarizes the features and inheritance of receptor-associated PSD, as well as other, rare, platelet disorders that are often associated with severe bleeding.

Mutations in the different genes responsible for causing platelet disorders range from single base missense and nonsense mutations or deletions, to frameshift mutations and whole gene deletions that affect protein structure and function [241]. Chromosomal abnormalities such as inversions or rearrangements are rarely found in platelet disorders, although they have been described for some cases of Glanzmann thrombasthenia and Wiskott-Aldrich syndrome [247,248]. Gene amplifications have not been described. For some platelet disorders, including some storage pool disorders (SPDs), the causative molecular defects have not yet been elucidated (see table 1.3) [239-242]. The studies done in Chapter 4 of this thesis were focused on identifying potential candidate genes for QPD, a SPD with as yet unidentified genetic causes.

Some platelet disorders are caused by genetic defects in transcription factors that are necessary for normal MK differentiation and maturation (reviewed causing in [249]). These include mutations in: GATA-1, X-linked thrombocytopenia [250,251]; Jacobsen/Paris-Trousseau Fli-1. causing thrombocytopenias [252,253]; and runt-related transcription factor/ acute myeloid leukemia-1 (RUNX/AML-1), causing familial platelet disorder with predisposition to acute myeloid leukemia [254]. Since these transcription factors act at both the early and late stages of megakaryopoiesis, defects in these proteins result in abnormal MK maturation and reduced platelet production [249] (see chapter 5 for further discussion).

Table 1.3. Inh	erited platelet	disorders.
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Disorder	Platelet	Structural	Phenotype	Inheritance	
	Count	abnormality/			
		Genetics			
Bernard-Soulier	Reduced	Mutations in GP	Decreased binding of	Autosomal	
Syndrome† [255,256]		Ibα, Ibβ, or IX	GPIb/IX/V complex to VWF	recessive	
Platelet-type von	Reduced	Mutations in GPIba	Spontaneous binding of	Autosomal	
Willebrand disease	- normal		GPIbα to VWF; clearance of	dominant	
[257]			v wF multimers from		
Glanzmann	Normal	Mutations in GP IIb	Dysfunctional/ deficient	Autosomal	
thrombasthenia		(α_{IIb}) or GP IIIa (β_3)	$\alpha_{IIb}\beta_3$; absent platelet	recessive	
[258, 259]			aggregation (all agonists); defective clot retraction		
Collagen receptor	Normal	Unknown molecular	Reduced collagen	Unknown	
defects [‡] [260]		defect	aggregation; GPVI or $\alpha_2\beta_1$ deficiency		
ADP receptor	Normal	Mutations in *P2Y ₁₂	Reduced ADP aggregation	Autosomal	
defects [‡]		(ADP) or $**P2Y_1$		recessive* or	
[245, 261]	Normal	Mutations in TVA	No response to TVA :	dominant**	
receptor defect [‡]	Normai	receptor	reduced response to collagen	dominant or	
[262]		receptor	reduced response to conagen	recessive	
Gray platelet	Reduced	Unknown molecular	Empty/indistinct α-granules;	Autosomal	
syndrome		defect	empty dense granules in	recessive or	
[263, 264]			autosomal dominant form	dominant	
Quebec platelet	Reduced	Unknown molecular	Increased uPA in platelets;	Autosomal	
[90, 243]	-normal	delect; <i>PLAU</i> linked	degraded a-granule proteins	dommant	
Dense granule	Normal	Unknown molecular	Quantitative deficiency of	Autosomal	
deficiency	· · · · · · · · · · · · · · · · · · ·	defect	dense granules	recessive or	
[203] Hermansky-	Normal	Mutations in HPS	Reduced number/ abnormal	Autosomal	
Pudlak/ Chediak-	Normai	genes, AP3B1, or	dense granules: associated	recessive	
Higashi syndromes		CHS1 (vesicle	with pigment abnormalities		
[266-268]		formation and			
W7:-1	Delevel	trafficking)	Deduced descention law	V Palad	
wiskott-Aldrich	Reduced	(signaling)	small platelets: defect in	X-linked	
X-linked		(signamig)	filopodia generation		
thrombocytopenia					
[269-271]				_	
αδ storage pool	Reduced	Unknown molecular	Combined α and dense	Autosomal	
[272]		defect	granule deficiency	dominant	
May-Hegglin	Reduced	Mutations in MYH9	Cytoskeletal protein	Autosomal	
anomaly; Fechtner/			abnormality	dominant	
Sebastian/ Epstein					
[273, 274]					
Scott syndrome	Normal	Unknown molecular	Impaired trafficking of	Autosomal	
[275-277]		defect; may involve	phosphatidylserine to platelet	recessive	
		ABCA1 activity	surface		

(*Table 1.3, continued*) Data summarized from recent reviews in [239-241,246], with selected references indicated in brackets. Primary secretion defects related to platelet agonist receptors are included (‡), as well as rare disorders of platelet function. †: also a giant platelet syndrome. Abbreviations: GP, glycoprotein; VWF, von Willebrand factor; ADP, adenosine diphosphate; P2Y₁₂, purinergic receptor P2Y G protein-coupled 12 (gene) ; P2Y₁, purinergic receptor P2Y G-protein coupled 1 (gene) ; TXA₂, thromboxane A₂; PLAU, urokinase plasminogen activator (gene); uPA, urokinase-type plasminogen activator; HPS1/3/4, Hermansky-Pudlak syndrome 1, 3 or 4 (genes); AP3B1, adaptor-related protein complex 3 beta 1 subunit ; CHS1, Chediak-Higashy syndrome 1 (gene) ; WAS, Wiskott-Aldrich syndrome (gene) ; MYH9, myosin heavy chain 9, non muscle (gene); ABCA1, ATP-binding cassette transporter A1 (gene).

1.4.3 Disorders of fibrinolytic proteins

Some rare bleeding disorders result from congenital defects in proteins of the fibrinolytic system. These disorders include quantitative and qualitative defects in α_2 -PI and PAI-1 [278-289], and conditions associated with excessive amounts of circulating plasminogen activators [243,290,291]. FXIII deficiency may also contribute to accelerated fibrinolysis because non- cross-linked fibrin has an increased affinity for plasminogen, and also because this deficiency reduces cross-linking of α_2 -PI to fibrin in clots [292]. Problems with fibrinolytic proteins are often suspected when there is a history of delayed-onset bleeding with hemostatic challenges without evidence of a coagulation factor deficiency. Bleeding symptoms of fibrinolytic bleeding disorders can include hemarthroses and hematoma formation (often after trauma), and sometimes intracranial hemorrhage [279,284,293]. In general, these disorders are treated with fibrinolytic inhibitor drugs [279-281,284,286,287], although fresh frozen plasma has also been effective in correcting α_2 -PI deficiency in some cases [288].

 α_2 -PI deficiency is a rare autosomal recessive condition that can manifest as both a quantitative or qualitative defect [283-289]. In this deficiency, systemic plasminemia is not observed (FDPs normal in plasma), suggesting that bleeding results from premature lysis of hemostatic plugs rather than from systemic dysregulation of fibrinolysis [283]. PAI-1 deficiency is also an autosomal recessive disorder that can result from a qualitative or quantitative defect in this protein [278-282]. In PAI-1 deficiency, plasminogen activator levels tend to be normal. Rarely, fibrinolytic defects have been attributed to increased levels of tPA, however the genetic cause was not elucidated [290,291]. QPD is the only known inherited disorder caused by increases in uPA [243]. The biochemical and clinical features of QPD are described in detail in section 1.6.

The molecular cause has been described for some inherited fibrinolytic disorders. Mutations reported for PAI-1 include missense and frameshift mutations affecting secretion or causing complete deficiency of PAI-1 (due to protein truncation), respectively [278,294]. For α_2 -PI deficiency, mutations include missense mutations [295], single or multiple base-pair deletions resulting in a premature stop codon or loss of a codon [296,297], insertions [298], and splice site mutations [299] (reviewed in [300]). The mutations responsible for causing increased plasminogen activator expression in blood have not been described. Chapter 4 describes investigations of the genetic cause for QPD using linkage analysis.

1.4.4 Bleeding disorders caused by gain-of-function defects

Most bleeding disorders are caused by qualitative or quantitative deficiencies of genes encoding coagulation, platelet or fibrinolytic inhibitor

proteins. Gain-of-function mutations or mutations that result in gene overexpression are rare, and include mutations causing platelet-type and type 2B VWD, as well as some forms of Glanzmann thrombasthenia [301-305]. Gain-offunction mutations causing platelet-type VWD can be single base pair substitutions [301,302] or a larger deletion [303] in GPIba, causing increased affinity of this receptor for VWF. Type 2B VWD is usually due to a single base substitution in VWF that causes spontaneous binding of VWF to platelets [304]. In one form of Glanzmann thrombasthenia, a point mutation in the β_3 integrin of the fibringen receptor $\alpha_{IIb}\beta_3$ causes expression of a constitutively active receptor [305]. When mRNA levels were explored in some of these cases, mRNA appeared to be well-transcribed, resulting in normal expression of the affected proteins [303]. The mutation causing OPD has not yet been described, but it causes a gain-of-function defect in fibrinolysis (see chapter 2) due to overexpression of uPA in platelets [243]. This defect appears to be restricted to the MK lineage, and is hypothesized to be linked to MK differentiation (see section 1.6 and Appendix I). Inheritance of QPD also causes a secondary increase in PAI-1 levels in platelets, likely as a physiological response to the increased levels of platelet uPA ([243] and see section 1.6).

Inherited gain-of-function mutations, especially those that cause gene overexpression, are rare in nature (reviewed in [306]). Table 1.4 summarizes the defects in some other known diseases characterized by gain-of-function abnormalities. A unique disorder of gene overexpression, Charcot-Marie-Tooth disease, is caused by a tandem duplication encompassing a region of chromosome 17 containing the gene for peripheral myelin protein-22 [307]. For this disorder, increased gene dosage, (rather than altered gene function), has been proposed as the most likely mechanism for gene overexpression [307]. Other diseases where gene overexpression is caused by gene amplification have also been described (reviewed in [308]). It is not known if QPD is caused by a similar mechanism (see chapter 5 for discussion).

Table 1.4. Examples of inherited human diseases caused by gain-of-function abnormalities in genes.

Defect	Gene	Disease
Gene overexpression	PMP22	Charcot-Marie-Tooth disease [307]
Receptor permanently on	GNAS	McCune-Albright disease [309]
Receptor acquires new substrate (Pittsburg allele)	P1	A1-antitrypsin deficiency [310]
Ion channel inappropriately open	SCN4A	Paramyotonia congenita [311]
Structurally abnormal multimers	COL2A1	Osteogenesis imperfecta [312]
Increased protein aggregation	HD	Huntington disease [313]

Reviewed in [299]. Gene abbreviations: *PMP22*, peripheral myelin protein 22; *GNAS1*, GNAS complex locus; *P1*, proteinase inhibitor (α_1 -antitrypsin); *SCN4A*, sodium channel voltage-gated type IV α -subunit; *COL2A1*, collagen type 2, alpha 1; *HD*, Huntington disease (huntingtin).

1.4.5 Determination of the genetic causes of bleeding disorders

The genetic determinants of some congenital bleeding disorders have been identified using genetic linkage studies (for examples see [314-322], and chapter 4). For such studies, DNA and clinical data are usually obtained from pedigrees with multiple affected individuals, and polymorphic microsatellite markers across the genome are genotyped to look for genomic regions that may be associated with the disease [323,324]. Association is determined using logarithm of odds (LOD) scores [325]. The LOD score is a statistical estimate of whether two genetic loci are likely to have been inherited together during meiosis, when chromosomes recombine [326]. Individuals who have inherited a particular disease allele will also (almost always) have inherited a set of common alleles for genetic regions close to the disease locus [325]. The non-random association of alleles at different loci is a phenomenon known as linkage disequilibrium, and this can be used to identify genetic regions that are closest to a locus harboring a genetic mutation [327,328]. When LOD scores are calculated, values of > +3 are suggestive of linkage and scores of < -2 suggest non-linkage of genetic markers with a particular disease [326]. LOD score calculations take into account the pedigree structure, affected/ unaffected status of individuals, and the observed genotypes for the microsatellite markers tested [325]. As the LOD score value is logarithmic, a score of +3 suggests a 1:1000 likelihood that the observed correlation between affected status for a disease and a particular genotype for a microsatellite marker has occurred by chance (1:10,000 for LOD +4; 1: 100,000 for LOD +5, etc.) [326]. Linkage data points to a putative locus (or loci) for candidate genes that can then be sequenced to look for DNA variations. Detailed maps of single nucleotide polymorphisms (SNPs) are available to help assess associations of DNA variations with disease [327,328]. Genome-wide linkage studies continue to be useful in evaluating susceptibility loci for diseases with asvet unidentified genetic causes.

1.5 UROKINASE PLASMINOGEN ACTIVATOR

uPA, also known as urinary-type plasminogen activator, was initially named because it was responsible for the fibrinolytic activity of urine [329,330]. uPA is a ubiquitously-expressed serine protease that has physiological roles in fibrinolysis, ECM degradation, immunity and angiogenesis [331]. uPA is upregulated in some pathological conditions, including cancer, where it contributes to growth and metastasis [332-336]. This section summarizes knowledge about the uPA gene (*PLAU*) and its regulation, the biological characteristics of uPA and uPAR (the uPA cellular receptor), and current concepts on uPA functions in health and disease.

1.5.1 The urokinase plasminogen activator gene, PLAU

PLAU is located on the long arm of chromosome 10 at position q24 [337,338] (NCBI human reference sequence assembly, March 2006: physical position 75,338,941- 75,347,272 [339]). It is flanked by the genes for vinculin (*VCL*) [100 kilobases (kb) downstream of *PLAU*; telomeric side] and calcium/calmodulin-dependent protein kinase II γ (*CAMK2G*) (36.5 kb, upstream of *PLAU*; centromeric side) [339]. A gene of unknown function with an open reading frame (orf), C10orf55, exists on the antisense strand, that overlaps with a large portion (2.8 kb) of the *PLAU* genomic sequence [339]. The entire sequence of *PLAU* was published in 1985 and evidence suggests it is a single copy gene, with no pseudogenes [340].

Ten alternative mRNA variants encoding different isoforms have been described for PLAU [341]. Eight of these isoforms encode good quality proteins and have been isolated from several tissues, while the other two appear to encode non-functional proteins [341]. The dominant mRNA isoform (isoform "bApr07"; from NCBI AceView, April 2007 assembly [341]) of this gene spans 6.4 kb, contains 11 exons and 10 introns, and encodes a 2.4 kb mRNA (431 amino acids, including a 20 residue- long signal peptide) [340,342,343] (figure 1.5A) (full sequence available on Genbank: contig NT 008583.16; GI: 37551286). The 5' untranslated region (UTR) of the dominant isoform includes the first exon and part of the second (which contains the start codon ATG). The 3' UTR, which is approximately 1 kb in length, includes a large portion of exon 11 (~800 bases) [343]. The intron-exon organization of PLAU closely resembles that of the tPA gene. with which it has ~40% homology [344]. Table 1.5 summarizes the structural motifs encoded by each exon of the dominant isoform of *PLAU* and their respective functions. Analysis of the exon- intron organization strongly suggests that PLAU arose by recombination events whereby exons encoding single functional domains were exchanged between different genes [340]. The intervening sequences of the catalytic portion are also similar to the conserved intron-exon pattern described for several other serine protease genes [340].

Human *PLAU* shows a high degree of homology with the mouse and pig uPA genes, with respect to the intron- exon organization, the nucleotide sequence of the coding region and the introns, and the 5' flanking region up to several kb upstream of the transcription start site (+1) [345-348]. *PLAU* also has substantial structural and sequence homology with the cow, rat and chicken uPA genes, especially within the protein-coding portions of the exons and the proximal promoter elements [349-351]. Conserved sequences in the flanking regions of uPA genes in different species suggest regulatory functions, and the presence of these elements may be necessary for full induction of gene transcription.

Several SNPs have been reported within PLAU, as well as substitutions or base deletions in the 3' and 5' UTRs, that do not have functional effects [340]. A

complete list of *PLAU* SNPs is available at http://www.ncbi.nlm.nih.gov/SNP/ (build #129). Alu repeat elements are present in two of *PLAU*'s introns, and in the region 200 nucleotides downstream from the polyadenylation (poly-A) site [340]. These elements are liable to frequent internal deletions and duplications due to mispairing between the repeats [306]. It has also been suggested that some Alu elements may promote recombination events [306].

EXON	STRUCTURAL MOTIF	FUNCTION				
Ι	5' UTR	promoter and regulatory region				
II	signal peptide start codon	targeting to endoplasmic reticulum translation start site				
III	first 9 amino acids	part of mature protein				
IV	EGF-like domain	interaction with uPAR				
V	part of kringle domain	stabilizes interaction with uPAR				
VI	part of kringle domain	stabilizes interaction with uPAR				
VII [†]	contains His 204	part of catalytic triad				
VIII [†]	contains Asp 255	part of catalytic triad				
IX^{\dagger}	contains Asn 302	O-glycosylation site				
X [†]	contains Asp 350	confers trypsin-like specificity				
XI^{\dagger}	contains Ser 356 3'UTR	part of catalytic triad site for polyadenylation of mRNA				

Table	1.5.	Structural	motifs	encoded	by	the	11	exons	of	PLAU	and	their	
respec	tive	functions [3	40].										

Information is based on the full-length transcript isoform "bApr07" of uPA mRNA, encoding a 431 amino acid protein [341]. Each exon is designated by a roman numeral (I to XI). [†]indicates exons encoding parts of the urokinase plasminogen activator (uPA) molecule that become the B-chain (catalytic chain) when latent single-chain uPA is converted to the active serine protease, two-chain uPA. Organization of several exons into single functional domains suggests they originated from recombination events between several different genes [340]. Abbreviations: UTR, untranslated region; EGF, epidermal growth factor; uPAR, urokinase plasminogen activator receptor; His, histidine; Asp, aspartic acid; Asn, asparagine; Ser, serine; mRNA, messenger ribonucleic acid.



Figure 1.5. Isoforms of uPA mRNA, showing alignment of exons and introns. Alternative mRNA isoforms of uPA are aligned in a 5' to 3' orientation. 10 isoforms have been described: 6 are alternatively spliced (isoforms a to f), and 4 exist in an unspliced form (isoforms g to j). Variants a to h are predicted to encode good quality proteins, while i and j are not. Isoform a could be a target for nonsense-mediated mRNA decay. Panel A shows the dominant isoform (isoform b) and the sizes of each exon (boxes; roman numerals I-XI) and intron (lines) are indicated. Hatched boxes indicate untranslated exons. Panel B shows the other 9 isoforms and their predicted exons and introns, aligned with respect to the exonintron structure of isoform b. (Figure modified from [341]).

1.5.2 Transcriptional regulation of PLAU

The *PLAU* proximal promoter contains the typical elements of human gene promoters and is highly conserved between mammalian species. A TATA box occurs 24 base pairs upstream, and there is a GC-rich region (containing repeats of GGGCGG) of about 200 bases immediately upstream from +1 [340,352]. The gene also contains one copy of a CAAT sequence (TGCAAGA) at -82 ('-' sign denotes an upstream element) [340,352]. The GC-rich and CAAT sequences are recognized by the ubiquitous transcription factors SP1 and CTF (also called nuclear factor 1; NF1), respectively, which results in constitutive, low levels of uPA expression in a variety of cells [353,354]. A RUNX/AML-1 element exists between +8 and +20 in the 5' UTR, but this element is not present in the mouse, rat, or pig uPA genes [346,347,350,355]. The function of this motif in *PLAU* regulation has not yet been determined, although it may influence the regulation of uPA expression in leukemic cells [355].

Many of the transcriptional elements of the PLAU promoter have been determined using promoter deletion and reporter gene expression assays, or by DNAase I footprinting assays. The most characterized regulatory region of the *PLAU* promoter is an enhancer located about 2 kb upstream of +1, which is highly conserved among mammalian species (human, pig, mouse), suggesting its importance in PLAU regulation [356-359]. This region is composed of an Ets/AP1_A site, a downstream AP1_B site, and a connecting cooperation mediator (COM) region of 74 bp that contains binding sites for the upstream enhancer factor (UEF) transcription factors 1 to 4 [358,360,361]. Another Ets/AP1 site, in the opposite orientation, is found further upstream of the enhancer at -5.36 kb and is also highly conserved [357]. Overexpression of transcription factors that bind Ets and AP1 sites (eg. Jun, Fos, Ets1, Ets2) in cells results in activation of the uPA promoter [357,362-364]. Ets and AP1 transcription factors are activated by members of the mitogen-activated protein kinase (MAPK) family, and MAPKs themselves can be activated by a variety of extracellular signals [365]. Thus, other signals can indirectly activate the *PLAU* promoter via Ets and AP1 sites, through the induction of signal transduction pathways, and these include: okadaic acid, growth factors, phorbol ester, hormones, cAMP, cytoskeletal reorganization, ultraviolet light, and tumor necrosis factor (TNF-a) [361,362,366-372]. The physical interaction between this key enhancer element and the PLAU promoter is enhanced by a loose chromatin structure that allows the intervening sequence to loop and bring the two elements close together [373].

Two other putative AP2 sites close to +1 are responsible for protein kinase A-dependent induction of uPA expression [374]. Other enhancer elements have been described at -112 to -123 (a cAMP-responsive element, CRE) [352], -308 to -536 [375] and -2144 [374]. Negative regulatory (silencer) elements exist at -536 to -660 (this is an enhancer-dependent silencer, with cell-specific activity shown in cervical carcinoma cells and fibroblasts, but not in prostatic carcinoma cells)

[375], -687 (a GATA4 transcription factor binding element), and -1572 to -1870 [356]. Two NF- κ B elements, that are highly conserved in mammals, occur at - 1583 and -1865 (this second one acts as a repressor) [376]. The former NF- κ B site is responsible for the induction of uPA expression by phorbol esters [376].

In the 3' UTR, which spans from +1368 to +2260, several fragments have been identified that exert a positive or negative influence on chimeric reporter genes. First, the region between +1999 and +2190 behaves as a transcriptional enhancer, while +1532 to +1723 behaves as an inhibitor of uPA expression [377]. A summary of the regulatory elements affecting *PLAU* transcription are summarized in figure 1.6.



Figure 1.6. Known regulatory elements 5' and 3' of *PLAU*. Regulatory elements are indicated relative to the *PLAU* transcription start site (+1). Well-characterized elements extend up to 2.3 kb upstream of *PLAU*, and the message instability region extends about 1 kb from the stop codon in exon 11. Abbreviations: COM, cooperation mediator; NF- κ B, nuclear factor κ B; CRE, cAMP-response element; RUNX/AML, runt-related transcription factor/acute myeloid leukemia; UTR, untranslated region.

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1.5.3 Post-transcriptional regulation of *PLAU*

Regulation of mRNA stability is thought to play an important role in eukaryotic gene expression by modulating the cytoplasmic abundance of mRNA. The variation in decay rates of different mRNAs is determined by *cis*-elements in the transcript. Instability-determining sequences can be found in 3' UTRs, or within protein coding regions of genes [378,379]. Furthermore, binding of specific proteins to mRNA can modulate mRNA stability [380,381]. The poly-A tail also appears to have a regulatory role, as removal of this tail precedes mRNA degradation [382].

The entire 3' UTR of *PLAU* is highly conserved between mammalian species [345-347.349.350.383]. uPA mRNA stability can be modulated either positively or negatively by a number of factors, which include protein synthesis inhibitors, Ca^{2+} , PKC down-regulation, glucocorticoids, TNF- α , and dioxin (reviewed in [384]). Three regions in the 3' UTR, are known to contribute independently to the rapid turnover (ie. instability) of uPA mRNA. These regions include a sequence with a stem structure, a region that requires ongoing transcription for its activity to destabilize the transcript, and an AU-rich element (ARE) that is responsible for mRNA stabilization induced by PKC downregulation [385]. AREs are the most widely studied mRNA instability elements. They usually contain pentameric AUUUA or nonameric UUAUUUA(U/A)(U/A) sequences [386]. The ARE in the 3' UTR of PLAU, which includes two separate AUUUA and AUUUUUA motifs, is immediately upstream of the poly-A signal and is about 50 nucleotides long [385]. The importance of this sequence in stabilization of mRNA is highlighted in some cancer cells, where impairment of ARE-mediated degradation mechanisms leads to very high levels of uPA mRNA expression (half life of mRNA increased from 70 min to 17 hours) [387]. Another group has shown that the 3' UTR of uPA mRNA contains a binding element for a novel protein (uPA mRNA binding protein: uPA mRNABp) that destabilizes the transcript [381].

Studies to determine the 3' instability elements and the mRNABp element of *PLAU* were done by inserting different parts of the 3'UTR of pig [385] or human [381] uPA mRNA at the 3' end of the β -globin gene, which normally produces a stable globin mRNA when expressed in cultured epithelial cells. The result of the addition of these 3' UTR elements was an unstable globin transcript, suggesting that all information governing rapid mRNA turnover resides in the 3' UTR. Protein synthesis inhibition using cycloheximide, PKC downregulation using phorbol ester, and cAMP induction using Ca²⁺ also rendered mRNA more stable, suggesting there are other pathways of mRNA destabilization in cells [388-390]. The presence of multiple instability- determining sites may be to ensure rapid depletion of uPA mRNA to avoid uPA overexpression. They may also provide the organism versatility and flexibility for regulating mRNA degradation in a tissue- and development-specific manner [385]. It is not known if there are additional sites in the 5' UTR or coding region of *PLAU* that could contribute to mRNA regulation [385].

1.5.4 Sites of uPA expression, and uPA protein structure

scuPA, or pro-urokinase, is a 54 kDa glycoprotein synthesized by many different cell types, including epithelial cells, endothelial cells, monocytes, neutrophils and macrophages [332-334,391]. High amounts of uPA (40-80 μ g/mL) are normally excreted in the urine [330]. Plasma levels are low (1-4 ng/mL) ([243,392,393] and see chapter 2), but the molar amount (20-70 pmol/L) is similar to that of tPA (70 pmol/L) [394]. Only trace amounts are detectable in normal platelets (up to 1.3 ng/10⁹ platelets) [79,243,395]. Ubiquitous expression of uPA is thought to be important for modulating fibrinolysis with tPA, and for normal tissue remodeling and cell migration [396,397]. Blood levels of uPA are known to increase in certain physical conditions, including after venous stasis [392], desmopressin infusion [398] and strenuous exercise [399], or in pathological conditions such as various types of cancer and liver disease [400-408]. In cancer, uPA is implicated in promoting invasion and metastasis, and uPA levels have been used as a prognostic indicator [409-412] (see section 1.5.7).

Like plasmin, uPA is made up of an A-chain that is responsible for binding to cell surfaces, and a catalytic B-chain. The N-terminal A-chain consists of one epidermal growth factor (EGF)-like and one kringle domain [331]. The EGF domain (residues 4-43) is responsible for uPA binding to cell-surface receptors, such as uPAR found on many cell types (see section 1.5.6), as well as other proteins [413]. The kringle structure in uPA (residues 47-135) has no affinity for fibrin, unlike the kringle domains of other proteins (eg. tPA and plasminogen) [414]. uPA does not directly bind to fibrin, likely because it lacks a LBS in the A-chain [4,415]. The kringle is involved in stabilizing the interaction between uPA and uPAR, as well as binding to other integrins including $\alpha_{v}\beta_{3}$ on endothelial cells and $\alpha_M \beta_2$ on neutrophils, which enhances plasminogen activation on cell surfaces [416-418]. The C-terminal B-chain (residues 144-411) contains the catalytic triad (His²⁰⁴, Ser³⁵⁶ and Asp²⁵⁵) necessary for the enzymatic activity of tcuPA [419]. Other important structural elements include the interaction between Lys³⁰⁰ with Asp³³⁵ in the flexible loop region of uPA, which stabilizes the conformation of scuPA [420], and a string of positively charged residues (amino acids 179 to 184) in a surface loop of the catalytic chain that are necessary for the interaction with PAI-1 [421].

Pro-urokinase undergoes several post-translational modifications, including glycosylation of Asp³⁰², phosphorylation of Ser^{138/303}, and fucosylation of Thr¹⁸ in the EGF-like domain [422,423]. Phosphorylation is induced by PKC activation and results in reduced interactions with cells (through uPAR) and PAI-

1 [422,424], and fucosylation affects the ability of uPA to trigger mitosis [423,425].

1.5.5 uPA activation and enzymatic activity

Unlike tPA, which is enzymatically active in its sc form, pro-urokinase has low intrinsic activity (0.4% the activity of tcuPA) [426-428]. scuPA is converted to tcuPA (also called high molecular weight uPA; HMW-uPA) by cleavage of the Lys¹⁵⁸-Ile¹⁵⁹ bond by plasmin, kallikrein, and FXIIa [4,429,430]. This yields two chains, linked by a single disulfide bond. Thrombin can also cleave scuPA at the Arg¹⁵⁶-Phe¹⁵⁷ bond, to create an inactive tc derivative, which can subsequently be activated by the release of the N-terminal dipeptide of its Bchain by cathepsin C or plasmin [429,431,432]. Low molecular weight uPA (LMW-uPA) is an active, processed form of the full-length protein, cleaved by plasmin at Lys¹³⁵-Lys¹³⁶ to release an amino terminal fragment (ATF) that includes the EGF-like and kringle domains [433]. Removal of the ATF results in a uPA molecule with a 21 amino acid residue A-chain and an intact B-chain, that retains its full ability to activate plasminogen but cannot bind uPAR [434]. scuPA is also converted to tcuPA by other proteins, including matrix metaloproteases (MMPs), and the type II transmembrane serine proteases matriptase, serase 1B and hepsin [435-438].

