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ORIGINAL ARTICLE

Thromboelastography and thrombin generation assessments for pediatric severe hemophilia A patients are highly variable and not predictive of clinical phenotypes

Natalie Mathews MD[1] | Fred G. Pluthero PhD[2] | Margaret L. Rand PhD[1,3,4] | Ann Marie Stain BHS, RN[1] | Manuel Carcao MD[1,5] | Victor S. Blanchette MB, BChir[1,5] | Walter H. A. Kahr MD, PhD[1,2,6]

[1]Division of Haematology/Oncology, Hospital for Sick Children, Toronto, Ontario, Canada

[2]Cell Biology Program, Research Institute, Hospital for Sick Children, Toronto, Ontario, Canada

[3]Translational Medicine Program, Hospital for Sick Children, Toronto, Ontario, Canada

[4]Departments of Laboratory Medicine & Pathobiology, Biochemistry, and Pediatrics, University of Toronto, Toronto, Ontario, Canada

[5]Department of Pediatrics, University of Toronto, Toronto, Ontario, Canada

[6]Departments of Pediatrics and Biochemistry, University of Toronto, Toronto, Ontario, Canada

Correspondence
Walter H. A. Kahr, Departments of Pediatrics & Biochemistry, University of Toronto, Division of Hematology/Oncology, Cell Biology Program, The Hospital for Sick Children, 555 University Avenue, Toronto, ON, M5G 1X8, Canada.
Email: walter.kahr@sickkids.ca

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Abstract

Background: Severe hemophilia A (SHA) patients vary in severity of bleeding, arthropathy, and requirements for replacement factor VIII (FVIII). Baseline hemostatic activity assays using calibrated automated thrombography (CAT) and thromboelastography (TEG) may offer insights into the physiological basis of clinical heterogeneity.

Objectives: Use CAT and TEG to measure baseline hemostatic activity in a cohort of 30 pediatric SHA patients with available clinical data. Determine effect of contact activation inhibition with corn trypsin inhibitor (CTI). Assess heterogeneity among patients for baseline hemostatic activity and examine correlations between assay results and clinical parameters including FVIII dosing regimen, von Willebrand factor level, and Pettersson arthropathy score.

Methods: SHA blood after FVIII washout was subjected to TEG, and platelet-rich (PRP) and platelet-poor plasma was used for CAT assays. Varying concentrations of tissue factor (TF) were used. Statistical analysis examined relationships between assay results, and clinical parameters.

Results: CTI treatment was required to obtain TEG and CAT results representative of baseline hemostatic activity. Weak activity was observed in assays with low TF concentrations (0.5–2 pM), and most but not all samples approached normal activity levels at high TF concentrations (10–20 pM). A significant positive correlation was observed between results of TEG and CAT-PRP assays. Correlations were not detected between hemostatic assay results and clinical parameters.

Conclusions: *In vitro* hemostatic assay results of samples containing platelets showed concordance. Assay results were not predictive of FVIII requirements or correlated with other clinical parameters. SHA patient heterogeneity is influenced by factors other than baseline hemostatic activity.

KEYWORDS
hemophilia, hemostasis, platelets, thrombin generation, thromboelastography

Essentials

- 30 severe hemophilia A boys were evaluated using TEG and calibrated automated thrombography.
- Corn trypsin inhibitor was used to prevent contact activation.
- Hemostasis assay results did not correlate with patient FVIII dosing requirements.
- TEG and CAT results were not correlated to phenotypic heterogeneity.

1 | INTRODUCTION

Patients diagnosed with severe hemophilia A (SHA) have endogenous factor VIII (FVIII) levels <0.01 IU/ml. The cumulative time spent at this level is predictive of joint bleeds and breakthrough bleeding[1]; thus, prophylactic recombinant factor VIII (rVFIII) therapy is considered the standard of care for SHA patients.[2] It has, however, been reported that 10%–15% of SHA patients exhibit a mild bleeding phenotype,[3,4] which may affect the age of first joint bleed and the severity of long-term morbidity secondary to arthropathy.[5,6] Patient variability may also affect treatment requirements because some patients bleed seldom and maintain normal joints with infrequent factor treatment, whereas others require alternate day or daily infusions of standard half-life factor to prevent spontaneous bleeds.[3,7] It has been proposed that rather than trying to maintain a particular minimum FVIII:C level, treatment of SHA patients should be tailored to individuals,[8] guided by clinical observations such as frequency of joint bleeds, Pettersson score,[9,10] Hemophilia Joint Health Score,[10,11] and magnetic resonance imaging evaluation.[10,12] Feldman et al. reported low arthropathy rates in SHA patients managed with a frequency-escalated prophylaxis regimen (Canadian Hemophilia Primary Prophylaxis Study)[13] in a cohort with a high adherence rate.[14] However, structural joint changes have been detected in patients managed by tailored primary prophylaxis,[15] and soft-tissue changes on magnetic resonance imaging were predictive of future osteochondral changes.[16] It would thus be ideal if individualized treatment approaches could be guided by laboratory assessments of potential patient requirements, which would facilitate optimal factor dosing to prevent bleeding while limiting exposure to rVFIII. This would be expected to potentially lower the risk of inhibitor formation,[17,18] as well as reduce treatment costs that can currently exceed $300,000 USD/y/patient.[19]

