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ORIGINAL ARTICLE

Exploring the utility of a novel point-of-care whole blood thrombin generation assay following trauma: A pilot study

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Abstract

Introduction: Plasma thrombin generation kinetics as measured by the calibrated automated thrombogram (CAT) assay is a predictor of symptomatic venous thromboembolism after trauma. We hypothesized that data from a new prototype assay for measurement of thrombin generation kinetics in fresh whole blood (near patient testing of thrombin generation), will correlate with the standard CAT assay in the same patients, making it a potential tool in the future care of trauma patients.

Methods: Patients were enrolled from June 2018 to February 2020. Within 12 hours of injury, blood samples were collected simultaneously for both assays. Variables compared and correlated between assays were lag time, peak height, time to peak, and endogenous thrombin potential. Data are presented as median with interquartile range (IQR). Spearman and Pearson correlations were estimated and tested between both assays; a P value of <0.05 was considered to be significant.

Results: A total of 64 trauma patients had samples analyzed: injury severity score = 17 (IQR), 10-26], hospital length of stay = 7.5 (IQR), 2-18) days, age = 52 (IQR, 35-63) years, 71.9% male, and 42.2% of patients received a transfusion within 24 hours of injury. Thrombin generation parameters between plasma and whole blood were compared and found that all parameters of the two assays correlate in trauma patients.

Conclusion: In this pilot study, we have found that a novel point-of-care whole blood thrombin generation assay yields results with modest but statistically significant correlations to those of a standard plasma thrombin generation assay. This finding supports studying this device in a larger, adequately powered study.

KEYWORDS
calibrated, kinetics, plasma, thrombin, trauma, venous thromboembolism

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Mayo Clinic, Rochester, Rochester, MN (Primary Site of Research).

Essentials

- A novel device has been developed to measure thrombin generation kinetics in whole blood.
- Accelerated thrombin generation is a predictor of venous thromboembolism after trauma.
- Thrombin generation values correlate between plasma calibrated automated thrombogram assay and the novel whole blood assay.
- Novel whole blood thrombin generation assay should continue to be explored as a future tool.

1 | INTRODUCTION

Traumatic injury remains a leading cause of morbidity and mortality worldwide. In addition to physical and psychological trauma, these patients also face potential morbidity associated with trauma-induced coagulopathy (TIC).[1-4] While effective resuscitation can treat the hemorrhagic effects of TIC, many of these patients suffer persistent hypercoagulability following trauma, even after hospital discharge. Prior work from Dr Park's lab has shown that up to 40% of symptomatic venous thromboembolisms (VTEs), including deep vein thrombosis (DVT) and pulmonary embolism (PE), occur after hospital discharge, and that this risk remains elevated up to 3 months after trauma.[5] Understanding an individual's coagulation profile is essential in the care of trauma patients including initial resuscitation efforts, chemoprophylaxis administration, and postdischarge monitoring.

Laboratory tests that rapidly and accurately quantify TIC have the potential to augment care for trauma patients. Plasma-based assays, such as the calibrated automated thrombogram (CAT), have been developed that assess thrombin generation kinetics in real time.[6,7] This assay is able to quantify an individual's plasma thrombin generation profile in response to tissue factor (TF) and procoagulant phospholipid. Plasma-based CAT describes several parameters of thrombin generation that will be discussed here: lag time (LT), which is the time (minutes) to the start of thrombin generation; peak height (PH), which is the maximum thrombin concentration (nM) at a given time point during the assay; time to peak (ttPeak), which is the time (minutes) to peak rate of thrombin generation; and endogenous thrombin potential (ETP), which is the total thrombin that can be generated during the assay (nM × minute). Prior studies have shown enhanced thrombin generation after trauma and that certain derangements in an individual's plasma thrombin generation profile may be independent predictors of VTE after trauma.[8-10] Unfortunately, plasma thrombin generation assays are not amenable to point-of-care (POC) use because they are typically run in batches of several patient samples at a time. Additionally, the absence of platelets and other cellular components in a plasma-based assay potentially limits the approximation of physiologic conditions.