In the presence of fibrin, scuPA has a high affinity for plasminogen bound to newly exposed C-terminal lysines on partially degraded fibrin, but not to plasminogen bound to internal lysines on intact fibrin [415,439], unlike tPA, which can bind to internal lysine residues on non-degraded fibrin [440]. Thus, the two plasminogen activators have a synergistic role in fibrinolysis, where tPA is important for the initiation of clot lysis, which exposes C-terminal lysines for plasminogen to bind, and then bind scuPA [440]. scuPA is locally converted to tcuPA by small amounts of plasmin generated on the fibrin surface, and the newly generated plasmin feeds back to further convert more scuPA to tcuPA [441,442]. Although uPA primarily interacts with fibrin-associated plasminogen to initiate fibrinolysis in vivo, scuPA has also been shown in vitro to bind directly to fibrin through a site that is expressed in the presence of a divalent metal cation, $Zinc^{2+}$ [443]. tcuPA can convert plasminogen to plasmin, both in the presence and absence of fibrin, unlike tPA which requires fibrin for its enzymatic activity [80,444], which is consistent with the many fibrin-independent roles of uPA in the body (reviewed in [336]) (see section 1.5.7). The uPA produced by endothelial cells could contribute to their angiogenic properties, as it facilitates proteolysis of ECM, allowing endothelial cells to proliferate, migrate, and form capillary-like structures in vitro [445]. Many uPA- mediated events involve binding of uPA to its receptor, uPAR (see section 1.5.6); however, uPA does not require the presence of uPAR for all its functions, including clearance of fibrin deposits and

wound healing [446]. In addition to plasminogen, uPA directly activates prohepatocyte growth factor (involved in motility, invasion, and growth of epithelial and endothelial cells), and it can cleave fibronectin, as well as its own inhibitor PAI-1 in a plasminogen-independent manner *in vitro*, suggesting uPA may contribute to other processes *in vivo* [447-449]. uPA can also cleave uPAR *in vitro* to release a uPAR fragment that is known to have chemotactic activity [450-453] (see section 1.5.7).

PAI-1 interacts irreversibly with tcuPA, but not scuPA, and this interaction is involved in uPA clearance from the circulation [454-456]. PAI-1 inhibits tcuPA by first forming a 1:1 reversible complex, followed by covalent binding of the reactive site of PAI-1 with the active site serine residue of uPA [454,457]. After tcuPA is neutralized, it binds to uPAR and is cleared through a mechanism that involves binding to the low-density lipoprotein receptor-related protein (LRP) (a2-macroglobulin receptor) [458]. uPAR may also bind scuPA, but it does not internalize scuPA unless it is activated to tcuPA and complexed with PAI-1 [458-461]. Internalized uPA-PAI-1 complexes are engulfed and digested in lysosomes. uPAR itself is not degraded when it is internalized, but is recycled back to the cell surface where it can bind new uPA [462]. The process of receptormediated endocytosis involves cAMP-dependent protein kinase and PKC signal transduction pathways, and is much faster than fluid-phase endocytosis of uPA/PAI-1 complexes [461,463]. PAI-1 can also bind to uPAR-bound tcuPA to inhibit ECM degradation, cell adhesion and migration that is initiated by cellassociated uPA [464]. In addition to its physiological inhibitor PAI-1, other inhibitors have been shown to block uPA, including protease nexin, α_2 -PI, α_2 macroglobulin, α_1 -antitrypsin, C1-inhibitor, PAI-2, and PAI-3 (APC inhibitor) [454,465]. Receptor-mediated endocytosis of uPA and other serpin complexes also occurs [466].

1.5.6 The uPA receptor, uPAR

uPAR is a 270 amino acid polypeptide, first identified on human monocytes, that acts to localize uPA-catalyzed events on cell surfaces [467-469]. It is anchored to plasma membranes by a glycosylphosphatidylinositol moiety and has three structural domains (D1, D2, D3), which are all involved in uPA binding, creating a high-affinity interaction [470,471]. uPAR is expressed on several cell types, including monocytes and neutrophils (which migrate into thrombi and increase local fibrinolytic activity) [76,333,472], and platelets, where it may contribute to some inflammatory processes [473,474]. On the plasma membrane, approximately 25% of uPAR is localized to lipid rafts [475], but it can also move freely on the plasma membrane in its unbound form and may associate with various membrane-associated molecules containing cytoplasmic domains (eg. integrins), which can influence movement of the cell cytoskeleton to causes cell

adhesion and spreading [476,477]. In addition to its involvement in fibrinolysis, cell adhesion and inflammation, uPA-uPAR interactions also contribute to pathologic fibrinolysis and tumor cell metastasis in cancer [478,479].

ScuPA, tcuPA, and the ATF all bind to uPAR [480], through several domains of the uPAR N-terminal A-chain [481]. Several regions of uPA are important, including amino acids 23 to 30, as mutations in these amino acids result in complete loss of uPAR binding [413,482]. When scuPA binds to uPAR, the activation of scuPA is enhanced, and this is followed by increased plasminogen activation [483]. The activation of plasminogen is enhanced because uPAR colocalizes uPA and plasminogen on cell surfaces, and not because of a catalytic effect of uPAR [468,483-486]. Upon binding, uPA induces a conformational change in uPAR, allowing it to interact with cellular components to transmit intracellular signals (reviewed in [335]). uPA induces uPAR-mediated activation of various kinases and transcription factors, and this may influence cytoskeletal structure, cell morphology, adhesion and migration [487-491].

In addition to uPAR, some cells, including platelets and neutrophils have other receptors capable of binding uPA [418,492,493]. For example, $\alpha_M\beta_2$ (an integrin on neutrophils) can alter fibrinolytic activity in the absence of uPAR, as it binds both uPA and plasminogen [418].

1.5.7 Current concepts on uPA function and its clinical implications

Mouse models of uPA deficiency or overexpression

There are no reports of humans with deficiencies of uPA or tPA, and so mouse studies have been used to determine the importance of plasminogen activators (reviewed in [95]). uPA deficiency (uPA -/-) in mice is not lethal and it does not impair reproduction or life span [494]. The most prominent phenotype of uPA deficiency is increased fibrin deposition in both normal and inflamed tissues, including the liver, skin, and intestine [494]. uPA -/- mice also have a higher incidence of endotoxin-induced thrombosis, and their macrophages show deficient plasmin-mediated breakdown of fibrin and matrix components, although invasion was unaltered [494]. Furthermore, the inflammatory cell recruitment (T-cells and macrophages) of these mice is greatly impaired, which predisposes them to bacterial infections [495]. In mice with a combined loss of uPA and tPA, the fibrinolytic impairment results in spontaneous fibrin deposits, retarded growth, and reduced fertility and survival, similar to plasminogen -/- mice [494,496]. These mice also display an almost total inability to lyse ¹²⁵I-labeled fibrin plasma clots [494]. The increase in severity of the phenotype of double-deficient mice suggests that tPA and uPA have complementary roles in normal biology.

QPD is the only known inherited disorder associated with increased uPA (see section 1.6). A transgenic mouse model of uPA overexpression in MKs, using a cell-specific promoter, has been developed to investigate the effects of tissue-specific uPA overexpression [497]. Mice that overexpress uPA in MKs exhibit a severe bleeding phenotype and like humans with QPD, they have α -granule protein degradation, without systemic fibrinogenolysis. However, unlike in QPD, where women have good outcomes with pregnancy [293], mice overexpressing uPA suffer from increased bleeding-related mortality with pregnancy and pregnancy loss [497]. Interestingly, these mice display some protection against arterial and venous thrombosis [497]. uPA has also been overexpressed in mouse macrophages by a transgenic approach, which results in the development of cardiac fibrosis, atherosclerosis, coronary artery occlusions, and premature death [498,499]. Overexpression of uPA in the liver results in spontaneous bleeding in mice, and systemic fibrinogenolysis [500].

The effect of uPA gene knockout on the pathogenesis of atherosclerosis is currently under investigation because it has important human implications (see below). When genetically-engineered mice at an increased risk for developing atherosclerotic lesions (apolipoprotein E knockout; ApoE) [501]. are induced to overproduce uPA in monocytes (cells that normally infiltrate into atherosclerotic plaques) the result is accelerated plaque formation, leading to vessel occlusion and death, suggesting an important role for uPA in the pathogenesis of atherosclerosis [499,502]. Combined ApoE and uPA deficiency results in reduced plasmin generation, which reduces MMP activation and protects mice from developing atherosclerotic plaques.

Role of uPA in cell remodeling and cancer

High levels of uPA and uPAR have been observed in non-malignant cellremodeling processes, such as normal embryogenesis, wound healing and postlactational mammary cell involution [503-505]. uPA is also highly expressed by many tumors and it is thought to play a role in cancer pathogenesis [94]. Plasminogen activator production in malignant tumors is believed to be part of the complex processes that stimulate tumor growth *in vivo* [506]. The *PLAU* promoter is hypomethylated in some cancer cells that overexpress uPA, suggesting that malignant transformation alters uPA regulation [178]. In cancer, uPA is thought to induce plasmin generation, which leads to basement membrane and ECM degradation, allowing cancer cells to migrate to distant sites [507]. Invading cancer cells express the highest levels of uPA and uPAR [508]. Metastasis is always associated with poor prognosis and high mortality [509], and for some tumors, uPA levels have been used as a prognostic marker [94,510-514]. Although uPA is a key player in malignancy, tPA is not thought to play any important role [94].

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Invasion and metastasis of tumor cells can be down-regulated by uPA inhibitors, anti-uPA antibodies, antisense uPAR expression and uPAR antagonists, or increased by uPA overexpression [515-517]. uPA -/- mice have reduced tumor development [518]. uPA can also directly, or through plasmin generation, lead to the release of angiogenic factors such as basic fibroblast growth factor and vascular endothelial growth factor, that are important for angiogenesis [519,520]. Paradoxically, PAI-1 also positively influences tumor invasion and angiogenesis, and has been correlated with poor prognosis in many cancers [412,521]. Loss of PAI-1 is linked with reduced tumor growth, invasion and metastasis [518,522].

Role of uPA in chemotaxis, cell adhesion, and apoptosis

The proteolytic activity of uPA is not required for some of its biological effects, including its influences on chemotaxis, cell adhesion and apoptosis. When uPA binds to uPAR, it can cleave the receptor to expose a small peptide of uPAR with chemotactic activity for several cell types, including activated neutrophils and macrophages [451-453]. The chemotactic epitope on uPAR can also be exposed by binding of inactive uPA or the ATF [451]. Binding of uPA to uPAR also exposes the vitronectin-interacting surface of uPAR, which through interactions with integrin receptors, can modulate rearrangement of the actin cytoskeleton and cell spreading [523,524]. uPA bound to uPAR also activates signal transduction pathways that lead to the expression of anti-apoptotic proteins in cells [525].

Role of uPA in other diseases

In addition to its roles in cardiovascular events and cancer, the uPA/uPAR system has been implicated in other human pathologies including HIV infection [526-529], atherosclerosis [530-532] and Alzheimer's disease [533-535]. Both scuPA and the ATF have been shown to have a negative regulatory effect on cells infected with HIV, which are known to produce both uPA and uPAR. uPA and uPAR levels are good predictors of HIV progression, independent of CD4⁺ T cell counts and viremia. The effects of uPA on HIV have been documented to include: cleavage of the HIV GP120 envelope; inhibition of virion-associated reverse transcriptase activity; and increased entrapment of virions in intracytoplasmic vacuoles [526-529]. These effects occur at lower concentrations than those needed for thrombolysis, and thus pro-uPA may represent a previously unclassified class of antiviral agents mimicking interferons in their inhibitory effects on HIV expression and replication [527]. The inhibition of uPA and its association with uPAR can trigger a signaling pathway that leads to inefficient release of HIV from monocytic cells [529].

uPA is also involved in atherosclerosis. All components of the uPA/uPAR/plasmin system are present in the artery wall, suggesting they could influence atherogenesis [530-532]. High levels of uPA and uPAR have been detected in atherosclerotic human arteries, and expression levels of both molecules correlate directly with disease severity [531,532].

A functional SNP (rs2227564) within exon 6 of *PLAU* has recently been linked to the pathogenesis of late-onset Alzheimer's disease [533,534]. This SNP causes a missense change in an amino acid (part of the kringle domain) that has been associated with age-dependent elevations of amyloid β , the main protein component of senile plaques. Plasmin degrades soluble and aggregated forms of amyloid β [536,537]. However, the relationship of *PLAU* sequence changes to Alzheimer's disease is uncertain as other groups have not confirmed the association of this *PLAU* SNP to the disease [538-540].

1.6 QUEBEC PLATELET DISORDER

QPD is a unique inherited bleeding disorder caused by increased levels of uPA in platelets. The following section contains a review entitled "Quebec Platelet Disorder: Features, Pathogenesis, and Treatment", that summarizes the family history, clinical presentation, biochemical features, and treatment approaches for QPD. This review contains findings from one of the manuscripts included in my thesis (Chapter 2: "Insights into abnormal hemostasis in the Quebec platelet disorder from analyses of clot lysis"), and has been reproduced with the publisher's permission.

As the senior author of this review, Dr. Catherine P.M. Hayward supervised the manuscript writing and wrote the section entitled "Practical approach to the diagnosis and treatment of Quebec platelet disorder". My specific roles (along with my co-first author Dr. D. Kika Veljkovic) were the collection and synthesis of the literature on QPD, and preparation of the manuscript, including generation of figures and tables. I thank Dr. Elisabeth Maurer-Spurej for contributing her data on QPD aggregation abnormalities, including generation of figure 1. I am grateful to Dr. Georges E. Rivard for his contributions to the writing of this manuscript.

Quebec Platelet Disorder: Features, Pathogenesis, and Treatment

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1.6.1 Summary

Quebec platelet disorder (QPD) is a rare, autosomal dominant, inherited bleeding disorder that is associated with unique abnormalities in fibrinolysis. Its hallmark features are delayed-onset bleeding following hemostatic challenges that responds to fibrinolytic inhibitor therapy and increased expression and storage of the fibrinolytic enzyme urokinase plasminogen activator in platelets, without increased plasma urokinase plasminogen activator or systemic fibrinolysis. The increased urokinase plasminogen activator in QPD platelets is only partially inhibited, and, as a result, there is intraplatelet generation of plasmin, and secondary degradation of many platelet α -granule proteins. During clot formation, the urokinase plasminogen activator released by QPD platelets leads to plateletdependent increased fibrinolysis, and this is postulated to be a major contributor to QPD bleeding. The focus of the present review is to summarize the current state of knowledge on QPD, including the history of this disorder, its clinical and laboratory features, and recommended approaches for its diagnosis and treatment.

1.6.2 Introduction

Congenital bleeding disorders have provided important insights into the factors important for regulating hemostasis and thrombosis. Quebec platelet disorder (QPD) is a rare, autosomal dominant, inherited bleeding disorder that is associated with unique changes to platelet proteins and striking platelet-dependent profibrinolytic abnormalities that can be difficult to detect using routine coagulation laboratory tests. Typically, this bleeding disorder manifests as delayed-onset bleeding following hemostatic challenges that responds to fibrinolytic inhibitor therapy, with or without the following symptoms: increased bruising, episodic joint bleeds, spontaneous hematuria and other bleeding symptoms [1-4]. The disorder's hallmark laboratory features include: a modest reduction (approximately 50%) in platelet counts, resulting in reduced to lownormal counts; defective platelet function in aggregation tests characterized by reduced platelet aggregation responses to epinephrine, with or without reduced aggregation with ADP and collagen; and a marked increase in the expression and storage of the fibrinolytic enzyme urokinase plasminogen activator (u-PA) in blood platelets, without systemic fibrinolysis or increased u-PA in plasma [2,3,5-7]. The increased u-PA in QPD platelets is associated with intraplatelet generation of plasmin, and this is thought to trigger secondary proteolytic degradation of megakaryocyte-synthesized and plasma-derived platelet a-granule proteins, including factor V - the first platelet protein identified to be abnormal in this condition [1-3,5,6,8].

Initially, the increased bleeding in this condition was thought to reflect defective platelet factor V-dependent prothrombinase function [1]. More recently,

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increased fibrinolysis has been implicated in the hemostatic defect, based on observations that QPD bleeding only responds to fibrinolytic inhibitor therapy, and that QPD platelets, when activated, release large amounts of u-PA that accelerate fibrinolysis [4–7]. The focus of the present review is to summarize the current state of knowledge on QPD, including the history of this disorder, its clinical and laboratory features, and recommendations on its diagnostic evaluation and management.

1.6.3 History of Quebec platelet disorder

QPD was first described in 1984 [1] as an autosomal dominant bleeding diathesis affecting members of a family from the Province of Quebec in Canada. The index cases had moderate to severe, delayed-onset bleeding with exposures to hemostatic challenges, without evidence of a coagulation factor deficiency from analyses of plasma [1]. The index cases were identified to have impaired platelet factor V-dependent prothrombinase function, due to a qualitative abnormality affecting their platelet but not plasma factor V [1]. The observation that platelet factor V was abnormal in this condition led to its initial designation as 'factor V Quebec' [1].

In 1996, further investigations of the index cases and other family members led to the discovery that, besides factor V, many other stored platelet proteins were abnormal [2,8]. The proteins abnormally degraded, or deficient, were identified to include α -granule proteins endocytosed from plasma (fibrinogen and factor V) and α -granule proteins made by megakaryocytes (multimerin 1, von Willebrand factor, thrombospondin-1, P-selectin, osteonectin, fibronectin) [2,8]. Studies of another Quebec family, with identical platelet abnormalities, confirmed these features and that the condition was associated with a reduction (of about 50%) in platelet counts to reduced or low-normal values and reduced platelet aggregation responses to epinephrine, with or without reduced aggregation with ADP and collagen [3]. The complexity of abnormalities in platelet number, function, and stored proteins in this disorder led to its redesignation as the 'Quebec platelet disorder' to reflect its complex phenotype [3].

Immunoelectron microscopy studies of the proteins degraded in QPD platelets excluded a defect in targeting proteins to platelet α -granules as the cause of their degradation [3]. As a result, QPD platelets were postulated to contain unregulated protease activity [3]. The abnormal protease activity was suggested to be within a secretory compartment as proteins on the external membrane and in the cytosol of QPD platelets were not degraded [2,3]. Unregulated protease activity was subsequently detected, and it was enhanced by plasminogen [5]. The protease abnormality was characterized as an increased expression and storage of the fibrinolytic enzyme urokinase-type plasminogen activator (u-PA) in QPD

megakaryocytes/platelets [5]. Later, increased intraplatelet (without systemic) plasmin generation was documented in the QPD [6], implicating platelet u-PA-triggered activation of plasmin as the cause of QPD bleeding and α - granule protein degradation.

A common ancestor was found recently [4] for the two families with the QPD. Since that report, additional cases have been identified that are related to this family (E. Maurer-Spurej, C.P.M. Hayward, and G.E. Rivard, unpublished observations). As a result, there are now more than 30 individuals in Quebec and other provinces of Canada who are known to have QPD. This corresponds to an overall prevalence of the QPD of approximately one per million in Canada and about one per 300 000 in the Province of Quebec. QPD has been traced back seven generations and it is not yet known whether this disorder originated before or after immigrants from France settled in the region of North America that became the Province of Quebec within Canada [3]. No cases have been reported outside Canada.

1.6.4 Clinical features of Quebec platelet disorder

The most comprehensive information on the clinical features of OPD comes from a recent study that used a standardized bleeding history questionnaire to evaluate the medical histories of 127 relatives in the QPD family [4]. This study used specific tests for OPD (i.e. assessments for increased platelet u-PA and degraded platelet fibrinogen) to determine which family members had the QPD, before determining risks for bleeding, as odds ratios, due to inheritance of the QPD by comparing unaffected and affected family members. The odds ratios for different QPD bleeding symptoms and problems are summarized in Table 1.6. Not surprising for an autosomal-dominant disorder, QPD is associated with a strong family history of bleeding, as all affected family members knew about relatives with bleeding problems [4]. Many individuals with QPD have changed their lifestyle due to their bleeding problems [4]. Their most serious bleeding episodes occur typically after exposures to haemostatic challenges, without fibrinolytic inhibitor therapy [4]. All affected individuals exposed to hemostatic challenges report increased, delayed-onset bleeding after each invasive dental or surgical procedure unless treated with a fibrinolytic inhibitor [4]. If these individuals are not treated, the excessive bleeding typically becomes evident 12– 24 h after exposure to hemostatic challenge, although sometimes it does not occur until 3-4 days following a challenge [1-3]. Untreated, QPD bleeding can persist for days or weeks [1-3].

Other common bleeding manifestations of QPD include bruises that are very large or track downward [4]. Approximately one-half of the affected individuals experience joint bleeds, which can lead to typical hemophilic arthropathy [2,4]. Approximately one-half experience episodic spontaneous hematuria that resolves without therapy, and this is more common among those with relatively higher platelet u-PA [4]. Other symptoms associated with QPD include excessive bleeding after cuts and delayed wound healing (odds ratio = 5), which is more common among those with lower platelet counts [4]. Risks for nose bleeds in QPD are only modestly increased [4].

For women with QPD, there is a modest increase in risk for experiencing prolonged menses [4] (Table 1.6). Most pregnancy outcomes are good, without increased risks for bleeding during pregnancy or an uncomplicated childbirth [4]. Fetal outcomes also appear to be good, as the numbers of offspring born to affected individuals with QPD, and the proportion of affected offspring, are not significantly reduced [4].

QPD life expectancy appears to be similar to other family members since fatal bleeding episodes are rare, particularly in those who receive treatment [4]. Although intracranial bleeding is not a common QPD bleeding manifestation, it caused the death of at least three affected family members [2].

Interestingly, none of the individuals with QPD, or their affected ancestors, are known to have suffered from thrombotic stroke, myocardial infarction or angina [4], suggesting that increased platelet u-PA in humans offers some protection against arterial thrombosis, as it does in mice [9].

Bleeding Symptom	Prevalence (proportion with symptom)	Odds Ratio (95% confidence interval)
Family history of bleeding	100 % (23/23)	137 (37 - 1000)
Excessive bleeding when challenged without	100% (19/19)	NE
fibrinolytic inhibitor therapy		
No excessive bleeding when challenged on	100% (12/12)	NE
fibrinolytic inhibitor therapy		
Prolonged bleeding after dental extractions ($> 24h$) ^b	94 % (16/17)	176 (18 - 4250)
Bleeding that has led to a change in lifestyle	60 % (12/20)	∞ (1.9 - ∞)
Experience nosebleeds	57 % (13/23)	4.0 (1.4 - 12)
Bleeding continuing for days after a deep cut	56 % (9/16)	37 (5.6 - 320)
History of transfusions	52 % (12/23)	9.8 (3.1 - 32)
Hematuria	50 % (11/22)	7.7 (2.4 - 25)
Menses lasting longer than 7 days	50% (3/6)	14 (1.6 - 147)
Joint bleeds	43 % (10/23)	∞ (7.4 - ∞)
Required transfusions after surgery(ies) ^b	38 % (5/13)	21 (2.9 - 195)
Required other treatments for bleeding after	38 % (5/13)	22 (2.9 - 198)
surgery(ies) ^b		
Bruises that spread downward or size of an orange or	32 % (7/23)	∞ (4.6- ∞)
larger		
Problems healing after an injury	26 % (6/23)	4.9 (1.3-19)

Table 1.6 Bleeding symptoms associated with Quebec platelet disorder, ranked by prevalence^a.

^aModified from published data [4]. Higher odds ratios (OR) reflect a higher risk for bleeding problems in individuals with Quebec platelet disorder compared with their unaffected family members. ∞ odds ratios estimated as infinite. NE, odds ratios that could not be evaluated as the information was not collected for unaffected relatives. ^bAbnormal bleeding with challenges was only reported by family members with Quebec platelet disorder who had not received fibrinolytic inhibitor therapy.

1.6.5 Overview of urokinase plasminogen activator and changes to this protease in Quebec platelet disorder

To understand the changes to u-PA in QPD, a brief review on this plasminogen activator is presented. Like tissue-type plasminogen activator, u-PA is a serine protease that functions to convert the zymogen plasminogen to plasmin, a broad-specificity enzyme that degrades fibrin, fibrinogen, and a variety of protein substrates (reviewed in [10–12]). u-PA and tissue-type plasminogen activator appear to have complementary roles in generating sufficient plasmin for fibrinolysis, as combined deficiency of these proteins results in greater impairment of fibrinolysis [10,12–21]. The overexpression of u-PA in mouse hepatocytes results in elevated u-PA in plasma and systemic fibrinolysis, whereas overexpression in mouse platelets triggers intraplatelet protein degradation without systemic fibrinolysis, similar to QPD [9,17].

In humans, u-PA is encoded by a single copy gene, PLAU, on chromosome 10 [22]. PLAU contains a number of regulatory elements that include the TATA box, the CAAT sequence, and the GC-rich regions of the proximal promoter, and other transcription factor-binding elements including COM, Ets/AP1, nuclear factor-kB, RUNX/AML and GATA-4 sites (reviewed in [23]). The u-PA gene also contains enhancer and silencer elements in the 5' regulatory region [24-26], and an AU-rich element in its 3'-untranslated region that influences u-PA mRNA turnover and stability [27]. The regulatory elements important for physiological regulation of u-PA are not well understood as most studies on u-PA expression have evaluated cultured cells. Nonetheless, the factors that are thought to modulate u-PA regulation in vivo include: transcription factors, such as AP1, Ets and Sp1 [28-30]; inflammatory modulators, such as interleukin-1. lipopolysaccharide and transforming growth factor- β [31–33]; growth factors, such as fibroblast growth factor-2 and hepatocyte growth factor [34,35]; hormones, including calcitonin and vasopressin [36,37]; and oncogenes, such as Ras/ERK [38]. Investigations are in progress to determine whether QPD is associated with a mutation in the u-PA gene.

Normally, u-PA is ubiquitously expressed at low levels in many different tissues [39], which is thought to be important for modulating fibrinolysis with tissue-type plasminogen activator (reviewed in [10–12,20]), and in proteolysis of the extracellular matrix, which influences cell migration [40,41]. Normally, large amounts of u-PA (40–80 μ g/ml) are found in urine [42]. Although normal plasma contains only small amounts of u-PA (1–4 ng/ml, 20–70 pmol/l) [5,7,43–45], the molar amount is similar to that of tissue-type plasminogen activator (70 pmol/l) [46]. Normal platelets also contain small amounts of u-PA (up to 1.3 ng/10⁹ platelets) [5,47–49]. Monocytes and neutrophils contain additional u-PA, although the precise amounts are unknown [50,51]. Secondary increases in u-PA expression have been implicated in the pathogenesis of a variety of cancers (including breast cancer, gastric cancer, colorectal cancer and ovarian

malignancies) [52]. QPD, however, is the only inherited disorder known to increase u-PA expression [5].

The translation of u-PA message in different tissues generates the singlechain form of u-PA, which has only low intrinsic catalytic activity [10,13,53]. The active forms of u-PA are generated by proteolytic cleavage and include two-chain u-PA and low-molecular-weight u-PA. Plasmin is important for converting singlechain u-PA to two-chain u-PA, although other proteases (such as matrix metalloproteinases) have also been implicated (reviewed in [10,54]). The activation of single-chain u-PA is enhanced by the u-PA receptor, u-PAR [54– 56], which also increases u-PA synthesis by endothelial cells and monocytes [57]. Low-molecular-weight u-PA is generated by further proteolysis of two-chain u-PA by plasmin, which removes the epidermal growth factor- like and kringle domains [10].

In vivo, the profibrinolytic effects of two-chain u-PA are inhibited by plasminogen activator inhibitor 1 (PAI-1) and protease nexin I [58], with PAI-1 having the more important role (reviewed in [10]). Two-chain u-PA is downregulated by binding to low-density lipoprotein receptor- related protein, and to the very-low-density lipoprotein receptor on hepatocytes, which triggers u-PA internalization and degradation (reviewed in [10]). The effects of u-PA are further down-regulated by inhibitors of plasmin, which include α_2 -plasmin inhibitor and α_2 -macroglobulin [59]. Of the two, α_2 -plasmin inhibitor is the more important inhibitor and it rapidly inhibits plasmin by forming a stable plasmin-inhibitor complex [60,61]. The fibrinolytic inhibitor drugs, tranexamic acid and ε aminocaproic acid, are lysine analogue compounds that competitively block the actions of plasmin, by blocking the lysine binding sites essential for fibrin binding [62].

The overexpression of u-PA in megakaryocytes, and its storage in QPD platelets, is thought to protect individuals with QPD from developing systemic fibrinolysis [5–7,63]. In mice, the overexpression of u-PA in megakaryocytes causes bleeding and it imparts resistance to arterial and venous thrombosis [9]. These mice, however, have some problems that are not features of QPD [4]. Unlike women with QPD, who have good outcomes with pregnancy [4], these mice suffer from increased bleeding related mortality with pregnancy, and pregnancy loss, unless treated with fibrinolytic inhibitors [9]. These differences could reflect the higher platelet counts, or platelet content of u-PA, in mice compared with humans with QPD [4]. Nonetheless, these mice illustrate that increased platelet u-PA can trigger increased bleeding and α -granule protein degradation, while also increasing resistance to venous and arterial thrombosis [9].

1.6.6 Laboratory features of Quebec platelet disorder

QPD can be difficult to diagnose unless definitive biochemical features of the disorder are assessed. The laboratory findings for this disorder are summarized in Table 1.7.

Abnormalities in Quebec platelet disorder platelet numbers and function

Individuals with QPD typically have mild thrombocytopenia to lownormal platelet counts due to an approximate 50% reduction in circulating platelet counts (range of observed values, $80-245 \times 10^9$ platelets/l) [2–4]. The reason for this reduction is unclear. The platelet counts of mice that overexpress u-PA in megakaryocytes were not reported [9]. QPD platelet survival, evaluated using Cr51, was determined to be normal [1].

In general, screening tests of primary hemostasis are not helpful for the diagnostic assessment of QPD as bleeding times range from normal to mildly prolonged [2,3], and closure times measured by the Platelet Function Analyzer 100 (PFA-100; Siemens Medical Solutions Diagnostics, Deerfield, Illinois, USA) are typically normal (C.P.M. Hayward and G.E. Rivard, unpublished data). QPD is associated with abnormal platelet function in light transmission aggregation studies of platelet-rich plasma without evidence of dense granule deficiency [2,3]. Individuals with OPD typically have absent platelet aggregation in response to low concentrations (e.g. 6-10 µmol/l) of epinephrine, and with higher concentrations (e.g. 30-40 µmol/l) the typical pattern is absent or very delayed aggregation [2,3]. Some affected individuals in the QPD family, however, have been documented to have a primary wave response to epinephrine, without secondary aggregation (Fig. 1.7). With other agonists, OPD platelet aggregation responses can be normal, although some affected individuals have reduced aggregation with ADP (Fig. 1.7) and collagen (data not shown) [2.3]. As the aggregation abnormalities of QPD (reduced aggregation with epinephrine and/or ADP and collagen) are not specific for this disorder, and similar abnormalities are commonly seen in inherited platelet secretion defects [64], more definite tests are required to diagnose OPD (assays for increased platelet u-PA and α -granule protein degradation), as outlined in the sections that follow. QPD platelets are functionally normal in assays of fibrin adhesion, although this is followed by accelerated fibrinolysis [7].
Table 1.7 Laboratory test findings for Quebec platelet disorder.

