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Thrombin generation abnormalities in Quebec platelet disorder

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Abstract

Introduction: Calibrated automated thrombograms (CAT) with platelet-poor (PPP) and platelet-rich plasma (PRP) have provided useful insights on bleeding disorders. We used CAT to assess thrombin generation (TG) in Quebec platelet disorder (QPD)—a bleeding disorder caused by a *PLAU* duplication mutation that increases platelet (but not plasma) urokinase plasminogen activator (uPA), leading to intraplatelet (but not systemic) plasmin generation that degrades α-granule proteins and causes platelet (but not plasma) factor V (FV) deficiency.

Methods: Calibrated automated thrombograms was used to test QPD (n = 7) and control (n = 22) PPP and PRP, with or without added tranexamic acid (TXA). TG endpoints were evaluated for relationships to platelet FV and uPA, plasma FV and tissue factor pathway inhibitor (TFPI) levels, and bleeding scores.

Results: Quebec platelet disorder PPP TG was normal whereas QPD PRP had reduced endogenous thrombin potential and peak thrombin concentrations (P values < .01), proportionate to the platelet FV deficiency ($R^2 \geq 0.81$), but unrelated to platelet uPA, plasma FV, or bleeding scores. QPD TG abnormalities were not associated with TFPI abnormalities and were not reproduced by adding uPA to control PRP. TXA increased QPD and control PRP TG more than PPP TG, but it did not fully correct QPD PRP TG abnormalities or improve TG by plasminogen-deficient plasma.

Conclusion: Quebec platelet disorder results in a platelet-specific TG defect, proportionate to the loss of platelet FV, that is improved but not fully corrected by TXA. Our study provides an interesting example of why it is important to assess both PRP and PPP TG in bleeding disorders.

KEYWORDS

coagulation, factor V, fibrinolysis, platelet function, procoagulant activity

1 | INTRODUCTION

Assessments of thrombin generation (TG) by calibrated automated thrombograms (CAT) have provided insights on how bleeding disorders alter blood coagulation.[1,2] An important advantage is the ability to test TG with both platelet-poor (PPP) and platelet-rich plasma (PRP).[1,2] Recently, tissue factor pathway inhibitor (TFPI) levels have emerged to be an important determinant of TG

and bleeding.[3-5] We postulated that using CAT to study TG and measuring TFPI levels in Quebec platelet disorder (QPD) might provide new insights on a disorder with unique fibrinolytic abnormalities and platelet but not plasma factor V (FV) deficiency.

Quebec platelet disorder was initially designated as "Factor V Quebec" when it was discovered to cause platelet but not plasma FV deficiency.[6] Later, QPD was identified to result from a *PLAU* duplication mutation that selectively increases (>100-fold) production of normal *PLAU* transcripts by the disease chromosome in megakaryocytes but not leukocytes, with minimal effects on plasma or urinary uPA.[7-10] The result is >100-fold increased uPA in QPD megakaryocytes/platelets, leading to consumption of active plasminogen activator inhibitor-1 (PAI-1) and intraplatelet (but not systemic) plasmin generation that degrades diverse α-granule proteins, including FV and its binding protein, multimerin 1 (MMRN1).[10-15] Fibrinolytic inhibitors, such as tranexamic acid (TXA), are the only effective treatment for QPD.[16]

TG in QPD has not been assessed since 1984,[6] and the disorder raises questions about the importance of platelet vs plasma FV. In normal human blood, ~25% of FV is contained within platelet α-granules, complexed to MMRN1, for regulated release with platelet activation.[17,18] Plasma FV circulates in a single chain form, whereas platelet FV is partially activated.[17] Animal studies indicate that plasma FV is more important for hemostasis than platelet FV.[19] However, platelet FV contributes to hemostasis when plasma FV is deficient.[20,21]

Some fibrinolytic disorders do not alter TG, and adding tissue plasminogen activator (tPA) to normal PPP has minimal effects on TG.[22,23] In TG assays with added tPA, severe PAI-1-deficient PPP shows a shortened lag time, shortened time to peak TG, and reduced peak thrombin concentration that have been attributed to increased activation, and inactivation, of multiple coagulation factors by plasmin.[24] As QPD increases platelet uPA and depletes platelets of active PAI-1,[10] it might similarly alter TG.

The goal of our study was to reexamine QPD TG, using CAT, and determine if: (a) platelet but not plasma FV deficiency impairs PRP TG; (b) TFPI levels are normal in QPD, and their association to QPD TG endpoints; and (c) TXA improves TG in QPD.

2 | MATERIALS AND METHODS

The study was carried out in accordance with the revised Helsinki protocol, with written informed consent and approval by the Hamilton Integrated Research Ethics Board, and the Centre Hospitalier Universitaire Sainte Justine ethics review board. Participants (identities anonymized) included: n = 7 with QPD (median ages in years, range: 45, 32-78; 85% male) confirmed by mutation analysis,[9] and general population controls (n = 22, including n = 7 age and sex-matched for QPD participants; some tested multiple times; median age in years, range: 35, 21-68; 55% female).