A novel method of measuring thrombin generation kinetics in fresh whole blood has been developed that allows for near patient testing of thrombin generation (NPT-TG). This POC assay has the potential to provide information about an individual's coagulation profile that could be applied at the bedside. Additionally, the testing of whole blood rather than plasma includes platelets and other cellular components involved in clot formation. The novel whole blood assay measures thrombin generation kinetics in a manner similar to the standard plasma-based assay. A TF agonist initiates a clotting reaction in the presence of a fluorogenic substrate, which changes as thrombin is generated. The results are standardized against a calibrator of known activity, and a curve is produced showing thrombin activity over time.

In this pilot study, our main objective was to compare thrombin generation parameters from this novel whole blood assay with those of the well-established plasma-based assay. We hypothesized that thrombin generation kinetics measured in whole blood will correlate with plasma thrombin generation parameters. We anticipate that these correlations will likely be modest due to the presence of platelets and cellular components in whole blood, with these two assays offering complementary views of an individual's coagulation profile.

2 | METHODS

2.1 | Study design, setting, and population

This study was approved by the Mayo Clinic Institutional Review Board and conducted using a waiver of informed consent. Adult patients presenting to the Mayo Clinic Emergency Department as trauma activations, from June 2018 through February 2020 were considered for study inclusion. Exclusion criteria included age <18 years, ongoing systemic anticoagulation at presentation (eg, heparin, warfarin, or novel oral anticoagulants) other than antiplatelet agents, known preexisting coagulopathy, cirrhosis, active malignancy, sepsis, renal failure requiring dialysis, burn injuries, or recent major surgery or another significant trauma in the past year, as well as pregnant women or prisoners. Trauma patients or their legal authorized representative (LAR) gave consent for this study after the collection of one or more blood samples. If the patient or LAR could not be consented for study participation or declined consent, the sample was destroyed and the patient was excluded. The time of injury (TOI) was determined by the prehospital medical providers based on information at the injury scene. We collected demographic and clinical characteristics for each patient from the electronic medical record.

2.2 | Blood sample collection

Blood samples were collected from both trauma patients and healthy volunteers, who were recruited as outpatients specifically for this study. Healthy volunteers provided full written informed consent before any sample collection. Two separate samples were obtained

from each subject: one for the whole blood thrombin generation assay and one for plasma thrombin generation, as described below. Blood was collected simultaneously for both assays through venipuncture or from indwelling catheters. Samples were collected upon patient arrival, up to 12 hours from the TOI for the trauma patients.

2.3 | Platelet-poor plasma collection

A total of 4.5 mL of whole blood was collected by venipuncture or via existing indwelling catheters into citrated Vacutainer tubes (0.105 M of buffered sodium citrate, 3.2%; Becton Dickinson, East Rutherford, NJ, USA). Within 20 minutes of collection, blood was processed to platelet-free plasma by double centrifugation (3000 g, 15 minutes), as recommended by the International Society on Thrombosis and Haemostasis vascular biology Scientific and Standardization Committee Collaborative Workshop,[11] and stored in multiple aliquots at −80°C until subsequent batch analysis.

2.4 | Whole blood collection

Whole blood was collected in 4-mL evacuated tubes containing both sodium citrate (3.2%) and corn trypsin inhibitor (CTI; 100 µg/mL; Hematologic Technologies, Essex Junction, VT, USA). The whole blood thrombin generation assay was run within 30 minutes of the blood draw. If the whole blood assay was unable to be run within 30 minutes of the blood draw, the sample was discarded.

2.5 | Calibrated automated thrombogram analyses

Thrombin generation kinetics were measured with the CAT assay (Thrombinoscope BV, Maastricht, Netherlands), utilizing a Fluoroskan Ascent plate reader (390 nm excitation, 460 nm emission, Thermo Electron Corp, Vantaa, Finland), as previously described by Hemker et al.[6,7] Assays of trauma patient samples were performed in triplicate. CTI was added to 800 µL of plasma for a final concentration of 50 µg/mL CTI before sample analysis. Thrombin generation was initiated using two different reagents: addition of 20 µL of platelet-poor plasma (5 pM relipidated human TF and 4 µM phospholipids, Diagnostica Stago Inc, Parsippany, NJ, USA) reagent. Then, 80 µL of citrated plasma was added to each well of U-bottom 96-well microtiter plates (Nunc; Thermo Fisher Scientific, Waltham, MA, USA) using a single-channel pipette. After an incubation period (10 minutes at 37°C), 20 µL of warmed FluCa reagent (Fluca kit, Diagnostica Stago Inc), which contains the fluorogenic substrate and calcium chloride ($CaCl_2$) was added to each well via an automated dispenser. Thrombin generation curves were recorded continuously for 90 minutes at a rate of three readings per minute. Separate wells containing the thrombin calibrator, which corrects for inner filter effects and quenching variation among individual plasmas were also measured in parallel. A dedicated software program, Thrombinoscope (Thrombinoscope BV) was used to calculate thrombin activity over time. The parameters derived were LT, PH, ttPeak, and ETP.