Test	Expected result
Platelet count	Normal to mildly reduced (80 - 245×10^9 platelets/L)
Bleeding time	Normal to mildly prolonged
PFA-100 closure time	Normal
Platelet aggregation:	
6-10 μM epinephrine	Typical: absent response
	Less common: primary wave only
30-40 µM epinephrine	Typical: absent or very delayed responses
	Less common: primary wave only
ADP	Normal or reduced
Collagen	Normal or reduced
Arachidonic acid	Normal
Ristocetin	Normal
Plasma factor V coagulant activity	Normal to mildly reduced
Serum fibrin(ogen) degradation products evaluated by polyclonal antibody assays:	Elevated in serum from whole blood (20-80 µg/mL) or from platelet rich plasma (levels dependent on platelet concentration)
	Normal (< 5 µg/mL) in serum from platelet poor plasm
Plasma D-dimer	Normal
Dilute Whole Blood Clot Lysis Time	Normal
Plasmin - α_2 plasmin inhibitor complexes	Normal in plasma
	Increased in platelets
Tests Useful for Definitive Diagnosis:	
Platelet u-PA ELISA	Increased uPA > 100 fold (approximately 400 to 600 n u -PA/10 ⁹ platelets)
Platelet u-PA Western blot	Increased u-PA, predominantly as tcu-PA
Western blot assays for degraded α -granule proteins such as fibrinogen, von Willebrand factor, thrombospondin-1, osteonectin, fibronectin, or P-selectin	Characteristic pattern of α -granule protein degradation



Figure 1.7 Quebec platelet disorder (QPD) platelet aggregation abnormalities. Light transmission aggregation responses for control (C) and QPD (Q) platelet-rich plasma, with ADP (final concentrations: C, 5 μ mol/l; Q1 and Q3, 20 μ mol/l; Q2, 30 μ mol/l) and 30 μ mol/l epinephrine (all samples). In QPD, responses to ADP can be reduced (Q1- Q3) or normal (data not shown), and responses to epinephrine are commonly absent (Q1) although primary aggregation without a secondary wave has been observed in some affected individuals (Q2, Q3).

Fibrinolytic abnormalities in Quebec platelet disorder

QPD is associated with an unusual but characteristic pattern of abnormalities in tests of fibrinolysis, related to the increased platelet u-PA (Table 1.7). Most tests of fibrinolysis performed in clinical laboratories, however – including plasma D-dimer levels, euglobulin clot lysis times, plasma plasmin– α_2 antiplasmin complexes, and dilute whole-blood clot lysis times – are normal in QPD [6,7,63]. QPD platelets (but not QPD plasma) contain degraded fibrinogen, and therefore serum prepared from QPD whole blood, or platelet-rich plasma, tests positive in commercial polyclonal antibody assays for fibrin(ogen) degradation products (FDP), such as the serum FDP assay by Siemens Medical Solutions Diagnostics (values: 20–80 µg FDP/ml serum) [6,7,63]. The positive results in serum FDP assays, in contrast to negative results in plasma D-dimer tests, help to distinguish QPD abnormalities from disseminated intravascular coagulation [63].

The most definite and specific test for QPD is an assessment for increased platelet u-PA antigen by enzyme-linked immunosorbent assays (ELISA) or by western blotting analysis (Table 1.7 and Fig. 1.8). In QPD platelets, the quantities of stored u-PA range from about 400 to 600 ng u-PA/10⁹ platelets, which is well above the normal level of less than 1.4 ng u-PA/10⁹ platelets [5]. Forty-five percent to 100% of the u-PA in QPD platelets is released in response to activation by thrombin *in vitro* [7]. Most OPD plasmas contain normal amounts of u-PA (up to 4 ng/ml), particularly when prepared using platelet activation inhibitors to prevent artifactual increases from platelet u-PA release [5,7]. While the quantities of u-PA in normal platelets are often too low for detection by western blot analyses [4,5], QPD platelet u-PA is readily detected - and it migrates predominantly as active two-chain u-PA, and smaller amounts of low-molecularweight u-PA and high-molecular-weight forms complexed to PAI-1 (Fig. 1.8) [5]. In QPD platelets, the stores of active PAI-1 are depleted by the increased u-PA, which requires additional PAI-1 for full inhibition [5]. The proportion of platelet PAI-1 that is bound to u-PA in QPD platelets in vivo (approximately 5%) [5] corresponds to the proportion of PAI-1 estimated to be active (<10%) in normal platelets [65–67]. The presence of unregulated, active u-PA in OPD platelets is associated with conversion of platelet plasminogen to plasmin, which can be detected by western blot assays [5] or by ELISA for plasmin- α_2 antiplasmin complexes in platelets [6].

Dilute whole-blood clot lysis time assays, and thromboelastography of whole blood or platelet-rich plasma clots, are not sufficiently sensitive to detect the increased fibrinolytic potential in QPD blood [7,63]. When QPD platelets are incorporated into forming clots *ex vivo*, however, extracellular plasmin generation and D-dimer generation are measurably increased [7]. Clots rich in QPD platelets are initially normal in appearance, but over time they become abnormally thin and small, due to increased plasmin generation that leads to premature disruption of

fibrin strands, platelet aggregates, and platelet–fibrin contacts. The perfusion of a preformed fibrin clot with QPD blood results in a normal amount of platelet adhesion; however, this is followed by accelerated fibrin degradation that can be inhibited by fibrinolytic inhibitors and by drugs that attenuate platelet activation (Fig. 1.9) [7]. These experimental models suggest that, at sites of injury, QPD platelets adhere and release u-PA that triggers accelerated fibrinolysis, akin to the fibrinolytic defect in mice that overexpress u-PA in platelets [9]. This 'gain of function defect in fibrinolysis' offers an explanation for the characteristic delayed-onset bleeding that occurs when individuals with QPD are exposed to a hemostatic challenge, unless treated with a fibrinolytic inhibitor. The loss (due to proteolysis) of prohemostatic, normal α -granule proteins, including platelet factor V, however, may possibly further contribute to QPD bleeding. The proposed, central role of increased platelet u-PA in QPD bleeding is illustrated in Figure 1.10.



Figure 1.8 Diagnostic abnormalities in Quebec platelet disorder (QPD) platelet proteins. Western blot analyses illustrate key diagnostic features of QPD: unlike control platelets (C), QPD platelets (Q) contain large amounts of urokinase plasminogen activator (u-PA), predominantly in the form of active, two-chain u-PA (tcu-PA) (a), and they also contain degraded megakaryocyte-synthesized and plasma-derived α -granule proteins (b). \blacktriangleleft , degradation products; LMW u-PA, low molecular-weight urokinase plasminogen activator; scu-PA, single-chain urokinase plasminogen activator.

A



Figure 1.9 Profibrinolytic effects of Quebec platelet disorder (QPD) blood on fibrin clots. Data (reproduced with permission) from reference [7] illustrate the breakdown of preformed fibrin in chambers perfused with normal and QPD whole blood. QPD blood triggers rapid degradation of fluorescently-labeled fibrin (* P < 0.01) (A) unless the fibrinolytic inhibitor tranexamic acid (AMCA) is added. (B) Similar experiments, evaluating the degradation of biotin-labeled fibrin in the chambers (degradation products indicated by arrows), illustrate that, like AMCA, platelet activation inhibitors [aspirin and prostaglandin E₁ (PGE₁)] attenuate the profibrinolytic effects of QPD blood. C, control plasma.



Figure 1.10 The proposed role of urokinase plasminogen activator (u-PA) in Quebec platelet disorder (QPD) bleeding. The increased expression of u-PA in QPD (Q), but not control (C), platelets results in increased levels of the u-PA protein in QPD platelets. QPD platelets contain predominantly active, two-chain u-PA (tcu-PA) that is partially inhibited by plasminogen activator inhibitor-1 (PAI-1). Excess amounts of active u-PA lead to intraplatelet plasmin generation [heavy chain (HC) is shown] with QPD platelets. Plasmin degrades several α -granule proteins (e.g. von Willebrand factor is shown) to characteristic degradation products, seen on western blot analyses (\blacktriangleleft). Furthermore, stored u-PA is released into the hemostatic plug at sites of injury, which triggers further plasmin generation and clot lysis. The accelerated clot lysis results in delayed bleeding, as illustrated by the delayed-onset hematoma a QPD individual suffered after a bumpy snowmobile ride.

Abnormalities in Quebec platelet disorder α -granule proteins

QPD α -granule proteins show a characteristic pattern of partial degradation (Fig. 1.8) that is not evident in other platelet storage pool disorders [2,3,6,68,69]. As a result, western blot assays for degraded α -granule proteins can be used for the diagnostic evaluation of QPD [2-6] (Fig. 1.8 and Table 1.7). The proteins degraded in QPD platelets include megakaryocyte-synthesized soluble agranule proteins (e.g. von Willebrand factor, thrombospondin-1, osteonectin), plasma-derived soluble α -granule proteins (e.g. factor V and fibrinogen), and the α -granule membrane protein P-selectin (Figs 1.8 and 1.10) [2,3,8]. The changes to these proteins can be mimicked by adding two-chain u-PA to normal platelet proteins, which also results in a quantitative loss of multimerin 1, an α -granule protein degraded by plasmin that typically appears quantitatively deficient in QPD platelets, assessed by ELISA and western blot analyses [2,3,6]. QPD platelet factor V degradation [2,3,5,6,8] is thought to be the cause of the defective platelet factor V-dependent prothrombinase function, which can be corrected ex vivo by plasma factor V [1]. Unlike platelet factor V, plasma factor V is not degraded in QPD; however, some affected individuals have mildly reduced plasma factor V coagulant activity [1,2,8].

The loss of normal α -granule proteins may possibly contribute to QPD bleeding; however, the delayed bleeding problems of QPD are quite different from the bleeding problems associated with severe α -granule protein deficiency [4,64,68].

1.6.7 Principal approach to the diagnosis and treatment of Quebec platelet disorder

At present, all known cases of QPD have been linked to a single family. A careful assessment of the bleeding history, family history and ancestry is important to the diagnostic evaluation of QPD. The failure to recognize QPD as a potential cause of delayed bleeding problems has resulted in delayed or missed diagnosis. In some affected individuals, the reduction in platelet count below the reference range has been mistaken for more common conditions, such as immune thrombocytopenia. Others were initially thought to have an unspecified platelet function defect, due to the aggregation test abnormalities before their relationship to the family was documented (E. Maurer-Spurej, C.P.M. Hayward and G. E. Rivard, unpublished observations).

The medical history for an assessment of QPD should ask about bleeding problems with high odds ratios for QPD (Table 1.6). First, the history should include question about problems with significant, delayed-onset bleeding following each hemostatic challenge and about responses to treatments, as response exclusively to fibrinolytic inhibitor therapy is a key feature of the disorder. Second, the history should also include questions about a family history of bleeding and of ethnic origin (i.e. French Canadian ancestors) and a personal or family history of joint bleeds; spontaneous hematuria and intracranial hemorrhage. Third, the history should contain queries about other bleeding symptoms (e.g. very large bruises) as this can be helpful to define the spectrum of the individual's bleeding problems, although these symptoms may be less specific for QPD.

Laboratory tests are required for the diagnostic assessment of QPD (Table 1.7), including the evaluation of family members who have not yet had exposures to hemostatic challenges (e.g. children) or have bleeding symptoms that suggest a different kind of bleeding problem (e.g. immediate bleeding with a hemostatic challenge). Aggregation assays and tests for increased serum FDPs can be useful for an initial clinical laboratory assessment, but the most definitive laboratory tests for QPD are assays of platelet u-PA (ELISA or western blot analyses) and assessments for α -granule protein degradation by western blot analyses (Fig. 1.8 and Table 1.7).

Table 1.8 outlines a practical approach to the treatment of QPD. The treatment of choice for QPD is fibrinolytic inhibitor drug therapy with tranexamic acid or ε -aminocaproic acid, as both are quite effective for preventing and treating QPD bleeding [4]. Aprotinin therapy may be appropriate for some rare circumstances, as hemostasis was achieved in one individual with QPD who received this drug during liver transplantation for transfusion-acquired hepatitis C [4]. Other therapies, including plasma, platelet transfusion, and desmopressin ([1,2,4] and G.E. Rivard, unpublished observations), have been tried – but as they are not effective for managing QPD bleeding, they are not recommended.

Fibrinolytic inhibitor therapy is indicated for preventing QPD bleeding with surgery and dental extraction, and for treating QPD joint bleeds and bleeds from trauma. Fibrinolytic inhibitor therapy is not recommended during pregnancy or uncomplicated childbirth as increased bleeding is uncommon in these situations. Fibrinolytic inhibitor therapy should be used with caution in situations with high risks for thrombosis. Pulmonary embolism developed in an individual with QPD who was on appropriate fibrinolytic therapy for a hematoma after a complicated childbirth that necessitated a Caesarian section [4]. Long-term (several months) administration of tranexamic acid seems to decrease frequency of bleeding in individuals with QPD and synovitis associated with 'hemophilic' arthropathy (G.E. Rivard, unpublished observations). Gastrointestinal bleeding is not increased in QPD. Individuals with QPD who present with gastrointestinal bleeding therefore require the usual investigations for this problem.

Table 1.8 Treatment recommendations for Quebec platelet disorder bleeding.

Indication	Recommended length of fibrinolytic inhibitor treatment with tranexamic acid or ε-aminocaproic acid at standard doses
Minor bleeding except spontaneous hematuria (e.g. prolonged bleeding after cuts, spontaneous hemarthrosis, loss of primary teeth)	3 - 4 days
Bleeding after major trauma	5 - 7 days
Intracranial bleeding (spontaneous or traumatic)	10 - 14 days
Minor surgery (e.g. biopsy)	3 - 4 days
Dental extractions	4 - 5 days
Major surgery	5 - 7 days with an intravenous dose before surgery
Spontaneous hematuria, pregnancy and uncomplicated childbirth	Treatment not indicated
Menorrhagia	Treatment on days of heavy menses

1.6.8 Genetic cause of Quebec platelet disorder

The genetic defect that causes QPD is under investigation. The condition is inherited as an autosomal dominant trait with high, and perhaps complete, penetrance [4]. As QPD platelets contain increased u-PA mRNA [5], we postulate that the condition results from aberrant transcriptional regulation of u-PA. The normal levels of u-PA in QPD plasma suggest that the QPD is associated with a lineage-restricted defect in u-PA regulation, possibly the result of mutations in regulatory elements of the PLAU gene, or in transcription factors that regulate u-PA production in megakaryocytes. We postulate that the resulting increase in u-PA expression leads to the production of profibrinolytic QPD platelets, as outlined in Figure 1.11. Although QPD platelets appear to account for the major fibrinolytic abnormalities in QPD blood [7], overexpression of u-PA in other cells involved in vascular injury responses, such as endothelial cells or leukocytes [50,51,57,70–73], is not yet excluded. Identification of the genetic cause of QPD will be an important next step forward in understanding this disorder.

1.6.9 Discussion

A vast amount of knowledge has been gained since QPD was first described over 20 years ago. The bleeding risks associated with the OPD are now well defined, and the reason for the delayed-onset bleeding is better understood. The laboratory tests most useful for the diagnostic evaluation of the QPD are not available in most clinical laboratories, but aggregation tests and serum FDP assays are often helpful to exclude the disorder. It should be possible to differentiate QPD from other, more common, disorders of platelet function provided that the bleeding history is carefully reviewed for the presence of delayed-onset bleeding after challenges that respond only to fibrinolytic inhibitor therapy, with or without a personal or family history of joint bleeds and spontaneous hematuria. Although a functional defect in platelet factor V is part of QPD, increased fibrinolysis at sites of injury, due to increased platelet u-PA, is now thought to be a major contributor to QPD bleeding. Identification of the genetic mutation that causes the QPD defect, and when and how it increases u-PA expression during megakaryopoiesis, will provide further important insight into the mechanisms that can transform normal, antifibrinolytic platelets into cells with powerful clot lytic activity.



Figure 1.11 Generation of profibrinolytic Quebec platelet disorder (QPD) platelets: a hypothesis. Comparison of the relative cellular contents of urokinase plasminogen activator (u-PA), plasminogen activator inhibitor-1 (PAI-1) and plasminogen as normal and QPD hematopoietic stem cells differentiate into megakaryocytes and platelets. Normally, u-PA levels remain low, and the increased PAI-1 expression (tied to differentiation) generates antifibrinolytic platelets. In QPD, u-PA expression and production is increased and this consumes PAI-1 and generates profibrinolytic platelets. The endocytosis of plasminogen occurs late in megakaryopoiesis and, in QPD megakaryocytes (MKs), its exposure to u-PA triggers plasmin-mediated α -granule protein degradation.

1.6.10. References

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1.7 THESIS HYPOTHESES AND OBJECTIVES

The clinical presentation and biochemical defect in QPD have been well characterized (reviewed in section 1.6). However, several unanswered questions remain about this unique bleeding disorder. First, the effects of increased QPD platelet uPA on clot lysis have never been formally investigated. Second, although QPD significantly alters uPA levels in platelets, without changing plasma levels of uPA, no studies have been done to evaluate if QPD changes the levels of uPA at other sites, such as urine. Finally, the nature of the genetic mutation responsible for the high levels of uPA in QPD platelets is not known. This section outlines the questions, hypotheses and specific objectives that were generated, based on the background knowledge presented in sections 1.1 to 1.6 of this chapter, to study these aspects of QPD.

1.7.1 What are the effects of increased QPD platelet uPA on clot lysis?

Rationale

Normal platelets are known to have antifibrinolytic properties, as they release large amounts of PAI-1 into forming clots that contributes to the resistance of platelet-rich thrombi to fibrinolysis [139,141,142]. Evaluation of platelet-rich clots by electron microscopy has shown that platelets have a protective effect on plasmin-induced clot lysis *in vitro*, as they increase the density of fibrin strands in areas containing platelets [139]. Platelet-fibrin interactions contribute to clot retraction, which further reduces the binding of plasminogen activators and plasmin generation in platelet-rich regions [136,138,143]. Thus, platelet-poor plasma clots are much more susceptible to lysis than normal platelet-rich clots. As QPD platelets contain large amounts of stored uPA (that are released upon platelet activation), coupled with consumption of active forms of platelet PAI-1 [243], this suggests that QPD platelets may induce accelerated lysis when they are incorporated into clots. The release of stored platelet uPA from activated platelets could contribute to increased local generation of plasmin to promote premature clot breakdown. Based on this knowledge, the following hypothesis was generated:

Increased uPA in QPD platelets leads to accelerated lysis of forming or preformed fibrin clots in vitro. These effects can be attenuated by platelet activation inhibitors or fibrinolytic inhibitors.

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

- 1. To determine if the incorporation of QPD platelets into forming clots causes premature clot lysis *in vitro*.
- 2. To evaluate if clots containing QPD platelets have altered morphology.

3. To determine if the effects of QPD platelets on clot lysis can be attenuated when clots are made in the presence of platelet activation inhibitors or fibrinolytic inhibitors.

1.7.2 Does QPD alter uPA levels in urine?

So far, uPA levels in QPD individuals have been evaluated only in platelets and plasma [243]. QPD has been shown to alter both the expression and storage of uPA in platelets, while plasma uPA levels are normal [243]. This information suggests that QPD may be restricted to the MK lineage, raising the possibility that QPD may not increase uPA in other cell types. Clinical data has shown that QPD individuals are at risk for experiencing a variety of bleeding symptoms, including hematuria, which is associated with higher levels of platelet uPA [293]. This suggests that hematuria in QPD individuals might be a consequence of increased platelet uPA, rather than changes in urinary uPA levels. Thus, for this part of my thesis, I generated the following hypothesis:

QPD does not increase uPA levels in urine.

To test this hypothesis, the specific objective of this study was:

1. To measure uPA in 24 hour urine collections from QPD and normal individuals.

1.7.3 What is the genetic locus harboring the QPD mutation? Is the causative mutation for QPD linked to *PLAU*?

Unlike most bleeding disorders, which are caused by haemostatic protein deficiencies or dysfunction, QPD is caused by a mutation that increases expression of uPA in platelets [243], and it is the only known inherited disorder that leads to uPA overexpression. Increased expression of uPA in QPD suggests that PLAU might be the candidate gene for QPD. The types of mutations that could cause overexpression of genes are discussed in detail in chapter 5. Some general modes of uPA overexpression that need to be considered as potential molecular causes of QPD, include mutations within PLAU or its regulatory regions, or mutations in another gene (eg. a transcription factor) that could cause dysregulated PLAU transcription. Briefly, these may be:

- a) Mutations within *PLAU* exons or introns that potentiate transcriptional activation.
- b) Mutations in *PLAU* promoter elements or other transcription factor binding elements, resulting in increased binding of transcription factors and increased expression.

- c) Mutations in *PLAU* enhancer elements that potentiate enhancer binding and increase transcription.
- d) Mutations in *PLAU* silencer elements that prevent binding of a gene silencer, leading to reduced transcriptional silencing.
- e) Mutations in instability elements in the 3' region of *PLAU*, resulting in increased mRNA stability.
- f) Deletions, insertions or rearrangements of DNA affecting transcriptional control of *PLAU*.
- g) *PLAU* gene duplications or amplifications causing increased "gene dosage".
- h) Mutations in the gene for a transcription factor or regulatory protein that increases its affinity for a *PLAU* regulatory element where it can induce transcription.

Increased uPA mRNA in QPD has previously been evaluated by a semiquantitative approach [243]. However, it is not known if QPD is caused by overexpression of one or both *PLAU* alleles in platelets. To investigate the genetics of QPD, I generated the following hypotheses:

The genetic mutation that causes QPD is a cis-acting mutation linked to PLAU. This mutation results in specific overexpression of one PLAU allele (the linked allele) in MKs.

The specific objectives for addressing this hypothesis were:

- 1. To map the genetic locus of the QPD mutation and determine if inheritance of QPD is linked to *PLAU*.
- 2. To sequence *PLAU* exons, introns and known 5' and 3' regulatory regions in QPD and healthy individuals to identify possible mutations in QPD DNA.
- 3. If a putative mutation is not identified as part of objective 2, to extend sequencing further 5' and 3' of *PLAU*, and to use Southern blotting to look for larger-scale mutations (insertions, deletions, rearrangements).
- 4. To quantitate expression of each of the two uPA mRNA alleles in platelets, CD34⁺ MK precursors (early megakaryopoietic cells), and salivary cells (used as a control).

1.8 THESIS OUTLINE

This thesis contains three published manuscripts that represent my work to address the objectives outlined in section 1.7. These manuscripts have all been reproduced with permission from the publishers.

Chapter 2 contains the manuscript entitled "Insights into abnormal hemostasis in the Quebec platelet disorder from analyses of clot lysis", and focuses on determining whether the incorporation of QPD platelets into clots contributes to clot lysis in QPD. In this study, I used *in vitro* experiments to show that QPD platelets can accelerate clot lysis, and this is evident by the release of fibrinolytic markers into clot sera and by morpholgical changes in clots over time. The effects of QPD platelets are attenuated by fibrinolytic or platelet-activation inhibitors, suggesting an important role for platelet activation and induction of the fibrinolytic cascade in QPD clot lysis.

Chapter 3 focuses on determining if uPA levels are altered in QPD urine, and is comprised of the manuscript "Evaluation of urokinase plasminogen activator in urine from individuals with Quebec platelet disorder". This study revealed that individuals with QPD do not have increased levels of uPA in their urine, supporting the hypothesis that QPD is a MK/platelet- specific defect. The findings also indicate that hematuria experienced by some individuals with QPD is a consequence of having high levels of platelet uPA, rather than increased urinary uPA.

Chapter 4 focuses on determining the genetic locus and putative mutation associated with QPD, and if QPD is caused by overexpression of one *PLAU* allele in MKs. The manuscript "Quebec platelet disorder is linked to the urokinase plasminogen activator gene (*PLAU*) and increases levels of the linked *PLAU* allele in megakaryocytes" contains studies of linkage analysis with chromosome 10 microsatellite markers that showed a 2 MB region containing *PLAU* is linked to QPD, but no mutations were identified by DNA sequencing of a 48 kb region around *PLAU*, or by Southern blot for a 25 kb region containing *PLAU* and its known regulatory elements. Quantitative mRNA studies show that QPD selectively increases levels of the linked allele of *PLAU*, which suggests that QPD is caused by a *cis*-acting mutation on *PLAU*.

Chapter 5 contains a discussion of the thesis findings and their implications, as well as some possible future directions for determining the QPD mutation. Appendix I contains an additional manuscript entitled "Increased urokinase plasminogen activator in Quebec platelet disorder platelets is linked to megakaryocyte differentiation", which contributed to knowledge about the genetic defect in QPD. For this manuscript, I participated in the studies that showed that the two genes adjacent to *PLAU* on chromosome 10, *VCL* and *CAMK2G*, do not have altered expression in QPD. This suggests that the QPD genetic defect may specifically affect *PLAU* expression, which is important to better understand the nature of the causative mutation, once it is identified.

CHAPTER 2

STUDIES OF THE CONTRIBUTION OF QPD BLOOD TO IN VITRO CLOT LYSIS

2.1 PREFACE

The goal of this chapter was to gain insights on the contribution of QPD platelet uPA to clot lysis in vitro. In this study, clots made from whole blood, platelet-rich or platelet-poor plasma were analyzed by the following methods: 1) whole blood TEG, to follow clot formation and lysis after initiating coagulation with TF; 2) electron microscopy, to visualize fibrin and platelets in clots and changes to clot ultrastructure over time; 3) biochemical assays (ELISA) of fibrinolysis, to measure uPA, PAP complex, and D-dimer levels in clot sera over time; and 4) perfusion experiments, to evaluate the effects of OPD blood on preformed fibrin. By TEG, the fibrinolytic abnormalities in QPD were not detectable, as this test was not sensitive to the quantities of uPA in OPD blood. However, when clots were formed at low shear, significant increases in plasmin generation and fibrinolysis occurred, and this was associated with increased uPA levels in clot serum. By microscopy, clots containing QPD platelets had progressive disruption of fibrin and platelet aggregates unless a fibrinolytic inhibitor drug was added. Perfusion studies further showed that QPD whole blood accelerated the degradation of preformed fibrin and that this could be blocked by fibrinolytic or platelet activation inhibitors. Experiements measuring uPA levels in whole blood versus platelet-rich clot sera further showed that QPD whole blood contained higher levels of uPA than could be accounted for by the predicted amount stored in platelets, suggesting there may be an additional source of uPA in QPD blood. In summary, this study demonstrated that QPD is associated with a "gain-of-function" abnormality that increases the lysis of forming or preformed clots, and this abnormality is dependent on platelet recruitment and activation. This suggests that accelerated fibrinolysis may be an important contributor to QPD bleeding, and provides a potential explanation for the delayed-onset bleeding that is characteristic to OPD.

The senior author for these studies was Dr. Catherine P.M. Hayward, who supervised the study design, experimental work and manuscript writing. I contributed to study design, experimental work, data analysis, and writing of the manuscript. Dr. Frederic Adam, with whom I share first authorship, contributed to study design, manuscript writing, and performed and analyzed platelet adhesion and perfusion experiments. I am grateful to Drs. Walter H.A. Kahr, Kathy A. Chorneyko, and A. Larry Arsenault for their contributions to study design, data interpretation and manuscript writing. I thank Ping Wang and Angelique Hofer for their technical help on the studies, and Francine Derome for her contribution to patient recruitment and sample collection. Finally, I gratefully acknowledge Dr. Georges E. Rivard for his guidance and contributions to study design, patient recruitment, data interpretation, and manuscript writing.

CHAPTER 2.2

INSIGHTS INTO ABNORMAL HEMOSTASIS IN THE QUEBEC PLATELET DISORDER FROM ANALYSES OF CLOT LYSIS

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Haemostasis.

2.2.1 Summary

Background: The Quebec platelet disorder (QPD) is inherited and characterized by delayed-onset bleeding following hemostatic challenge. Other characteristics include increased expression and storage of active urokinase-type plasminogen activator (u-PA) in platelets in the setting of normal to increased u-PA in plasma. There is also consumption of platelet plasminogen activator inhibitor-1 and increased generation of plasmin in platelets accompanied by proteolysis of stored a-granule proteins, including Factor V. Aims and Methods: Although fibrinolysis has been proposed to contribute to QPD bleeding, the effects of QPD blood and platelets on clot lysis have not been evaluated. We used thromboelastography[®] (TEG[®]), biochemical evaluations of whole blood clot lysis, assessments of clot ultrastructure, and perfusion of blood over preformed fibrin to gain insights into the disturbed hemostasis in the OPD. Results: Thromboelastography was not sensitive to the increased u-PA in QPD blood. However, there was abnormal plasmin generation in QPD whole blood clots, generated at low shear, with biochemical evidence of increased fibrinolysis. The incorporation of QPD platelets into a forming clot led to progressive disruption of fibrin and platelet aggregates unless drugs were added to inhibit plasmin. In whole blood perfusion studies, OPD platelets showed normal adherence to fibrin. but their adhesion was followed by accelerated fibrinolysis. Conclusions: The QPD is associated with 'gain-of-function' abnormalities that increase the lysis of forming or preformed clots. These findings suggest accelerated fibrinolysis is an important contributor to QPD bleeding.

2.2.2 Introduction

Congenital bleeding disorders usually result from defects or deficiencies in cellular or plasma components needed for hemostasis [1–4]. Some of these conditions, although rare, have provided important insights into the factors that limit bleeding. During normal hemostasis, platelets adhere at sites of vessel injury, and their activation and release of prothrombotic substances such as adenosine 5'-diphosphate (ADP) and thromboxane serve to enhance thrombin generation and hemostatic plug formation [5,6]. Platelets also help protect the hemostatic plug from premature lysis by releasing large quantities of plasminogen activator inhibitor-1 (PAI-1) at sites of injury [7–9].