2.1 | Sample preparation

Blood (20-40 mL) was collected by venipuncture and anticoagulated with 0.109 mol/L buffered sodium citrate (1:9 volume/volume; for preparation of PPP and PRP) and acid citrate dextrose (ACD, 1:6 volume/volume; for preparation of gel-filtered platelets [GFP][25] and platelet lysate[7,8]), as described.

2.2 | Thrombin generation

Calibrated automated thrombograms analyses of PPP and PRP TG were performed as recommended, using autologous PPP to adjust PRP to 150×10^9 platelets/L.[1] For some experiments, gel-filtered platelets (GFP) were tested after resuspension in commercial FV-deficient plasma (George King Bio-Medical), as described,[25] using platelet count-matched controls if samples contained $<150 \times 10^9$ platelets/L. TG was also assessed with purchased, plasminogen-deficient plasma (Affinity Biologicals Inc.).

Thrombin generation (triplicate estimates) was assessed in accordance with ISTH recommendations and completed within 3-4 hours of sample collection.[1] TG was quantified using a Fluoroskan plate reader (Thermo Fisher Scientific AG) and Thrombinoscope reagents and software (Stago Canada Ltd.).[26,27] PPP test wells contained: 80 μL PPP and 20 μL PPP reagent (containing 4 μmol/L phospholipids, 5 pmol/L TF). PRP test wells contained: 80 μL PRP and 20 μL PRP reagent (containing 0.5 pmol/L TF). Assays were done with or without added 10 mmol/L TXA (final, Sigma-Aldrich), which fully blocks the profibrinolytic effects of QPD platelets.[15] TXA effects were also tested by replicate determinations (n = 14 sets of triplicate determinations, with and without drug) of plasminogen-deficient plasma. For some experiments, control PRP was tested with or without added 300 ng uPA per 10^9 platelets (Research and Diagnostic Systems, Inc.) to mimic complete uPA release by QPD platelets (2 sets of triplicate determinations). Ten minutes before adding the FluCa regent, some PRP were preincubated with platelet agonists (combination of [final concentrations]: 10 μ g/mL Horm collagen, Helena Laboratories; 10 μ mol/L ADP, Sigma-Aldrich; and 50 mmol/L SFLLRN, Bachem Bioscience Inc). For all tests with additives, additives were added to both test and calibrator wells. TG was started by dispensing 20 μL of FluCa reagent (Stago Canada Ltd.) into each well. TG measurements were taken at 20-second intervals for 60 minutes at 37°C to evaluate: endogenous thrombin potential (ETP, nmol/L × min), peak thrombin concentration (nmol/L), time to peak (minutes), and lag time (minutes).[1] Areas under the curve were manually calculated for TG curves that did not return to baseline.

2.3 | Protein and RNA analyses

Double-spun citrated PPP and platelet lysates were prepared for enzyme-linked immunosorbent assays (ELISA) of: plasma and platelet FV antigen (in house assay, as described earlier[12,14]); and platelet

uPA (uPA ELISA kit, R&D Systems)[10] (n = 5 QPD; n = 5 controls). Plasma (n = 6 QPD, n = 14 controls) and platelet (n = 5 QPD, n = 11 controls) TFPI levels were assessed by an ELISA that uses a monoclonal antibody capture and a polyclonal antibody for detection and recognizes natural and recombinant TFPI (Human TFPI DUO SET ELISA, R&D Systems). Total platelet protein (DC protein assay; Bio-Rad Laboratories) was determined as described.[7] Megakaryocyte TFPI was evaluated using previously generated megakaryocyte RNA-seq data (n = 3 QPD, n = 3 controls).[8]

2.4 | Statistical analyses

Quebec platelet disorder data were compared to age- and sex-matched controls, and to all controls after analyzing control data for age and sex differences. First sample data were analyzed for participants with multiple determinations. Two-tailed Mann-Whitney tests, with Bonferroni correction for multiple comparisons, were used to assess differences in TG endpoints and protein levels, and % differences in TG endpoints for simultaneous tests ± TXA. Linear regression was used to assess relationships between: (a) bleeding scores and TG endpoints in PPP and PRP; (b) plasma FV and TG endpoints in PPP and PRP; (c) platelet FV and TG endpoints for PRP and for GFP in FV-deficient plasma; (d) platelet uPA levels and TG endpoints in QPD PRP; and (e) plasma TFPI and TG endpoints for PRP and PPP. Multiple regressions were used to assess relationships between platelet FV, uPA levels, TFPI levels, and PRP TG endpoints in QPD samples. For platelet uPA comparisons, values below assay detection were rounded up to the lowest detectable level. Coefficients of variation (CV) were calculated for participants and pooled plasmas with ≥3 determinations. Differential analysis of RNA-seq data was previously performed[8] using DESeq2[28] (statistics available at: https://doi.org/10.1371/journal.pone.0173991.s005). FPKM (fragments per kilobase million) values were calculated from raw counts using the *fpkm* function in DESeq2. Raw sequencing data are available in the European Genome-phenotype Archive with controlled access under accession EGAD00001006053.