2.6 | Near patient testing of thrombin generation

Thrombin generation kinetics in whole blood was measured using the NPT-TG, a prototype machine designed by Stago (Diagnostica Stago, Asnières-sur-Seine, France) specifically for this purpose. This device is not US Food and Drig Administration approved but is currently under investigation at three trauma centers. The NPT-TG assay operates on the same principle as plasma-based CAT, measuring the change of a thrombin-specific fluorogenic substrate over time, and comparing this measurement against a calibrator with a known value of thrombin activity. Two activating solutions are prepared in 0.5% bovine serum albumin (BSA). One is a control without TF. The other is a solution of 26-pM relipidated human TF in 0.5% BSA buffer with $CaCl_2$ added as an agonist to initiate clotting reaction, with the final concentrations of 6.5 pMTF, 16.7 mM $CaCl_2$, and CTI 50 µg/mL. The fluorogenic substrate (1.67 mM) is added. The test condition is run in two individual channels, alongside a third control channel with no TF. Thrombin generation curves are recorded continuously at a rate of three readings per minute for about 75 minutes. These channels are each measured in triplicate and the results averaged. The results are measured using software provided by Stago (Diagnostica Stago). The rate of thrombin generation is measured over time and produces a curve analogous to that generated by the plasma thrombin generation assay, allowing comparison of LT, PH, ttPeak, and ETP. Per Stago's recommendation, control tests are performed weekly using a control reagent provided by Stago (NPT-TG Coag Control) in lieu of whole blood. Coefficient of variation (CV, %) ranges for the individual whole blood assays included in this series (n = 64) as well as control tests (n = 44) were calculated and are reported below.

2.7 | Statistical analyses

Categorical variables were summarized as n (%). Continuous variables were summarized as median with interquartile ranges (IQRs) and compared using the Kruskal-Wallis test. Pearson correlations and Spearman rank correlations were estimated and tested for significance between plasma and the corresponding whole blood variables. The assessment of association was based on the more robust Spearman correlation, while Pearson correlations were estimated primarily for descriptive purposes. A P value of <0.05 was considered to be statistically significant. Trauma patients were also analyzed with and without a subgroup of patients taking antiplatelet medications, and both Pearson and Spearman rank correlations were estimated between the plasma and whole blood parameters for each of those groups. All analysis was performed using SAS, version 9.4 (SAS Institute, Cary, NC, USA).

3 | RESULTS

Eighty-nine patients were screened for participation, 5 patients met exclusion criteria, 17 patients declined consent, and 3 patients were excluded due to technical errors with the whole blood thrombin generation assay (one instance of blood collected in the wrong tube, one error preparing reagents, and one mechanical error with machine after the assay had begun). The remaining cohort of 64 trauma patients had a median age of 52 (IQR, 35-63), median injury severity score of 17,[10,24] 71.9% were male, and 96.9% had blunt mechanism of injury. As described in Table 1, 27 (42.2%) required blood product transfusion, and 10 (15.6%) required massive transfusion defined as >3 units of red blood cells within any 60-minute period.[12] Of the patients who received blood product transfusion at any point in their treatment course, 18 of 27 (66.6%) were transfused before the blood draw for this study. Six patients (9.4%) developed VTE during hospitalization. One sample from a patient with VTE clotted in a sodium citrate tube before it could be processed, so plasma thrombin generation data are available for 63 of the 64 patients, while whole blood thrombin generation data are available for all 64 trauma patients.