The Quebec platelet disorder (QPD) is an autosomal dominant bleeding disorder, with increased risks for delayed-onset bleeding (12–24 h) after hemostatic challenges, that responds to fibrinolytic inhibitor therapy (tranexamic acid and epsilon-aminocaproic acid) but not to platelet or plasma transfusions [10–13]. The genetic basis of the QPD is not yet known. The QPD induces complex alterations in hemostatic and fibrinolytic proteins that may accelerate

fibrinolysis and impair platelet function. Features of the OPD include: markedly increased expression and storage of active, urokinase-type plasminogen activator (u-PA) in platelets despite normal to modestly increased u-PA in plasma; normal plasma levels of other fibrinolytic and coagulation proteins; chronic activation of the fibrinolytic cascade in platelets, but not in plasma, with secondary plasminmediated, proteolysis of stored platelet α -granule proteins (including platelet factor V [FV], von Willebrand factor, fibrinogen, and others); defective, platelet FV-dependent prothrombinase function, because of proteolysis of platelet FV; reduced to normal platelet counts (range: 120-245 x 10⁹ L⁻¹); impaired platelet aggregation with epinephrine, and normal to impaired aggregation with ADP and collagen [10,11,13–17]. OPD platelets have a normal profile of external membrane glycoprotein receptors, and their ability to promote clot retraction is normal, likely because the intracellular activation of the fibrinolytic cascade in the QPD spares platelet surface proteins from degradation [11,13]. Although QPD α granule proteins are abnormally degraded, they are secreted in response to agonist stimulation, like normal platelet α -granule proteins [11,13,14,16].

The effects of QPD blood and platelets on fibrinolysis are largely unknown although dilute whole blood clot lysis times are normal in the QPD [11]. Platelet-mediated increased fibrinolysis has been proposed to contribute to QPD bleeding, based on: (i) the abnormally high levels of u-PA that are stored in QPD platelets and released upon activation by agonists such as ADP and thrombin [16]; (ii) the normal QPD plasma levels of fibrinogen, plasminogen, tissue-type plasminogen activator, α_2 - plasmin inhibitor, α_2 -plasmin inhibitor–plasmin complexes, and D-dimer [13,15–17]; (iii) differences between the delayed-onset bleeding problems of the QPD [12] and the immediate onset bleeding problems of gray platelet syndrome (a more severe α -granule protein deficiency) [2,18,19]; and (iv) the bleeding diathesis induced in mice when megakaryocytes are genetically engineered to express u-PA [20].

To gain insights into the changes to fibrinolysis in the QPD, we compared the lysis of normal and QPD clots generated in vitro, using methodologies that included: whole blood TEG to follow clot formation and lysis after initiating coagulation with tissue factor; ultrastructural analyses of fibrin and platelet aggregates in platelet-poor and -rich plasma clots; and finally whole blood perfusion experiments to determine if QPD blood accelerated lysis of preformed fibrin. We report that the QPD is associated with 'gain-of-function' abnormalities that increase the lysis of forming or preformed clots.

2.2.3 Methods

Participants

This study was carried out in accordance with the recently revised Helsinki Protocol for research on human subjects and institutional ethics review board approval from Hamilton Health Sciences, McMaster University and Hôpital St. Justine. All study participants provided informed consent. Subjects included nine representative adults with the QPD and 12 healthy, unrelated, adult controls of similar age. As previously reported [11,13,15,17], all QPD subjects tested were verified to have: platelet counts ranging from reduced to normal (values: 114–193 x 10⁹ L⁻¹); normal plasma levels of fibrinogen, plasminogen, and α_2 -plasmin-inhibitor (not shown); and normal platelet-rich plasma clot retraction (prepared using 300 x 10⁹ platelets L⁻¹, and evaluated kinetically, as described [21]).

Blood collection and sample preparation

Blood (200 mL) was collected from each subject into buffered 3.2% sodium citrate (for whole blood clot lysis and perfusion studies) or acid citrate dextrose (ACD) anticoagulant (for isolation of platelets). At least three QPD individuals and three controls were used for each type of experiment. Methods described [11,16] were used to harvest platelets and to prepare platelet-poor plasma (from citrated blood) and platelet lysates. Platelets pelleted from ACD anticoagulated plasma were resuspended in whole blood, autologous plasma or Tyrode's buffer to prepare samples with standardized platelet counts.

Thromboelastography

Thromboelastography[®] (TEG[®]) was performed using citrate anticoagulated whole blood (kept in capped tubes at 37°C for 0.5 h after collection) similar to methods described [22,23], with simultaneous monitoring of reactions in four TEG[®] units (Haemoscope Corporation, Niles, IL, USA; eight plastic reaction cups) for 180 min (the maximal period of monitoring). Cups were preloaded with 20 µL of 200 mM CaCl₂, and 10 µL of recombinant tissue factor (Innovin, Dade-Behring, Deerfield, IL, USA; 1.5 mg mL⁻¹ stock diluted 1/500 in 4% phosphate buffered saline (PBS)/albumin, pH 7.4). Coagulation was initiated by adding whole blood samples (310 or 320 µL to maintain a 360 µL final reaction volume). Testing was carried out with or without 10 μ L of 1.0 mg mL⁻¹) recombinant tissue-type plasminogen activator (rt-PA) (Genentech Inc., San Francisco, CA, USA), which produced minimal to no lysis of most control whole blood samples. Some studies were carried out using blood samples adjusted to $200-2000 \times 10^9$ platelets L⁻¹, by addition of autologous platelets or replacement of

platelet-rich with platelet-poor autologous plasma. The sensitivity of TEG[®] to u-PA was evaluated using normal blood, spiked with exogenous u-PA (final: 0– 1500 ng mL⁻¹ blood). Platelet-dependent effects on fibrinolysis were also evaluated by TEG[®], using normal or QPD platelets (final: up to 2000 x 10⁹ platelets L⁻¹) that were resuspended in aliquots of group AB whole blood to provide the plasma fibrinolytic proteins. Some TEG[®] sera were harvested for biochemical analyses (see below).

Preparation of clots for visual, biochemical, and microscopic analyses

Citrated whole blood samples, from individuals with the QPD (n=3) and controls (n= 5), with or without added tranexamic acid (10 mM final) (Sigma-Aldrich Canada Ltd, Oakville, Canada), were clotted by adding 1 U mL⁻¹ human thrombin (Enzyme Research Laboratories, South Bend, IN, USA) and 20 mM CaCl₂ (final concentrations). Additional samples were clotted using 20 mM CaCl₂ or the mixture of tissue factor and CaCl₂ used for TEG[®]. Samples were clotted while stirring at low-shear rates in aggregometer cuvettes (1000 rpm Chronolog 560VS model; Chronolog, Co., Harveston, PA, USA), because pilot experiments indicated the mixing resulted in more consistent and enhanced fibrinolysis. After 3 h (comparisons with TEG[®] clots only) or 18 h incubations at 37°C, residual clot sizes were recorded by two observers before centrifuging samples to harvest sera (12 000 x g, for 10 min). For biochemical analyses, the harvested sera and platelet-poor plasma were supplemented with protease inhibitors (final concentrations: 0.3 µM aprotinin, 2.8 µM E64, 10 mM EDTA, 1 µM leupeptin, 5 mM N-ethyl maleimide, 4 mM AEBSF, 2 µM pepstatin, 100 µM 1,10phenanthroline monohydrate, 100 µg mL⁻¹ soybean trypsin inhibitor; sources as described) [16]. Samples were stored at -70°C until analyzed. Enzyme-linked immunosorbent assays (ELISA) were used to quantify u-PA levels (DakoCytomation, Carpinteria, CA, USA) in serum of clots prepared from whole blood and platelet-rich plasma. ELISA were also used to quantify generation of plasmin- α_2 plasmin inhibitor (PAP) complex and D-dimer (American Diagnostica of Canada, LP, Montreal, QC, Canada) in samples. Results were compared with basal levels in platelet-poor plasma. Data on platelet counts, hematocrits, and the u-PA content of platelet lysate, plasma, and serum were used to evaluate OPD platelet contributions to increased levels in serum.

For electron microscopy (EM), clots without red blood cells were prepared from control (n= 3) and QPD (n= 3) platelet poor and concentrated, platelet-rich plasma samples (final 1200 x 10^9 platelets L⁻¹ for visualization of aggregates) by similar procedures, except samples were mixed and incubated overnight in sterile culture chambers. Positive controls for these analyses included clots made with normal plasma (with or without platelets) with added recombinant two-chain u-PA (tcu-PA, gift from Dr Jack Henkin, Abbott Laboratories, Abbot Park, IL,

USA), at a concentration similar to the average amount of u-PA antigen released by 18 h into sera of clots prepared with concentrated OPD platelet-rich plasma (final: 550 ng mL⁻¹ plasma). Clots were photographed to document their gross appearances after overnight fixation in 2% glutaraldehyde in 0.2 M, pH 7.4 sodium cacodylate buffer (NaCac), as this optimized detection of altered clot density. After fixation, samples for scanning (SEM) and transmission (TEM) electron microscopy were washed with NaCac buffer and postfixed with osmium tetroxide (1% in NaCac, 0.1 M, pH 7.4), before stepwise ethanol gradient dehydration (50%, 70%, 95%, then 100%). For SEM, samples were critical point dried, and sputter coated with gold palladium, before examination on a JEOL JSM-840A scanning electron microscope (Japanese Electron Optics Laboratories, Peabody, MA, USA). Samples for TEM were embedded using propylene oxide and Spurr's resin and thin sections were stained with uranyl acetate and lead citrate before examination on a JEOL JSM-1200EX transmission electron microscope (Japanese Electron Optics Laboratories). EM images were selected to be representative of overall clot morphology.

Static platelet adhesion assays

The adhesion of control and QPD platelets to different adhesive proteins was compared using a modification of an established assay [24]. Briefly, microtiter plates were coated with 100 μ L of 10 μ g mL⁻¹ fibrinogen (Enzyme Research Laboratories), 10 μ g mL⁻¹ fibronectin (Enzyme Research Laboratories), or 20 μ g mL⁻¹ collagen (Horm, Nycomed, Munich,Germany). Platelet adhesion to fibrin was tested using fibrinogen-coated wells, treated with 0.2 U mL⁻¹ thrombin for 15 min. Wells were blocked with BSA, as described [24], before incubating with 100 μ L of washed platelets (300 x 10⁹ L⁻¹ in Tyrode's buffer; 60 min, room temperature). Next, wells were gently washed with Tyrode's buffer before quantifying adherent platelets by phosphatase activity, as described, using p-nitrophenyl phosphate (Sigma-Aldrich Canada Ltd) and separate standard curves for each platelet sample tested [25].

Platelet perfusion studies

Perfusion experiments were used to further assess platelet adhesion to fibrin and the effect of QPD and control blood on preformed fibrin degradation. Briefly, testing was carried out by drawing blood (with or without additional platelets), using a Harvard syringe pump (Pump 22; Harvard Apparatus Inc., Holliston, MA, USA) without recirculation, through fibrin precoated glass microcapillary tubes (Vitrocom Hollow Rectangle capillaries, 100-mm length and 0.2 x 2-mm cross section; Fiber Optic Center, New Bedford, MA, USA). Some studies were carried out using blood with added 10 mM tranexamic acid

(AMCA), or 500 μ M aspirin (Aspegic; Sanofi-Synthélabo, France) and 3 μ M prostaglandin E1 (PGE1; Sigma-Aldrich Canada Ltd).

The fibrin-coated chambers were prepared by serial incubations and washes with: 1 mg mL⁻¹ of fibrinogen (37°C, 2 h); a phosphate buffered saline PBS wash; an incubation 0.2 U mL⁻¹ thrombin (15 min); followed by a final wash with 1 U mL⁻¹ of unfractionated heparin (Leo Pharma Inc., Ajax, ON, Canada) in PBS. For some studies, the chambers were coated with labeled fibrinogen that had been conjugated with AlexaFluor594 using a purchased kit (Molecular Probes Inc., Eugene, OR, USA) or with biotin (final concentration 2.5 mM; Pierce, Rockford, IL, USA), as recommended by the suppliers. Prior to use, labeled proteins were dialyzed (4°C, overnight). Fibrinogen labeling by AlexaFluor594 was confirmed, according to kit instructions. Fibrinogen labeling by biotin was confirmed using streptavidin peroxidase. The labeled proteins contained 98–99% clottable fibrinogen.

To evaluate platelet adhesion to fibrin in whole blood perfusion chambers. the platelet-rich plasma fraction of control and QPD citrated blood (centrifuged 150 x g, 15 min) were harvested, and platelets were added or removed to adjust the final platelet count. Samples were mixed with, 1/10 volume ACD, 3 µM PGE₁, 100 mU mL⁻¹ apyrase (Sigma-Aldrich Canada Ltd), before labeling the platelets (37°C, 30 min) with 2.5 µM calcein acetoxymethylester (calcein-AM; Molecular Probes Inc.). To reconstitute whole blood, platelets were pelleted (1500 x g, 12 min) and mixed with the removed volume of autologous citrated plasma, and red cells from the original sample. Fibrin-coated chambers were perfused at low shear rates (500 s⁻¹) and platelet adhesion and fibrin fluorescence were recorded, using an inverted epifluorescence microscope (Nikon Eclipse TE 300; Nikon, Tokyo, Japan), coupled to Nikon ACT-1 software. Data were analyzed using Adobe[®]Photoshop[®] 7 (Adobe, San José, CA, USA). Briefly, fluorescent images of the chamber surface were converted into white and black pictures, and after threshold setting, the number of black (background) and white (fluorescent platelets or fibrin) pixels were calculated to determine percentage of the total area covered by platelets or the quantity of residual labeled fibrin.

Each perfusion experiment was carried out with samples from 3–4 representative individuals with the QPD and 5–6 controls. In initial analyses of QPD platelet adhesion to fibrin, chambers were perfused for 5 min before adding 5 μ M SFLLRN (Bachem Bioscience Inc., Prince of Prussia, PA, USA) to the perfusing blood to trigger platelet activation (without thrombin or fibrin generation), followed by an additional 5 min of perfusion. After quantifying the adhesion, the chambers were sealed, and incubated overnight at 37°C, before harvesting the chamber blood plasma (centrifuged at 13 000 x g, 5 min) to evaluate D-dimer generation from the preformed fibrin. All other studies were carried out without added SFLLRN. To assess the morphology and spreading of platelets on fibrin, chambers were perfused for 10 min, washed (1 U heparin mL⁻¹

PBS), fixed (2% formaldehyde, 30 min; room temperature) and treated with 0.1% Triton X-100 (10 min; room temperature) before labeling with 1 μ M AlexaFluor488 phalloidin (Molecular Probes Inc.) to visualize F-actin.

Perfusion studies were also used to evaluate degradation of AlexaFluor594-labeled fibrin over 80 min of perfusion with whole blood (unlabeled; with or without adjustment to 200 x 10^9 platelets L⁻¹ final). To evaluate the mechanisms of labeled fibrin loss over time, some studies were carried out using chambers coated with biotin-labeled fibrin, and perfused with whole blood, with or without 10 mM AMCA, or 500 μ M aspirin and 3 μ M PGE₁. After 10 min of perfusion (to allow platelet recruitment to the fibrin), chambers were sealed and incubated overnight before assessing the labeled fibrin degradation by non-reduced sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8% gel) and Western blotting with streptavidin conjugated with horseradish peroxidase (Molecular Probes Inc.), as described [13,15].

Statistical analysis

Quantitative data were compared using two-tailed Student's t tests. Chisquared analysis was used to compare observed to expected levels of u-PA in QPD serum if platelets were the source of releasable u-PA.

2.2.4 Results

TEG[®] assessment of clot lysis

TEG[®] analyses were carried out using whole blood with standardized platelet counts, as the platelet content of blood influenced the maximal clot amplitudes (MA) and clot susceptibility to lysis by t-PA (Fig. 2.1 and data not shown). Like normal blood samples, QPD samples clotted rapidly, and showed variable fibrinolysis with t-PA (Fig. 2.1), despite release of 30–57% of QPD platelet stored u-PA into serum by the end of the 3 h of TEG[®] monitoring. The amounts of exogenous u-PA required for full lysis of control blood samples by 3 h of TEG[®] with added t-PA were approximately 250 ng u-PA mL⁻¹ blood, or 500 ng u-PA mL⁻¹ plasma (Fig. 2.2A and data not shown). This amount exceeded the u-PA concentrations in QPD serum, even for blood samples adjusted to 1000–2000 x 10⁹ platelets L⁻¹ (all values <375 ng mL⁻¹). Nonetheless, mixing studies suggested QPD platelets had abnormal effects on clot lysis as they failed to prolong the lysis of normal whole blood clots by t-PA (Fig. 2.2B shows representative samples). However, very large amounts of QPD platelets (e.g. 1000–2000 x 10⁹ L⁻¹ final or higher, depending on the QPD platelet u-PA content)

were required to illustrate this effect in mixing studies, especially when the control blood used for mixing was more resistant to t-PA lysis (not shown).



Figure 2.1. Thromboelastography[®] (TEG[®]) of Quebec platelet disorder (QPD) and control whole blood clots analyzed with added tPA. Samples were adjsted to final platelet counts of 200 and 1000 x 10^9 L⁻¹, as indicated. Tracings compare changes in the recordings of clot tensile strength over time (horizontal axis, 180 min in total) for representative control (n=2) and QPD blood samples (n=3). The initial phase of clot formation is indicated below one tracing, and values indicate the recorded maximal clot amplitudes in millimetre. The arrow indicates complete lysis.



Fig. 2.2. TEG[®] analyses, comparing the effects of exogenous u-PA (A), and the influence of adding control or QPD platelets (B) on t-PA induced lysis. Panel A compares the effects of 150, 250, and 500 ng mL⁻¹ u-PA on clot lysis. Panel B compares shifts in the time to lysis for a control sample, mixed with or without exogenous control or QPD platelets (final: 400 x 10^9 L⁻¹). Arrows indicate complete lysis.

Visual, biochemical and ultrastructural analyses of clot lysis

Biochemical assays of clot sera were used to determine if OPD blood had an ability to accelerate fibrinolysis without addedt-PA. Samples for these analyses were prepared while mixing at low-shear rates, followed by a 3h or an overnight incubation. After an overnight incubation, none of the OPD whole blood clots was visibly lyzed, although biochemical assays indicated that fibrinolysis was increased in the OPD samples. In the sera from OPD clots, prepared at low shear, with or without added tissue factor (not shown) or thrombin (Table 2.1), u-PA levels were increased above basal plasma u-PA concentrations (Table 2.1 shows data for citrated plasma, processed without platelet activation inhibitors). In platelet-rich plasma clots, 45-100% of the QPD stored u-PA was released into serum by 3h, and 100% was released by 18h. Plasma u-PA levels were much lower ($<5 \text{ ng mL}^{-1}$) when OPD blood was processed with added PGE₁ and aspirin to prevent platelet activation. After 18h of incubation, OPD whole blood sera (final: 200 x 10^9 platelets L⁻¹) contained more u-PA than predicted, based on the levels of stored platelet u-PA (observed values for three QPD subjects 81-176 ng mL⁻¹, compared with expected, 76–81 ng mL⁻¹, $\chi^2 = 21$, P< 0.001). The opposite was found for sera prepared from platelet-rich plasma (data not shown, $\chi^2 = 73$, P< 0.001). Unlike control sera and QPD plasma samples, QPD sera also contained log-fold increased quantities of PAP and D-dimer (Table 2.1 and additional data from 3h and overnight clots prepared with calcium only, or with calcium and tissue factor, not shown). Although the fibrinolytic inhibitor tranexamic acid did not reduce u-PA secretion into QPD serum, it markedly attenuated D-dimer generation in QPD clots (>3 log reductions, data not shown).

Ultrastructural analyses were used to evaluate the architecture of plasma and concentrated, platelet-rich clots in QPD. QPD plasma clots had the same visual appearance as control plasma clots (Fig. 2.3, insets) and their ultrastructure, by TEM and SEM, was normal and characterized by a dense network of elongated fibrin strands (Fig. 2.3 shows SEM images; TEM images not shown).The concentrated, OPD platelet rich-plasma clots initially looked normal, and at 5h, they showed the same pattern of clot retraction as control samples (not shown). After overnight incubation, the concentrated, QPD platelet-rich clots appeared retracted but smaller and/or thinner, with some containing holes (Fig. 3 insets), and others showing full disintegration. The concentrated, platelet-rich QPD clots resembled control plasma clots prepared with 550 ng mL⁻¹ added u-PA, which was equivalent to the average u-PA concentration in the sera of concentrated, QPD platelet-rich plasma clots after overnight incubation (not shown). The serum levels of PAP and D-dimer for these samples confirmed that OPD platelets increased plasmin generation and fibrinolysis whereas OPD plasma did not (not shown). By TEM, the clots containing normal platelets contained large platelet aggregates linked to fibrin strands (Fig. 2.4A,B), and initially, the clots containing QPD platelets looked quite similar (Fig. 2.4A). However, by 18h, the clots
containing QPD platelets showed homogeneous dispersion of clot elements, evident by a loss of long, spindlelike fibrin strands and an increased content of more globular fibrin structures because of reduced integrity of individual fibrin fibers (Fig. 2.4B). By 18h, the QPD clots also contained fewer and smaller platelet aggregates, and fewer platelet-fibrin contacts (Fig. 2.4B). Similar abnormalities in clot fibrin and platelet aggregates were also seen after 18h in clots containing normal platelets and exogenous u-PA (not shown). In both QPD and control clots evaluated at 18h, platelets lacked the well-defined internal structures and external membranes that were evident in earlier samples (Fig. 2.4B). The abnormalities in QPD platelet-rich clots appeared to reflect plasmin generation, as adding the fibrinolytic inhibitor tranexamic acid protected QPD clots from time-dependent disruption of fibrin strands and platelet aggregates (Fig. 2.4C).

Table 2.1 Biochemical markers of fibrinolysis in plasma and serum from control and Quebec platelet disorder (QPD) whole blood samples, clotted at low-shear rates.

Subjects	Sample	Urokinase-type plasminogen activator (u-PA) (ng mL ⁻¹)	D-dimer (ng mL ⁻¹)	Plasmin-α ₂ plasmin inhibitor (PAP) complexes (ng mL ⁻¹)
Controls n=5	Plasma WB serum	$\begin{array}{c} 0.7 \pm 0.1 \\ 0.8 \pm 0.2 \end{array}$	350 ± 210* 18 600 ± 3000	230 ± 110 279 ± 120
QPD n=3	Plasma WB serum	15 <u>+</u> 2* 47 <u>+</u> 14**	570 ± 10* All values > 2,000,000**	200 ± 40* All values > 18,000**

Values are given in mean \pm SD. Results compare basal levels of u-PA, D-dimer, and PAP complexes in citrate-anticoagulated plasma (prepared without platelet activation inhibitors) to the quantities in serum after an overnight incubation. Significant differences (P < 0.05 for u-PA; P < 0.001 for PAP and D-dimer) between plasma and whole blood (WB; no added platelets) sera (*), and between control and QPD samples (**) are indicated.



Figure 2.3 Appearance and ultrastructure of control and QPD clots. Representative macroscopic (insets) and scanning electron microscopy images (taken at 2500x magnification) show control and QPD fibrin clots, after 18 h of incubations. Images show samples with and without 1200×10^9 platelets L⁻¹.



Figure 2.4 Effects of time and the fibrinolytic inhibitor drug AMCA on the ultrastructure of control and QPD clots. Transmission electron microscopy images compare clots containing $1200 \times 10^9 \text{ L}^{-1}$ platelets at different time points after clot formation, without or with (panel C only) added AMCA. Panels show electron-dense fibrin strands (arrowheads) interspersed among less electron-dense platelets (asterisks). Boxed areas indicate platelet-fibrin aggregates imaged at low magnification. Panel A compares control and QPD clots at 0.5 h. Panel B compares clots at 18 h. In both A and B, higher magnification images are shown to the right of each micrograph. Panel C shows the effects of AMCA on clots containing QPD platelets.

Platelet adhesion and flow chamber assessments of clot lysis

Static adhesion assays (using washed platelets, resuspended to 300 x 10^9 L⁻¹) were used to evaluate if QPD platelets adhered normally to fibrinogen, fibrin, and the control proteins fibronectin and collagen. QPD platelet adhesion (relative to controls, mean % ±SD) to fibrinogen (QPD: $253\% \pm 40\%$, C: $100\% \pm 21\%$, P < 0.001) and fibrin (QPD: $153\% \pm 41\%$, C: $100\% \pm 43\%$, P < 0.01) was significantly increased whereas it was normal with fibronectin (QPD: $123\% \pm 41\%$, C: $100\% \pm 38\%$, P = 0.2) and collagen (QPD: $133\% \pm 28\%$, C: $100\% \pm 34\%$, P = 0.06).

In whole blood perfusion chambers, QPD and control platelets adhered to fibrin, predominantly as a monolayer of adherent, spread platelets showing pseudopod extensions, with some small aggregates, consistent with platelet activation (Fig. 2.5A). The adhesion of QPD platelets to fibrin was quantitatively reduced in chambers perfused with whole blood samples without added platelets (Fig. 2.5B), possibly because of the lower QPD blood platelet counts (ranges, in 10^9 platelets L⁻¹: QPD: 128–158; controls: 223–317). QPD platelet adhesion to fibrin normalized when the whole blood samples were adjusted to a standardized platelet count (Fig. 2.5C). Like normal platelets, QPD platelets showed significantly reduced adhesion to fibrin (P < 0.001) when platelet activation inhibitors (PGE₁ and aspirin) were added to the blood (Fig. 2.5C).

Because the assays of D-dimer from blood incubated in fibrin-coated perfusion chambers suggested QPD platelets accelerated fibrinolysis (range: 3238–3579, vs. 266– 1405 ng mL⁻¹ D-dimer for controls), further perfusion studies were carried out using chambers coated with AlexaFluor594- labeled fibrin. The perfusion of QPD whole blood, with (Fig. 2.6A) or without (not shown) adjusted platelet counts, over AlexaFluo594-labeled fibrin accelerated fibrin loss, relative to control blood samples. The accelerated loss of AlexaFluo594- labeled fibrin in chambers perfused with QPD blood was blocked by the fibrinolytic inhibitor AMCA (Fig. 2.6A). Similar analyses, using biotin-labeled fibrin, confirmed QPD blood accelerated fibrin degradation (Fig. 2.6B) in a manner that was blocked by the fibrinolytic inhibitors of platelet activation (aspirin and PGE₁, Fig. 2.6C). These data confirmed QPD blood increased the lysis of preformed fibrin, in a manner dependent on platelet activation.



Figure 2.5 Adhesion of control and QPD platelets to preformed fibrin clots, evaluated in perfusion chambers. Panel A shows representative images (63x magnification, oil immersion) of adherent, control (C) and QPD platelets, labeled with AlexaFluor 488 phalloidin to visualize the cytoskeleton F-actin. Panel B compares adhesion of control (n=6) and QPD (n=3) platelets to fibrin using whole blood samples, without added platelets (* indicates values significantly different from control platelets, P < 0.05). Panel C compares adhesion of control (n=6) and QPD (n=4) platelets to fibrin for samples adjusted to 200 x 10⁹ platelets L⁻¹, with or without added 10 mM AMCA, or the combination of 3 μ M prostaglandin E₁ (PGE₁) and 500 μ M aspirin (*** indicate significant inhibition by drug, P < 0.001). Bars in panels B and C indicate SD.



Figure 2.6 Degradation of fibrin clots, evaluated in perfusion chambers. The testing was carried out using control (C) or QPD whole blood with (panels A, C) or without (panel B) adjustment of the platelet count to 200 x 10^9 L⁻¹. Panel A shows fibrin degradation, monitored by the loss, over time, of AlexaFluor594-labeled fibrin (bars indicate SD) from the surface of chambers perfused with control (n=5) or QPD (n=4) blood, tested with or without added 10 mM AMCA. Significant differences (*) were found between C and QPD after 50 min (P < 0.01) and for QPD samples, tested with and without AMCA (P < 0.05 at 55 min, and P < 0.01 at 60-80 min). Panels B and C show the degradation of biotin-labeled fibrin induced by control or QPD whole blood, evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8% gel) and Western blotting. In B, arrows indicate degradation products present in increased quantitites in QPD samples. Panel C shows the effects of drugs (10 mM AMCA or the combination of 3 μ M PGE₁ and 500 μ M aspirin) on fibrin degradation by QPD blood (data for one subject, representative of four individuals, are shown).

2.2.5 Discussion

Studies of blood from individuals with bleeding problems have provided insights into the nature of their hemostatic problem, and the factors important for controlling bleeding in vivo. Recently, platelet-mediated accelerated fibrinolysis was postulated to contribute to QPD bleeding, because this condition is associated with marked increases in platelet u-PA, without large increases in plasma u-PA or increased systemic fibrinolysis [16], and because QPD bleeding responds to fibrinolytic inhibitor therapy [12]. We tested this possibility by modeling fibrinolysis with QPD blood in vitro. By TEG®, QPD fibrinolytic abnormalities were difficult to detect, as the test (even with added t-PA) was not very sensitive to the quantities of u-PA in OPD blood, even when the blood was spiked with more platelets. However, in OPD whole blood clots formed at low shear without added t-PA, there was biochemical evidence of increased plasmin generation and fibrinolysis. associated with increased serum u-PA. Furthermore. the incorporation of OPD platelets into clots also induced progressive disruption of fibrin and platelet aggregates unless a fibrinolytic inhibitor drug was added. The perfusion of QPD whole blood over fibrin, in the absence of a stimulus for thrombin generation, was followed by the accelerated fibrin degradation. These observations confirm that the increased levels of plasminogen activators in QPD blood [16] induce a 'gain-of-function' defect in the lysis of forming and preformed clots. These abnormalities, coupled with the responses of QPD bleeding to fibrinolytic inhibitors [12], suggest that accelerated fibrinolysis is an important contributor to OPD bleeding.

Studies of clot architecture have indicated that normal platelets protect fibrin by releasing PAI-1 that inhibits plasmin generation and renders clots more resistant to fibrinolysis, particularly in regions closest to platelets [26,27]. Exogenous plasminogen activators are known to alter the architecture of fibrin clots [26–31], but these analyses have been carried out using purified proteins, and not the complex mixture of proteins found in blood, plasma, and platelets, or in clinical samples. Nonetheless, the ultrastructure of the concentrated, plateletrich clots that we prepared was quite similar to clots made from purified proteins [26,27,29]. Like normal platelets, QPD platelets adhered and attached to forming or preformed fibrin. However, over time, the incorporation of QPD platelets into clots (like exogenous u-PA) disrupted clot fibrin, platelet aggregates, and plateletfibrin contacts, unless drugs were used to neutralize plasmin. In perfusion experiments, OPD blood accelerated the lysis of fibrin, in a manner blocked by fibrinolytic inhibitor drugs and dependent on platelet activation. These data suggest the fibrinolytic inhibitor drugs are helpful to controlling QPD bleeding [12] because they protect QPD hemostatic plugs from an accelerated loss of structural integrity.