3 | RESULTS

ELISA (data as median [range] for QPD vs controls) verified that QPD platelets were deficient in FV (μg FV/mg platelet protein: 0.35 [0.18-0.46] vs 0.89 [0.63-1.54]; $P < .001$) and contained increased uPA (ng uPA/mg platelet protein: 57 [24-110] vs 0.15 [below detection-0.16]; $P < .0001$); and QPD plasma contained normal amounts of FV (μg/mL: 8.4 [7.0-10.2] vs 7.7 [6.3-10.7]; $P = .33$). QPD and control participants had similar TFPI levels in their plasma (ng/mL: 35.7 [20.6-40.0] vs 24.1 [11.4-48.3], $P = .06$) and platelets (ng TFPI/mg platelet protein: 10.5 [7.9-28.1] vs 16.7 [8.6-22.8], $P = .57$). In QPD and control megakaryocytes, TFPI mRNA expression was within the top quartile of megakaryocyte-expressed genes (FPKM: 9.15 [4.83-12.14] vs 12.40 [7.96-14.97]) and was not significantly

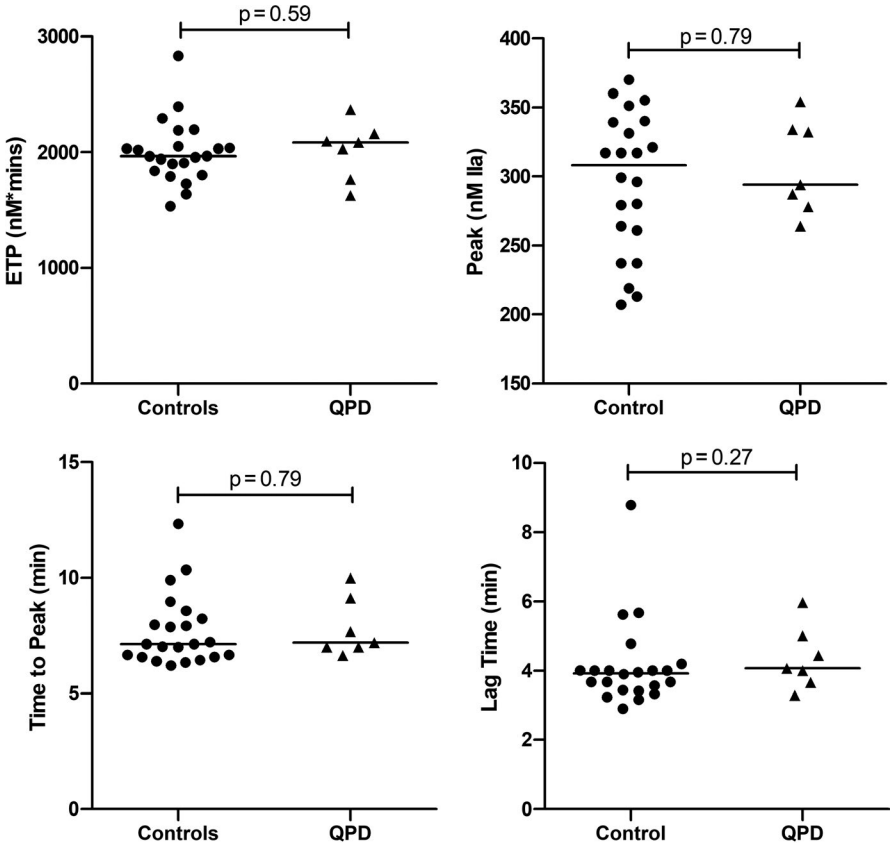

FIGURE 1 Thrombin generation findings for Quebec platelet disorder (QPD) and control platelet-poor plasma samples. Panels compare QPD and control data for ETP, peak thrombin concentration, lag time, and time to peak (*P* values as indicated)

