Whole blood thrombin generation profiles of patients with cirrhosis explored with a near patient assay

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Abstract

Background and Aims: Patients with cirrhosis have a rebalanced hemostasis, often with normal or elevated thrombin-generating (TG) capacity in plasma. Whole blood (WB) TG allows faster determination and, importantly, includes the influence of all circulating blood cells. We aimed to study the TG profile of patients with cirrhosis in WB and in platelet poor plasma.

Methods: Thrombin-generating capacity in WB and plasma were assessed with a near-patient WB-TG assay and the calibrated automated thrombinography assay, respectively. TG assays were tested in presence and absence of thrombomodulin. Conventional coagulation tests were also performed.

Results: Thirty-four patients with cirrhosis and twenty-two controls were analyzed. Compared with controls, patients had substantially deranged results in conventional coagulation tests. Comparable WB-TG capacity (endogenous thrombin potential until peak, ETPp) but significantly lower peak thrombin were found in patients, and these results persisted when thrombomodulin was present. TG of the patients was more resistant to thrombomodulin than controls in both WB and plasma, although the inhibitory effect of thrombomodulin was drastically weaker in WB than in plasma. The peak of WB-TG in patients correlated moderately with their hematocrit and platelet count. Significant correlations were found between TG results in WB and plasma.

Conclusions: The WB-TG assay shows a normal to hypocoagulable state in patients with cirrhosis with a decreased anticoagulant activity of TM compared to plasma-TG. The clinical value of this assay needs further validation.

Keywords
chronic liver disease, coagulation, thrombin generation, bleeding, thrombosis
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1 | INTRODUCTION

Patients with liver dysfunction may acquire substantial alterations in their hemostatic system because the liver is responsible for the synthesis of the majority of hemostatic proteins. Conventional coagulation tests, such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT), are frequently prolonged in patients with cirrhosis, suggesting a generalized tendency toward bleeding. However, PT and APTT are only sensitive to changes in the procoagulant pathways, therefore reflecting only the abnormalities of the pro-coagulant proteins in cirrhosis, but providing little information about the anticoagulant pathways including antithrombin and proteins of the protein C pathway. Anticoagulant pathways may also be impaired in cirrhosis. Clinical data show that cirrhotic patients are not “auto-anticoagulated,” but rather have equal or even higher thrombotic risk than patients without liver disease. These observations have led to the concept of re-balanced hemostasis to explain the complex coagulation profiles in patients with cirrhosis. Although a hemostatic balance is reached in cirrhosis, this equilibrium is more fragile and may easily tip toward a hypo- or hypercoagulable state; however, traditional coagulation tests are not suitable to assess the functioning coagulation profiles in these patients.

Thrombin generation (TG) is a sophisticated laboratory test capable of assessing the complex interplay between pro- and anti-coagulant pathways, thus reflecting the function of the global hemostatic system. Modern TG assays, such as the calibrated automated thrombomicrography (CAT) assay, enable continuous TG measurement in plasma and have been shown to be predictive of thrombotic or bleeding risk in various coagulation disorders. The onset, rate, and total capacity of TG in a given sample are represented by different parameters of a TG curve, ie, the lag time, peak thrombin, and endogenous thrombin potential (ETP), respectively. By adding an activator of the anticoagulant protein C pathway, eg, thrombomodulin (TM) or Protac, a TG assay reflects the balance between all pro- and anticoagulant drivers. Studies using the TM-modified TG assays have revealed that the impaired procoagulant system is compensated for by the reduced anti-coagulant function in liver disease. This is reported to occur in both chronic liver disease and acute liver injury/failure. These observations are in agreement with the clinical observations that there is no increased hemostasis-related bleeding risk for most patients.