Trauma patients were compared with 13 healthy volunteers, who had a median age of 35[2539] and were 61.5% female (Table 1). All of the female controls were premenopausal and 3 of 8 (37.5%) were on the Nexplanon (etonogestrel 68 mg) hormonal contraceptive implant at the time of sample collection. As described in Table 2, healthy volunteers had significantly shorter LT and ttPeak as compared to trauma patients in the whole blood assay. However, in the plasma thrombin generation assay, the healthy volunteers showed no difference in LT and had a significantly longer ttPeak compared to the trauma patients. Healthy volunteers also had significantly lower ETP than trauma patients using the whole blood assay, but this difference was not significant with the plasma thrombin generation assay. When the healthy volunteers were evaluated by sex, there were no differences in the thrombin generation profiles between males and females.

TABLE 1 Demographics and clinical characteristics for the trauma cohort (n = 64) and healthy volunteers (n = 13)

	Trauma patients	Healthy volunteers
Age, y	52 (35-63)	35 (25-39)
Male, n (%)	46 (71.9)	5 (38.5)
Female, n (%)	18 (28.1)	8 (61.5)
ISS	17 (10-24)	…
Blunt mechanism (%)	62 (97%)	…
Any blood product transfusion, n (%)	27 (42.2)	…
Massive transfusion requirement, n (%)[a]	10 (15.6)	…
VTE, n (%)	6 (9.4)	…

Note: Results are presented as median with interquartile range.
ISS, injury severity score; VTE, venous thromboembolism.
[a]Massive transfusion threshold as defined by Savage et al.[12]

Among the trauma cohort, the absolute values for each thrombin generation parameter were different between the whole blood– and plasma-based assays as described in Table 2. However, there was statistically significant correlation between the two assays for each thrombin generation parameter (Table 3). Additionally, when select patients with outlier values for individual thrombin generation parameters (Figure 1A-1D) were removed from the analysis, the correlation between the two assays was persistent.

Six patients within the trauma cohort developed symptomatic VTE during hospitalization, with two PEs and four DVTs. Thrombin generation characteristics of these patients are described in Figure 1A-1D.

We also compared both the plasma and whole blood thrombin generation assay values against the international normalized ratio (INR) for each of the trauma patients. INR was selected as a comparison because this is a standard measure of coagulation that is collected on all trauma patients on arrival at our institution, and is widely used at most medical centers. The median INR for all patients was 1.1 with a range of 1.0-1.6. There was no statistically significant Spearman or Pearson correlation between INR and any parameter of either the plasma or whole blood thrombin generation assay.

At the time of injury, 13 patients in our trauma cohort were taking antiplatelet medications, with 12 on aspirin alone and one on both aspirin (81 mg) and clopidogrel (Plavix, 75 mg/d). Of the patients on aspirin alone, two were on full dose (325 mg), one was on an unknown dose, and the rest were on low-dose aspirin (81 mg). When reviewed separately from the rest of the cohort, there were no statistically significant correlations between the plasma and whole blood thrombin generation parameters for these 13 patients. However, when the remaining trauma patients who are not on any antiplatelet agents (n = 50) were analyzed alone, the correlation coefficients between plasma and whole blood thrombin generation increased (Table 3). Overall, trauma patients on antiplatelet medications have significantly decreased PH (nM) as compared to those who are not on those medications in both the plasma (265.8 [IQR, 218.8-309.3] vs 229.1 [IQR, 203.4-241.7]; $P = .02$) and whole blood assays (111.2 [IQR, 100.9-128.7] vs 98.0 [IQR, 81.6-110.9]; $P = .05$). Trauma patients on antiplatelet medications also had a significantly decreased ETP (nM × minute) as compared to those not on those medications with the plasma assay (1383 [IQR, 1222-1549] vs 1241 [IQR, 1103-1358]; $P = .03$), but this difference did not reach statistical significance in the whole blood assay (816.3 [IQR, 714.4-899.9] vs 687.6 [IQR, 617.9-732.1]; $P = .05$).