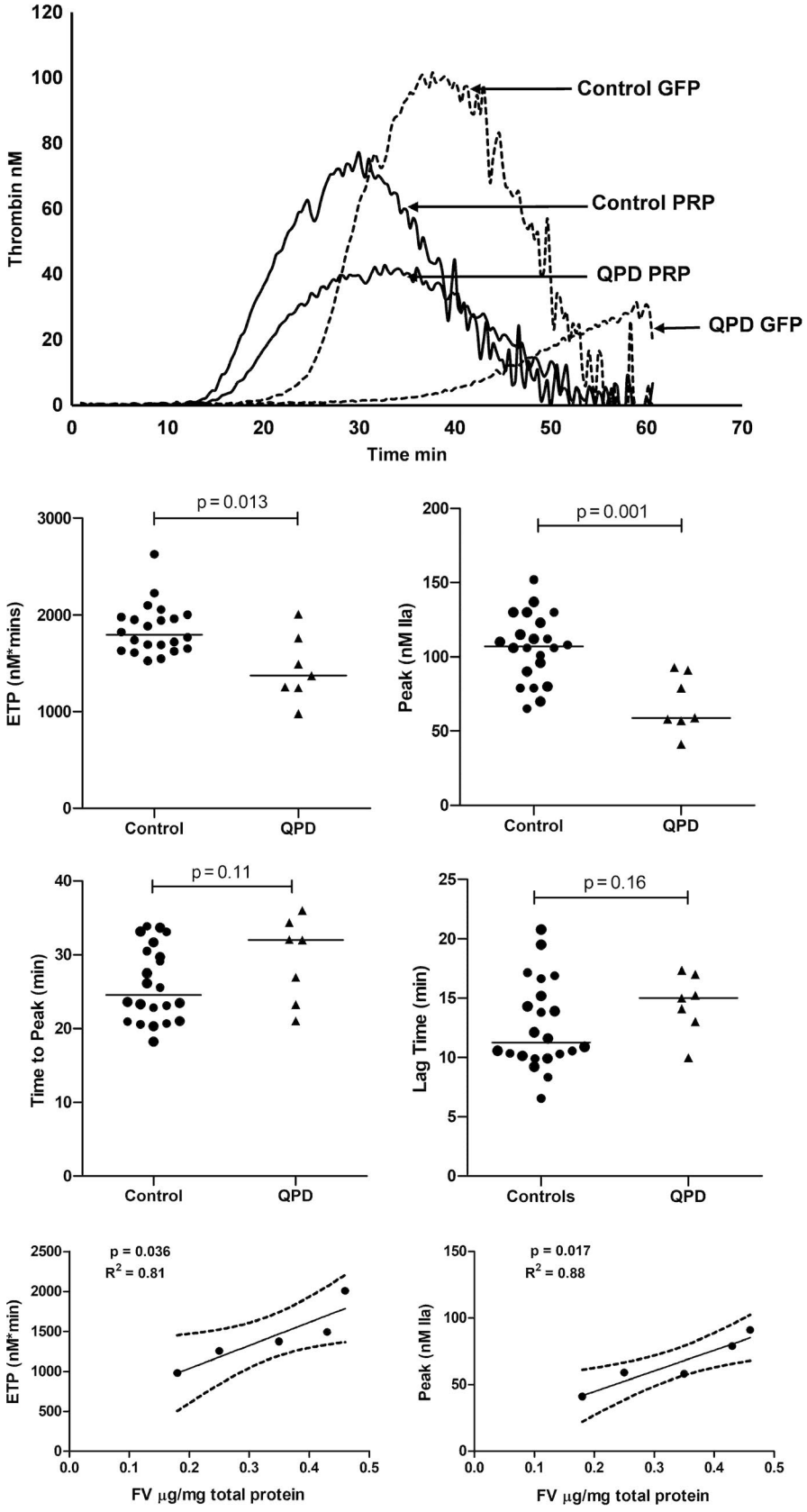

FIGURE 2 Thrombin generation findings for Quebec platelet disorder (QPD) and control platelet-rich samples. Top and middle panels respectively compare QPD and control thrombograms for PRP and GFP tested in FV-deficient plasma (all tested at platelet counts of 150×10^9/L), and QPD and control PRP TG ETP endpoints (*P* values as indicated). Lower panels summarize associations (R^2, *P* values and 95% confidence limits, as indicated) between QPD platelet FV antigen and QPD PRP TG findings for ETP and peak thrombin concentration

different by differential expression analysis (1.34-fold, $P > .34$, Wald test).

Repeat determinations with normal pooled plasma (14 sets, triplicate estimates) verified acceptable within-run CV for TG endpoints (ETP: 5.0%; peak thrombin concentration: 5.9%; time to peak: 3.2%; and lag time: 3.9%). Between-run CV estimates (as medians [range]) were also acceptable for: (a) PPP (based on 19 replicates for normal pooled plasma, 3 replicates for 1 QPD and 3-5 replicates for 3