In vitro hemostasis assays have long been featured in the diagnosis, management, and physiological study of bleeding disorders, owing to the insights they provide into hemostatic function.[20–25] For example, thrombin generation assays have been used to examine factor activity levels and bleeding severity in adult HA patients[26] and patients with other rare bleeding disorders,[27] and to monitor the pharmacokinetics of FVIII[26] and the response of SHA patients to FVIII-bypassing agents[28–31] and emicizumab.[32] Such studies have primarily used calibrated automated thrombography (CAT), which allows accurate high-throughput testing of both platelet-rich plasma (PRP) and platelet-poor plasma (PPP).[33] CAT assays are typically initiated by the addition of low concentrations of tissue factor (TF), which is also required for blood samples from SHA patients to yield useful results in thromboelastography (TEG) assays.[34] TEG has been used to detect variations in the clotting of blood samples from SHA patients in response to FVIII inhibitors,[35] activated prothrombin concentrate,[36] recombinant forms of FVIII[37,38] and FVIIa,[36,39] and to exercise.[40] Corn trypsin inhibitor (CTI) treatment of blood samples at the time of collection inhibits contact pathway activation,[41,42] which is reported to be necessary for the accuracy and reproducibility of TEG and CAT assays performed with low TF concentrations.[43,44]

This study examined a cohort of 30 pediatric SHA patients with an assortment of *F8* null and missense variants, who were receiving normal half-life rFVIII replacement with dosing regimens ranging from on demand to 3 times/wk. CAT and TEG assays were used to measure baseline *in vitro* hemostatic function after a minimum 72-h washout period, following guidelines for limiting preanalytical and analytical variables proposed by the SSC of the ISTH.[45] Data were analyzed to examine the utility of CTI treatment, and to look for potential correlations among assay results, and between them and various patient clinical parameters. A major focus was to identify a potential relationship between patient baseline hemostatic activity and rFVIII requirements.

2 | MATERIALS AND METHODS

This study was performed with prior approval by the Research Ethics Board at The Hospital for Sick Children in Toronto, Canada.

2.1 | Participants

Informed consent was obtained from all participants. SHA patients aged 1–17 years had a baseline FVIII level of ≤0.01 IU/ml at diagnosis. Exclusion criteria were a known congenital bleeding disorder in addition to SHA; FVIII-inhibitor level exceeding 0.6 Bethesda assay units at the time of testing; and signs of illness or active bleeding during the rFVIII washout period of 72 h minimum. Race/ethnicity of patients was not available, and thus not described here. However,

see Section 4, explaining that this likely had no influence on the outcome.

2.2 | Sampling

Blood samples were collected via peripheral venipuncture and transferred to collection tubes without vacuum. After an initial draw of 5 ml for clinical laboratory testing (blood cell counts, activated partial thromboplastin time, prothrombin time, FVIII level), aliquots for hemostasis assays were collected in 2.7 ml 3.2% citrate tubes (Vacutainer; Becton-Dickenson) with or without added CTI (Haematologic Technologies) to a final concentration of 20 μg/ml. Blood was gently mixed by inversion, transported within 15 min to the laboratory, and incubated at 37°C for at least 30 min before testing. Plasma samples were frozen for batch testing of von Willebrand factor (VWF) antigen, indicative of blood group and FVIII clearance rate.[46]

2.3 | Calibrated automated thrombography

PRP and PPP were prepared from blood samples by sequential centrifugation: 150*g* for 10 min for PRP, 2500*g* for 10 min followed by 14,000*g* for 2 min for PPP. Samples showing visible hemolysis were discarded. Autologous PPP was used to dilute PRP samples to a standard platelet concentration of 150/nl. CAT assays were performed using a Thrombinoscope system in a Fluoroskan Ascent FL automated fluorometer. PRP was assayed with TF concentrations of 2, 5, 10, and 20 pM TF (Innovin, Dade; TF concentration provided by K. Mann) resuspended in HEPES 20 mM, NaCl 140 mM, bovine serum albumin 5 mg/ml, pH 7.35. PPP samples were assayed at TF concentrations of 1, 5, and 20 pM (Thrombinoscope PPP CAT reagents). CAT assays were allowed to proceed until a thrombogram curve was completed, or for 120 min. Thrombinoscope version 3 software was used to derive the CAT parameters peak thrombin concentration (PT, nM), endogenous thrombin potential (ETP, area under thrombogram), lag time (min), and time to peak thrombin (min); data were exported to Microsoft Excel for further analysis.

2.4 | Thromboelastography

Whole blood samples were assayed in a Haemoscope TEG 5000 Analyzer (Haemonetics Corp.) with recalcification and activation with TF (Innovin, Dade) resuspended in 4% albumin phosphate buffered saline buffer, pH 7.4 at a final concentration of 0.5 or 1 pM. Assays were allowed to run until a stable maximum amplitude (MA, mm) value was recorded (up to 180 min). Haemoscope TEG version 4 software was used to generate the TEG parameters MA, reaction time (R, min), maximum rate of thrombus generation (MRTG, mm/min), and time to reach MRTG (min); data were exported to Microsoft Excel for further analysis.