Blood perfusion studies have been used previously to illustrate the profibrinolytic effects of recombinant t-PA and the antifibrinolytic effects of

normal platelets on clot lysis induced by exogenous plasminogen activators [32]. Unlike this study, we observed that some fibrin degradation occurred with perfusion of normal blood samples, possibly because we followed clot lysis for a longer time and used citrate (not ACD) as the anticoagulant to maintain a more physiologic pH during the perfusion. The fibrin surface was verified to recruit platelets, and to trigger platelet spreading, pseudopod extension and formation of small aggregates, consistent with platelet activation. The combination of PGE1/aspirin partially inhibited the control and QPD platelet adhesion to fibrin, consistent with their known attenuating effects on platelet adhesion to fibrin [33,34]. We confirmed that QPD platelet recruitment to a fibrin surface was associated with increased fibrin degradation, likely mediated, at least in part, by platelet delivered u-PA, as platelet activation inhibitors reduced the fibrin degradation. Although OPD platelets are profibrinolytic (Table 2.1, Figs 2.3 and 2.4), it is possible that other cells in QPD blood (such as leukocytes) contribute to the profibrinolytic effects of QPD blood. Further studies are needed to determine if the QPD alters u-PA in leukocytes as more u-PA was detected in QPD serum that could be accounted for, based on amounts stored in QPD platelets. Polymorphonuclear leukocytes bind to fibrin and platelets [35], and contain u-PA and other proteases [36-40] that may enhance the proteolysis of fibrin by normal and/or QPD blood. Given the complex cellular regulation of u-PA [41,42], genetic studies will be important to unravel how the OPD alters u-PA regulation.

Over the years, a number of abnormalities have been proposed to cause OPD bleeding. The problem was first attributed to defective platelet FV [10], and plasmin-mediated degradation of QPD platelet FV [17] appears to be the cause of abnormal, platelet FV-dependent, thrombin generation in the absence of plasma FV [10]. More recently, OPD bleeding has been postulated to result from the abnormal, platelet-mediated delivery of u-PA into clots [16,17]. We were unable to show any abnormalities in QPD clot formation by TEG[®] (using tissue factor to initiate FV-dependent coagulation), likely because plasma FV was present to support thrombin generation. Other data indicated that the fibrinolytic abnormalities detected in OPD blood were not attributable to abnormal platelet FV. Firstly, abnormal plasmin generation and increased fibrinolysis occurred in QPD whole clots, formed with exogenous thrombin to bypass any platelet FV procoagulant defect. Secondly, fibrinolysis was increased when anticoagulated QPD blood was perfused over a fibrin clot, without a stimulus for further thrombin generation. It is nonetheless possible that the loss, from proteolysis, of platelet FV and other QPD a-granule proteins also contribute to an impaired hemostasis in the OPD in vivo.

Unlike stored α -granule proteins, the major adhesive glycoproteins on the surface of resting QPD platelets are not degraded [13]. In our current study, we analyzed QPD platelet adhesion in static adhesion assays without plasma, and in whole blood, flow-based assays at 5 min (when adhesion was maximal) before

lysis of the fibrin-coated surface, which occurred after 25 min. These analyses indicated that QPD platelets are able to bind to adhesive proteins prior to initiation of fibrinolysis. It is unclear why QPD platelets showed an increased ability to adhere to fibrin and fibrinogen in static adhesion assays using washed platelets, but normal adhesion to fibrin in whole blood perfusion experiments, where plasma proteins are present. Some potential explanations include plasmin-mediated activation of QPD platelets during the more lengthy preparation of washed platelets [43,44] and/or an effect of QPD α -granule adhesive protein degradation on platelet adhesive function in assays without plasma. Nonetheless, we cannot exclude the possibility that QPD platelets have impaired adhesive function in vivo, because of their stored adhesive protein degradation [11,14,16,17] and perhaps an increased proteolysis of glycoprotein Ib-IX-V and other membrane receptors by plasmin within clots.

Our present study, modeling fibrinolysis *in vitro*, confirms that the increased plasminogen activator content of QPD blood is associated with 'gain-of-function' abnormalities in the fibrinolytic balance that were not previously detected using routine clinical laboratory tests of fibrinolysis [11,13,15]. Similar 'gain-of-function' abnormalities in clot lysis, and negative hemostatic consequences, have been demonstrated to occur with storing u-PA in murine platelets [20], although there are differences in the hemostatic problems of these mice and humans with the QPD [12]. Our in vitro assessment illustrates that with a stimulus for clot formation, and/or platelet recruitment to fibrin, fibrinolysis is accelerated by the imbalance in fibrinolytic proteins in QPD blood, and we suspect this also occurs in vivo after a hemostatic challenge. It would be interesting to apply TEG[®], biochemical assays of whole blood clot lysis, and whole blood fibrin perfusion analyses to assess fibrinolysis in other congenital and acquired disorders.

2.2.6 References

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CHAPTER 3

STUDIES OF URINARY uPA IN QPD

3.1 PREFACE

Approximately 50% of QPD individuals have experienced hematuria, and this has been associated with having higher levels of platelet uPA [293]. The study presented in this chapter investigated whether individuals with QPD have increased amounts of uPA in urine compared to healthy individuals, in order to determine if this may be a contributor to urinary bleeding in QPD. 24 hour urine samples were collected from QPD and control individuals, and urinary uPA was measured by ELISA. The control samples were used to establish a normal range for the studies. To account for any incomplete sample collections, urinary uPA to creatinine ratios were also calculated for each individual. No differences in urinary levels of uPA were detected in QPD individuals compared to controls, suggesting that hematuria in QPD is a hemostatic consequence of having high amounts of platelet uPA. Furthermore, this study is consistent with the hypothesis that the biochemical defect in QPD is specific to MKs [541].

As a senior author on the manuscript, Dr. Catherine P.M. Hayward supervised the study design, experimental work, and manuscript writing. My specific roles included study design, subject recruitment and sample collection, data acquisition and analysis, and manuscript writing. I am grateful to Dr. D. Kika Veljkovic and Francine Derome for their help with subject recruitment and sample collection, and to Dr. Georges E. Rivard for his scientific guidance.

CHAPTER 3.2

EVALUATION OF UROKINASE PLASMINOGEN ACTIVATOR IN URINE FROM INDIVIDUALS WITH QUEBEC PLATELET DISORDER

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3.2.1 Letter to the editor

Quebec platelet disorder (QPD) is an autosomal dominant bleeding disorder associated with increased risks for bleeding and the expression and storage of urokinase plasminogen activator (u-PA) in platelets, despite normal u-PA in plasma (reviewed in [1]). In the recent article on QPD published in this journal [1], QPD was proposed to result from defective u-PA regulation by megakaryocytes. However, the levels of u-PA in other QPD cells and urine have not been reported. Individuals with QPD are at increased risk of experiencing bleeding following hemostatic challenges, and approximately 50% experience episodic, spontaneous hematuria, which is associated with higher levels of platelet u-PA [2]. As urine normally contains significant amounts of u-PA (40–80 mg/ml) [3], we investigated whether individuals with QPD have an increased urinary u-PA as a potential contributor to their urinary tract bleeding.

The study was conducted with institutional research ethics board approval, in accordance with the revised Helsinki Declaration on human research. To assess urinary u-PA excretion, 24 hour urine samples were collected, with informed consent, from four subjects with confirmed QPD and 18 healthy control subjects (equal numbers of men and women). Hematuria was evaluated by dipstick analysis. An enzyme-linked immunosorbent assay (ELISA) was used to quantify u-PA antigen in urine (Siemens Medical Solutions, Munich, Germany). Urinary creatinine was measured by the Hamilton Regional Laboratory Medicine Programme, using the Jaffe' method (Roche Diagnostics, Indianapolis, Indiana, USA). Urinary u-PA to creatinine ratios (ng/ng x 10^{-5}) were calculated for each individual to correct for the effects of any incomplete urine collections on estimates of urinary u-PA. Reference intervals (RI) for urinary u-PA, and u-PA: creatinine ratios, were derived from the mean ± 2 SD of healthy control data, evaluated after log transformation to normalize the distribution.

At the time of the study, no subjects had visible hematuria and one subject, with QPD, had hematuria on dipstick analysis. The 24 hour, total urinary u-PA excretion was similar for QPD and healthy control subjects (mean, range, in μ g: QPD: 100, 63–250; control: 79, 20–316; P value = 0.6), as were the urinary u-PA: creatinine ratios (mean, range, x 10⁻⁵; QPD: 9, 3–13; control: 6, 1–16; P = 0.4).

QPD is associated with increased levels of u-PA in platelets, which triggers intraplatelet activation of the fibrinolytic cascade and accelerates fibrinolysis at sites of injury [4–6]. Although hematuria is a common manifestation of QPD, our study indicates that QPD does not increase urinary u-PA. This suggests that QPD spontaneous hematuria is a hemostatic consequence of increased platelet u-PA. Our findings are consistent with the hypothesis that QPD results from a megakaryocyte-specific defect in u-PA regulation [1]. We are investigating the genetic mutation that causes QPD, to determine how this

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disorder alters u-PA expression and storage in megakaryocytes and platelets, without increasing urinary or plasma u-PA.

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CHAPTER 4

GENETIC STUDIES OF QUEBEC PLATELET DISORDER

4.1 PREFACE

The studies presented in this chapter investigated several aspects of the genetic defect responsible for causing OPD. PLAU was considered as the best candidate gene for this disorder, as QPD selectively increases uPA expression in MKs [243]. First, linkage analysis was done using microsatellite markers spanning chromosome 10 to map the genetic locus for QPD. The results indicated that a 2 Mb region that contained PLAU and 22 other genes was the most likely locus for the mutation. DNA sequencing was then done to evaluate if mutations within the exons, introns or known regulatory regions (5' and 3') of PLAU existed that may explain the changes in uPA expression in QPD. Southern blotting was also done to investigate whether a larger DNA change (deletion, insertion, or rearrangement) has occurred in the region encompassing PLAU and its known regulatory regions in QPD. No mutations were identified in the regions tested, even when sequencing was expanded to cover the region between PLAU and the neighboring gene on the 5' end, CAMK2G. Further studies, using mRNA from platelets. CD34⁺ MK progenitor cells, and salivary cells, were then done to determine if QPD is caused by a cis-effect on PLAU. An expressed SNP in exon 11, and qRT-PCR, were used to show that QPD distinctly increases the uPA transcript from the linked PLAU allele in platelets, but not in CD34⁺ cells and saliva, which had minimal increases of the linked *PLAU* allele.

An additional study (included in Appendix I) was used to show that QPD specifically alters PLAU expression in MKs/platelets, but not the expression of the genes flanking PLAU on chromosome 10 (CAMK2G and VCL). Furthermore, this study showed that the defect in uPA overexpression in QPD occurs with MK differentiation, as early MKs *in vitro* did not contain the >100-fold increases in uPA seen in more mature cultured MKs. Together, these two studies have shown that QPD is caused by a *cis*-acting mutation that increases expression of the linked *PLAU* allele in MKs as they differentiate, and that this mutation is somewhere within the 2 Mb region identified by linkage analysis.

As a senior author on this paper, Dr. Catherine P.M. Hayward supervised the study design, subject recruitment, experimental work, and manuscript writing. My specific roles included study design, experimental work, analysis of data, and preparation of the manuscript. I am very grateful to Dr. Andrew D. Paterson for his participation in study design, data analysis, and manuscript writing. I thank Drs. Johanna M. Rommens, Dennis E. Bulman, John S. Waye, and Georges E. Rivard for their help with study design, interpretation of results and manuscript writing. I also thank Dr. D. Kika Veljkovic and Jessica Blavignac for their contributions to the experimental work and manuscript writing, and Francine Derome for her help with QPD subject recruitment.

CHAPTER 4.2

QUEBEC PLATELET DISORDER IS LINKED TO THE UROKINASE PLASMINOGEN ACTIVATOR GENE (*PLAU*) AND INCREASES EXPRESSION OF THE LINKED ALLELE IN MEGAKARYOCYTES

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4.2.1 Summary

Quebec platelet disorder (QPD) is an autosomal dominant disorder, with high penetrance, that is associated with increased risks for bleeding. The hallmark of QPD is a gain-of-function defect in fibrinolysis due to increased platelet content of urokinase plasminogen activator (uPA), without systemic fibrinolysis. We hypothesized that increased expression of uPA by differentiating QPD megakaryocytes is linked to *PLAU*. Genetic marker analyses indicated that QPD was significantly linked to a 2 Mb region on chromosome 10q containing *PLAU* with a maximum multi-point logarithm of odds (LOD) score of +11 between markers D10S1432 and D10S1136. Analysis of *PLAU* by sequencing and Southern blotting excluded mutations within *PLAU* and its known regulatory elements as the cause of QPD. Analyses of uPA mRNA indicated that QPD distinctly increased transcript levels of the linked *PLAU* allele with megakaryocyte differentiation. These findings implicate a mutation in an uncharacterized *cis* element near *PLAU* as the cause of QPD.

4.2.2 Introduction

Most inherited bleeding disorders result from genetic defects that reduce hemostatic protein expression, secretion or function.¹ Inherited conditions that cause bleeding by increasing gene expression are uncommon and include Quebec Platelet Disorder (QPD),^{2,3} an autosomal dominant disorder, with high and possibly complete penetrance,⁴ and a prevalence in Quebec of 1:300,000.⁴ QPD is associated with a unique, gain-of-function abnormality in fibrinolysis, due to increased platelet stores of urokinase plasminogen activator (uPA), without systemic fibrinolysis or increased uPA in plasma,^{2,5} urine⁶ or CD34⁺ hematopoietic progenitors.⁷ QPD increases risks for a number of bleeding symptoms including delayed-onset bleeding after hemostatic challenges that responds only to fibrinolytic inhibitor therapy.⁴ Diagnostic tests for QPD include assays for increased platelet uPA and α -granule protein degradation from intraplatelet plasmin generation.^{2,4}

We considered *PLAU*, the uPA gene on chromosome 10q24, as a candidate gene for QPD as QPD selectively increases (about 100- fold) *PLAU* expression during megakaryocyte differentiation.⁷ Additionally, increased *PLAU* expression in mouse megakaryocytes results in a QPD-like disorder, characterized by abnormal bleeding that responds to fibrinolytic inhibitor therapy, stored platelet protein degradation and accelerated lysis of thrombi.⁸ *PLAU* contains 11 exons, 10 introns and its conserved regulatory elements include a 3' untranslated region (UTR) that affects mRNA stability (reviewed in Diamandis *et.al.*²) (Figure S1) and a 2.5 kilobase (kb) upstream region that is known to bind transcription factors

produced during megakaryopoiesis (reviewed in Kaushansky ⁹). These observations led us to investigate *PLAU* as a candidate gene for QPD.

4.2.3 Methods

This study was carried out in accordance with the Helsinki Protocol for research on human subjects and institutional ethics review board approval from Hamilton Health Sciences, McMaster University and Centre Hôspitalier Universitaire Sainte Justine. Participants were 28 affected and 110 unaffected individuals who were descendants of a common QPD ancestor,⁴ and 110 healthy unrelated controls. Subjects provided written informed consent, and if under 18 years of age, parental consent was obtained. DNA was collected from all subjects for linkage analysis, DNA sequencing and Southern blotting. *PLAU* allelic expression was assessed using RNA harvested from platelets, peripheral blood CD 34⁺ cells and saliva cells of selected individuals (4 QPD, 5 controls). A detailed description of materials and methods is provided in Document S1.

4.2.4 Results and Discussion

The 41 individuals selected for initial genotyping of chromosome 10 microsatellites were predicted to be informative, with estimated average (maximum) LOD score of 8.4 (10.9) compared to 10.0 (14.1) for all family members. Genotyping of the 41 subjects (Table 4.1) indicated significant linkage of GATA121A08 and D10S1432 to QPD (respective LOD scores = +3.6 at θ = 0.05 and +3.3 at θ = 0.1; not shown). Genotyping of all QPD family members, for these and additional markers (Table 4.1), confirmed that the region containing *PLAU* was strongly linked to QPD (LOD scores 3.0 to 8.4), with a common haplotype in QPD individuals.

Multipoint linkage analysis with the markers closest to PLAU (Figure 4.1A), indicated that the most likely site for the disease gene was near PLAU (maximal LOD score= 11). Data for D10S195 excluded part of this region, and defined a 2 Mb region of chromosome 10q as the most likely location for the QPD mutation. This region contains 22 additional genes (Table S1), including two transcription factors (*HSGT1* and *MYST4*) that are not expressed by megakaryocytes or known to regulate PLAU.¹⁰

13.7 kb upstream of the *PLAU* transcription start site, we identified a potentially informative marker for QPD (designated QPD-1) - a SNP (T/G) (Build 36.1 position: 75,327,173). Genotypes of all 28 affected and 4/110 unaffected QPD family members were T/G whereas other unaffected family members were T/T. Among 105 unrelated French-Canadians, the G allele was uncommon (3)

individuals: T/G, 102: T/T). SNP QPD-1 was strongly linked with QPD (LOD score= +11.8) (Table 4.1). This LOD score, and the maximal LOD score from multipoint analyses, were close to the maximum predicted by linkage simulations, excluding the possibility of another locus for QPD in this pedigree.

Southern blotting for a 25.2 kb region of chromosome 10 containing PLAU and its known regulatory elements excluded major alterations from QPD. DNA sequencing excluded the possibility that QPD results from mutations in the exons, introns and characterized 3' and 5' regulatory elements of PLAU, or in the entire 24 kb upstream of the PLAU transcription start site and 2 kb downstream of the 3' UTR. Complete sequencing of both PLAU alleles for these regions was confirmed as the overlapping PCR products demonstrated SNP heterozygosity in at least 1 QPD subject (Table S2).

To test for cis or trans effects of QPD on *PLAU* expression, the expressed exon 11 SNP rs4065 (T/C; T allele linked to QPD) was studied in heterozygous individuals. Unlike control platelets (T:C ratio=1.5), QPD platelets contained an abundance of the T-allele transcript (all ratios >150) (p=0.016) (Figure 4.1B). In QPD CD34⁺ cells and saliva cells (which contained normal amounts of uPA mRNA, p = 0.36), the abundance of the T allele transcript was more similar to controls (approximately 4-fold and 2-fold increases, respectively, compared to controls) (p values= 0.016) (Figure 4.1B). As sequencing excluded mutated *PLAU* mRNA stability elements, these findings indicated that QPD results from a cis regulatory defect that distinctly increases transcription of the linked *PLAU* allele during megakaryocyte differentiation.

Genetic disorders with altered tissue-specific patterns of gene transcription appear to be rare.¹¹ We had anticipated that QPD might result from mutations in conserved, 3' message stability elements or in the conserved, 2.5 kb, 5' regulatory region of *PLAU* that binds hematopoietic transcription factors, and other silencers and enhancers (reviewed in Diamandis et. al.² and Nagamine et. al.¹²). As these possibilities were fully excluded, the search for the cis regulatory mutation that causes QPD must now extend beyond PLAU to identify the unique sequence changes that markedly increase transcription of the linked PLAU allele during megakaryocyte differentiation. In some disorders, the causative mutation that alters transcription is quite far (5' and 3') from the dysregulated gene,^{13,14} and it can be in an unrelated, neighboring gene.^{15,16} Regulatory elements, with cis or trans effects, have been identified as far as 1 Mb away from the genes transcribed.¹⁷⁻²⁰ Interestingly, the nature of cis mutations that alter gene transcription in other blood disorders (e.g. thalassemia), include deletions within locus control regions²¹ and point mutations that create promoter-like sequences for binding transcription factors.²²

Future identification of the mutation in the linked region of chromosome 10 that increases *PLAU* transcription in QPD may be helpful to develop improved

QPD therapy that targets the cause of uPA overexpression rather than limiting the consequences. The information may also suggest novel ways to upregulate PLAU expression to protect against arterial and venous thrombosis.⁸

Marker Name	Rutgers	Build 36.1	No. of QPD	LOD score at θ (recombination fraction)				
	position	position	family members genotyped	0.00	0.01	0.05	0.1	0.2
D10S1453*		6 615 628	41	-00	-16.04	-7.74	-4.26	-1.35
D10S189*	19.62	6 761 879	41	-00	-4.69	-2.98	-2.11	-1.05
D10S2325*	31.35	12 832 951	41	-00	-2.52	0.07	0.93	1.23
D10S1423*	44.78	19 477 909	41	-00	-2.98	0.09	1.06	1.46
D10S1426	57.44	30 535 790	41	-00	-1.99	1.05	1.94	2.12
D10S1208*	62.04	35 297 637	41	-00	-5.2	-0.25	1.33	1.97
D10S1221*		57 199 892	41	-00	-10.04	-4.71	-2.6	-0.83
D10S1225	80.04	64 425 004	41	-00	-0.92	0.31	0.65	0.66
GATA121A08†	85.32	70 182 554	138	-00	4.39	4.55	4.17	3.14
rs12356915*	and the second second	73 931 323	138	-2.41	-2.15	-1.14	-0.66	-0.28
D10S1432†	93.7	74 329 402	138	-00	4.69	6.65	6.75	5.56
D10S188†	93.7	75 075 749	138	-00	2.98	3.23	2.95	2.11
D10S1737†	93.7	75 259 301	138		1.06	3.75	4.2	3.42
QPD-1†	191 Sec. 201.50	75 327 173	138	11.8	11.59	10.71	9.5	6.8
PLAU		75 340 896	×					
rs4065	93.7	75 346 470	138	0.76	0.72	0.59	0.45	0.23
rs1908339†		75 513 106	138	2.88	3.0	3.0	2.72	1.97
D10S535†	93.7	76 010 712	138	-00	1.64	3.15	3.31	2.69
rs7899137†		76 338 468	138	3.0	2.86	2.58	2.27	1.62
D10S556	94.38	76 922 909	138	-00	1.38	2.33	2.37	1.81
D10S195*	94.38	77 289 176	138	-00	-3.38	-1.02	-0.15	0.4
D10S1136†	95.77	78 009 813	138	-00-	7.83	8.42	7.94	6.17
D10S1730	96.85	78 600 935	41	0	-0.4	1.69	2.03	1.86
D10S2327*	98.55	80 382 014	41	-11.4	-3.88	-1.49	-0.56	0.06
D10S1419		92 354 576	41	-00	-1.3	-0.06	0.32	0.43
D10S677*	114.71	95 954 428	41	-00	-8.1	-2.33	-0.29	0.98
D10S1239*	121.96	103 186 224	41	-00	-3.5	-0.51	0.44	0.87
D10S1237	133.64	116 109 968	41	-00	1.04	2.09	2.22	1.83
D10S1230*	145.08	122 732 623	41	-00	-13.29	-6.56	-3.9	-1.59
D10S1213	150.11	125 396 627	41	0.26	0.24	0.15	0.06	-0.02
D10S1248*	166.09	130 982 451	41	-00	-7.09	-3.82	-2.06	-0.53
D10S212*	174.19	134 299 489	41	-8.5	-2.61	-0.27	0.51	0.85

Table 4.1. Two-point linkage analysis of chromosome 10 microsatellite markers and SNPs.

Markers were evaluated for 41 selected individuals or all 138 QPD family members, as indicated. SNPs are designated by rs numbers, except for the newly identified SNP, QPD-1. *PLAU* indicates the location of the urokinase plasminogen activator gene. Rutgers map positions are in centimorgans, except for 6 markers whose genetic map positions are unknown. Build 36.1 positions indicate physical distances on chromosome 10. *Markers excluding linkage. †Markers giving the best evidence for linkage.



Figure 4.1 Multipoint linkage and allelic expression analysis of *PLAU* in QPD. (A) Results of multipoint linkage using 4 microsatellite markers (positions are shown relative to GATA121A08). The genetic location of *PLAU* between markers D10S1432 and D10S1136 is indicated on the x-axis, which is the region with the highest LOD score for this analysis (+11). (B) Graph of reverse transcriptase-quantitative PCR (RT-qPCR) analyses of *PLAU* alleles for SNP rs4065 in different tissues and indicates the measured ratios of T/C alleles in saliva cells, CD34⁺ cells, and platelets in samples from 5 controls and 4 individuals with QPD. T/C ratios were significantly different for QPD compared with control samples for all tissues (P=.016).

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4.2.6 Supplementary Information

Document S1: materials and methods

Sample collections for molecular analyses

EDTA anticoagulated blood (5 mL) was collected from all subjects for genotyping. Additional blood (200 mL, collected into acid citrate dextrose) and saliva (collected using Oragene RNA collection kits, DNA Genotek Inc., Ottawa, ON, Canada) were collected from some subjects (QPD: n= 4; controls: n= 5) to prepare RNA from platelets, peripheral blood CD34⁺ cells, and saliva cells.

DNA isolation for genetic studies

Genomic DNA was isolated using DNA Blood Midi kits (QIAGEN, Mississauga, ON, Canada) and quantified by spectrophotometry (Eppendorf, Mississauga, ON, Canada) or nanodrop methods (Thermo Fisher Scientific Inc., Waltham, MA, USA).

RNA isolation for allelic expression studies

RNA was harvested from platelets, peripheral blood CD34⁺ cells (with minimal platelet contamination, prepared as described⁷) and saliva using RNeasy Mini kit (QIAGEN), and the RNase Free DNase Set (QIAGEN), to digest any contaminating DNA. RNA sample quality and quantity was assessed using RNA 6000 Nano assay kit and a 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada), which confirmed that the samples used for analysis had an acceptable concentration (range: 5-550 μ g/mL). All samples were stored at -70°C until use.

Genotyping of chromosome 10 microsatellites and single nucleotide polymorphisms

Initial genotyping of QPD family members was undertaken using 21 microsatellite markers on chromosome 10, separated by an average of 7.7 cM based on positions on the Rutgers genetic map. Five additional microsatellite markers (all within a 2.2 cM region) and 4 SNPs were selected for additional mapping analysis around *PLAU*. Sequencing of DNA from 5 unaffected and 5 affected family members was used to screen the regions immediately upstream and downstream of *PLAU* for additional, potentially informative genetic markers (see section on sequencing).

Primers used for microsatellite marker genotyping were designed (after obtaining sequences online at http://genome.ucsc.edu/cgi-bin/hgGateway), or purchased with fluorescent tags (infrared 700 and 800; Li-Cor Biosciences, Lincoln, NE, USA) for multiplex reactions. Marker genotyping was performed by PCR in 96-well plates using 50 ng of DNA sample, 1 µL 10x PCR buffer (containing 15 mM MgCl₂, 100 mM Tris-HCl, pH 8.3), 0.091 uL of each primer (20 µM), 0.1 µL M13 fluorescent dye (20 µM) (Li-Cor Biosciences), 1.5 µL dNTPs (2.5 mM) (GE Healthcare), and 1 unit Taq polymerase (GE Healthcare) (10 µL final volume in water). Multiplex PCR (up to 4 markers per pool) was performed using the two types of fluorescently labeled primers, to assess PCR fragments that differed in expected sizes, and a Tetrad PTC-225 Peltier Thermal Cycler (MJ Research Inc., St. Bruno, QC, Canada) under the following conditions: initial denaturation 94°C for 45 seconds; 30 cycles of 94°C for 30 seconds, 50°C for 30 seconds, 72°C for 30 seconds; and final extension 72°C for 10 minutes. PCR products were run on the 4000 DNA analysis system (Li-Cor Biosciences), using 8% acrylamide gels (SequaGel-8) (National Diagnostics, Atlanta, GA, USA) as recommended by the manufacturer. Alleles were assigned using Saga Generation 2 software (Li-Cor Biosciences).

SNP genotyping for rs12356915, rs4065, rs1908339 and rs7899137 was performed using the Taqman® 5'-nuclease assay (Roche Molecular Systems, Pleasanton, CA, USA), with custom-made primers and fluorescently labeled 6-carboxy-fluorescein (FAM)TM or VIC® probes (Applied Biosystems, Foster City, CA, USA) specific for each allele. Reaction mixtures contained: 10 ng of DNA, 5 μ L 2x Master mix (Roche), 0.45 μ L of each primer (20 μ M), 0.4 μ L of each detection probe (5 μ M) and water to a final volume of 10 μ L. PCRs were carried out in 96-well plates using the ABI 7900HT sequence detection system (Applied Biosystems) under the following conditions: 50°C for 2 minutes, 40 cycles of 94°C for 15 seconds, and 60°C for 1 minute. Genotypes were assigned based on fluorescence measurements read at 60°C for 2 minutes using Sequence Detection Software (SDS) 2.2.1 (Applied Biosystems).

Data collection for genotyping analysis

Genotype information was entered together with the pedigree information and the affection status for all subjects. Individuals who had previously tested positive for QPD were considered affected, while all other family members were designated as having an unknown affection for the purpose of the linkage analysis. Entries were verified by two individuals.

Linkage analysis with microsatellite markers and SNPs on chromosome 10

For linkage analysis, QPD was assumed to be an autosomal dominant disease with complete penetrance, no phenocopies and a disease allele frequency of 0.0001.

To determine if the collected family member samples were sufficient to detect linkage, computer simulations were performed using the SLINK program with either 41 individuals (26 affected) carefully selected from all branches of the pedigree, or all 138 family members. Mendelian inheritance was evaluated using the pedmanager program (http://www.wi.mit.edu/). Two-point and multipoint linkage analysis of marker data was performed using the MLINK and LINKMAP programs to calculate LOD scores. LOD scores of \geq 3 were accepted as showing linkage and scores \leq -2 were accepted as excluding linkage.

Multipoint linkage analysis was performed around the *PLAU* region using shifting multipoint analysis due to the large size of the pedigree and computational limitations. Genetic distances were obtained from the Decode genetic map. The region with the highest positive LOD scores was then aligned with Build 36.1 (March 2006 assembly) of the UCSC genome browser (Genome Bioinformatics Group of UC Santa Cruz, http://genome.ucsc.edu/cgi-bin/hgGateway) to identify genes in the vicinity of the critical interval for QPD.

Southern blot analyses of PLAU and its proximal regions

Southern blot analyses were used to screen for large insertions, deletions or rearrangements within a 25.2 kb region containing *PLAU* and its known regulatory elements. Analyses were done using genomic DNA (3 μ g) from an unrelated healthy control, representative unaffected and affected QPD family members. DNA samples were digested using the restriction enzymes *Hind*III (Roche), *BgI*II (Invitrogen, Carlsbad, CA, USA) and *Eco*RV (Boehringer Mannheim, Gaithersburg, MD, USA), as recommended by the manufacturer, before separation on a 0.7% agarose gel, along with a molecular weight marker (DNA marker II; Fermentas Life Sciences, Burlington, ON, Canada). After transfer to a HybondTM-N⁺ nylon membrane (Amersham Biosciences, Piscataway, NJ, USA), blots were hybridized with a radiolabeled probe for the marker and another probe of 670 nucleotides (labeled by random priming method with ³²P dATP, Amersham Biosciences) corresponding to intron 1, exon 2, intron 2, and exon 3 of *PLAU*.