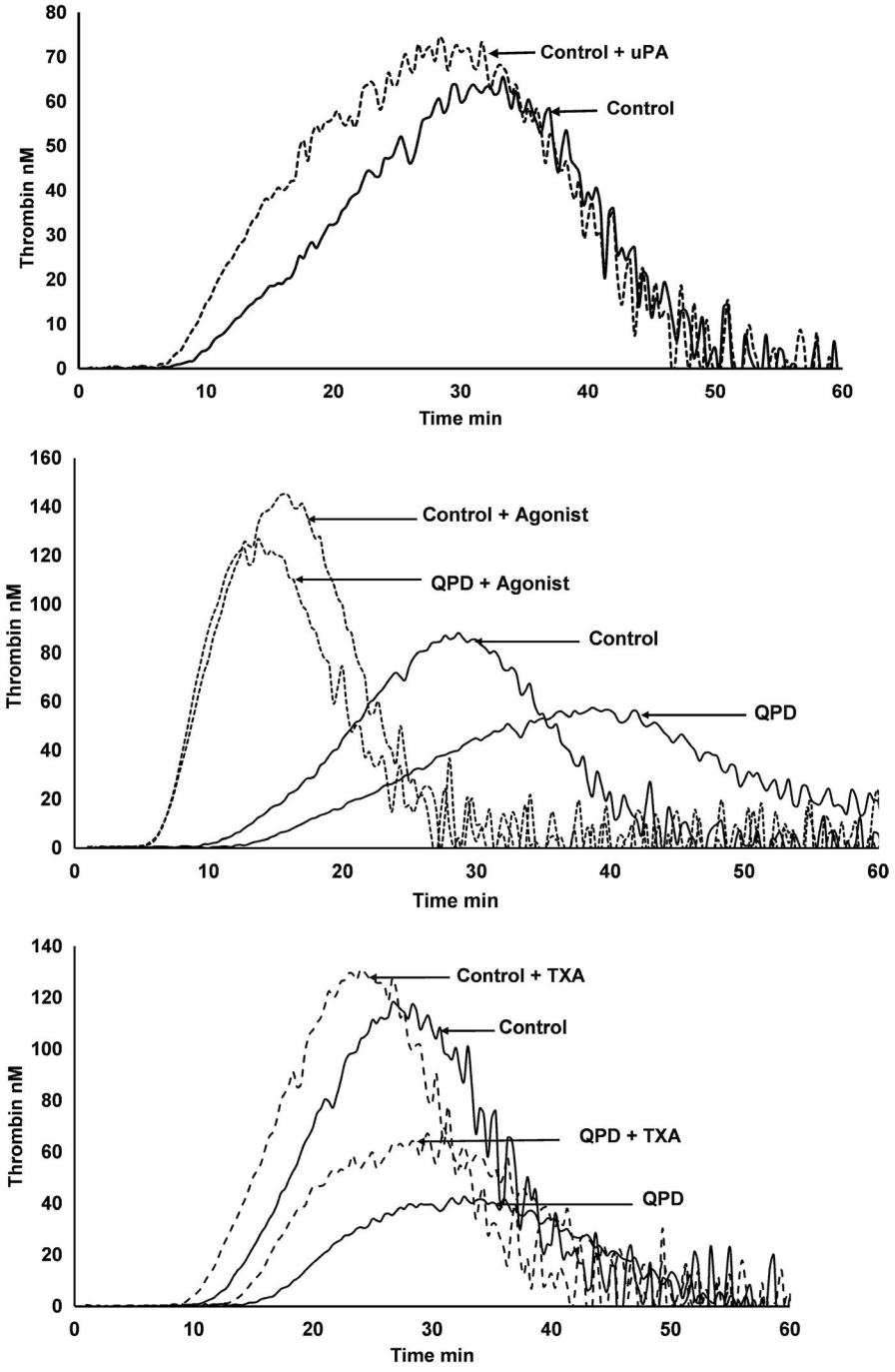

FIGURE 3 The effects of added uPA, platelet-activating agonists, and tranexamic acid on platelet-rich plasma thrombin generation. Representative thrombograms show the effect of adding uPA on control samples (upper panel), and the effects of adding agonists (middle panel) or tranexamic acid (TXA; lower panel) on QPD and control samples

controls): ETP: 11.9% [6.3%-16.2%]; peak thrombin concentration: 15.4% [3.7%-19.5%]; time to peak: 7.5% [2.7%-12.2%]; and lag time: 8.8% [4.6%-13%]; and (b) PRP (based on 3 replicates for 1 QPD and 3-5 replicates for 3 controls): ETP: 13.9% [9.8%-23.1%]; peak thrombin concentration: 24.1% [16.5%-28.2%]; time to peak: 18.0% [17.3%-23%]; and lag time: 23.6% [21.4%-36.2%]. As control PRP and PPP TG data showed no significant sex differences in TG endpoints ($P > .13$) and no relationship to age ($R^2 < 0.55$, $P > .29$), QPD TG findings were compared to all control data.

Quebec platelet disorder and control PPP showed comparable TG (Figure 1) whereas QPD PRP (n = 7) showed reduced ETP and peak thrombin concentrations ($P \leq .02$) without significant prolongations in lag time or time to peak TG ($P \geq .13$; Figure 2). QPD GFP tested in FV-deficient plasma showed reduced and delayed TG relative to platelet count-matched controls (Figure 2).