2.5 | Data analysis

Data derived by CAT and TEG software were analyzed using Microsoft Excel and GraphPad Prism. N values <30 in data sets represent loss from one or more of: (1) failure of samples to generate a thrombogram (mostly at low TF concentrations); (2) hemolysis in plasma; (3) insufficient sample volume for all assays; (4) technical failure during assay. Correlation analysis was based on Spearman's nonparametric rank correlation coefficient (*r*); *p* values <0.05 were considered significant. Scatter diagrams are shown with means and 95% confidence intervals, which serve as a general indication of statistically significant difference when they do not overlap between groups.

3 | RESULTS

3.1 | Genetic and clinical variation in the study population

The patient cohort is described in Table 1. All participants were male, mean age was 11.6 years. Twenty-four boys had known null *F8* variants, five had predicted non-null missense variants, and and lacked genomic diagnosis at the time of study. Patients received on average 2.1 doses of rFVIII/week, with regimens ranging from on demand to three doses/week. FVIII levels in samples at time of testing are listed. Mean Pettersson score was 4.2 (range 0–46); VWF:Ag levels ranged from 0.35 to 1.5 IU/ml.

3.2 | Effects of CTI treatment

CAT peak thrombin (PT, in nM) values for PPP samples derived from blood collected with and without CTI tested at 3 TF concentrations are shown in Table S1. There was a consistent trend for CTI samples to have lower values in assays with 1 pM TF, as shown in Figure 1A, and a paired *t* test gave a significant difference ($p < 0.01$). The coefficient of variation (CV, Table S1) was also somewhat higher for CTI-treated samples. A similar CTI treatment effect was observed in CAT assays of PRP (not shown). CTI treatment also had a marked effect on the results of TEG assays of citrated blood with 0.5 pM TF with MRTG being consistently lower (Figure 1B; paired *t* test $p < 0.01$) for CTI-treated samples. These results indicate that contact activation influenced the results of CAT and TEG assays, hence only results from CTI-treated samples will be considered from here onwards.

3.3 | Thrombin generation in PPP

CAT assays of PPP from SHA patients showed considerable variation within the cohort, and a dramatic increase in thrombin generation (i.e., PT values) with increasing TF concentration (Figure 2A). All individual samples followed this increasing trend (Figure 2B). Results from Table S1 are summarized in Table S2; mean PT was 33.2 and

TABLE 1 Clinical description of the patient cohort

Study ID	Age (y)	*F8* Var	Null Variant?	rFVIII /wk	P score	VWF:Ag (IU/ml)	PTT (s)	FVIII (IU/ml)
HH01	9.6	I22 INV	Yes	2	0	0.67	75	≤0.01
HH02	15.3	NON	Yes	3	18	0.46	76	≤0.01
HH03	6.6	FS	Yes	3	0	0.62	60	≤0.01
HH04	16.1	I1 INV	Yes	2	0	0.64	66	≤0.01
HH05	15.8	I22 INV	Yes	3	0	0.54	62	≤0.01
HH06	15.3	I22 INV	Yes	2	0	0.58	67	≤0.01
HH07	15.3	NON	Yes	3	13	1.09	75	≤0.01
HH08	13.7	FS	Yes	2	5	0.64	82	≤0.01
HH09	7.3	NON	Yes	3	0	0.65	73	≤0.01
HH10	15.1	FS	Yes	2	0	0.72	71	≤0.01
HH11	16.9	MIS	No	3	2	0.57	64	≤0.01
HH12	15.8	FS	Yes	2	0	0.89	60	0.04
HH13	10.9	I22 INV	Yes	3	4	0.76	58	0.04
HH14	7.0	MIS	No	1	2	0.72	66	≤0.01
HH15	9.7	I22 INV	Yes	3	0	0.48	85	≤0.01
HH16	14.8	I22 INV	Yes	3	15	0.51	81	≤0.01
HH17	9.2	MIS	No	2	0	0.60	60	0.03
HH18	16.9	I1 INV	Yes	2	0	1.10	59	0.03
HH19	13.9	I22 INV	Yes	3	0	0.52	73	≤0.01
HH20	8.0	MIS	No	2	2	0.90	75	≤0.01
HH21	17.3	UNK	N/A	3	0	0.71	65	≤0.01
HH22	13.3	INS	Yes	3	10	0.75	61	0.02
HH23	10.2	FS	Yes	3	0	0.35	80	≤0.01
HH24	17.4	FS	Yes	3	46	1.13	57	≤0.01
HH25	3.9	MIS	No	0	0	1.50	74	≤0.01
HH26	17.9	DEL	Yes	1	9	1.33	71	≤0.01
HH27	1.9	NON	Yes	1	0	0.83	92	≤0.01
HH28	5.7	I22 INV	Yes	1	0	0.80	75	≤0.01
HH29	2.5	I22 INV	Yes	0	0	0.50	75	≤0.01
HH30	6.2	I22 INV	Yes	0	0	0.80	84	≤0.01

Note: Subjects are listed by study identification. *F8* variants (Var) included chromosomal deletions (DEL) and inversions (INV) in introns (I) 1 and 22, nonsense (NON), frameshift (FS) and missense (MIS) variants, and an unknown (UNK). Null variants had no endogenous FVIII. Recombinant FVIII regimen is listed as doses/week or 0 for on demand. Also listed are Pettersson (P) score, and VWF:antigen (Ag) levels, partial thromboplastin time (PTT) and FVIII level for patient blood samples tested.