Although the TM-modified TG assay was a crucial step forward in understanding the net effects of the multiple alterations in coagulation in cirrhosis, there are still several questions that remain to be answered. Standard TG assays are performed with platelet poor plasma (PPP) in the absence of blood cells, with synthetic phospholipids (PL) added to provide surfaces for the assembly of tenase and prothrombinase. These conditions may be different from physiological coagulation, which depends on the surfaces provided by activated platelets and most probably also erythrocytes. During physiological coagulation there is a continuous interplay among coagulation initiation, platelet activation, propagation of coagulation, and erythrocyte suppletion. In cirrhosis, anemia and thrombocytopenia are common and this could lead to reduced availability of procoagulant surfaces, which could potentially limit the rate and even capacity of TG. A study assessing TG in the platelet rich plasma (PRP) of cirrhotic patients demonstrated that platelet count is an important determinant of TG. Although PRP-TG reflects the influence of platelets, it does not measure the additional impact of other blood cells. On the contrary, whole blood (WB)-TG includes the influence of all circulating blood cells. Another practical advantage of performing WB-TG over plasma TG is that no centrifugation is needed, avoiding potential pre-analytical variation and allowing faster results, which is crucial for point-of-care applications in critically ill or actively bleeding patients.

We have previously developed a near patient WB-TG assay by using a miniaturized TG device and a microfluidic chip. This assay was shown to be predictive of postoperative blood loss in patients undergoing elective cardiac surgery. The short turn-around time and compact size of the near patient WB-TG assay system allows possible deployment outside a specialized laboratory environment. In this study, for the first time, we studied WB-TG profiles of a cohort of patients with cirrhosis by using the near patient assay.

2 | MATERIALS AND METHODS

2.1 | Patients and healthy controls

Patients with cirrhosis and healthy controls were recruited at King’s College Hospital NHS Foundation Trust, London. The study was approved by the London (Camden & Islington) Research and Ethics Committee (REC number: 11/LO/0706) and the local Research and Development department at King’s College Hospital NHS Foundation Trust. All participants provided written informed consent prior to study entry; where this was not possible, consultee permissions were obtained from their next of kin. Subjects were excluded from the analysis if they had acute liver failure or had received any anticoagulant or anti-platelet therapy.
2.2 | Blood collection and plasma preparation

Blood was collected into vacuum tubes (1 volume 0.109 mol/L trisodium citrate to 9 volumes blood; VACUETTE®, Greiner Bio-One). The blood was kept at room temperature and used within 4 hours after collection. PPP was prepared by double centrifugation of citrated WB at 2840 g for 10 minutes. Plasma was aliquoted and frozen at −80°C before analysis.

2.3 | WB-TG as measured with a modified near patient assay

WB-TG was measured on a previously described miniaturized TG device and a newly modified microfluidic chip. The previous chip, which was made from methyl methacrylate-acrylonitrile-butadienestyrene (MABS), suffered from occasional fluid leakage. We adopted a new design for the chip, which consisted of two pieces of polydimethylsiloxane (PDMS) with a porous matrix disc of 5 mm in diameter and 190 µm in thickness (grade 589/1; Whatman) in between. The two PDMS parts were prepared in house by mixing 10 parts of polymer with 1 part of curing agent (Sylgard 184, Dow Corning) and then solidified in an aluminum mold at 100°C for 90 minutes. After treatment in a plasma cleaner (PDC-32G-2, Harrick Plasma) for 15 minutes, the two PDMS parts were then assembled with filter paper in the center of the chamber between the two PDMS parts. Permanent bonds formed between the two PDMS parts, hence leakage was effectively prevented.

Citrated blood (15 µL) was mixed with 15 µL trigger solution containing 600 µmol/L fluorogenic thrombin substrate (ZGGR)$_2$-Rhodamine 110 (P$_2$Rho; Mercachem BV), 5 pmol/L recombinant human tissue factor (TF; Siemens Healthcare), and 33.4 mmol/L CaCl$_2$ in buffer containing 20 mmol/L HEPES, 140 mmol/L NaCl, and 5 g/L bovine serum albumin (Sigma) with a pH of 7.35. Fifteen microliters of the above mixture was immediately injected into the inlet of the microfluidic chip and the fluorescence measurement was started. An additional measurement was also done with soluble TM (U-protein Express) added into the TF-containing trigger solution. The final concentrations were 50% volume blood, 2.5 pmol/L TF, 16.7 mmol/L CaCl$_2$ and 300 µmol/L P$_2$Rho in the presence or absence of 150 nmol/L TM. This TM concentration was chosen to inhibit TG by 50% in normal pooled plasma (NPP). TG parameters were calculated with Thrombinscope software version 5.0, and TG parameters including the lagtime, TTP, peak, and ETP were chosen for further analyses.