Quebec platelet disorder platelet FV levels showed strong associations to PRP ETP and peak thrombin concentration endpoints (Figure 2), whereas uPA levels did not ($R^2 \leq 0.23$; $P \geq .41$). Control platelet FV levels showed no significant association to TG endpoints for PRP or PPP ($R^2 < 0.15$, $P > .12$) or GFP in FV-deficient plasma ($R^2 < 0.55$, $P > .06$). Plasma FV levels did not show association to any QPD or control PPP or PRP TG endpoints ($R^2 < 0.03$, $P \geq .51$). Plasma TFPI levels showed association to the lag time for QPD PRP ($R^2 > 0.81$, $P = .013$) but not to other QPD or control PRP or

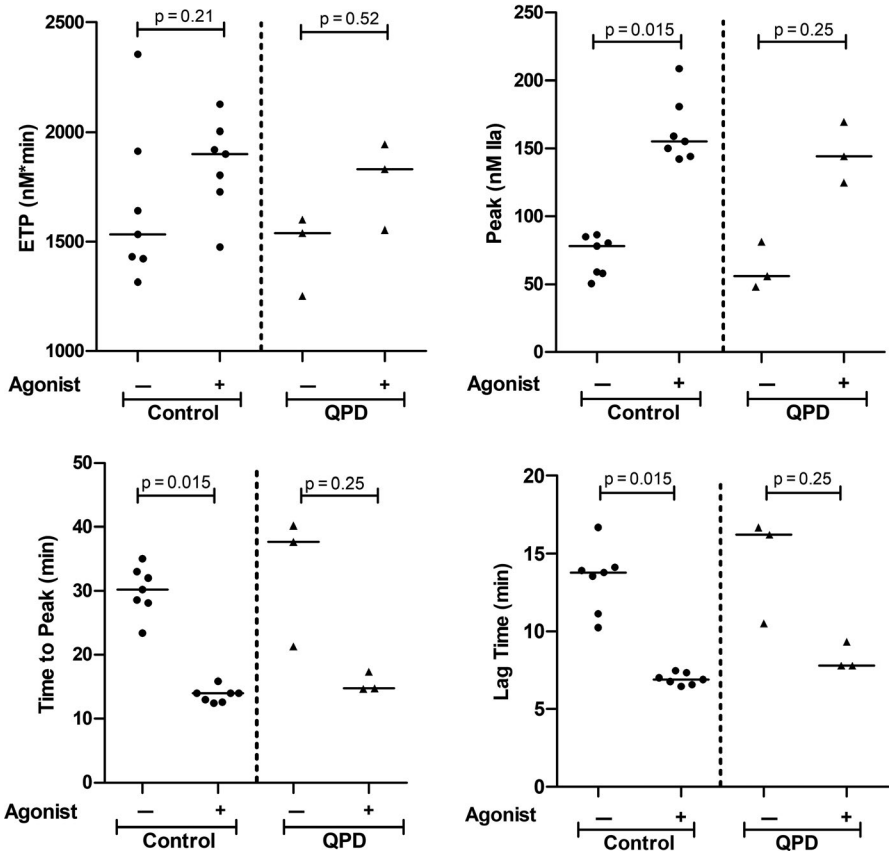

FIGURE 4 The effect of platelet-activating agonists on thrombin generation endpoints for Quebec platelet disorder (QPD) and control platelet-rich plasma. Panels compare control and QPD data for different endpoints (*P* values indicated)

PPP TG endpoints ($R^2 < 0.57$, $P \geq .08$). Although QPD participants had elevated ISTH-BAT scores (median: 8, range: 4-27), the scores did not show significant association to: PRP or PPP TG endpoints ($R^2 < 0.16$; *P* values ≥ .28); plasma or platelet TFPI levels ($R^2 < 0.47$; *P* values ≥ .19); platelet FV levels ($R^2 = 0.22$; $P = .42$); or platelet uPA levels ($R^2 = 0.44$; $P = .22$).

The addition of exogenous uPA to control PRP samples did not reproduce QPD PRP TG findings (Figure 3). Platelet-activating agonists increased TG by control and QPD PRP, without accentuating QPD TG abnormalities (Figures 3 and 4). Although TXA did not significantly improve TG by plasminogen-deficient plasma (respective *P* values: ETP $P = .062$, peak thrombin concentration $P = .12$, time to peak $P = .49$, lag time $P = .64$), it significantly improved TG by both QPD and control PRP, without correcting QPD abnormalities in ETP and peak thrombin concentrations (Figure 3 and 5). TXA improved TG by QPD PRP more than PPP, but this was also evident with control samples, for all endpoints except ETP (Figure 5).

PRP from the QPD participant who was tested twice on TXA (several weeks and months after starting 1 g orally, every 8 hours for a spontaneous, subcapsular renal hematoma), and again, several months after stopping TXA, showed similar findings (1st/2nd determination "on-TXA" vs "off-TXA": ETP: 2092/1564 vs 1764 nmol/L × min; peak thrombin concentration: 107/90 vs 93 nmol/L; time to peak: 25.5/25.3 vs 21.0 minutes, lag time: 11.6/12.3 vs 13.0 minutes; "off-TXA" findings included in Figures 1,3 and 5). The improvements in peak thrombin concentration with added TXA appeared greater for the "off-TXA" sample (18% vs 4% for the 2nd sample drawn "on-TXA").