For each of the whole blood patient and control assays (NPT-TG Coag Control), CV was calculated among the three thrombin generation curves. For the whole blood assays, CV range for LT was 0.93%-7.37%, CV range for ttPeak was 0.60%-7.48%, CV range for PH was 0.12%-15.8% (3/64 samples had a CV > 10% for PH), and CV range for ETP was 0.93%-19.2% (21/61 samples a CV > 10% for ETP). For the control assays, CV range for LT was 1.69%-14.85% (1/44 tests with CV > 6%), CV range for ttPeak was 1.05%-6.72%, CV range for

TABLE 2 Comparison of individual plasma and whole blood thrombin generation parameters for trauma patients and healthy volunteers expressed as median (interquartile range)

Test parameter	Trauma patients (n = 64[a])	Healthy volunteers (n = 13)	*P* value
Lag time (minutes)			
Plasma	3.6 (3.2-4.7)	3.6 (3.2-3.6)	.36
Whole blood	4.3 (3.5-4.8)	3.2 (2.9-3.5)	**<.01**
Peak height (nM)			
Plasma	248.9 (214.1-302.0)	164.8 (130.4-211.9)	**<.01**
Whole blood	109.1 (97.4-128.2)	110.3 (101.3-125.4)	.69
Time to peak, min			
Plasma	6.4 (5.7-7.7)	7.5 (7.0-8.2)	**.02**
Whole blood	7.3 (6.3-8.2)	5.6 (5.3-5.8)	**<.01**
Endogenous thrombin potential (nM × min)			
Plasma	1, 358 (1168-1521)	1206 (1129-1472)	.29
Whole blood	784.0 (685.0-892.0)	566.0 (505.0-743.0)	**<.01**

[a]Plasma thrombin generation results are missing for one trauma patient.

Bold values indicate a *P*-value of < .05 and are considered significant.

TABLE 3 Spearman and Pearson correlations between plasma and whole blood thrombin generation parameters in all trauma patients (n = 63) presented in the top half of the table

Thrombin generation parameters (all trauma patients)	Pearson correlation coefficient	*P* value	Spearman correlation coefficient	*P* value
Lag time, min	0.55	**<.01**	0.40	**<.01**
Peak height, nM	0.44	**<.01**	0.466	**<.01**
Time to peak, min	0.555	**<.01**	0.356	**<.01**
Endogenous thrombin potential, nM × min	0.095	.46	0.54	**<.01**
Thrombin generation parameters (patients not on antiplatelet)				
Lag time, min	0.65	**<.01**	0.47	**<.01**
Peak height, nM	0.57	**<.01**	0.53	**<.01**
Time to peak, min	0.67	**<.01**	0.42	**<.01**
Endogenous thrombin potential, nM × min	0.22	.13	0.68	**<.01**

Note: Spearman and Pearson correlations between plasma and whole blood thrombin generation parameters for the trauma patients who are not on any antiplatelet agents (n = 50) presented in the bottom half of the table.

Bold values indicate a *P*-value of < .05 and are considered significant.

PH was 1.19%-108.24% (7/44 tests with CV > 7%), and CV range for ETP was 1.30%-58.93% (8/44 tests with CV > 10%, and 2 of those with CV > 15%).

4 | DISCUSSION

In this pilot study, we sought to compare and correlate thrombin generation parameters from the well-established plasma thrombin generation assay with a novel POC prototype assay for whole blood thrombin generation. We found a modest but statistically significant correlation between the plasma and whole blood assays for all thrombin generation parameters in trauma patients.

The raw values for each thrombin generation parameter from these two assays differ, as anticipated, given that they employ different tools, methodologies, and sample types (whole blood vs plasma) to measure the same pathway. Some of the differences in these values may be explained by the presence of platelets and cellular components