In each run of TG measurement, NPP was also measured on the same plate. The ETP and peak values of the study subjects were normalized as the percentage of the ETP and peak of the NPP tested without TM in the same run, respectively. For better standardization and comparison, the PPP-TG parameters of samples measured in the presence of TM, including peak$_{+}^{+}$ and ETP$_{+}^{+}$, were also normalized as the percentage of the ETP and peak of the NPP tested without TM in the same run. The preparation of the NPP has been described previously. Blood from 116 healthy adult volunteers who gave written consent and did not take any anticoagulant or antiplatelet drugs for at least 2 weeks before blood draw was collected at Maastricht University Medical Center. After an initial centrifugation step (2500 g, 5 minutes) plasma was pooled, followed by ultracentrifugation (100 000 g, 10 minutes). Aliquots of 500 µL were snap-frozen in liquid nitrogen and stored at −80°C until analysis.

2.4 | PPP-TG measurement with the CAT assay

TG in PPP was measured with the CAT technique on a Fluoroskan Microplate Fluorometer (Thermolabsystems OY) as originally described. The final concentration of TF was 5 pmol with 4 µmol/L PL in the absence or presence of 10 nmol/L TM. The concentration of TM was chosen to inhibit TG by 50% in normal pooled plasma (NPP). TG parameters were calculated with Thrombinscope software version 5.0, and TG parameters including the lagtime, TTP, peak, and ETP were chosen for further analyses.

2.5 | Standard laboratory measurements

All coagulation assays were performed on the automated analyzer STA-R as previously described. Reagents were obtained from Diagnostica Stago for all coagulation assays which were performed on the automated analyzer STA-R Evolution (Diagnostica Stago). APTT and international normalized ratio (INR) were measured by coagulation-based assays, with STA-Cephascreen and STA-Neoplastine, respectively. Clauss methodology was utilized to quantify fibrinogen with STA-Fibrinogen. Antithrombin activity was measured by a chromogenic substrate method, with STA-Stachrom
ATIII. Factor (F) II, VIII, and X were quantified with STA-deficient II, VIII, X immune-depleted plasmas using coagulation-based assays. Full blood cell count analysis was performed by a flow cytometry method and hemoglobin by a colorimetric method, with reagents from Bayer Diagnostics and the Advia 2120 automated hematology analyzer (Bayer Diagnostics).

2.6 | Statistics

Statistical analyses were performed with SPSS version 25 and graphs were generated using GraphPad Prism software version 6. Normality of the data was assessed using the Shapiro-Wilk test. Data are represented as median with interquartile range (IQR). Comparisons between two independent groups were performed with the Mann-Whitney test. Fisher’s exact test was used to compare nominal data between groups. The Spearman test was used for the correlation analysis. A two-sided P-value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Characteristics of patients and controls

In total 34 patients with cirrhosis and 22 healthy donors were included in the analyses. As shown in Table 1, the patient group was older (P < .001) but had similar gender distribution compared with controls. The etiology of cirrhosis included alcohol related liver disease (n = 17), non-alcoholic fatty liver disease (n = 7), viral hepatitis (n = 3), and primary sclerosing cholangitis (n = 3). In addition, drug induced liver injury, primary biliary cirrhosis, non-alcoholic steatohepatitis, and autoimmune disease were each found in one patient.

Four patients were classified as Child-Pugh class A, 18 patients were in class B, and 12 patients in class C. The median Model for End-Stage Liver Disease (MELD) score of the patients was 17.5 (IQR: 14.3-23.8). Twenty-six patients had acutely decompensated (AD) cirrhosis, with a CLIF-AD score of 51 (45-53). Six patients fulfilled criteria for chronic liver failure (ACLF), of which three patients had grade 3 (ACLF). The peak and ETPp™ values of the patient plasma were significantly higher than the controls (35.8% and 58.7% higher in median values; P = .009 and P < .001, respectively; Table 2 and Figure 1C).

3.2 | Conventional coagulation tests and cell counts

Patients with cirrhosis had significantly distorted coagulation profiles compared to healthy controls, as shown in Table 1. Patients had significantly higher INR and longer APTT (P < .001 for both). Lower levels of both pro- and anticoagulant factors were found in the patients, such as FII, FX, and antithrombin, except FVIII, which was higher (P < .001 for all factors compared with control). Clauss fibrinogen was comparable between the patients and controls.