Sequencing of PLAU and regulatory regions

Genomic DNA sequencing was performed for 3 QPD and 3 control individuals to analyze the 11 exons and 10 introns of PLAU, and the known regulatory elements of PLAU, which included the adjacent 5 kb upstream region (containing the promoter and other characterized regulatory elements) and 2 kb region downstream from the PLAU 3' UTR. The affected individuals, chosen for sequencing analysis, were known to share a common haplotype based on genotypes of microsatellite markers for the linkage studies. Sequencing was later extended to cover 24 kb upstream of the PLAU transcription start site for a representative individual with QPD, and at least 5 more QPD and 5 controls were sequenced when new variations were identified. Primers used to amplify DNA for subsequent sequencing were designed using Primer3 (http://frodo.wi.mit.edu/cgibin/primer3/primer3 www.cgi) to generate overlapping fragments of 1.1 to 4.5 kb that contained at least one previously characterized SNP to verify that each PLAU allele was amplified and sequenced for individuals with QPD. PCRs were performed with the High Fidelity FastStart PCR Master protocol (Roche), with each reaction containing: 25 µL FastStart PCR Master mix, 1.5 µL of each primer (10 µM), and 1.5 µL genomic DNA (20-50 µg/mL) (50 µL final volume in water). For regions containing long poly-A or poly-T repeats, the high fidelity enzyme Pfu polymerase (Stratagene, La Jolla, CA, USA) was used. Cycling conditions were as follows: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 2 min, 60°C for 30 seconds, 72°C for 2 minutes; final extension at 72°C for 10 minutes. PCR products were run on 1% agarose gels, and then purified using QIAEXII or QIAquick PCR purification kits (QIAGEN) before sequencing the forward and reverse strands using non-overlapping primers and a 3730 DNA Analyzer (Applied Biosystems).

Sequence data was aligned with contig NT_008583.16 (GI: 37551286) using the BLASTN algorithm (http://www.ncbi.nlm.nih.gov/blast/) to look for regions of mismatch. Polymorphisms were confirmed using dbSNP build #129 (http://www.ncbi.nlm.nih.gov/SNP/). Two individuals reviewed all sequencing data.

Assessment of uPA allelic expression

RT-qPCR was performed using a TaqmanTM 5' nuclease assay (Applied Biosystems, Assays on Demand kit c_3155393_10) and RNA from platelets, $CD34^+$ cells and saliva cells of subjects verified (by genomic DNA sequencing) to be heterozygous for SNP rs4065 in exon 11 of *PLAU*. Homozygous individuals were tested as controls to verify the assay specificity. All individuals tested provided samples of all 3 sources. To avoid amplification of contaminating genomic DNA, primers spanning intron-exon boundaries were used. Alleles were

multiplexed using the fluorescent probes FAMTM for the T allele, VIC® for the C allele and Tamra (TET) (Applied Biosystems) for the housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) to normalize measurements. Reactions were carried out in 96- well plates, in triplicate, using the following reagents: 12.5 µL RT-PCR master mix (Roche), 0.625 µL 40x RT-PCR primer/FAM/VIC fluorescent probe mix (Applied Biosystems), 0.5 µL TET probe, 0.25 µL of each PCR primer (10 µM), 0.25 µL quantitated multiplier RT mix (Qiagen), and 40-250 ng RNA (25 µL final volume with water). Each plate was run on the SDS 7900 machine under the following conditions: 50°C for 20 minutes (RT step), 95°C for 15 minutes (PCR activation step for Tag polymerase), and 40 cycles of 94°C for 45 seconds, 60°C for 75 seconds (PCR step). SDS 2.2.1 software was used to calculate: the average threshold cycle (Ct) values for each set of triplicates: Ct values normalized to GAPDH (Δ Ct); and Δ Ct values. normalized to a control sample containing equal amounts of the T and C alleles ($\Delta\Delta$ Ct values). The relative quantities of the two alleles were expressed as $\Delta\Delta$ Ct values for each sample. Control and OPD samples were compared using the Mann-Whitney rank test to look for differences in the ratios of the rs4065 alleles in each tissue type tested (expressed as P values).

Because total uPA mRNA had been previously assessed for CD34⁺ cells and platelets⁷, but not for saliva, total uPA mRNA in saliva was assessed by RTqPCR using a TaqmanTM 5' nuclease assay and the equipment used for analysis of uPA alleles. Reactions were multiplexed using the fluorescent probes FAMTM for uPA and Tamra (TET) for GAPDH, and performed in triplicate using the following reagents: 12.5 μ L RT-PCR master mix (Roche), 0.625 μ L 40x RT-PCR primer/FAM fluorescent probe mix (Applied Biosystems), 0.5 μ L TET probe, 0.25 μ L of each PCR primer (10 μ M), 0.25 μ L quantitated multiplier RT mix (Qiagen), and 40-250 ng RNA (25 μ L final volume with water). The conditions used for runs were identical to uPA allele analyses. Ct values of serially diluted, pooled control samples (1:5 – 1:3125) were used to generate a standard curve to determine relative uPA mRNA levels, normalized to GAPDH.



Figure S1. The known regulatory regions of PLAU.
Table S1. Genes found in the 2 Mb region on chromosome 10 linked with QPD.

GENE	DESCRIPTION: Name and Function	Start Position	End Position
OIT3	Oncoprotein-induced transcript 3; calcium ion binding	74,323,345	74,362,793
PLA2G12B	Phospholipase A2, group XIIB; role in lipid catabolism	74,364,944	74,384,516
P4HA1	Procollagen-proline 2-oxoglutarate 4-dioxygenase (praline- 4-hydrolase), alpha polypeptide I; collagen synthesis	74,436,986	74,504,679
NUDT13	Nudix (nucleoside diphosphate linked moiety X), motif 13; hydrolase activity	74,540,216	74,561,587
HSGT1	Suppresor of S.cereviciae gcr2; transcription coactivator activity	74,564,349	74,593,701
DNAJC19	DNAJ (Hsp40) homolog, subfamily C, member 9; heat shock protein binding	74,672,588	74,677,031
MRPS16	Mitochondrial ribosomal protein S16; mitochondrial protein synthesis	74,678,607	74,682,457
TTC18	Tetratricopeptide repeat-containing isoform A; no information on function	74,683,527	74,784,599
ANXA7	Annexin A7; voltage-sensitive calcium channel activity	74,805,195	74,843,847
PPP3CB	Protein phosphatase 3 catalytic subunit beta isoform (calcineurin A beta); cell cycle regulation	74,873,566	74,925,960
USP54	Ubiquitin specific proteinase 54; cysteine-type endopeptidase activity	74,965,965	74,971,502
MYOZ1	Myozenin 1; binds alpha-actinin and gamma-filamin in skeletal muscle Z lines	75,061,418	75,071,521
SYNPO2L	Synaptopodin 2-like; actin binding	75,074,650	75,085,838
SEC24C	Sec24-related gene family, member C; vesicle trafficking	75,174,138	75,199,239
FUT11	Fucosyltransferase; metabolism of fucosylated glycans	75,202,055	75,205,982
CHCHD1	Coiled-coil helix-coiled-coil helix domain containing 1; nuclear protein	75,211,814	75,213,412
NDST2	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2; N-sulphation	75,232,438	75,236,638
CAMK2G	Calcium/calmodulin-dependent protein kinase 2 gamma; calcium signaling	75,242,265	75,304,349
PLAU	Urokinase-type plasminogen activator; converts plasminogen to plasmin	75,340,924	75,347,261
VCL	Vinculin; cytoskeletal protein anchoring F-actin to membrane	75,424,956	75,549,920
AP3M1	Adaptor-related protein complex 3, mu 1 subunit; vesicle budding and protein sorting	75,550,021	75,580,832
ADK	Adenosine kinase; transfer of gamma-phosphate from ATP to adenosine	75,955,007	76,139,066
MYST4	MYST histone acetyltransferase (monocytic leukemia 4); transcription regulation	76,268,447	76,462,645

Descriptions, functions, and physical locations from Build 36.1 are indicated (Source: UCSC genome browser). Genes known to be expressed in platelets are italicized.⁹

PCR primers	PCR fragment	SNPs identified	Contig position	Build 36.1 position
E. SLACC ACC TOT CAC COT	2020 hr			
F. J-AGG AGC ICI CAG CCI	2039 bp	***********	24109646	75217407
R. 5'- AAT GGC GAC TTA ACA		rs2675677 (G/T)	24198040	75318255
TCG GG-3'		rs2675676 (G/T)	24199442	75318293
F: 5'-CTT GGC TAG AGG TGC	2053 bp	102010010 (011)		10010000
CAG AG-3'	1	rs2675675 (C/T)*	24200203	75319054
R: 5'-GCC CAG CTT GTC TCA		rs5786125 (-TTCT)	24200346	75319198
AAC TT-3'		rs4998818 (C/T)	24200398	75319249
		not known (T/C)	24200464	75319315
		rs10824043 (A/G)	24200625	75319476
		rs10824044 (C/T)	24200707	75319558
E. S'ATT CCA CGA AAC TCC	2150 hn	rs5/86126 (-/A)	24201084	75319935
AGT AAG-3'	2139 bp	rs2633304 (C/T)*	24201805	75320656
R. 5'-TGG TTG TGA CCC TGG		rs2688616 (C/G)*	24201805	75320658
GGC A-3'		rs2688615 (C/G)	24202654	75321255
		rs2633305 (A/G)	24203278	75322129
		rs2633306 (A/C)	24203602	75322453
F: 5'-TAC TCA AAC CCG ACC	4534 bp			
ACA CA-3'		rs2675674 (C/T)	24203837	75322688
R: 5'-CAA CAT GGA TGA CCC		rs2688614 (C/T)*	24204031	75322882
TTG AA-3'		rs2688613 (A/G)	24204200	75323051
		rs2688612 (A/C)	24204360	75323211
		rs2688611 (A/C)	24204527	15323378
		rs2675669 (C/T)*	24204933	75323800
		rs2633307 (A/G)*	24205027	75323887
		rs2633308 (A/T)*	24205059	75323910
		rs2633309 (A/G)	24205241	75324092
		rs11000802 (C/G)*	24205322	75324176
		rs2688610 (C/T)*	24206086	75324937
		rs2675670 (G/C)*	24206783	75325634
		not known $(C/T)^*$	24207480	75326331
		rs10824045 (A/G)*	24207698	75326549
		rs2633300 (G/T)*	24207802	/5326653
		$(A/C)^*$	24207909	75326730
E. 5'-CCA GAC AGA ACC TCC	2318 hn	not known (1/O)	24207970	75520811
ACC TG-3'	vp	not known (G/T)*	24208322	75327173
R: 5'-TCT GTG GAA TGC AGC		not known (T/G)*	24208578	75327429
AGA AC- 3'		rs2948689 (A/G)	24209015	75327866
		rs2688609 (C/T)*	24209336	75328187
		rs2688608 (A/C)*	24209504	75328355
		rs2675679 (A/G)*	24209736	75328587
E: S' TTG GTC AGG AGT CCC	2258 hp	rs20/3080 (C/1)*	24210019	15328870
TGT TC-3'	2256 Up	rs2461864 (A/T)	24211027	75329878
R: 5'-ACA AAC GCA GCA GAA		rs1815076 (A/G)*	24211041	75329892
GTT CA-3'		rs2675663 (G/T)*	24211425	75330276
		rs34502829 (-AC)	24211491	75330342
		rs11000804 (C/G)	24211512	75330363
		not known (AG	24211522	75330373
		repeat variation)*		
		not known (37 bp	24211885	75330736
		deletion)*		

Table S2, continued	rs9971038 (C/T)*	24212304	75331155
	rs9971040 (C/G)*	24212317	75331168
	()		
F: 5'-TGG TGA AAC CCC TTG 2761 bp			
TTC TC-3'	rs4746155 (A/G)*	24212726	75331577
R: 5'-AGA TGG GGC CTA GAA	rs11385547 (-/T)	24213157	75332008
ATC TGA-3'	rs11000805 (C/G)*	24214506	75333357
F: 5'-AAC AGG GCA TAA TGG 2363 bp			
TGC AT-3'	rs2688607 (C/T)*	24214891	75333742
R: 5'-CTG CTC AGA GTT CTG			
GTT AC-3'			
F: 5'-TCA TCC CAG TCC TTT GTT 3475 bp	States and the second		
CC-3'	rs2633298 (C/G)	24217494	75336345
R: 5'-CCT GCT GGG GAA AGT	not known (T/G)*	24219666	75338517
ACA AG-3'	rs2459449 (C/T)	24220168	75339019
	rs2227551 (G/T)	24220345	75339196
F: 5'-CAG CTC AGC AAG CAC 1116 bp			10003 270
AGA AG-3'	rs2227552 (C/T)*	24220474	75339325
R' 5'- ACC AGT GGA ATG GAA	rs36228908 (-/GT)*	24220807	75339658
CAA AG-3'	rs4007240 (-/GT)	24220820	75339672
F: 5'-CAC GAC ACC TAA CCC 2303 bn	10100/210(/01)		10003012
AAT CC-3'	rs1916341 (A/C)*	24222317	75341168
R' 5'-CTA CCA TGC CAG ATT	rs2227558 (-/G/T)*	24223532	75342384
TTT GC-3'	rs34916472 (-	24223533	75342385
111 00 5	/G/T)*	21220000	10012000
E: 5'-CAT GGT GTA ACC CTG 1282 bn	10(1)		
CCT CT-3'	rs2227560 (A/G)	24223630	75342481
R' 5'-GTC CCA TGA AAT CTG	rs2227562 (A/G)*	24224116	75342967
CCA CT-3'	rs2227564 (C/T)	24224256	75343107
F: 5'-TCC TTC CCA GCA AAG 1591 bp	102227001(0/1)	21221200	10010101
TGT TC-3'	rs2227566 (C/T)*	24224886	75343737
R: 5'-GAA CAC TCA CCT GGC	rs2227568 (C/T)*	24225034	75343885
AGG AA-3'	rs2227571 (C/T)*	24225895	75344746
F: 5'-TGA AGA TCC GTT CCA 1676 bp			
AGG AG-3'	rs2227571 (C/T)*	24225895	75344746
R: 5'-CCC AGC TCA CAA TTC			
CAG TC-3'			
F: 5'-CTA GGG TCT GGG AGT 1444 bp			
GTG GA3'	rs4065 (C/T)*	24227619	75346470
R: 5' CAG TGC TGG TCA CAG			
GTC AT-3'			The second second
F: 5'-GGG GAG CAG AGA CAC 1545 bp			
TAA CG-3'	rs2227574 (-/G)*	24228322	75347173
R: 5'-GTC AAG CTG GCA TTC	rs2227575 (A/C)*	24228995	75347846
TGG AT-3'	not known (C/T)*	24229089	75347940
	rs2948691 (C/T)*	24229092	75347943
	rs2633315 (C/T)*	24229095	75347946
F: 5'-CGC CTG TAA TCC AGC 2198 bp			
ACT TT-3'	rs2227576 (T/G)*	24229431	75348282
R: 5'-TGT GAA ACT GGT TGC	rs2227578 A/G)*	24229870	75348721
TGG AG-3'			

Table S2. Summary of PCR primers, product sizes and SNPs identified after sequencing *PLAU* and surrounding areas. The SNPs listed are those that differed from the reference sequence (from contig NT_008583.16) in at least 1

individual tested (affected or unaffected). For SNPs with two possible alleles "(X/Y)", alleles are indicated in brackets. "(-/X or Y or XY)" indicates a deletion of one or two bases at the specified position. SNPs having two possible alleles that could also be deleted are indicated as "(-/X/Y)". SNPs that were found to be heterozygous in at least 1 QPD individual are indicated by a *. Build 36.1 positions were obtained using the UCSC genome browser. Information for previously reported SNPs was obtained from dbSNP (rs numbers) (see Materials and Methods). SNPs listed as "not known" are new DNA variations that were found in either affected or unaffected individuals tested.

CHAPTER 5

DISCUSSION AND FUTURE DIRECTIONS

OPD is a unique inherited bleeding disorder that affects both platelets and the fibrinolytic system by increasing the expression and storage of the fibrinolytic enzyme uPA in MKs (reviewed in [541]). This thesis aimed to extend the current knowledge on QPD by addressing some unanswered questions about the biochemical defect and genetic mutation in the disorder. The first goal was to evaluate the effects of QPD platelets on clot lysis in vitro, as platelet-mediated fibrinolysis has been postulated to contribute to bleeding in OPD [243]. The second goal was to investigate if the QPD biochemical defect extends to cells of the urinary system, to gain insights on why some individuals with QPD may experience urinary tract bleeding [293]. The third goal was to map the genetic locus for QPD, and to look for the putative mutation that is responsible for causing the greater than 100-fold increased uPA expression in QPD platelets [243]. My studies showed that: 1) QPD causes a gain-of-function defect in fibrinolysis when platelets are incorporated into clots [542]; 2) the QPD defect does not extend to cells of the urinary tract system [543]; 3) the genetic locus for QPD lies in a 2 MB region of chromosome 10 that harbors PLAU [544]; and 4) the mutation causing QPD has cis-effects on PLAU expression [544]. The next sections discuss these key findings and implications for future work to study the tissue-specificity of QPD and identify the precise mutation that generates profibrinolyic platelets.

5.1 Insights on the contribution of QPD platelet uPA to clot lysis

Normal platelets confer protection to thrombi from fibrinolysis by releasing large amounts of PAI-1 [139,141,142], and by increasing local concentrations of fibrin in platelet-rich areas, making clots less permeable to fibrinolytic proteins [137,138,140]. Recently, platelet-mediated accelerated fibrinolysis was postulated to contribute to QPD bleeding, as this condition is associated with marked increases in platelet uPA, without large increases in plasma uPA or systemic fibrinolysis [243], and because QPD bleeding responds to fibrinolytic inhibitor therapy [293]. The studies presented in Chapter 2 aimed to investigate this possibility using in vitro assays of clot formation and clot lysis with QPD blood. These studies demonstrated that QPD is associated with a gainof-function abnormality that accelerates the lysis of forming or preformed clots, when there is a stimulus for clot formation or platelet recruitment to fibrin [542]. The importance of OPD platelets to clot lysis was highlighted by the finding that fibrinolysis was attenuated in the presence of fibrinolytic or platelet activation inhibitors [542]. These findings have important implications for understanding the mechanism of QPD bleeding.

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Most platelet disorders are characterized by defects in primary hemostasis that cause immediate-onset bleeding [239-241]. OPD is an exception, as individuals with OPD experience bleeding that is characteristically delayed in onset [293,541]. I used several different assays to show that clot formation in OPD occurs normally in vitro, but the incorporation of OPD platelets into clots accelerates clot lysis [542]. First, TEG [171] was used to measure the viscoelastic changes in OPD clots during clot formation. TEG showed that clot formation in QPD whole blood was similar to controls, and the fibrinolytic abnormalities in OPD blood were not detectable within the 3 hour test period, even when the platelet count was increased (Chapter 2, figure 2.1) [542]. Clot architecture (including the presence of elongated fibrin strands, platelet aggregates and platelet-fibrin contacts), assessed by electron microscopy, was also normal in QPD at early time points after clot formation (Chapter 2, figure 2.4) [542]. Analysis of clots over time indicated that clots made with QPD whole blood or platelet-rich plasma were lysed prematurely, compared to control clots [542]. Biochemical assays of clot sera showed increases in serum uPA levels, reflecting uPA release from activated platelets, and this correlated with increased plasmin generation (measured by PAP complexes) and D-dimer (Chapter 2, table 2.1) [542]. Visually, QPD clots were partially or fully disintegrated within 18 hours of clot formation (Chapter 2, figure 2.3), and normal clot architecture was disrupted in QPD (Chapter 2, figure 2.4) [542]. The effects of QPD blood on the breakdown of preformed fibrin, assessed by flow assays, were also marked by the release of degraded fibrin into the flow-through (Chapter 2, figure 6) [542]. These changes in clots were not observed when QPD platelet-poor plasma was used to generate fibrin [542]. Furthermore, the effects of QPD blood on clot lysis could only be mimicked in normal blood by adding high amounts of exogenous uPA [542]. Previous studies using purified proteins to assess the effects of plasminogen activators on fibrin clot architecture, also reported similar findings [138,139,148]. Collectively, my findings implicate a platelet-dependent defect in fibrinolysis in OPD, that offers an explanation for why QPD individuals suffer from delayedonset, abnormal bleeding after hemostatic challenges [293].

The release of uPA from activated QPD platelets is the key event that mediates accelerated clot lysis in QPD blood [542]. To highlight this, the studies in Chapter 2 were carried out both in the absence and presence of platelet activation (PGE₁, aspirin) and fibrinolytic (AMCA) inhibitors [37,38,113]. Biochemical assays (ELISA, Western blot), electron microscopy, and flow assays all confirmed that the effects of QPD platelets on clot lysis could be attenuated in the presence of these inhibitors [542]. The presence of AMCA resulted in: 1) preservation of normal platelet-platelet and platelet-fibrin contacts (Chapter 2, figure 2.4); 2) attenuation of the increased degradation of cross-linked fibrin within QPD clots, as measured by D-dimer release into clot sera; and 3) reduced lysis of preformed fibrin by flowing QPD blood (Chapter 2, figure 2.6) [542]. PGE₁ and aspirin similarly reduced the breakdown of preformed fibrin to FDPs,

suggesting that inhibition of platelet activation resulted in reduced release of uPA into the clot milieu, and reduced plasmin generation (Chapter 2, figure 2.6) [542]. This is clinically significant, as the only effective treatment or prophylaxis for QPD bleeding has been the use of fibrinolytic inhibitor therapy [293]. The findings of this study indicate that increased expression and storage of uPA in QPD platelets causes a gain-of-function abnormality in fibrinolysis, that accelerates QPD clot lysis, and this may also occur in vivo after a hemostatic challenge [542]. Similar gain-of-function abnormalities in clot lysis have been observed in a mouse model of uPA overexpression in platelets (driven by a MKspecific promoter) [497]. In these mice, pulmonary venous thrombi are rapidly lysed by transgenic platelets, and mice also have increased resistance to arterial thrombosis, suggesting that local release of uPA by transgenic platelets can cause rapid lysis of nascent thrombi [497]. Some parallels can be made between these mice and individuals with QPD, including increased bleeding, α -granule protein degradation, and a favorable response to fibrinolytic inhibitors [293,497]. However, these mice have other problems that are not features of QPD, including increased bleeding-related mortality with pregnancy, and pregnancy loss [497]. Nonetheless, the increased resistance of transgenic mice to venous and arterial thrombosis [497] raises an important implication for individuals with QPD. So far, there are no reports of an individual with QPD having suffered from thrombotic stroke, myocardial infarction or angina, suggesting that their gain-offunction defect in fibrinolysis may confer some protection against arterial thrombotic events [293]. Only one individual with QPD has experienced a pulmonary embolism, but this occurred while she was on fibrinolytic inhibitor therapy, suggesting that QPD may also confer some protection against venous events [293]. The low number of thrombotic events that have occurred so far in the QPD family (in both affected and unaffected individuals) prevents us at this time from determining the precise contribution of increased platelet uPA to protection against thrombosis.

QPD platelets contain degraded forms of adhesive proteins (fibrinogen, thrombospondin-1, VWF) and the coagulation factor FV, which may contribute to defective hemostasis *in vivo* [90,243,545-548]. The studies presented in Chapter 2 did not evaluate the contributions of degraded α -granule proteins to QPD bleeding. However, clinical evidence from individuals with Gray platelet syndrome, a SPD characterized by a near total loss of granule proteins and little to no bleeding [549], suggests the absence of α -granule proteins likely contributes minimally. Defective platelet FV was previously thought to contribute to QPD bleeding by causing abnormal thrombin generation on the platelet surface [548]. Indeed, when plasma FV is absent (~80% of total FV in blood [550]), QPD individuals do have abnormal thrombin generation, which is what led to the initial designation of the disease as "Factor V (Quebec)" [548]. The studies in Chapter 2 suggest that degraded platelet FV is not the main contributor to bleeding in QPD [542]. TEG analyses did not show abnormalities in clot formation with QPD

whole blood when TF was used to initiate FV-dependent coagulation [542], suggesting that in QPD, functional plasma FV may be sufficient to support prothrombinase assembly [546-548]. Also, when the platelet FV defect was bypassed (exogenous thrombin was used to initiate clotting, or studies were done with preformed fibrin), the fibrinolytic abnormalities in QPD (increased plasmin generation and fibrinolysis) were still detected [542]. An additional possibility that was not explored in these studies, is that uPA released from activated OPD platelets and local plasmin generation could result in the degradation of important clot components, such as adhesive protein receptors (eg. GPIb, $\alpha_{IIb}\beta_3$). These GPs are not degraded on the surface of resting OPD platelets [546]. However, they are known plasmin substrates in vivo and in vitro [91,551]. Local plasmin generation caused by the release of stored uPA from activated platelets into clots made in vivo, could thus affect normal platelet adhesion and aggregation, leading to a weak hemostatic plug that is more susceptible to fibrinolysis. Evaluation of these other potentially-degraded proteins in QPD clots using biochemical assays (Western blot, ELISA) could provide additional information on how the QPD platelet defect could contribute to accelerated fibrinolysis. For example, a wellestablished ELISA assay exists for glycocalicin, a degradation fragment of GPIb generated by plasmic lysis [91]. The methods used in these studies could further be applied to studying clot lysis in other fibrinolytic defects also characterized by delaved-onset bleeding (a₂-PI and PAI-1 deficiencies; plasma tPA overexpression) [282,283,290,291]. The absence of fibrinolytic inhibitors would likely result in premature clot lysis, without increased PAP or D-dimer generation. Studies of clots made from plasma with increased tPA would be expected to show similar consequences for clots as the QPD defect when plateletpoor plasma is used. However, unlike QPD, this defect may show more delayed effects on clot lysis, as normal platelets would be present to confer some protection against fibrinolysis.

5.2 Studies of uPA expression in urinary tract cells and other tissues

The elevated levels of uPA in QPD platelets, and normal to modest increases of uPA in plasma, suggest that there might be a MK-specific dysregulation of uPA in QPD [243,541]. uPA is normally ubiquitously expressed [391], and urine is one site that normally contains high amounts of uPA [330]. In QPD, about 50% of individuals have experienced spontaneous hematuria that is associated with having higher levels of platelet uPA [293]. However, individuals with QPD have not been evaluated for increased uPA in the urinary tract. In the study presented in Chapter 3, an ELISA assay for uPA was used to quantitate 24 hour urinary uPA excretion in QPD and control subjects [543]. The study demonstrated that there is no significant difference in 24 hour urinary excretion between QPD subjects and controls, which indirectly suggests that QPD MKs and

platelets [543]. The findings suggest that episodic, spontaneous hematuria in QPD is most likely a consequence of increased platelet uPA [543].

So far, the highest levels of uPA in OPD have been measured in platelets [243]. Two studies included in this thesis provide additional data on the tissuespecific nature of uPA dysregulation in QPD [544,552]. In Chapter 4, total uPA mRNA was measured in cells collected from saliva, a biological fluid which does not contain platelets [544]. In Appendix I, uPA expression by CD34⁺ hematopoietic progenitor cells and by differentiating MKs grown in culture (tested on days 7 and 13), were evaluated [552]. The levels of uPA mRNA in QPD saliva were normal [544]. In contrast, uPA mRNA levels in day 7 (early differentiation) and day 13 (late differentiation) QPD MKs were increased by 4fold and 100-fold, respectively, compared to controls, but they were not significantly increased in QPD CD34⁺ progenitors [552]. Together, these data indicate that the large increase in uPA in QPD platelets results from uPA overexpression during MK differentiation [544,552]. We previously hypothesized that the overexpression of uPA during QPD megakaryopoiesis might follow the same temporal pattern as other α -granule proteins (Chapter 1, section 1.6, figure 1.11), whose expression increases at the late stages of MK maturation, and this was confirmed [541]. It is thus likely, that the QPD mutation confers to PLAU some characteristic of a gene whose expression increases as MKs differentiate [552]. Alternatively, the removal of an element that normally represses PLAU expression, could also be implicated [552]. In normal MKs, uPA mRNA and protein levels remain fairly consistent during megakaryopoiesis [552], suggesting that MK differentiation normally has little effect on potentiating PLAU expression.

MK differentiation is governed by the timed expression of various transcription factors, that either promote or limit differentiation (reviewed in [249]). Mutations affecting these transcription factors have significant effects on MK maturation, both *in vitro* and *in vivo* [249]. Many α -granule proteins have well-characterized binding motifs for different MK transcription factors that turn on their expression during megakaryopoiesis (see below). PLAU regulatory elements have been well characterized in the region up to 2.5 kb upstream of the gene, and several binding motifs exist that are capable of interacting with MKspecific transcription factors (eg. Ets-1, RUNX1) (see Chapter 1, figure 1.6) (reviewed in [541]). Key transcription factors that promote normal megakaryopoiesis and their relevance to PLAU and other genes encoding α granule proteins are summarized in table 5.1. Additionally, the expression of other transcription factors at certain stages of hematopoiesis or megakaryopoeisis (c-Myb, PU.1, Tel, EKLF, ETO2) can have negative regulatory effects on MK differentiation, which is important for correct timing of expression of some MKspecific genes [553-557]. Future studies of the QPD mutation will provide

important insights on which transcription factor(s) may be implicated in causing the surge in uPA expression in maturing MKs (see section 5.5).

Table5.1.Majortranscriptionfactorsinvolvedinmegakaryocytedifferentiation.