4 | DISCUSSION

The main goal of our study was to reassess QPD TG abnormalities. We found that QPD is associated with significant PRP but not PPP TG abnormalities, with strong associations between QPD platelet (but not plasma) FV levels and QPD PRP ETP and peak thrombin concentration. Interestingly, platelet-activating agonists improved PRP TG, as anticipated,[29] without accentuating the QPD PRP TG defect. The addition of uPA to control PRP, to mimic full uPA release by QPD platelets, appeared to accelerate TG rather than recapitulating QPD abnormalities, possibly because the added uPA increased plasmin generation that enhanced platelet activation[30] and unlike QPD platelets, the control platelets were not deficient in FV or active PAI-1. We unexpectedly observed that TXA significantly improved TG of both control and QPD PRP samples, more than it improved PPP TG. As TXA did not improve TG by plasminogen-deficient plasma, we suggest that TXA improves TG by reducing plasmin generation during *ex vivo* assessments of TG. We noted that TFPI (which has emerged to be an important determinant of TG[3,4,31]) is normal in QPD plasma and platelets and that plasma TFPI showed association to the lag time for QPD PRP samples. The QPD TFPI findings are interesting as plasma FV serves as a carrier for TFPI and FV-TFPI binding inhibits FV activation and prothrombinase activity.[5,32-34] We did not find any associations between

	PRP (— TXA)			PRP (+TXA)		
	Control (n = 15) median (range)	QPD (n = 7) median (range)	P value	Control (n = 15) median (range)	QPD (n = 7) median (range)	P value
ETP nM*min	1769 (1444-2746)	1366 (979-2012)	0.02	1986 (1619-2910)	1546 (1343-2099)	0.024
Peak nM	107 (65-144)	59 (41-93)	0.006	115 (75-158)	71 (64-114)	0.005
Time to peak min	26.1 (18.2- 33.6)	32.0 (21.0-36.0)	0.105	22.3 (17.0-29.8)	28.6 (20.1-32.8)	0.244
Lag time min	12.1 (9.0-20.7)	15.0 (9.9-17.3)	0.18	10.8 (8.0-16.8)	11.8 (9.3-15.0)	0.23