3.3 | WB-TG profiles

Figure 1A shows representative WB-thrombograms of the patients and controls. As summarized in Table 2 and shown in Figure 1B, patients had significantly longer lagtime and TTP than controls (P < .01 for both; Figure 1B). The peak thrombin value was substantially lower in patients than in controls (median values were 118 and 179 nmol/L for patients and controls, respectively; P < .001). However, the ETPp was not significantly different between the patients and controls (230 [196-294] versus 247 [222-280] nmol × min/L, respectively; P = .356).

The addition of 150 nmol/L TM into the WB-TG assay substantially prolonged the lagtime in both patients and controls to a similar extent of 37% (P = .926). Conversely, the added TM only exhibited a weak inhibitory effect on ETPp. TM-induced inhibition on the ETP was slightly lower in patients at 10.3 (2.0-19.9) % than in controls at 18.9 (12.7-27.5)% (P = .048). In the presence of TM, the ETPp™ was still similar between patients and controls (P = .709), whereas the peak™ remained lower in patients (P < .001; Table 2 and Figure 1C).

3.4 | PPP-TG profiles

We obtained TG data from 28 out of the 34 patients, because PPP was not collected from 5 patients, and no TG curve was obtained from 1 of the patients. When PPP-TG was measured in the absence of TM, comparable lagtime and TTP values were found in patients and controls. The peak and ETP were slightly but significantly lower (12.2% and 14% lower in median values, respectively) in patients (Table 3 and Figure 2A). When PPP-TG was tested in the presence of TM, the peak™ and ETP™ values of the patient plasma were significantly higher than the controls (35.8% and 58.7% higher in median values; P = .009 and P < .001, respectively; Table 3 and Figure 2B).

3.5 | Correlations of TG parameters with other factors

We explored a possible relation between TG parameters and other factors. Age of the study subjects was counterintuitively correlated with longer lagtime (r = .435) and TTP (r = .405), as well as lower peak (r = -.301) of WB-TG, but not with ETPp (r = .021, P = .879; Table 4).
The PPP-TG parameters in the presence of TM also showed moderate to weak correlations with age, including the lagtime$^{\text{TM}}$ ($r = .394$) and ETP$^{\text{TM}}$ ($r = .284$).

Moderate to weak correlations were found between WB-TG parameters in the absence of TM with corresponding PPP-TG parameters in absence of TM, including the lagtime ($r = .282$), peak ($r = .468$), and ETP ($r = .415$; Table 4).

Positive correlations were found between the hematocrit of patients and their WB-TG parameters, including the peak (Spearman $r = .434$), ETPp ($r = .417$) (Table 4), and peak$^{\text{TM}}$ ($r = .374$). The peak value was also positively correlated with the platelet count of the patients ($r = .559$). The platelet count showed weaker correlation with the ETPp ($r = .304$, $P = .085$).

In addition, the platelet count was positively correlated with some parameters of the PPP-TG, including the lagtime ($r = .529$, $P = .004$), ETP ($r = .45$, $P = .016$), and lagtime$^{\text{TM}}$ ($r = .528$, $P = .004$). No significant correlation was found between hematocrit and PPP-TG parameters.

Cirrhosis severity, as represented by the Child-Pugh classification and MELD score, was correlated with longer lagtime and lower peak of WB-TG, both in the absence and presence of TM ($P < .05$ for all correlations). The above scores were also correlated with lower peak of PPP-TG ($P < .05$ for both correlations).
DISCUSSION

We studied the TG profiles of patients with cirrhosis in WB using a near patient WB-TG assay and in PPP with the standard CAT assay. WB-TG results showed normal TG capacity but lower TG rate in patients with cirrhosis, suggesting normal- to hypocoagulability; whereas TM-modified PPP-TG suggested hypercoagulability, with both higher TG capacity and rate found in cirrhosis. The WB-TG parameters showed moderate correlations with platelet count and hematocrit, as well as the standard PPP-TG parameters. We also surprisingly found that WB-TG is less sensitive to the anticoagulant function of TM than PPP-TG.