203 nM for 1 and 5 pM TF, respectively. The latency of thrombin generation, indicated by lag time and time to peak, decreased as TF concentration increased (Table S2). The CV for PT and ETP declined (e.g., from 0.49 to 0.24 for PT) with increased TF concentration, as did the high/low ratio for PT, which almost halved at each step (Table S2). This is consistent with higher TF concentrations overcoming the effects of FVIII deficiency, as was the >50% increase in mean PT observed for SHA samples when TF concentration rose from 5 to 20 pM, which was substantially greater than the 10% increase seen for the normal donor sample (Table S2).

PT values for PPP samples tested with 1 and 5 pM TF showed a strong positive correlation (Figure S1A), whereas comparisons of the proportionate change in PT (i.e., the difference divided by the value for the lower TF concentration) showed significant negative correlations for the step from 1 to 5 pM TF (Figure S1B) and from 5 to 20 pM TF (Figure S1C). This indicates that the samples with the weakest thrombin generation tended to respond the most strongly to increased TF activation.

3.4 | Thrombin generation in PRP

Complete thrombogram data were obtained for PRP samples from 25 of 30 patients assayed at 2, 5, 10, and 20 pM TF. As with

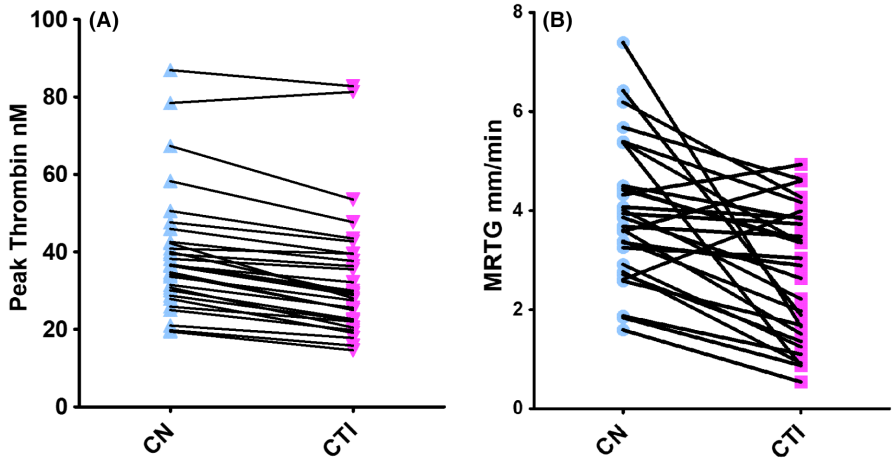
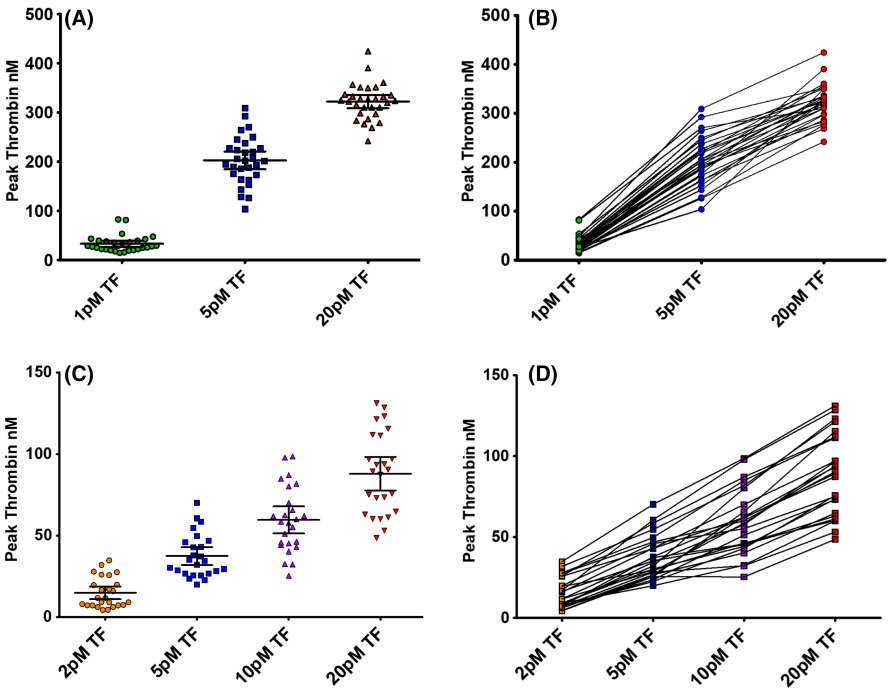


FIGURE 1 Corn trypsin inhibitor (CTI) treatment decreased readouts from thrombin generation and thromboelastography assays. (A) Peak thrombin values from CAT assays with 1 pM tissue factor (TF) of platelet-poor plasma derived from citrated only (CN) and CTI-treated blood (complete data in Table S1). (B) Maximum rate of thrombus generation (MRTG) values from TEG assays of CN and CTI-treated blood with 0.5 pM TF. The consistent trend toward lower values for CTI-treated samples indicates contact activation influenced results of both assays, with an especially marked effect on TEG

FIGURE 2 Platelet-poor (PPP) and platelet-rich (PRP) plasma samples from SHA patients show wide variation in thrombin generation at all concentrations (pM) of tissue factor (TF) used. (A) Peak thrombin values in nM for PPP samples; (B) lines track results for individual patient samples. (C) Peak thrombin values for PRP samples; (D) lines track results for individual patient samples. Bars in A, C show means and 95% confidence intervals