Factor	Expression and role	Binding motif at <i>PLAU</i> locus?	Binding motif at α-granule protein gene locus?	Effect of mutations (including known human diseases)
GATA-1 [†]	All stages of differentiation [558,559]	N/A (Other GATA motif present)	Yes [560-568]	Increased number of MKs with arrested differentiation and ultrastructural abnormalities; defective erythropoiesis; X-linked thrombocytopenia; acute megakaryoblastic leukemia in Down syndrome [250,251,558,569-573]
GATA-2 [†]	Early stages of differentiation [559,574]	N/A (Other GATA motif present)	Yes [575,576]	Arrested MK differentiation [577-579]
FOG-1 [†]	Cofactor for GATA-1 and GATA-2 [559,580]	N/A	Yes [562,565- 567,581]	Complete absence of MK progenitors [570,582]
Fli-1 [†] (an Ets factor)	Interacts with GATA-1 (all stages of differentiation) [566,567]	N/A (Other Ets motif present)	Yes [560,565- 567,583]	Increased number of MK progenitors/ ultrastructural abnormalities; reduced number of α-granules (giant size); Jacobsen/Paris-Trousseau thrombocytopenic syndromes [252,253,584,585]
Ets-1	All stages of differentiation [586]	Yes [356- 358]	Yes [560,561, 568,581, 587,588]	Thrombocytopenia; may be deleted in Paris-Trousseau syndrome (same genetic locus at Fli-1) [252,589]
Ets-2	Early differentiation [590]	N/A (Other Ets motif present)	Yes [560]	Immature MKs [590]
GABPa	Early differentiation [591]	N/A (Other Ets motif present)	Yes [591]	Reduced expression of early MK genes [591]
Runx/ AML-1	Early differentiation; binds GATA-1 [592]	Yes [355]	Yes [592-595]	Reduced number and size of MKs; qualitative and quantitative platelet defects; familial platelet disorder with progression to AML [254,596,597]
NF-E2	polyploidizatio n proplatelet formation [558,598]	N/A	Yes [599,600]	Increased number of immature MKs; reduced number of α -granules; reduced proplatelets/platelets; [588,600-603]

SCL/ tal-1 [†]	Early differentiation [604]	N/A	Yes [605]	Reduced number of early progenitors; thrombocytopenia [606]
GFI-1b	Early differentiation [607]	N/A	N/A	Arrested MK development [607]
Sp1	Early differentiation [608]	Yes [353, 354]	Yes [565,581, 609,610]	Arrested MK development; reduced response to Mpl ligand [611]
ZBP-89	binds GATA-1 [612]	N/A	Yes [612]	Absent hematopoiesis [613]

Reviewed in [249]. The timing of expression of each transcription factor during megakaryopoiesis is indicated, as well as interactions (for some) with other transcription factors. Many α -granule proteins have binding motifs for these transcription factors, and some motifs also exist at the *PLAU* locus (see Chapter 1, figure 1.6). The last column summarizes data from human, cell line, or animal studies that have evaluated the effects of mutant forms of these transcription factors (indicated by a [†]) can result in embryonic lethality in animal models, suggesting the importance of these transcription factors to life.

The studies of uPA expression in platelets, CD34⁺ progenitors, differentiating MKs and saliva all support the hypothesis that QPD is a MKspecific defect in uPA dysregulation [243,544,552]. However, data obtained from the studies presented in Chapter 2 now raises a new question about the tissuespecificity of uPA in QPD [542]. During clot lysis studies with QPD blood, the release of uPA into the sera of clots made either from platelet-rich plasma or whole blood was measured. Sera from QPD platelet-rich clots contained 100% of the amount of uPA predicted to be stored in QPD platelets [542]. However, QPD whole blood clot sera contained more uPA than predicted from the amounts potentially released by QPD platelets, suggesting an additional source of uPA may exist in OPD whole blood [542]. This finding suggests that there may be other cells in QPD whole blood that can release uPA during clot formation. Leukocytes are one cell type that normally expresses uPA, and this has been implicated in the activation of fibrinolysis on the surface of these cells [76,153,333]. Various classes of leukocytes, including monocytes and granulocytes, are derived from the same myeloid stem cell progenitor as MKs [183], which suggests that they may have similar pathways of transcriptional regulation for their expressed genes. This makes them an attractive cell type for further investigations on uPA dysregulation in QPD. Future studies with these, and other cell types that associate with thrombi, are further discussed in section 5.5.

5.3 Mapping of the QPD genetic locus- insights on the causative mutation and implications for other platelet disorders of unknown genetic cause

Linkage analysis has been effective for determining the genetic loci of several inherited platelet disorders [317-321], and I postulated that such an analysis would provide important insights on the pathogenesis of OPD. The aim of the study presented in Chapter 4 was to determine whether QPD is linked to inheritance of PLAU (the candidate gene for the disorder), and if the overexpression of uPA in OPD is caused by cis- or trans- effects on PLAU expression [544]. QPD was linked to a 2 Mb region on chromosome 10 (LOD scores up to +11 by multipoint linkage analysis) that includes PLAU and 22 other genes (Chapter 4, Table S1) [544]. No mutations were identified in a region of ~48 kb encompassing PLAU and all of its known regulatory elements [544]. However, a shared haplotype (including inheritance of a rare QPD-linked SNP) for QPD individuals in this region suggested that *PLAU* is the most likely locus for QPD [544]. In the study presented in Appendix I, changes in expression in platelets and/or MKs for the two genes flanking PLAU, CAMK2G and VCL, were not evident, suggesting that the QPD mutation specifically alters PLAU regulation [552]. It is not certain if the QPD mutation affects any of the other 20 genes within the linked region, since expression of these other genes was not evaluated in my studies [544].

QPD platelets were previously found to contain increased amounts of uPA transcript [243], but it was not known if mRNA was transcribed from one or both *PLAU* alleles in MKs. In my studies, an expressed exon 11 SNP was used to address this issue [544]. The mRNA allele linked to QPD was increased up to 150-fold compared to the unlinked allele in QPD platelets, whereas CD34⁺ cells and salivary tissue contained minimally increased levels of the linked transcript (< 4-fold increase) [544]. These findings, coupled with linkage data, and the finding that QPD individuals share a common haplotype in the region of *PLAU* on chromosome 10, indicate that QPD is caused by a *cis*-acting mutation that affects *PLAU* expression. Because the QPD genetic defect likely arose as a founder effect in one common ancestor, and due to the dominant inheritance pattern [541], it is almost certain that all QPD individuals have inherited the same mutation with minimal or no divergence.

The study presented in Chapter 4 has mapped the genetic locus for QPD and confirmed *PLAU* as a candidate gene for this disorder [544]. QPD has been classified as a SPD, due to the unique changes in α -granule proteins that are present in platelets. Studies using linkage analysis have previously mapped genomic regions associated with two other SPDs [614,615]. However, several other SPDs still have no defined genetic cause, including Gray platelet syndrome, δ -granule deficiency and $\alpha\delta$ -SPD [240-242]. It may be possible to apply the methods described in this thesis to identify regions of the genome linked with other SPDs. However, the extreme rarity of most of these disorders (most families have only one or two affected individuals), could make linkage studies difficult [264,272,616-619]. Furthermore, unlike QPD, which has only been identified in one French-Canadian family, these disorders have been described in several families around the world [616,617,619]. QPD is unique from other SPDs even in its pathogenesis, as other SPDs are usually characterized by reduced numbers of granules and a deficiency or absence of stored granule proteins [240-242]. QPD is not caused by a defect in targeting proteins to α -granules, and only multimerin 1 is quantitatively reduced [90,545,546]. The underlying defect of other SPDs appears to be related to granule biogenesis, protein compartmentalization, maturation, or trafficking during megakaryopoiesis [620-624], implicating one or more genes (and possibly different mutations), as the cause. This study has added important new knowledge on the genetic locus for one SPD whose genetic cause had been in question for many years.

5.4 Mechanisms of normal and aberrant gene expression: relevance to QPD

The findings from genetic analyses suggest that the QPD mutation is outside the region that was sequenced or evaluated by Southern blot, or that the mutation could not be detected by the methods used in these studies [544]. Many different types of DNA alterations could account for the biochemical change induced in QPD, and these are discussed below.

Normal gene expression is mainly controlled by the promoter region, enhancer and silencer elements, and the local chromatin environment, including locus control regions (LCRs; long-distance enhancer elements that are often tissue-specific) (reviewed in [625,626]). The minimal promoter is often enough to promote normal gene expression, although more distal cis-acting genomic elements (enhancers and silencers) contribute to spatiotemporally correct expression [627]. High level transcription is best achieved when chromatin is in an open (euchromatic) rather than closed (heterochromatic) conformation [628]. Alternative promoters and exons, as well as very distant regulatory elements (up to 1 MB away, and often within the coding regions of other genes) may also affect normal gene transcription [629,630]. Overexpression of genes can occur by several different mechanisms, including cis- or trans- effects [625]. The studies presented in Chapter 4 have already excluded mutations in a transcription factor regulating PLAU (a trans- effect) as the QPD mutation, since the linkage data and analysis of PLAU transcripts show that QPD causes a cis-regulatory effect on PLAU expression [544]. The types of mutations implicated in cis-regulatory genetic defects are summarized in table 5.2, along with a summary of whether such mutations were excluded by the studies presented in this thesis. Briefly, no small-scale mutations were identified by DNA sequencing in the known regulatory elements of PLAU (including mRNA stability elements), and a major alteration encompassing PLAU and its known regulatory elements was excluded

by Southern blotting [544]. The results of Southern blotting suggest that either: 1) a large genomic change has not occurred in QPD; 2) a change has occurred in an area outside of the region evaluated; or 3) a change has occurred, but it encompasses the complete area evaluated, and thus was not detected by the selected Southern blot probes or by DNA sequencing. As my studies did not evaluate if there were copy number abnormalities of *PLAU* in individuals with QPD, this needs to be investigated further (see section 5.5), as other diseases have been described where copy number changes of a gene lead to gene overexpression [307].

Another interesting aspect of the QPD mutation is that it causes a lineagespecific defect in uPA expression. Various mutations, including rearrangements [631,632], point mutations [633], and deletions/insertions [632,634,635] have previously been described that cause changes in the tissue expression patterns of genes in disease. These mutations can cause: 1) changes in the timing of gene expression during development, without altering tissue-specificity of expression [633,636]; 2) a shift from restricted tissue expression of a gene to ubiquitous expression [631,632,634]; or 3) a switch from one tissue-specific expression pattern to another distinct tissue-specific pattern [637]. Identification of the putative QPD mutation will provide important clues about why uPA overexpression is differentiation-dependent and lineage-specific in this disorder.

Table 5.2. Some potential *cis*-acting mutations that could result in gene overexpression and their possible relevance to QPD.

Type of cis-acting mutation	Relevance of this type of mutation to QPD
Promoter mutation resulting in increased TF binding or creation of new TF binding element	No mutations identified by sequencing of <i>PLAU</i> promoter
Enhancer mutation (including LCRs) resulting in increased enhancer binding, or creation of new enhancer binding element	No mutations identified by sequencing enhancer elements of <i>PLAU</i> (up to 2.5 kb upstream) If this was the cause of QPD, mutation would have to be in an uncharacterized, distant enhancer or LCR (> 36.5 kb upstream or 5 kb downstream of <i>PLAU</i>)
Silencer mutation resulting in reduced/absent silencer binding	No mutations identified by sequencing silencer elements of <i>PLAU</i> (up to 2.5 kb upstream) If this was the cause of QPD, mutation would have to be in an uncharacterized, distant silencer element (>36.5 kb upstream or 5 kb downstream of <i>PLAU</i>)
Duplication or amplification resulting in increased copy number of gene and/or regulatory elements	No breakpoint identified by Southern blot in 25 kb region containing <i>PLAU</i> and known regulatory elements If this was the cause of QPD, mutation would have to include <i>PLAU</i> and the region more than 13 kb upstream and more than 5 kb downstream of <i>PLAU</i> , or affect a nearby genomic region
Gross deletion, insertion or rearrangement bringing distant regulatory element close to gene or creating new regulatory element	No breakpoint for insertion/rearrangement identified by Southern blot in 25 kb region containing <i>PLAU</i> and known regulatory elements; no deletion up to 36.5 kb upstream [†] If this was the cause of QPD, the insertion/ rearrangement would have to be in a region more upstream than 13 kb and more downstream than 5kb of <i>PLAU</i> ; for a deletion, mutation would have to be >36.5 kb upstream of <i>PLAU</i>
Gross deletion, insertion or rearrangement moving gene from hetero- to euchromatic genomic region	This possibility was not evaluated in these studies
mRNA instability element mutation resulting in increased stability/half life	No mutations identified by sequencing the known instability elements of <i>PLAU</i>

Reviewed in [625,626]. Hypotheses about the relevance of each type of mutation to QPD (column on right) are based on results of DNA sequencing and Southern blotting [544]. [†]indicates this was confirmed by DNA sequencing of both *PLAU* copies (heterozygous SNPs present). LCR, locus control region.

5.5 Future directions for studies on QPD

This thesis project has made important contributions to understanding the MK-specific, gain-of-function fibrinolytic defect in QPD, and the underlying genetic cause. During the course of these studies, new questions arose, and these will now be the focus of new work to expand our knowledge about the disorder. General questions that need to be addressed are: does the QPD biochemical defect extend to other cells in the body? What is the specific genetic mutation that causes QPD, and how does this mutation cause *PLAU* overexpression? Why does the dysregulation of uPA in QPD show lineage-restriction? This section summarizes some approaches to answering these questions.

Studies of uPA overexpression in other cells

The biochemical defect in QPD was previously thought to be restricted to MKs. The studies presented in Chapter 2 showed that QPD whole blood clot sera contained more uPA than could be accounted for by the total amount present in OPD platelets [542]. This suggests that OPD whole blood contains an additional source of uPA that may contribute to accelerated clot lysis. A good candidate cell type to investigate for such a defect is QPD leukocytes, as these cells originate from the same hematopoietic stem cell as MKs, and various types of leukocytes are known to express uPA [76,153,333]. Methods bases on those described in Appendix I, which investigated changes in uPA in differentiating MKs [552], can be used to investigate the possibility of uPA dysregulation in QPD leukocytes. First, different types of leukocytes can be isolated from QPD blood based on their unique cell-surface antigens. The following assays could then be used to evaluate various aspects of uPA in these cells: 1) ELISA, to quantitate uPA protein; 2) qRT-PCR, to quantitate uPA expression; 3) Western blot, to evaluate the size and activation state (sc or tc) of uPA; and 4) immunostaining, to localize uPA in cells (like platelets, leukocytes contain secretory granules [638]) [552]. Assays similar to ones used in Chapter 2, which investigated platelet contributions to clot lysis in QPD [542], but using platelet-free plasma and adding back isolated QPD leukocytes, could also be done to investigate the effects of QPD leukocytes on plasmin generation and fibrin clot lysis in vitro. To address whether the genetic defect in QPD induces a myeloid progenitor-specific defect in uPA expression, cells of the lymphoid lineage (eg. lymphocytes and natural killer cells [639]) could also be evaluated [542,552].

Normal endothelial cells are also known to synthesize and secrete uPA, suggesting a possibility for altered uPA regulation in this cell type in QPD [334]. Unlike leukocytes, which can be isolated from peripheral blood, endothelial cells may be more difficult to obtain from QPD subjects, as it would require, for example, a harvest of human umbilical vein endothelial cells [640] from a

newborn that has inherited QPD. Determining the tissue expression of uPA in QPD will provide important insights into regulatory mechanisms contributing to uPA over-production (eg. transcription factors involved in tissue-specific gene regulation), and give clues about the mechanism of action of the QPD mutation. Furthermore, studies of uPA changes in QPD leukocytes may provide important insights on the role of leukocytes in QPD (and normal) clot lysis, and whether leukocytes contribute to the gain-of-function defect in fibrinolysis in QPD.

Identification of the QPD causative mutation

The studies presented in Chapter 4 have shown that QPD is linked to a 2 Mb region on chromosome 10 harboring *PLAU* and 22 other genes, and have excluded the possibility of another locus for QPD in this pedigree [544]. For this region, *PLAU* presents as the best candidate gene for QPD, as none of the other genes in the region are known to regulate *PLAU* [544], and the two that are transcription factors (*HSGT1* and *MYST4*) are not known to be expressed by MKs [202]. Although changes in expression in *CAMK2G* and *VCL* were not detected in QPD MKs and/or platelets, the other 20 genes in the linked region cannot be excluded as being altered in QPD, and further studies measuring the expression of their transcripts should be done. It is possible that the QPD genetic defect, which may lie anywhere within the 2 Mb linked region, not only affects *PLAU* expression, but expression of other genes in the region as well.

Since these studies have shown that QPD is caused by a *cis*-effect on *PLAU* expression, new strategies should now be focused on determining the precise *cis*-mutation that causes this alteration. Little is known about distal elements that may contribute to *PLAU* transcriptional regulation, including alternate promoters, exons, transcription factor binding sites, enhancer and silencer elements or LCRs, as studies of *PLAU* regulation have mostly focused on the regions most proximal to the gene (~ 5 kb upstream of +1) (reviewed in [641]). Such elements could very likely exist within the 2 Mb linked region, which has not been completely sequenced [544], and a mutation at such sites could produce the QPD phenotype. Alternatively, a larger-scale mutation, such as a gene amplification event or creation of a new element with regulatory properties could result in *PLAU* overexpression.

To identify unique interactions between transcription factors and their regulatory elements affecting PLAU in QPD, it would be necessary to obtain cellular material from QPD MKs, since these cells aberrantly express uPA, as well as expressing the necessary factor(s) that can turn on PLAU transcription. Binding of transcription factors to *cis*-elements on DNA can be determined *in vitro* by various assays, including: 1) DNA footprinting, a method for identifying a region of DNA protected by a bound protein from DNAse digestion; 2) gel-shift

assays, which monitor the change in mobility when DNA and a protein are bound; 3) Southwestern blotting of both DNA and protein; and 4) reporter constructs, to evaluate the effects of different genetic sequences on gene expression in specific transcription factor-expressing cell lines (reviewed in [642]). New technologies chromosome conformation such as capture (3C)and chromatin immunoprecipitation and microarray assays (ChIP-chip) have also been developed to look for genome-wide protein-DNA interactions in a highthroughput manner [643-646]. In silico methods can also be used to evaluate if a region of genomic DNA interacts with a particular transcription factor. Several collections of experimentally-defined transcription factor binding sites have been assembled, such as TRANSFAC, a database that catalogues eukaryotic transcription factors and their known binding sites [647]. Other tools, such as MatInd, MatInspector, MATRIX SEARCH, SIGNAL SCAN, and rVista can be used to search an input sequence for potential transcription factor binding sites [648-651].

If the QPD mutation creates a novel transcription factor binding element, the unique DNA change would have to be identified before in silico analyses of the new DNA sequence can be done. Southern blots could first be used to look for large-scale DNA changes spanning across the 2 Mb linked region. It is unlikely that QPD is caused by a large deletion within the 36.5 kb region between PLAU and CAMK2G, as sequencing analysis confirmed the presence of both PLAU alleles across this region [544]. Genome-wide SNP arrays (offered by Affymetrix, Santa Clara, CA; or Illumina, San Diego, CA) could also be used to evaluate changes in copy number or polymorphisms across the genome that could give clues about possible deletions or amplifications spanning large areas [652]. including the PLAU locus. This strategy would facilitate evaluation of the large 2 Mb linked region, without having to sequence DNA across the whole region. Fluorescence in situ hybridization (FISH) could also be used to look for large changes amplifications, chromosomal (DNA insertions. deletions. rearrangements) [653] by using probes specific for parts of the 2 Mb linked region, particularly those nearest to PLAU, and assessing for changes in probe binding or intensity. A combination of these techniques, coupled with highthroughput sequencing technologies [654], would provide the best strategy for identifying the specific genomic region harboring the putative QPD mutation.

Additional studies of uPA mRNA in QPD platelets

Studies of uPA mRNA in QPD MKs and platelets assumed that these cells expressed the dominant isoform of PLAU (Chapter 1, figure 1.5, isoform *b*), as this isoform is known to encode the normal 54 kD sc-uPA protein [341]. The primers used for RNA studies (spanned parts of exons 5, 10, and 11), however, would have detected all 3 of the full-length PLAU isoforms (isoforms *a*, *b* and *c*),

which all have well-characterized 3' ends and verified poly-A signals [341]. Thus, my studies did not evaluate whether the transcript isoform of uPA in QPD platelets is the dominant isoform, or one of the other less common isoforms, which could differ in their stability in various cell-types [341]. Future work can now be done identify the isoform of mRNA present in QPD platelets. It is also possible, that the QPD mutation results in the production of a novel isoform of *PLAU* that differs at the 5' or 3' end. This possibility can be investigated using rapid amplification of cDNA ends (RACE) to amplify the 5' and 3' ends of the uPA transcript [655]. cDNA sequencing could then provide information on whether QPD platelets contain a unique isoform as a consequence of the QPD mutation. A unique isoform of uPA mRNA could confer increased stability to the transcript, which could account for the large increases in mRNA levels measured in QPD platelets [552]. Changes in the chromosomal environment of *PLAU* could also potentiate gene transcription by increasing the accessibility of transcription factors to DNA binding sites [626].

Investigations of an alternative mode of PLAU regulation in QPD

The function of the C10orf55 gene, which overlaps with a significant portion of the *PLAU* genomic sequence, has not yet been determined [339]. This gene may in fact be involved in regulated alternate expression of *PLAU* [341], and mutations in *PLAU* in QPD could account for the increases in *PLAU* expression. To test the possibility that *PLAU* is regulated by C10orf55, C10orf55 could be overexpressed in a cell line that constitutively expresses uPA (eg. PC-3) [353,375], and changes in uPA expression in the presence or absence (empty vector) of C10orf55 could be evaluated. Alternatively, using a chIP experiment [656], C10orf55 could be tested for co-immunoprecipitation with a regulatory element of *PLAU* (eg. the promoter). The presence of C10orf55 transcript in MKs from QPD individuals could also be evaluated by qRT-PCR using total cellular RNA, as previously done for uPA [552], to determine if it is also overexpressed in QPD.

5.6 Clinical implications of mapping the QPD genetic locus

The number of known individuals with QPD in Canada (now more than 30) has increased in recent years with the identification of several new affected family members [541]. These individuals were traced back to the original QPD pedigree. The prevalence of QPD in Canada is 1 in 1,000,000 and 1 in 300,000 in the Province of Quebec. Individuals with QPD are typically diagnosed using research laboratory-based assays, such as ELISA for uPA and Western blots for degraded α -granule proteins [541]. These tests are not practical for use in the clinical lab and can be very time consuming. Other assays, such as platelet

function assays (eg. aggregation with different platelet agonists) can give variable results, and often do not show abnormalities in affected individuals [541]. Recent investigations on QPD using proteomics also revealed that this method cannot be used, on its own, as a specific test for QPD, as proteomics could detect α -granule abnormalities, but not the hallmark increases in uPA, in platelets [657]. The studies presented in this thesis have important clinical implications, as they have provided new molecular strategies for identifying individuals at risk for having QPD. These are: 1) a rare SNP (designated as QPD-1) [544] can be genotyped in individuals suspected of having QPD, and presence of the linked QPD allele would indicate a high likelihood of a person having inherited QPD (the linked allele is present in 100% of QPD individuals, <4% of unaffected family members, and <3% of unrelated French-Canadians); and 2) qRT-PCR analyses of platelet mRNA could be used to look for the >150-fold increase in the linked allele of uPA mRNA [544].

Identification of the QPD mutation, based on the studies done in this thesis, will provide important insights on normal *PLAU* regulation in a variety of cells, and highlight the unique mechanism whereby normally antifibrinolytic platelets can become profibrinolytic. This could eventually lead to the development of ways to upregulate *PLAU* expression in at-risk individuals to protect against arterial and venous thrombosis [544]. For QPD individuals, determining their mutation may lead to the development of new therapies that would target the cause of uPA overexpression, rather than limiting the consequences [544]. Identification of the QPD mutation in the future could lead to the development of a genetic diagnostic test for QPD. Such a test could be done rapidly and easily, using DNA isolated from a small amount of blood or saliva [544]. Furthermore, a diagnostic genetic test for QPD would provide clinicians with a method to screen for QPD among patients with undiagnosed bleeding disorders, which are encountered frequently in the clinical setting [658,659].

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APPENDIX I

INCREASED EXPRESSION OF UROKINASE PLASMINOGEN ACTIVATOR IN QUEBEC PLATELET DISORDER IS LINKED TO **MEGAKARYOCYTE DIFFERENTIATION**

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Summary

Quebec platelet disorder (QPD) is an inherited bleeding disorder associated with increased urokinase plasminogen activator (uPA) in platelets but not in plasma, intraplatelet plasmin generation and α -granule protein degradation. These abnormalities led us to investigate uPA expression by OPD $CD34^+$ progenitors, cultured megakaryocytes and platelets, and whether uPA was stored in QPD α -granules. Although QPD CD34⁺ progenitors expressed normal amounts of uPA, their differentiation into megakaryocytes abnormally increased expression of the uPA gene but not the flanking genes for vinculin or calcium/calmodulin-dependent protein kinase IIy on chromosome 10. The increased uPA production by cultured QPD megakaryocytes mirrored their production of α -granule proteins, which was normal. uPA was localized to OPD α - granules and it showed extensive colocalization with α -granule proteins in both cultured QPD megakaryocytes and platelets, and with plasminogen in QPD platelets. In QPD megakaryocytes, cultured without or with plasma as a source of plasminogen, α -granule proteins were stored undegraded and this was associated with much less uPA-plasminogen colocalization than in QPD platelets. Our studies indicate that the overexpression of uPA in QPD emerges with megakaryocyte differentiation, without altering the expression of flanking genes. and that uPA is costored with α -granule proteins prior to their proteolysis in QPD.

Introduction

Quebec platelet disorder (QPD) is an unusual inherited bleeding disorder, associated with increased expression and storage of the fibrinolytic enzyme urokinase plasminogen activator (uPA) in platelets and delayed-onset bleeding following trauma or surgery that responds only to fibrinolytic inhibitor therapy.¹⁻³ The genetic cause of OPD has recently been linked to inheritance of a region on chromosome 10 that contains the uPA gene (PLAU).⁴ The normal uPA in OPD urine and plasma (prepared with platelet activation inhibitors),⁵ and apparent increases in uPA message in platelets (based on Northern blot analysis),² suggest that the increased uPA in QPD platelets results from increased uPA expression by megakaryocytes.¹ However, the expression of uPA by CD34⁺ progenitors, and by normal and QPD megakaryocytes, at different stages of differentiation, has not been characterized or quantified. Furthermore, it has not been determined if OPD selectively increases uPA mRNA in platelets or if it also increases mRNA from the flanking genes on chromosome 10 that encode vinculin (a protein normally expressed in platelets)⁷ and calcium/calmodulin-dependent protein kinase IIy (CAMK2G), a protein expressed by T-lymphocytes⁸ that has not been studied in platelets.

Normally, blood contains similar molar amounts of uPA and tissue plasminogen activator (tPA) for converting plasminogen to plasmin and only small amounts of uPA in platelets (up to 1.3 ng uPA/ 10^9 platelets) (reviewed in Diamandis et al.¹). Unlike normal platelets, OPD platelets contain sufficient uPA (approximately 400 - 600 ng $uPA/10^9$ platelets)² to trigger extracellular plasmin generation and premature clot lysis when incorporated into forming or preformed clots.⁶ Within OPD platelets, single chain (sc) uPA is not evident as uPA is stored in active forms that include two chain uPA (tcuPA) and low molecular weight uPA (LMWuPA). In addition, OPD platelets contain uPA complexed with the active forms of platelet plasminogen activator inhibitor 1 (PAI-1), which are consumed in OPD.² uPA activation within QPD platelets is postulated to result from exposure to plasmin, as OPD platelets, but not plasma, contain elevated levels of plasmin- α_2 plasmin inhibitor complexes.⁹ uPA-induced, intraplatelet generation of plasmin is thought to trigger degradation of diverse stored α -granule proteins - a hallmark feature of QPD that affects proteins synthesized by megakaryocytes, including thrombospondin-1 (TSP-1), Pselectin, osteonectin, and von Willebrand factor (VWF), and proteins endocytosed from plasma, such as fibrinogen and factor V.¹⁰⁻¹² The loss of α -granule multimerin 1 (MMRN1) in QPD¹¹ is also thought to result from plasmin-mediated degradation. Heterogeneity in the protein contents of megakaryocyte and platelet α -granules is now recognized to result in some separation of proteins, such as fibrinogen from VWF,¹³ and antiangiogenic proteins, like TSP-1 and endostatin, from proangiogenic proteins, like vascular endothelial growth factor (VEGF) and basic fibroblast growth factor.¹⁴ However, uPA has never been demonstrated within OPD α -granules, and the extent of its colocalization with OPD platelet plasminogen and degraded α -granule proteins has not been evaluated.

To characterize uPA expression during normal and OPD megakaryopoiesis, and investigate uPA storage in QPD α -granules, we studied CD34⁺ progenitors, cultured megakaryocytes and platelets. We report that the increased expression of uPA in OPD is not evident in circulating hematopoietic stem cells and that it emerges as QPD megakaryocytes differentiate, resulting in production of platelets that contain increased uPA, but not increased vinculin or CAMK2G mRNA. We also report that uPA is contained within the a-granules of circulating OPD platelets, where it colocalizes with plasminogen and α -granule proteins known to be degraded in OPD, consistent with the proposed mechanism of QPD α -granule protein degradation.

Materials and Methods

All studies were conducted with approval of the institutional ethics review boards and in accordance with the Declaration of Helsinki, as last amended in 2004.

Sample collection

Peripheral blood samples (200 mL/donation) were collected from QPD and healthy control subjects with written informed consent. Samples were collected into sterile acid citrate dextrose anticoagulant (vol:vol = 1:6) containing 1 mM theophylline (Sigma-Aldrich, Oakville, ON, Canada), 3 μ M prostaglandin E1 (Sigma-Aldrich) and 3 μ M aprotinin (Roche Diagnostics, Laval, QC, Canada).

Isolation of cells from peripheral blood

Platelets were harvested from peripheral blood, as previously described (upper 2/3 of platelet rich plasma; minimal leukocyte contamination verified by cell counting of selected samples).¹¹ CD34⁺ hematopoietic stem cells with minimal platelet contamination (< 0.5 platelets per nucleated cell), were isolated from peripheral blood by a modification of the method described for isolating CD34⁺ cells from cord blood.¹⁵ Briefly, after removal of platelet rich plasma, and density gradient separation using Ficoll,¹⁵ mononuclear cells were further purified by an OptiPrep (Axis-Shield, Oslo, Norway, 200g, 12 minute centrifugation) density gradient separation before immunomagnetic isolation of CD34⁺ cells.¹⁵ Mononuclear cells, from Ficoll gradient separations, were also used to obtain control T-lymphocytes by immunomagnetic isolation (Human CD3⁺ selection kit, StemCell Technologies, Vancouver, BC, Canada) to quantify CAMK2G mRNA. The final CD34⁺ cell and T-lymphocyte purities, assessed by flow cytometry,¹⁵ were consistently over 90%.