TG assays have been extensively used to study the coagulation profiles of patients with liver disease, and studies using TM-modified plasma TG assay have shown intact or elevated TG potential in cirrhosis, supporting the concept that these patients have re-balanced hemostasis. However, until now all studies on TG profiles in these patients were performed in plasma samples, which has limitations, such as the requirement for a specialized laboratory environment, a longer turnaround time for results, and the inability to study the effect of most blood cells (except platelets) on TG. Until now there are no published data of WB-TG measurements in liver disease patients. Although whole blood viscoelastic assays have been tested in liver disease patients and shown additional value in reducing blood product administration, they do not assess the protein C pathway, and their ability to predict clinical outcome is still unclear.

Using the near patient WB-TG assay, we observed that the endogenous thrombin generating potential (ETPp) values of patients with cirrhosis, regardless of the presence/absence of TM, were in the same range as in healthy controls, suggesting that these patients had normal thrombin-generating capacity. The standard PPP-TG results add to the growing literature that hypocoagulable state is suggested when TG is tested without activating the protein C pathway, whereas hypercoagulable state is found when the function of

| Table 2: Whole blood thrombin generation profiles of patients and controls |
|-----------------------------|------------------|--------|
| WB-TG parameters in the absence of TM | | |
| Lagtime (min) | 5.1 (4.2–6.1) | 3.3 (2.8–3.9) | <.001 |
| TTP (min) | 8.4 (7.3–9.9) | 5.7 (5.1–6.8) | <.001 |
| Peak (nmol/L) | 118 (98–145) | 179 (164–192) | <.001 |
| ETPp (nmol × min/L) | 230 (196–294) | 247 (222–280) | .356 |
| WB-TG parameters in the presence of TM | | |
| Lagtime™ (min) | 6.6 (6.1–9.3) | 4.6 (4.1–5.2) | <.001 |
| TTP™ (min) | 10.9 (9.8–15) | 8.1 (7.1–8.9) | <.001 |
| Peak™ (nmol/L) | 79.7 (65.1–96.2) | 115 (104–127) | <.001 |
| ETPp™ (nmol × min/L) | 205 (181–238) | 195 (177–233) | .709 |

Note: Values are median (interquartile range).

Abbreviations: ETPp, endogenous thrombin potential until the thrombin peak; TG, thrombin generation; TM, thrombomodulin; TTP, time to peak; WB, whole blood
the anticoagulant protein C pathway is included in TG by adding TM into the test. These results indicate that cirrhosis does not necessarily result in a hypocoagulable state as suggested by their lower procoagulant factor levels and prolonged PT and APTT, thus reinforcing that prophylactic administration of fresh-frozen plasma necessarily result in a hypocoagulable state as suggested by their cending platelet counts.

Of note, although the TG capacity of cirrhotic patients is comparable to the controls, their peak thrombin level is discordantly lower, suggesting a hypocoagulable state. The ETP and peak are usually well correlated in PPP-TG, but their relation in WB has not been extensively explored. The above discordance might be due to a differential effect of low cell count on peak and ETPp. In our experience and in literature, the platelet count much more strongly affects the peak of PRP-TG than the ETP. In fact the ETP plateaued once the platelet count reached a certain level (approximately $10^9$ platelets/L) but the peak still increases with further ascending platelet counts. The influence of erythrocyte count on WB-TG is less explored, but a recent study found that at a normal platelet concentration of $200*10^9$/L the peak of WB-TG was significantly augmented by increasing hematocrit from 0.2 to 0.45, whereas the ETP was largely unchanged. Therefore, the lower counts of platelets and erythrocytes in cirrhotic patients might have induced differential impact on the peak and ETP of WB-TG, and caused the above-observed discordance. Platelets and erythrocytes are involved in TG via many mechanisms, including but not limited to their expression of procoagulant surface. The activation/function of platelets in cirrhosis remains controversial. It has been reported that platelets are defective in aggregate formation, have a storage pool defect and defective transmembrane signaling in cirrhosis, while other studies have reported that blood cells from cirrhotic patients have abnormally high levels of PS exposure and this may contribute to their hypercoagulable state. Moreover, erythrocytes, as the most abundant cells in circulation, may also indirectly impact TG by augmenting platelet activation. Overall the function of blood cells in TG of liver disease patients is still poorly characterized and this WB-TG assay has potential to improve insight into this topic.