PPP CAT assays, considerable variation was observed within the cohort for PT values at each TF concentration (Figure 2C), and all samples followed an increasing trend with rising TF concentration. Complete data are shown in Table S3 and summarized in Table S4; thrombograms for a normal donor sample and a representative subset of SHA patients are shown in Figure S2. When tested at TF concentrations <2 pM, several samples failed to resolve thrombograms (data not shown). As indicated by the value ranges for PT and ETP, along with values for CV and high/low (Table S4), somewhat greater variation was observed in thrombin generation for PRP samples relative to PPP. PT values for PRP rose with increasing TF concentrations (Figure 2C,D), but patient PRP samples consistently gave lower PT values than the normal donor control; even at 20 pM TF, seven PRP samples still had PT <50% of the normal value (Table S3). In contrast, when tested with 20 pM TF, all PPP samples had >67% of the normal sample PT value, and four matched or exceeded it (Tables S1, S2).

The proportionate changes in PT values for PRP tested at 2 and 5 pM TF (Figure S3A), and 5 and 10 pM TF (Figure S3B) were negatively correlated. This indicates that, as was seen with PPP, at each step, the samples with the weakest thrombin generation tended to have the strongest response to increasing TF concentration.

3.5 | TEG analysis of blood clotting

TEG assay results showed considerable variation among patient samples (MRTG values shown in Figure 3A), with a consistent increase in MRTG for samples tested with 1 pM TF compared with 0.5 pM (Figure 3B). Results are summarized in Table S5. The value ranges, CV values, and high/low ratios indicate considerable variation within the SHA cohort, which is evident in the MRTG values shown in Figure 3A,B. There was a positive response to increased TF, with mean MRTG of 2.69 and 4.33 for 0.5 and 1 pM TF, respectively, and consistent with established normal ranges the result for the normal donor sample showed little difference between TF concentrations (unlike most SHA samples, normal blood typically begins clotting spontaneously within 20 min without addition of TF). At 0.5 pM TF, all SHA samples had lower MRTG than the normal sample (Table S5), with 58% having values <50% of the normal sample and 17% being >70% of normal. At 1 pM TF, 50% of SHA samples had MRTG values >70% of the normal and 37% were <50% (Table S6). Thus, at both TF concentrations, the results of TEG assays were consistent with weak and highly variable clotting among SHA samples. The proportionate change in R time with increasing TF concentration was negatively correlated with R time at 0.5 pM TF (Figure S4A), indicating that the slowest clotting samples showed the strongest effect of increased TF concentration on clotting initiation. In contrast, MRTG at 0.5 pM TF showed no significant correlation with the response of samples to 1 pM TF (Figure S4B), although individual MRTG values at both concentrations (Table S5) showed a strong positive correlation (see the following section).

3.6 | Correlation of CAT assays of PPP and PRP

Comparisons were made between PPP and PRP datasets in which complete PT results were obtained at three different TF concentrations (Table S7, $n = 24$). Results of correlation analyses are shown in Table 2, top. PRP assay datasets positively correlated with each other, as did PPP datasets, whereas PPP and PRP correlated significantly at 20 pM TF, but not at lower TF concentrations. These results indicate that at relatively low TF levels, thrombin generation in these samples was strongly influenced by the presence of platelets, which provide the main source of phospholipids in PRP assays (phospholipids are present in the CAT reagent used for PPP assays).

3.7 | Comparisons of TEG and CAT assay results

The correlation analysis is summarized in Table 2, bottom; datasets used for comparisons are shown in Tables S8 (PPP PT and TEG MRTG; $n = 24$) and S9 (PRP PT and TEG MRTG; $n = 19$). No significant correlation was detected between TEG MRTG and CAT PT for assays with PPP at any TF concentration used. In contrast, comparisons of TEG MRTG (0.5 and 1pM TF) and CAT PT for PRP (2, 5, 10, and 20 pM TF) showed significant correlations for all pairings, with the exception of MRTG at 1 pM TF and PT at 2 pM. Correlation graphs of MRTG (0.5 pM TF) with PT (5pM TF) are shown for PPP and PRP in Figures S5A and S5B, respectively.

3.8 | Relationship of hemostasis assay results with clinical variables

The clinical variable expected to be of greatest potential relevance to baseline hemostatic function is the rFVIII treatment regimen. For the purpose of analysis, subjects were sorted into three subgroups according to FVIII doses received/week: 0–1, 2, or 3 (Table S10). The three groups showed differences in mean