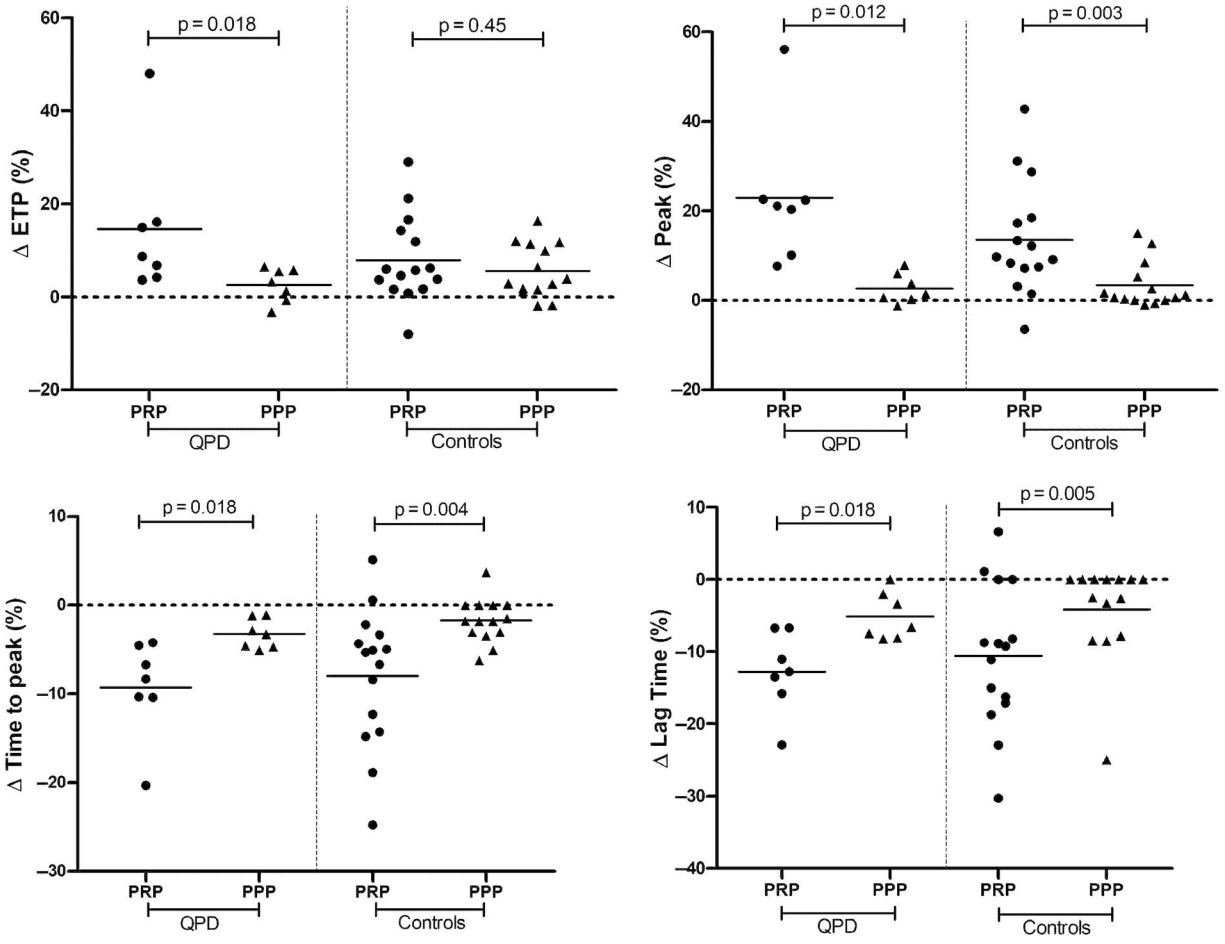

FIGURE 5 The effect of added tranexamic acid on thrombin generation by Quebec platelet disorder (QPD) and control platelet-rich plasma and platelet-poor plasma. The table inset compares TG endpoints for control and QPD PRP, tested with and without added drug (TXA). The panels compare the effects of TXA on PRP vs PPP as % differences in TG endpoints compared to baseline (*P* values indicated) for control and QPD samples

QPD ISTH-BAT scores and TG endpoints, platelet FV, platelet uPA levels, plasma or platelet TFPI levels, but this is not surprising as having QPD is the main predictor of QPD bleeding.[16] Our findings suggest that the impaired QPD PRP TG is largely due to the platelet FV deficiency. However, TXA (which is very effective in treating and preventing QPD bleeding) did improve how well QPD platelets support TG ex vivo. Furthermore, some QPD PRP TG abnormalities (eg, reduced peak thrombin concentration) resemble those of PAI-1-deficient PPP tested with added tPA.[24] It remains possible that other pathological changes to QPD platelets contribute to their TG abnormalities, including the deficiency of MMRN1. We studied TG without thrombomodulin, as recommended for bleeding disorder investigations.[1,2] Given the

complex abnormalities in QPD PRP, it would be challenging to address if QPD alters activated FV downregulation by activated protein C/protein S.[1,2,5]

It would be interesting to use other types of samples and CAT to test if platelet FV is important for maximal TG when plasma FV and TFPI levels are normal. Potentially, this could be done using severe FV-deficient platelets re-suspended in normal plasma and/or PRP from persons with gray platelet syndrome.[35] However, these conditions are quite rare.

FV is very susceptible to plasmin-mediated degradation,[36] which occurs in QPD α-granules.[6,12,14] TG assays are traditionally performed without inhibitors of fibrinolysis[1] and some add tPA to simultaneously assess plasmin generation.[22,23] The greater effects of TXA (a lysine analogue that inhibits fibrinolysis) on PRP compared to PPP TG are interesting and could reflect that platelets express uPAR[37] and release polyphosphate that also enhances plasmin generation.[38] It would be interesting to determine if TXA improves or corrects the TG abnormalities of severe PAI-1-deficient samples,[24] in TG assays of PPP and PRP, with or without added uPA and tPA. Both plasma and platelets contain plasminogen,[7] and we could not obtain plasminogen-deficient PRP to further verify that TXA improves TG by inhibiting plasmin generation. We noted that TXA therapy had little effect on QPD PRP TG findings, even when given orally for several months to treat a QPD bleed, likely because the drug does not alter QPD platelet uPA or α-granule protein degradation (Hayward and Rivard, unpublished). Could some therapeutic benefits of TXA for other conditions reflect improved platelet-dependent TG? PRP TG has not been evaluated during TXA therapy for surgical, traumatic, or postpartum bleeding. Nonetheless, TXA therapy for orthopedic surgery does not alter prothrombin fragment 1.2 levels[39] and TXA therapy for postpartum hemorrhage does not significantly alter PPP TG.[40]

We conclude that QPD results in a platelet-specific defect in TG, proportionate to the loss of platelet FV, suggesting that platelet FV, and possibly other α-granule proteins, are important to maximize TG, even when plasma FV is present. Additionally, the fibrinolytic inhibitor drug TXA improves TG for both control and QPD PRP and PPP samples, although TXA therapy appears to have little impact on QPD TG findings. Our study provides an interesting illustration of why it is important to test TG with both platelets and plasma present when evaluating TG in bleeding disorders.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to disclose.

AUTHOR CONTRIBUTION

All authors contributed to study design and the writing of the paper. CPMH supervised the project. JB, TS, ST, and ML performed experiments and analyzed data. ST, JB, and TS prepared figures.

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