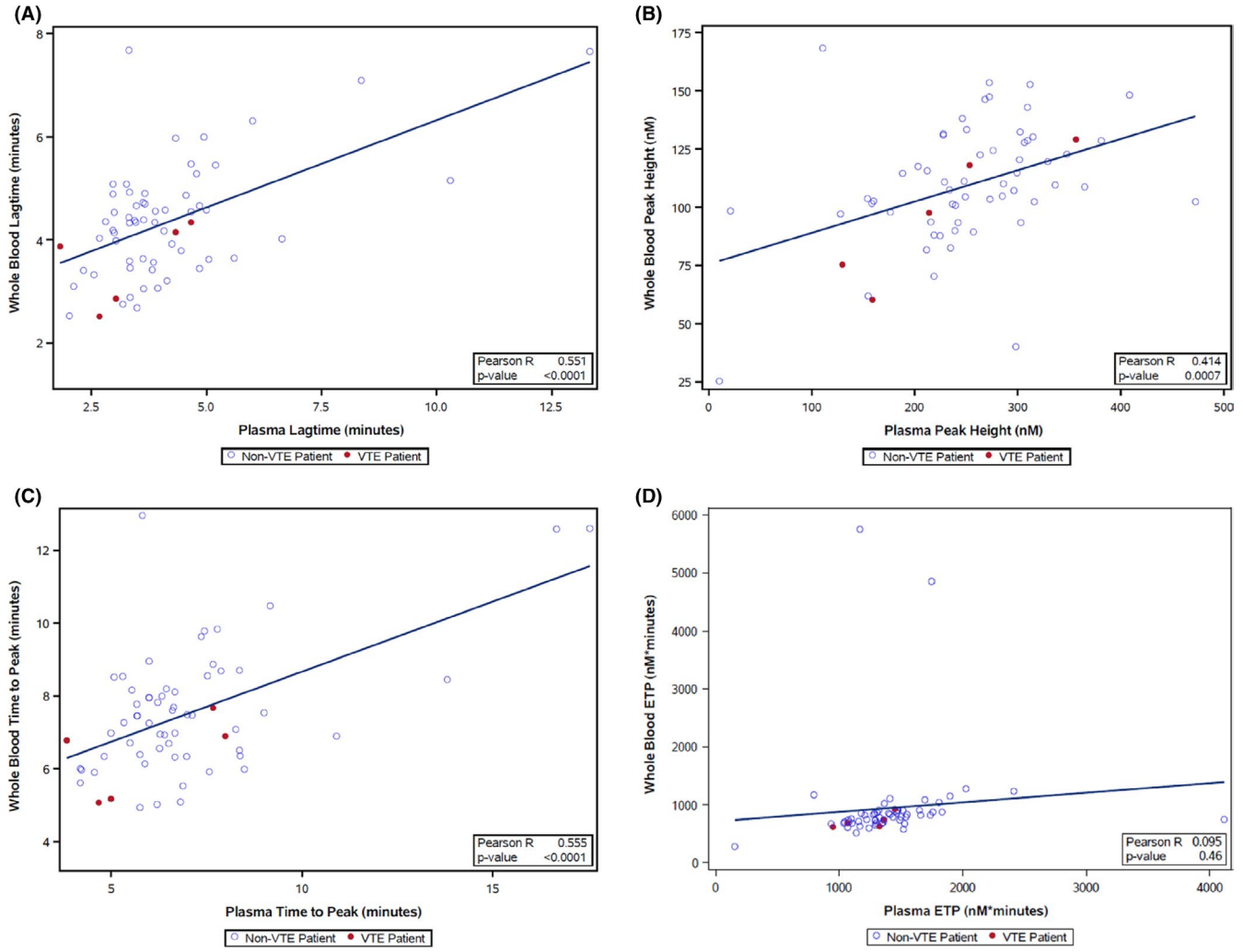

FIGURE 1 A, Linear regression models for individual plasma and whole blood thrombin generation parameters. Trauma patients who developed venous thromboembolism (VTE; n = 5) are shown in the filled-in circles, non-VTE trauma patients (n = 58) are shown in the open circles. One trauma patient who developed a VTE is not shown due to lack of plasma thrombin generation data

in the whole blood assay as compared to plasma. Platelets play an important role in controlling the thrombin burst that results in fibrin clot formation.[13] Additionally, red blood cells have been shown in vitro to augment prothrombin activation and bolster thrombin release through the meizothrombin pathway.[14,15] Differences in the levels of thrombin generation between whole blood and plasma due to the presence of differing cellular components was also hypothesized and demonstrated by Coleman et al[16] in another study of this novel whole blood thrombin generation assay, and our results are consistent with this. These differences in part explain why the correlations between the two assays are modest, as these assays are complementary; showing different aspects of an individual's clotting profile.

When the trauma cohort was analyzed with the 13 patients who were taking antiplatelet agents removed, correlations between the plasma and whole blood thrombin generation assays increased (Table 3). This emphasizes the important role of platelets in thrombin generation that is captured in the whole blood assay. The important role of platelets is also supported by the fact that the patients taking antiplatelet agents had lower PH than the rest of the trauma patients. This indicates that these patients may not make as much thrombin, likely due to thrombin generation being reduced by those medications.