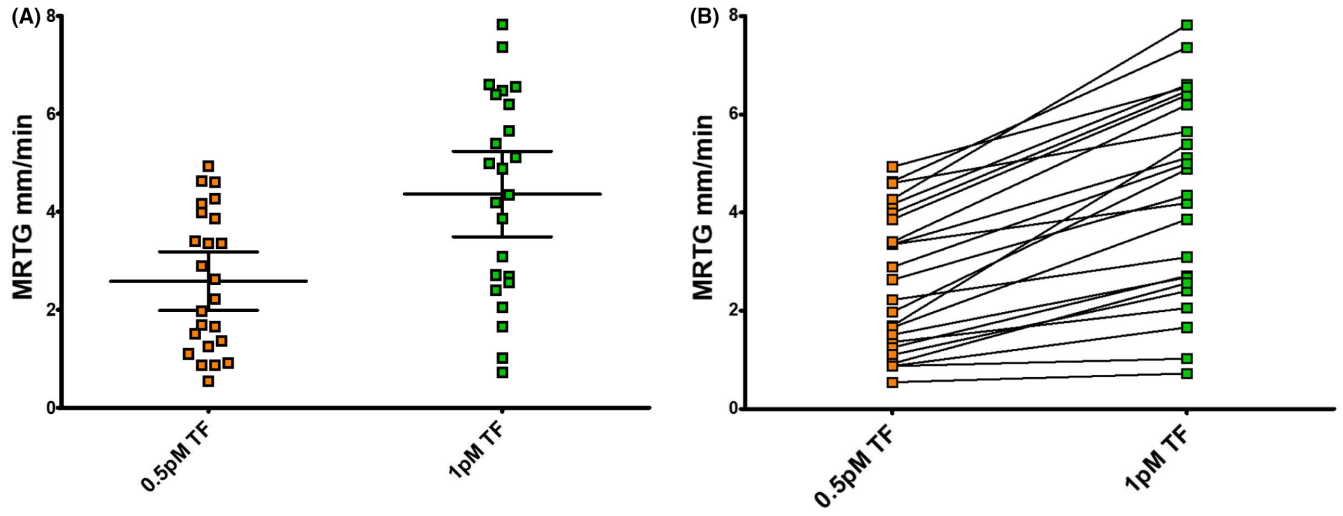


FIGURE 3 Thromboelastography assays of SHA patient blood samples show wide variation of maximum rate of thrombus generation (MRTG) with 0.5 pM and 1 pM tissue factor (TF). (A) Maximum rate of thrombus generation (MRTG) values in mm/min for individual samples (bars show means and 95% confidence intervals). (B) Lines show trends for samples from individual patients

TABLE 2 Results of hemostasis assays show intra- and inter-assay correlations

PRP and PPP peak thrombin (nM)							
		PRP			**PPP**		
***n* = 24**	**[TF]**	**5**	**10**	**20**	**1**	**5**	**20**
R							
PRP	2	0.827	0.599	0.444	−0.036	0.017	0.224
	5		0.758	0.647	−0.113	0.000	0.284
	10			0.923	0.166	0.221	0.473
	20				0.128	0.213	0.447
PPP	1					0.908	0.498
	5						0.571
P							
PRP	2	**0.000**	**0.002**	**0.030**	0.867	0.939	0.293
	5		**0.000**	**0.001**	0.600	1.000	0.179
	10			**0.000**	0.438	0.299	**0.020**
	20				0.551	0.319	**0.028**
PPP	1					**0.000**	**0.013**
	5						**0.004**
PRP peak thrombin (nM) and TEG MRTG							
		MRTG	**PRP PT**				
***n* = 19**	**[TF]**	**1**	**2**	**5**	**10**	**20**	
R							
MRTG	0.5	0.922	0.457	0.488	0.656	0.478	
MRTG	1		0.403	0.484	0.677	0.582	
P							
MRTG	0.5	**0.000**	**0.049**	**0.034**	**0.002**	**0.038**	
MRTG	1		0.087	**0.036**	**0.001**	**0.009**	

Note: Comparison matrices are shown with Spearman *r* values (R) at top and *p* values (P) below, with *p* values <0.05 indicated by bolding. *Top*: Peak thrombin (PT, nM) values for CAT assays show that PRP datasets positively correlated with each other, as did PPP datasets, whereas PRP and PPP values were positively correlated at high tissue factor (TF) concentrations (10 and 20 pM). *Bottom*: TEG maximum rate of thrombus generation (MRTG) results showed no correlation with CAT assay results for PPP (Figure S5), but they did correlate positively with PRP PT values at most TF concentrations.

age (6.4, 13.3, and 13.2 years, respectively) and Pettersson score (1.6, 0.8, and 7.7, respectively). However, when hemostasis assay results were plotted by group and assay TF concentrations, no evident differences were observed among groups for CAT PT values for PPP (Figure 4A) or PRP (Figure 4B), nor for TEG MRTG (Figure 4C). All comparisons showed $p > 0.05$ in nonparametric one-way ANOVA with Dunn multiple comparison test. Thus, the results of in vitro assays of baseline hemostasis were not predictive of patient rFVIII requirements and vice versa. A correlation analysis of TEG MRTG and CAT PT with three clinical parameters having continuous numerical values: age, VWF levels, and Pettersson score, also detected no significant correlations (Table S11), aside from a positive correlation between patient age and PRP PT. The clinical parameters did not correlate significantly with each other (Table S11).