Megakaryocyte cultures

CD34⁺ cells were cultured with thrombopoietin (TPO), as described,¹⁵ except 50 ng/mL recombinant human TPO (PeproTech, Rocky Hill, NJ, USA) was used. Cell expansion was quantified at different stages of culture, as described,¹⁵ with viability determined by Trypan blue exclusion. Cells were evaluated by flow cytometry, as described, to quantify the percentage that expressed the mature megakaryocyte marker CD41a (glycoprotein α IIb).¹⁵ Cells were cultured until day 13 when megakaryocyte maturation was evident and cell viability was still acceptable (> 70%). For some analyses, day 7 cultures were 7 supplemented with 10% (vol/vol) sterile, heparinized, platelet poor plasma (from blood group AB donors)¹⁶ as a source of exogenous plasminogen, as the addition of purified plasminogen led to a complete loss of viable cells in control and QPD cultures, and the addition of more plasma reduced megakaryocyte differentiation.

Analyses of mRNA by quantitative reverse transcription polymerase chain reaction (RT-qPCR)

Total cellular RNA was extracted from different cells of the same subjects, including CD34⁺ cells, cultured megakaryocytes and platelets, using RNeasy Mini Kits (QIAGEN, Mississauga, ON, Canada) as recommended. RNase-Free DNase (QIAGEN) was added to digest any contaminating DNA. Quality and quantity of extracted RNA was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Mississauga, ON, Canada). Isolated RNA was stored at -80°C until analyzed.

For reverse transcription (RT), 72 ng total RNA was incubated at 37°C for 60 minutes in a 20 μ L reaction mixture containing recommended Omniscript RT Kit components (QIAGEN), 2.5 μ M oligo (deoxythymidine)₂₀ primer (Invitrogen, Burlington, ON, Canada) and 20 units RNAse inhibitor RNaseOUT (Invitrogen). Reactions were stopped by a 5-minute incubation at 95°C, followed by rapid cooling on ice.¹⁷

RNA from CD34⁺ cells was used only for quantitative real-time polymerase chain reaction (qPCR) analysis of PLAU transcription, as the quantities harvested precluded other analyses. RNA from day-7 and -13 megakaryocytes and platelets was used for qPCR analysis of PLAU, VWF (control for increased mRNA during megakaryocyte differentiation¹⁸) and VCL^7 transcription. RNA from platelets was also used to evaluate CAMK2G transcription in QPD. Transcription of the housekeeping gene glyceraldehyde 3phosphate dehydrogenase (GAPDH) was evaluated simultaneously in all samples as an endogenous control to correct for potential variations in template RNA, RT and qPCR efficiencies. Supplies and instructions for qPCR were from Applied Biosystems (Foster City, CA, USA). The gene-specific sets of oligonucleotide primers and fluorescent probes for qPCR (from pre-developed TaqMan Gene Expression Assays) were: uPA: Hs00170182 m1; VWF: Hs00169795 m1; Hs00243320 m1; CAMK2G: Hs00538454 m1 vinculin: and GAPDH: 4333764T. All selected amplicons spanned intron-exon boundaries. qPCR singleplex reaction mixtures included: 5 µL cDNA, 12.5 µL TaqMan Gene Expression Master Mix (containing DNA polymerase), 1.25 µL 20x TagMan Gene Expression Assay reagents, and 6.25 µL RNAse-free water. Assays were done in 96-well TaqMan optical reaction plates in triplicate (or duplicate if there were limiting quantities of template RNA and cDNA) using an ABI PRISM 7900HT real-time thermal cycler, with the following thermal profile: 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. No template controls served to exclude contamination.

For each qPCR run, standard curves were obtained by amplifying serially diluted (1:5 - 1:3125) cDNA that was reverse-transcribed from pooled samples. For uPA, vinculin and VWF, a pool of total platelet RNA from 5 QPD subjects

was used. For CAMK2G, a pool of total T-lymphocyte RNA from 5 control subjects was used. Relative mRNA levels were obtained using the average value for the target gene threshold cycle (Ct), normalized to GAPDH Ct, as described.¹⁹

Protein analyses

Cell lysates for protein analysis were prepared from CD34⁺ cells, cultured megakaryocytes and platelets, by methods described.^{2,15} Cell-free supernatants were harvested from megakaryocyte cultures on day 7 and day 13, and for some studies daily from days 7 to 13, as described.¹⁶ Samples were stored at -80°C until analyzed.

Enzyme-linked immunosorbent assays (ELISAs) were used to quantify the following: uPA (modified to include a lower concentration standard of 12.5 pg/mL) and PAI-1 (Oncogene Science, Cambridge, MA, USA); uPA-PAI-1 complexes and platelet factor 4 (PF-4; Hyphen Biomed, Neuville-sur-Oise, France); tPA (lower standards used as described)²; uPA receptor (uPAR; American Diagnostica Inc., Montreal, QC, Canada); TSP-1¹¹; MMRN1¹¹; and VWF.¹⁵ Cell lysate and culture supernatant results were expressed in quantities per 10⁶ cells, or per mg total cellular protein (comparisons of megakaryocyte and platelet lysates).¹⁵ Due to the low numbers of CD34⁺ cells in peripheral blood, a pooled QPD CD34⁺ cell lysate from 3 donors was compared to 3 pooled control CD34⁺ cell lysates (each from 3 donors), to determine if the protein quantities for QPD samples were within the range for control samples.

Western blotting was used to evaluate the mobilities of uPA, TSP-1, VWF, P-selectin and PAI-1 in platelet lysates (10 μ L/lane) and cultured megakaryocyte lysates (45 μ L/lane; 10 pooled from 3 individuals to obtain sufficient material) after separation by sodiumdodecyl- sulfate polyacrylamide gel electrophoresis (SDS-PAGE), as described.^{2,9,11,15} Platelet VEGF was also analyzed by Western blotting (1 μ g/mL mouse anti-VEGF [Cedarlane, Burlington, ON]; secondary antibody 1:25 000 horseradish peroxidase-labeled donkey antimouse IgG [Jackson ImmunoResearch Laboratories, West Grove, PA]) after separation (reduced) on a 12% SDS-polyacrylamide gel.

Immunofluorescent microscopy

<u>Immunolabeling</u>. Cultured megakaryocytes and platelets were double labeled as described,¹⁶ except resting platelets (2 x 10^8 /mL, prepared as described²) were fixed in suspension (1% paraformaldehyde, room temperature, 10 minutes) and dried on aptex-coated slides overnight, before permeabilization, quenching and blocking as described.¹⁶ Primary antibodies included the following: mouse

antibodies to uPA (10 µg/mL, Monosan, Uden, The Netherlands), TSP-1 (CH-1, 3.5 µg/mL),²⁰ MMRN1 (JS-1, 10 µg/mL)²⁰, $\alpha_{IIb}\beta_3$ (10 µg/mL, BD Biosciences, Mississauga, ON), VEGF (2 µg/mL, Cedarlane), and osteonectin (5 µg/mL, Haematologic Technologies Inc, Essex Junction, VT); rabbit antibodies to uPA (10 µg/mL; Monosan), uPAR (6.4 µg/mL Abcam, Cambridge, MA), plasminogen (1:100, Biogenesis, Poole, Great Britain), fibrinogen (1:5000, Behring, Marburg, Germany), and VWF (1:300, Dako, Mississauga, ON); and sheep antibody to factor V (10 µg/mL, Affinity Biologicals, Ancaster, ON). Secondary antibodies (AlexaFluor 594- or AlexaFluor 488-conjugated) were used as described.¹⁶ Controls included: single labeled cells, cells labeled without primary or secondary antibodies, or with normal mouse, rabbit or sheep IgG. Additional controls included cells single labeled using the opposite secondary antibodies to ensure no 11 antibody cross-reactivity. Labeled cells were mounted with Permafluor mounting media (Beckman Coulter, Marseille, France).

<u>Image acquisition and processing.</u> Immunolabeled samples were viewed on a DMI6000B wide fluorescent microscope (Leica Microsystems, Richmond Hill, ON) with Orca ER-AG camera (Hamamatsu, Bridgewater, NJ) and Volocity 4 acquisition software (Improvision, Waltham, MA). Fluorescent crossover between channels was verified to be negligible. Z-series were acquired at 22°C with 100x/1.4NA DIC oil plan apo objective, using identical microscope settings for samples and negative controls. Images were restored in Volocity 4 using the iterative deconvolution function,²¹ before importing into MBF-ImageJ²² (McMaster University Biophotonics Facility, http://www.macbiophotonics.ca/imagej/) for linear, uniform brightness and contrast adjustments, background subtraction and scale bar labeling.

<u>Quantitative colocalization analysis.</u> The degree of labeled protein colocalization was assessed using the MBF-ImageJ Intensity Correlation Analysis plugin to determine the Pearson's correlation coefficient (r_p , range: -1 to 1, with 1 equivalent to complete colocalization)²³ and the Manders overlap coefficient (r, range: 0 to 1, with 1 equivalent to complete colocalization).²⁴ A minimum of 10 day-13 cultured megakaryocytes and 30 platelets were evaluated for each protein comparison and data were confirmed using cells from 2 additional subjects.

Immunoelectron microscopy

To determine the subcellular distribution of uPA, frozen thin sections of platelets were prepared and immunolabeled, as described¹⁵ with rabbit anti-uPA (Monosan) and a gold-coupled secondary antibody (British Biocell, Cardiff,

United Kingdom). After counterstaining with uranyl acetate, platelets were examined with a Philips CM10 electron microscope (Philips Healthcare, Surenes Cedex, France).

Statistical analysis

Most quantitative data were expressed as mean plus or minus SEM (range) and compared by 2-tailed unpaired *t* test. Immunolocalization data (r_p and r; expressed as mean \pm SD, range) were analyzed by Welch ANOVA with Satterthwaite *t* post-hoc analysis. Significance was established at *P* less than .05.

Results

Characteristics of control and QPD cultured megakaryocytes

QPD and control megakaryocytes showed the typical phenotype, and expansion of megakaryocytes grown in culture with TPO from peripheral blood progenitors²⁵ (fold expansion: 8.3 ± 0.8 [range, 5.3 - 10.0] for 6 QPD cultures; 7.4 ± 2.6 [range, 1.3 - 16.5] for 5 control cultures; P = .8). Although QPD cultures contained a lower proportion of cells expressing $\alpha_{IIb}\beta_3$ on day 7 (QPD: $5.2\% \pm 1.3\%$, [range 2% -10%]; control: $21\% \pm 4\%$ [range, 12% - 34%, P = .01), by day 13, the difference was not significant (QPD: $57\% \pm 6\%$ [range, 45%-76%]; control: $69\% \pm 4\%$ [range, 61% - 84%; P = .13).

Evaluation of gene expression in CD34+ cells, cultured megakaryocytes and platelets

RT-qPCR analyses indicated that uPA mRNA was not increased in QPD CD34⁺ cells (Figure A1A); however it was increased in day-7 (3.7 ± 0.5 -fold higher than controls) and day-13 cultured QPD megakaryocytes (101 ± 31 -fold higher than controls) (Figure A1A), and in QPD platelets (90.1 ± 18.6 -fold higher than control platelets; Figure A1A). Unlike uPA mRNA, VWF and vinculin mRNA were not increased in QPD platelets or megakaryocytes (Figure A1A). In addition, CAMK2G mRNA was not increased in QPD platelets (Figure A1A).

Production of uPA and other proteins during QPD megakaryopoiesis

Pooled QPD CD34⁺ cells contained approximately 60 pg uPA/10⁶ cells, which was within the range observed for pooled control samples (n = 3; 40 \pm 20

pg/10⁶ [range, 10–70 pg/10⁶]). Although day-7 and day-13 QPD megakaryocyte cultures contained normal amounts of PAI-1, PF-4, TSP-1, VWF and MMRN1, they contained increased amounts of uPA (Table A1). Some day-13 QPD megakaryocyte cultures also contained small amounts of uPA-PAI-1 complexes (Table A1). The increased uPA production by QPD megakaryocytes coincided with the increased production of PF-4, TSP-1 and VWF in QPD and control cultures (Figure A1B). By day 13, QPD megakaryocytes contained 19% to 27% of the QPD platelet uPA/mg cellular protein (paired analyses of samples from 4 QPD individuals). tPA was undetectable (< 1.6 ng/mL) in all CD34⁺ cells (n = 3 QPD and 3 control lysates) and megakaryocyte cultures (n = 4 QPD and 6 control day-7 and day-13 lysates and supernatants), indicating that uPA was the only plasminogen activator produced during megakaryopoiesis.

Plasminogen was undetectable in all megakaryocyte cultures (< 0.8 ng/mL in n = 3 QPD and 3 control day-13 lysates and supernatants) unless the cells were cultured in media containing plasma (ng/10⁶ cells, in lysate from cultures with plasma, n = 3: QPD: 73 ± 13 [range, 46 – 90], control: 27 ± 8 [range, 11 - 36]; *P* = .06).

The amounts of uPAR in cultured megakaryocytes were not quantified as there were similar, low amounts of uPAR antigen in control and QPD platelets $(ng/10^9 \text{ platelets}, n = 4: \text{QPD: } 0.7 \pm 0.1 \text{ [range, } 0.6 - 0.9]; \text{ control: } 0.4 \pm 0.1 \text{ [range, } 0.2 - 0.9]; P =.1), which were undetectable by immunostaining of platelets and megakaryocytes (not shown).$



Figure A1. Expression of uPA, α -granule proteins, vinculin, and CAMK2G in QPD (Q) and control (C) CD34⁺ cells, cultured megakaryocytes, and platelets. (A) RT-qPCR analysis of uPA, VWF, vinculin, and CAMK2G mRNA levels in platelets and/or CD34⁺ cells, and day-7 and -13 megakaryocytes, relative to controls (arbitrarily set to 1) to identify altered patterns of gene expression in QPD. (B) Comparison of uPA, PF-4, TSP-1, and VWF antigen in megakaryocyte culture supernatants, evaluated by ELISA at different stages of culture. Undetectable levels are presented as zero. Data represent mean values; error bars indicate SEM for data from 3 to 5 subjects. * Significant increases (P<.05) in QPD, compared with control.

			Mean \pm S.E.M. (range) /10 ⁶ cells				
			S	Cell-associated			
Protein, unit	Sample	n	Day 7	Day 13	Day 13		
uPA, pg	Q	4	460 ± 50 (320 - 550)*	16,000 ± 4,000 (7,000 - 26,000)*,†	$4,700 \pm 2,000$ (670 - 10,000)*		
	С	4	160 ± 80 (50 - 370)	$\begin{array}{c} 150\pm90\\ (40-440)\end{array}$	20 ± 7 (3 - 40)		
PAI-1, ng	Q	4	< 0.05	63 ± 19 (31 - 109)	25 ± 7 (7 - 43)		
	С	4	1 ± 1 (< 0.05 - 3)	68 ± 21 (16 - 105)	25 ± 8 (11 - 48)		
uPA-PAI-1 complexes, pg	Q	4	< 100	500 ± 300 (< 100 - 1100)	600 ± 300 (< 100 - 1300)		
	С	4	< 100	< 100	< 100		
PF-4, IU	Q	3	< 0.3	148 ± 60 (27 - 210)	317 ± 146 (31 - 513)		
	С	3	< 0.3	131 ± 46 (39 - 185)	245 ± 120 (46 - 460)		
TSP-1, % NPP	Q	3	< 0.03	2.3 ± 0.6 (1.1 - 3.2)	2.2 ± 0.8 (0.6 - 3.1)		
	С	3	< 0.03	3.4 ± 1.5 (0.8 - 6.0)	5.0 ± 2.2 (1.7 - 9.1)		
MMRN1, ng	Q	6	< 25	25 ± 20 (< 25 - 147)	154 ± 36 (33 - 260)		
	С	6	< 25	105 ± 39 (< 25 - 232)	255 ± 99 (75 - 761)		
VWF, ng	Q	3	< 8	140 ± 36 (75 - 202)	NE		
	С	3	< 8	85 ± 36 (21 - 145)	NE		

Table A1. Quantities of uPA and other proteins in day-7 and day-13 QPD and control megakaryocyte cultures, evaluated by ELISA.

Q indicates QPD; C, control; NPP, normal pooled platelet; and NE, not evaluated. * P < .05 for comparisons of Q versus C. †P < .05 for comparisons of day-13 versus day-7 samples.

Distribution of uPA in cultured QPD megakaryocytes and platelets

Immunofluorescent microscopy (Figure A2) indicated that there was strong uPA labeling of all differentiated QPD cells that showed strong labeling for $\alpha_{IIb}\beta_3$ or TSP-1 (representing 45% to 52% of all cells in culture). In contrast, control megakaryocytes showed only faint uPA immunolabeling (Figure A2), which was indistinguishable from background (not shown). Within QPD-cultured megakaryocytes, uPA colocalized with $\alpha_{IIb}\beta_3$, TSP-1 and MMRN1 in perinuclear and granular structures (Figure A2), which was confirmed by quantitative analysis (r_p : uPA and $\alpha_{IIb}\beta_3$: 0.93 ± 0.03 [range, 0.88–0.96; uPA and TSP-1: 0.92 ± 0.04 [range, 0.80 – 0.97]; uPA and MMRN1: 0.85 ± 0.04 [range, 0.79 – 0.90]) (r: uPA and 15 $\alpha_{IIb}\beta_3$: 0.97 ± 0.01 [range, 0.96 – 0.99]; uPA and TSP-1: 0.96 ± 0.02 [range, 0.88 – 0.98]; uPA and MMRN1: 0.90 ± 0.02 [range, 0.84 – 0.92]).

Immunoelectron microscopy of platelets confirmed that uPA was stored in QPD α - granules, without significant labeling of other platelet structures (Figure A3A) or control platelets (not shown). Further analyses by immunofluorescent microscopy indicated that there was extensive colocalization of QPD platelet uPA with TSP-1 (Figure A3B; Table A2) and plasminogen (Figure A4 Q plt; Table A2), and extensive but less complete colocalization of QPD platelet uPA with VWF, VEGF (Figure A3B; Table A2), osteonectin, fibrinogen and factor V (Table A2). Colocalization of QPD platelet uPA with MMRN1 could not be quantified, as unlike QPD megakaryocytes (Figure A2), QPD platelets showed only very faint immunolabeling for MMRN1 (not shown). Western blot analyses indicated that like many other α -granule proteins, QPD platelet VEGF was abnormally degraded as QPD platelets contained decreased amounts of VEGF (Figure A3C) that also had abnormally lower mobility (bands indicated by arrow in Figure A3C that were evident in all QPD samples on longer exposure not shown).

In day-13 QPD megakaryocytes cultured with plasma, plasminogen was found predominantly in peripheral structures and not in u-PA-containing granules (Figure A4 Q MK). Quantitative analyses confirmed that there was less colocalization of uPA and plasminogen in day-13 QPD megakaryocytes (r_p : 0.39 \pm 0.08 [range, 0.30 – 0.53]; r: 0.67 \pm 0.07 [range, 0.55 – 0.74]) compared with QPD platelets (Table 2; *P*< .001). These data indicated that there was incomplete trafficking of plasminogen into uPA-containing structures in cultured QPD megakaryocytes compared with QPD platelets.



Figure A2. Intracellular distribution of uPA compared with $\alpha_{IIb}\beta_3$, and α granule proteins TSP-1 and MMRN1 in day-13 QPD and control cultured megakaryocytes. Panels show deconvolved immunofluorescent images of megakaryocytes, double immunolabeled with rabbit anti-uPA (red, left panels) and mouse monoclonal antibodies to $\alpha IIb\beta 3$, TSP-1, or MMRN1 (green, middle panels) (merged images on right). Insets show magnified images of peripheral cytoplasm. Experiments were performed as outlined in "Immunofluorescent microscopy."



Figure A3. The uPA storage site in QPD platelets. (A) Immunogold labeling of uPA in QPD platelets, showing occasional uPA labeling into α -granules, with no significant labeling of the surface-connected cannalicular system (SCCS), mitochondria (m), or plasma membrane. Insets show a QPD platelet α -granule under higher magnification. Experiments were performed as outlined in "Immunoelectron microscopy." (B) Deconvolved immunofluorescent images of QPD platelets, double-immunolabeled with an antibody to uPA (red, left panels) and antibodies to TSP-1, VWF, or VEGF (green, middle panels) (merged images on the right). (C) Western blot of VEGF in pooled platelets from 20 healthy individuals (NPP), and in platelets from 4 QPD and 2 control individuals, evaluated after SDS-PAGE. Arrow indicates VEGF forms with lower mobility in QPD platelets.



Figure A4. Distributions of plasminogen and uPA in day-13 QPD cultured megakaryocytes compared with platelets. Deconvolved immunofluorescent images of QPD platelets (Q plt) and QPD megakaryocytes (Q MK) grown with plasma. Cells were double immunolabeled with antibodies to uPA (red) and plasminogen (Pg, green; merged images at right). Experiments were performed as outlined in "Immunofluorescent microscopy."

	Mean \pm S.D. (range)				
Protein colocalized with	Pearson's correlation	Manders' overlap coefficien			
uPA	coefficient	(r)			
	(\mathbf{r}_{p})				
Plasminogen	$0.98 \pm 0.01 \ (0.95 - 1.00)^*$	$0.99 \pm 0.01 \; (0.97 - 1.00)^*$			
TSP-1	$0.92 \pm 0.04 \; (0.81 - 0.99)$	$0.96 \pm 0.02 \; (0.93 - 0.99)$			
Osteonectin	$0.83 \pm 0.09 \ (0.64 - 0.96)^{\dagger}$	$0.88 \pm 0.07 \; (0.76 - 0.97)$ †			
Fibrinogen	$0.81 \pm 0.08 \ (0.52 - 0.88)^{\dagger}$	$0.91 \pm 0.03 \ (0.80 - 0.94)$ †			
VEGF	$0.69 \pm 0.14 \ (0.25 - 0.89)^{\dagger}$	$0.85 \pm 0.07 \ (0.61 - 0.96)$ †			
VWF	$0.68 \pm 0.14 \ (0.42 - 0.90)$ †	$0.86 \pm 0.06 \ (0.70 - 0.96)^{\dagger}$			
Factor V	$0.66 \pm 0.14 (0.32 - 0.89)$ †	$0.85 \pm 0.07 \ (0.68 - 0.96)^{+}$			

Table A2.	Extent	of uPA	colocalization	with	other	a-granule	proteins in	QPD
platelets.								

*Greater colocalization with uPA than other evaluated proteins; P < .001. †Less colocalization with uPA than plasminogen and TSP-1; P < .001.

Forms of uPA and a-granule proteins in QPD cultured megakaryocytes

In contrast to QPD platelets, which contained mostly tcuPA, day-13 QPD cultured megakaryocytes contained mainly scuPA (Figure A5A), even when cultured with plasma (not shown). Like QPD platelets, QPD megakaryocytes contained some high-molecular-weight uPA complexes (Figure A5A, bands indicated by * and **) that included forms (*) recognized by PAI-1 antibodies (data not shown), as suggested by uPA-PAI-1 complex ELISA (Table A1). Control megakaryocytes contained undetectable uPA by Western blotting (not shown).

Unlike the degraded α -granule proteins in QPD platelets, the TSP-1, VWF and P-selectin in day-13 QPD cultured megakaryocytes, grown with or without added plasma, had the mobility of the normal forms in control platelets (Figure A5B) and megakaryocytes (not shown). QPD megakaryocytes also contained normal quantities of MMRN1, even when cultured in media with plasma (Table A1 and data not shown). Analyses of cultures with plasma, by Western blotting, indicated that there was no detectable conversion of plasminogen to plasmin in QPD megakaryocytes (not shown), unlike QPD platelets.



Figure A5. Forms of uPA and α -granule proteins stored in day-13 QPD cultured megakaryocytes compared with platelets. Panels show proteins evaluated by Western blotting after SDS-PAGE. (A) Nonreduced (top panel) and reduced (bottom panel) uPA in QPD megakaryocytes (Q MK) compared with platelets (Q plt). Recombinant low-molecular-weight (LMW), single-chain (sc), and 2-chain (tc) uPA are shown for reference. High-molecular-weight complexes (* and **) and forms recognized by PAI-1 antibodies (*) are indicated. (B) TSP-1, VWF, and P-selectin in QPD megakaryocytes (Q MK), grown with (+) or without (-) plasma, compared with control (C plt) and QPD (Q plt) platelets.

Discussion

The magnitude and timing of increased uPA transcription and storage during QPD megakaryopoiesis has been uncertain. Our study indicates the differentiation of QPD CD34⁺ progenitors into megakaryocytes results in differentiation-dependent, log-fold increases in uPA message and protein, without increasing VWF, vinculin or CAMK2G expression (Table A1; Figure A1). Interestingly, the temporal increased production of uPA, during OPD megakaryopoiesis, mirrored the increased production of α -granule proteins, which were made in normal quantities (Table A1: Figure A1B) and costored with uPA within QPD α -granules (Figures A3, A4; Table A2). We found that unlike QPD platelets, QPD megakaryocytes contained undegraded a-granule proteins and scuPA (Figure A5), which has low catalytic activity.²⁶ These data, and the different distributions of plasminogen in cultured QPD megakaryocytes compared with platelets (Figure A4), suggest that the activation of uPA and proteolysis of α granule proteins occur late, after plasminogen trafficks into OPD α -granules and is converted to plasmin. Importantly, our study provides new evidence that profibrinolytic abnormalities of QPD platelets reflect increased expression of the uPA gene as hematopoietic progenitors differentiate into megakaryocytes, without altering expression of the flanking genes on chromosome 10 that encode vinculin and CAMK2G.

The expression of genes during hematopoietic stem cell differentiation into megakaryocytes is highly regulated and coordinated by transcription factors synthesized during megakaryopoiesis (for reviews, see Battinelli et al.²⁷, Pang et al.²⁸, and Chang et al.²⁹). This likely contributes to the sustained low levels of uPA expression, and increased expression of PAI-1 and other α-granule proteins during normal megakaryopoiesis,³⁰ that accompany production of normal, antifibrinolytic platelets.⁶ PLAU is normally expressed by many different cell types,³¹ and we found that its transcription remains fairly stable during normal megakaryopoiesis. Further studies are required to determine whether QPD increases uPA expression when hematopoietic stem cells differentiate along other lineages. The mechanism that leads to increased PLAU expression during QPD megakaryopoiesis, without changing α -granule protein production, or the expression of vinculin or CAMK2G (the genes that flank PLAU), is presently unknown. This selective increase suggests QPD results from a gain-of-function mutation in a binding site(s) for regulatory factors that increase the expression of genes encoding a-granule proteins and/or other platelet-restricted genes or that normally repress PLAU transcription during megakaryopoiesis. The transcription factors that increase gene expression during megakaryopoiesis include GATA-1; FOG-1; GATA-2; ETS family members ETS-1, FLI-1, TEL and GABPa; RUNX-1; NF-E2; SCL; GFI-1B; and ZBP-89.^{27-29,32}. In addition, c-MYB, ETS family member PU.1, and EKLF are negative regulators of megakaryocyte differentiation.^{29,33} and ETO-2 has recently been identified as a corepressor that

binds the GATA-1-containing pentameric complex to prevent early expression of some genes expressed late during megakaryopoiesis, such as PF-4.³⁴ The 2.5-kb upstream region of *PLAU* contains conserved elements that bind some of these transcription factors (e.g GATA, ETS, RUNX-1), and a number of other silencers and enhancers.³⁵ Recently, QPD was linked to a *cis* regulatory defect in a 2-megabase region of chromosome 10 that includes *PLAU*, however, DNA sequencing excluded mutations within *PLAU* and its characterized regulatory elements, including the binding sites for GATA proteins, ETS family members and RUNX-1.⁴ This suggests that QPD results from a mutation in more distal, uncharacterized regulatory element(s) of *PLAU* that binds one or more transcription factors to increase *PLAU* transcription during megakarypoiesis, leading to the production of platelets enriched in stored uPA.

Heterogeneity has been noted in the contents of normal platelet α granules,^{13,14} and we documented this in QPD platelets by rigorous, quantitative estimates of protein colocalization (Table A2), previously used to analyze other cell types.^{36,37} We identified that the increased production of uPA in QPD megakaryocytes results in uPA trafficking to the majority of α -granules, including the subpopulation of these granules that store proangiogenic (VEGF) and antiangiogenic (TSP-1) proteins,¹⁴ and the proteins fibrinogen and VWF.¹³ Immunoelectron microscopy confirmed uPA was stored in OPD a- granules (Figure A3) but we did not attempt to estimate its colocalization with other α granule proteins by immunoelectron microscopy as the amounts of uPA contained in QPD platelets (.400 ng/mg cellular protein) were challenging to detect. These findings, and the extensive costorage of uPA and plasminogen in QPD platelet α granules, offer an explanation for the diversity of the secretory proteins degraded within OPD platelets. The degree to which individual α -granule proteins colocalize with uPA and plasminogen likely influences their degradation in QPD, although this is difficult to formally evaluate as the proteolysis of some QPD α granule proteins (e.g. MMRN1 and factor V) $^{10-12}$ limits their detection in platelets. At present, the sorting mechanisms that lead to heterogenous platelet α -granule protein contents are uncertain but could be influenced by the timing of protein production, endocytosis and also homotypic and heterotypic binding interactions.^{13,14,38}

The exposure of plasminogen to scuPA is known to generate plasmin.²⁶ Our current study provides indirect evidence that the uptake of plasminogen for costorage with uPA is required to generate sufficient plasmin to trigger α -granule protein degradation in QPD, as the trafficking of plasminogen to forming α -granules was not recapitulated in cultures with or without added plasma. The mechanism of plasminogen uptake into α -granules is unknown, although bulk transport or receptor-mediated endocytosis (as demonstrated for other proteins)³⁹⁻⁴³ seem plausible given that plasminogen production by cultured megakaryocytes was undetectable. *In vivo*, the uptake of plasma proteins (e.g. fibrinogen) into α -

granules occurs late during megakaryopoiesis.⁴²⁻⁴⁴ It is possible that plasminogen and uPA costorage, and intra- α -granular plasmin generation, occurs in the bone marrow environment in QPD, where maturing megakaryocytes are exposed to plasma proteins throughout megakaryopoiesis. More definitive proof for a key role of platelet plasminogen in triggering QPD α -granule protein degradation might come from crossing plasminogen-deficient mice⁴⁵ with mice that have QPD-like platelet α -granule protein degradation from overexpression of uPA in megakaryocytes.⁴⁶

Our current study establishes that overexpression of uPA in QPD emerges as QPD hematopoietic progenitors differentiate into megakaryocytes without increasing expression of the flanking genes or the production of other α -granule proteins. An important next step will be to identify the QPD mutation that switches the pattern of uPA expression during megakaryopoiesis from sustained low levels to the dramatic (log fold) increased expression typical of an α -granule protein.

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