Prior work from our lab showed that plasma ttPeak is an independent predictor of VTE development up to 92 days after trauma.[10] As shown in Figure 1, among the 5 VTE patients who have thrombin generation data available, the majority appear to have shorter LT and ttPeak than trauma patients who did not develop VTE. This should continue to be examined in a larger cohort to evaluate if the whole blood thrombin generation assay has a similar predictive value for VTE after trauma to the plasma thrombin generation assay.

Interestingly, the healthy volunteers in this study showed more accelerated thrombin generation than trauma patients using the whole blood assay. In this small pilot study, we had only 13 healthy volunteers, the majority of which were premenopausal females, while the trauma cohort was predominantly middle-aged males. This is because enrollment of healthy controls had to be paused early due to the coronavirus disease 2019 pandemic. Though data are limited,

prior studies have shown that reproductive-age females have more accelerated thrombin generation than males and that this can be influenced both by menstrual cycle stage and use of hormonal contraceptives.[17-20] Additionally, prior studies have shown an acceleration of thrombin generation with age in healthy adults using the plasma CAT assay.[21] The impact of age on the whole blood thrombin generation assay has not yet been established, and it may be that varying levels of cellular components and hormones with age may impact this assay differently.

This study has several additional limitations. First, given that this is a pilot study with a small number of patients, selection bias is certainly a concern. Additionally, because blood samples were collected up to 12 hours from the time of injury, 18 of the 64 trauma patients received some blood product transfusion before sample collection. Blood product transfusion can influence thrombin generation by introducing exogenous clotting factors, thus altering an individual's thrombin generation profile. Additionally, prior studies have isolated large numbers of procoagulant microparticles from stored blood and have demonstrated the ability of microparticles to generate thrombin through a factor XIa–mediated pathway in the absence of TF, which could also impact thrombin generation results in patients who received blood product transfusion before sample collection.[22-24] Additionally, as described in the methods section, samples for the whole blood assay were collected in 3.2% citrated tubes containing 100 µg/mL CTI, while the samples from the plasma-based assay were collected in 3.2% citrated tubes that did not contain CTI. Because of this difference in collection technique, the samples used in the plasma assay could have had the intrinsic pathway stimulated before addition of CTI to plasma at the time of the assay. This may be one reason to explain why the correlations between the two assays were not more robust.

We observed that when patients taking antiplatelet medications were removed from the trauma cohort, correlation coefficients improved between the plasma and whole blood thrombin generation assays. These results should be interpreted cautiously as these patients were heterogeneous in terms of what medications and dosages they were taking, and we do not know to what extent they were taking these medications as prescribed. As such, these results may reflect varying degrees of platelet inhibition.

5 | CONCLUSION

The novel POC prototype whole blood thrombin generation assay has potential clinical utility in the care of trauma patients, given that it can be run near the patient as opposed to the standard plasma-based thrombin generation assay, which is not amenable to bedside use. Our pilot study shows that in trauma patients, the plasma and whole blood thrombin generation assays significantly correlate. A larger cohort is needed to assess if the novel whole blood thrombin generation assay is comparable to the plasma-based assay in predicting VTE and hemorrhagic complications, as we have shown previously in our laboratory.[25]

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RELATIONSHIP DISCLOSURE

KGM holds consulting positions for Stago Diagnostica and Baxalta/Shire; he owns stock with Novo Nordisk, Catalyst, Baxter, Allergen, and Alnylam, and is the former owner of Haematologic Technologies. All other authors, including MJF, TM, GS, JI, KRB, RK, SB, SH, EL, DS, and MSP have no relevant conflicts of interests or disclosures.

AUTHOR CONTRIBUTIONS

MJF: study design, data collection and analysis, critical writing, and revising of manuscript; TAM: data analysis and interpretation, critical writing, and revising of manuscript; SB: study design and critical revising of manuscript; KGM: study design and critical revising of manuscript; GMS: critical revising of manuscript and data analysis and interpretation; KRB: critical revising of manuscript,and data analysis and interpretation; RAK: study design and critical revising of manuscript; SFH: critical revising of manuscript; EAL: data interpretation and critical revising of manuscript; DS: study design, data interpretation, and critical revising of manuscript; MSP: study design, data collection, data analysis and interpretation, critical writing, and revising of manuscript.

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