4 | DISCUSSION

Our observations indicate that CTI treatment had a significant effect on the results of CAT and TEG assays of SHA patient samples (Figure 1A,B). This confirms the utility of contact pathway inhibition for assessment of baseline hemostatic function in these patients using *in vitro* assays, which is consistent with previous reports. Thrombin generation assays are sensitive to preanalytical variables (e.g., methods of blood collection) and to analytical variables that can be reduced by using calibrated assays (i.e., CAT) and standardized reagents (e.g., TF, phospholipids).[47] Inhibiting contact pathway activation with CTI[41,42] also improves the consistency of TEG and CAT assays, especially at low TF concentrations.[43,44] For example, van Veen et al.[48] reported that CAT assays of PPP from normal individuals and patients with clotting factor deficiencies, or

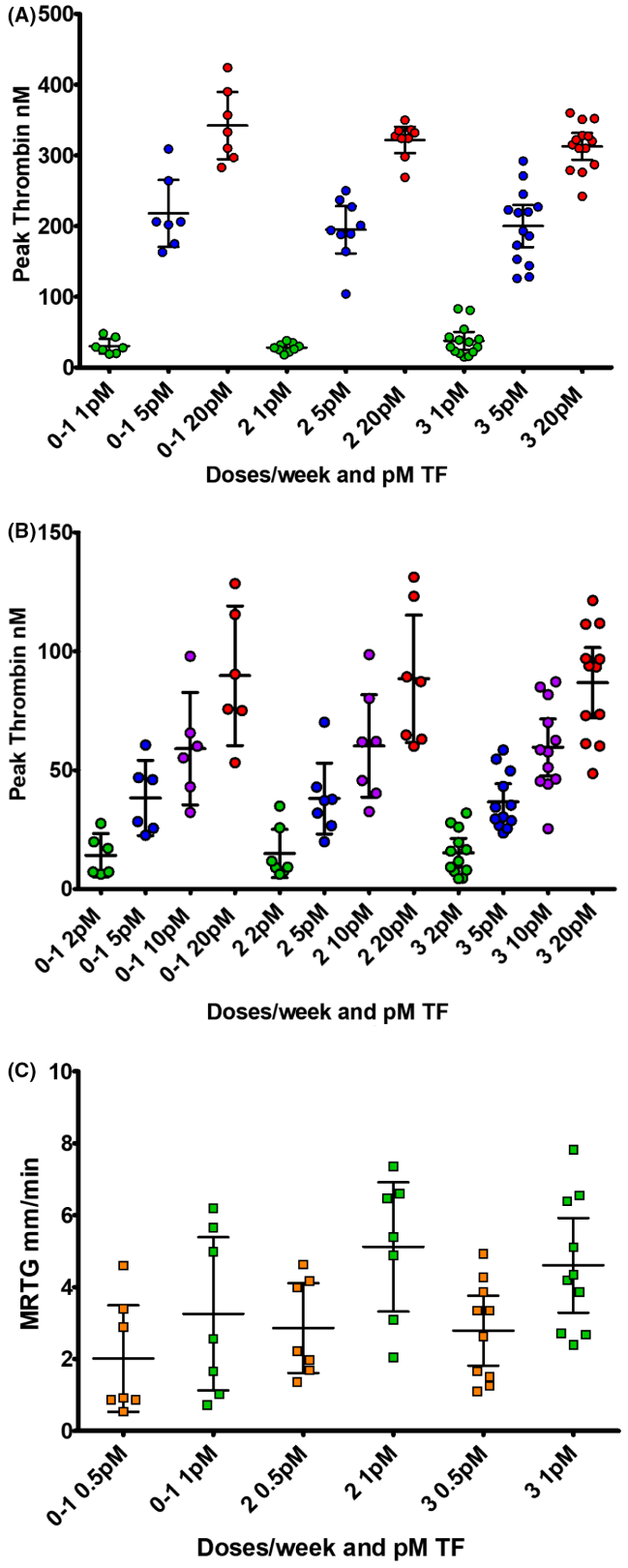


FIGURE 4 Results of CAT and TEG assays show no evident relationship with SHA patient FVIII dosage requirements. Samples are sorted on X-axes by patient FVIII requirement groups (0–1, 2, or 3 doses/wk) and pM tissue factor (TF) used in assays. (A) CAT peak thrombin values for PPP samples (see Figure 2A). (B) CAT peak thrombin values for PRP samples (see Figure 2C). (C) TEG MRTG values (see Figure 3A). Bars show means and 95% confidence intervals

receiving warfarin treatment, showed less intra-assay variation with CTI treatment.

We observed substantial inter-individual variation among SHA patient samples in CAT assays of PPP (Figure 2A,B) and PRP (Figure 2C,D), consistent with previous studies of pediatric patients.[33] For both PPP and PRP, the CV and high/low ratios for PT and ETP were markedly higher at the lowest TF concentration tested (1 pM for PPP, Table S2; 2 pM for PRP; Table S4). Although the source of TF differed for CAT assays of PPP (Thrombinoscope reagent) and PRP (Innovin), both preparations produced similar inter-individual variability of assay results. The results of TEG assays also indicated considerable variation within the SHA cohort (Figure 3A, Table S5), and a positive response to increased TF concentration (Figure 3B). Overall, these observations are consistent with the expectation that *in vitro* thrombin generation and blood clotting in SHA samples will converge toward normal levels as increased TF stimulation leads TF-FVIIa-mediated thrombin generation to become predominant. This effect may also account for our observation of a significant correlation between PT values for PPP and PRP at 10 and 20 pM TF, but not at the lower concentrations (Table 2), where these assays appear to measure different aspects of thrombin generation.