Interestingly, WB-TG showed less sensitivity to the anticoagulant function of TM than PPP-TG. A high dose (150 nmol/L) of TM only induced weak inhibition on WB-TG, as the ETPp in patients and controls was only inhibited by 10.3% and 18.9%, respectively. Although the WB-TG of cirrhotic patients showed increased resistance to TM
WAN et. al. compared to controls, the difference was only on the border of statistical significance ($P = .048$). Conversely, a much lower dose of TM (10 nmol/L) exhibited a much stronger inhibitory effect on the ETP of PPP-TG and the effect was five-fold weaker in the patients than in controls. The TM resistance in cirrhosis is most probably due to their reduced protein C and protein S, combined with the elevated FVIII levels. The high dose of TM, however, did substantially prolong the lagtime of WB-TG in both patients and controls, but this effect may be not via the anticoagulant function of the protein C pathway, but rather because TM prevented thrombin from activating platelets and consequently limited the availability of procoagulant phospholipids.

This study has several limitations. First, the number of study subjects was relatively low and a heterogenous group of patients was included. Second, the age of the cirrhotic patients was higher than the healthy controls. However, the effect of age on thrombin generation in the general population is modest, and in patients with cirrhosis this effect is even smaller as cirrhosis-related distortions of the coagulation system outweigh the effects of age on plasma levels of coagulation factors. This is further supported by our observation that older age was counterintuitively correlated with longer lagtime and lower peak of WB-TG. Furthermore, due to unknown reasons, WB-TG was more resistant to TM than PPP-TG despite being used at a high concentration. It is noteworthy that Lebretton et al. reported that plasmatic hypercoagulability of cirrhosis patients was detectable only when testing TG with the addition of TM but not activated protein C. Increased understanding of the function of TM in WB-TG is warranted to further optimize the TM-modified WB-TG assay for better evaluation of the protein C system. Future studies with larger sample size will be needed to explore the feasibility of this assay to assist coagulation management in liver disease patients.

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### CONFLICTS OF INTEREST

All authors state that they have no conflictS of interest related to this study.

### AUTHOR CONTRIBUTIONS

T. W. Ow, L. Rabinowich, and O. Barbouti recruited participants, and collected samples and patient data. J. Wan, W. Hendrix, and J. Konings performed thrombin generation experiments. R. Arya, W. Bernal, T. Lisman, L. N. Roberts, and M. Roest designed and supervised the study. V. C. Patel and B. de Laat assisted with analyses and writing of the paper. J. Wan, W. Bernal, T. Lisman, L. N. Roberts and M. Roest interpreted the data and wrote the manuscript. All authors approved the final version for submission.

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| TABLE 4 Correlations between WB-TG parameters and other factors | WB-TG parameters | Lagtime | Peak | ETPp | Lagtime™+ | Peak™+ | ETP™+ |
|---|---|---|---|---|---|---|---|
| Age | 0.435** | −0.301* | 0.021 | 0.408** | −0.207 | 0.033 |
| Platelet count | −0.147 | 0.559** | 0.304 | −0.346* | 0.342 | 0.027 |
| Hematocrit | −0.162 | 0.434* | 0.417* | −0.289 | 0.374* | 0.335 |
| PPP-TG parameters | | | | | | |
| Lagtime | 0.282* | 0.251 | 0.410** | 0.219 | 0.156 | 0.079 |
| Peak | −0.344* | 0.468** | 0.280* | −0.397** | 0.460** | 0.057 |
| ETP | −0.295* | 0.629** | 0.415** | −0.432** | 0.568** | 0.121 |
| Lagtime™+ | 0.449*** | 0.046 | 0.350* | 0.397** | 0.000 | 0.100 |
| Peak™+ | −0.008 | −0.250 | −0.178 | 0.042 | 0.006 | 0.009 |
| ETP™+ | 0.241 | −0.537** | −0.258 | 0.295* | −0.237 | 0.050 |

Note: Spearman correlation coefficients are shown.

Abbreviations: ETP, endogenous thrombin potential; ETPp, endogenous thrombin potential until thrombin peak; PPP, platelet poor plasma; TG, thrombin generation; TM, thrombomodulin; WB, whole blood

* $P < .05$.
** $P < .01$.
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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