Our CAT results indicate that for both PPP and PRP samples, those with the weakest thrombin generation (i.e., PT) showed the strongest response to increases in TF concentration, with the effect being greatest for the step up from the lowest TF concentration (1 pM for PPP, Figure S1; 2 pM for PRP, Figure S3). A similar effect was observed for TEG clot initiation (R time), with the latest clotting samples at 0.5 pM TF showing the strongest proportional response to 1 pM TF (Figure S4A). For TEG MRTG, however, no similar pattern was observed, indicating that clot initiation was more sensitive to TF activation than thrombus generation. Although a TF concentration of 0.5 pM is much greater than the femtomolar levels that trigger hemostasis *in vivo*,[49] we cannot rule out the possibility that some of the variation observed in TEG clot initiation with low TF concentrations reflects differences in the endogenous TF content of blood samples. This may also apply to CAT assay results with low TF concentrations.

As mentioned previously, CAT PT values for PPP and PRP showed no significant correlation at TF concentrations <10 pM. TEG MRTG also showed no significant correlation with PPP PT. We did observe significant correlations between PRP PT at all TF concentrations and TEG MRTG at 0.5 pM TF (Table 2). This indicates these assays likely measure similar aspects of hemostatic activity, with an obvious common factor being the presence of platelets at 150/nl for CAT PRP, and within normal ranges in citrated blood used for TEG. We conclude that these assays can be used individually or in tandem to obtain information regarding the hemostatic potential of blood samples that has a better chance of reflecting potential physiological hemostasis in SHA patients after factor washout than assays of plasma alone.

Our analysis did not detect significant correlations between *in vitro* assay results and values for the continuous clinical parameters VWF:Ag and Pettersson score (Table S11). Nor did we detect evidence of a relationship between assay results and patient rFVIII requirements (Figure 4A–C). These findings were not unexpected, given that other efforts to use global hemostatic assays to predict the therapeutic requirements of SHA patients have been unsuccessful.[50–52] This may be attributed to an assortment of non-exclusive factors, which include: (1) at low TF levels, even those SHA samples with the strongest relative hemostatic activity in CAT and TEG assays were still weak relative to normal donor samples, and thus indicated ineffective FVIII-mediated hemostasis *in vivo*; (2) all the patients in our cohort received rFVIII replacement therapy, which ensured they spent little or no time at the baseline levels of hemostatic function we assessed via CAT and TEG assays; (3) like many studies, ours was insufficiently powered to detect subtle relationships between hemostasis assay results and clinical parameters; and (4) key clinical outcomes such as joint bleeds are affected by many factors that show significant variation among patients and can act in combination, including patient physical activity,[53] body mass index,[54] and factor clearance rates.[55]

We consider it unlikely that race/ethnicity and other potential sociocultural determinants of health (which are not described for our participants) played a significant role in our findings. This is because in the Canadian health care system, clinic visits, as well as all aspects of hemophilia treatment (including FVIII replacement therapy), do not incur costs to the patient. Furthermore, The Hospital for Sick Children is a quaternary care facility, where patients are seen by a comprehensive care team that includes physician assessment, physiotherapy assessment, and social worker support, thus ensuring that socioeconomic factors do not affect patient care.

It is possible that the small sample size and within person variability of assays resulted in missed associations. Of obvious relevance to this and other studies is the limited ability of *in vitro* assays to replicate and/or capture the complexities of hemostasis *in vivo*, which involves interactions among platelets, proteins (e.g., FVIII and other clotting factors) and other biomolecules and tissues that ensure clots form when, where, and to the extent they are required. This complexity provides vast scope for variations in many aspects of hemostatic physiology. For example, factors we did not account for in this study that are known to affect hemophilia phenotypes include differences in inflammatory markers,[56] thrombophilia,[57,58] and defects in fibrinolysis.[59]

In conclusion, our observations confirm that contact pathway inhibition via CTI is required for effective measurement and assessment of variation in baseline hemostatic function of blood and plasma samples from SHA patients using TEG and CAT assays. Our results indicate that for low concentrations of added TF, TEG assays of citrated blood, and CAT assays of PRP yield concordant results for the potential of CTI-treated samples from SHA patients to form clots and generate thrombin in vitro. We therefore suggest that future studies use these assays in tandem to help detect and limit confounding effects of preanalytical and analytical variables. The same considerations apply to potential use of these assays in monitoring patient hemostatic function and response to treatment with standard half-life FVIII (still in use in many parts of the world)[60] and newer preparations.

AUTHOR CONTRIBUTIONS

W.H.A.K., F.G.P., and N.M. wrote the manuscript; M.L.R., V.S.B., and M.C. edited the manuscript. W.H.A.K., M.L.R., F.G.P., and V.S.B designed the research. A.M.S. procured patient samples. W.H.A.K. coordinated sample analysis. F.G.P. performed the *in vitro* assays and statistical analysis. All authors approved the final version of this manuscript.

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RELATIONSHIP DISCLOSURE

The authors have no conflicts of interests to disclose.

ORCID

Natalie Mathews https://orcid.org/0000-0002-9501-0468
Fred G. Pluthero https://orcid.org/0000-0002-0451-5840
Margaret L. Rand https://orcid.org/0000-0001-7671-1405
Manuel Carcao https://orcid.org/0000-0001-5350-1763
Victor S. Blanchette https://orcid.org/0000-0003-3341-5010
Walter H. A. Kahr https://orcid.org/0000-0002-2832-7158

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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