Impact of soluble thrombomodulin and activated protein C on dynamic hemostatic function in trauma: a focus on thrombin generation and clot lysis

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Abstract

Trauma-induced coagulopathy describes a complex set of coagulation changes affecting severely injured patients. The thrombomodulin-protein C axis is believed to be central to the evolution of trauma-induced coagulopathy. Soluble thrombomodulin (sTM) levels are elevated after injury. Our objectives were to explore whether sTM (at concentrations found in patients after injury) plays an important role in trauma-induced coagulopathy, and specifically to evaluate the effects of sTM and activated protein C (APC) on thrombin generation (TG) and clot lysis time (CLT). Plasma from healthy volunteers was spiked with rising concentrations of sTM and APC and the effects on TG and CLT were analyzed. Plasma samples from a cohort of trauma patients were evaluated using TG and CLT, and results correlated to clinical parameters and factor VIII, factor V, APC, sTM and fibrinolytic measures. Increasing sTM concentrations in volunteer plasma led to reductions in endogenous thrombin potential and prolongation of 50% CLT, in a dose-dependent manner. No effect on TG or CLT was seen with rising APC concentrations. In 91 trauma patients, higher sTM values were associated with greater, rather than reduced, endogenous thrombin potential (median 1,483 *vs.* 1,681 nM/min) and longer 50% CLT (41.9 *vs.* 54.0 mins). In conclusion, sTM concentrations, across ranges found after trauma, affect both TG and 50% CLT, unlike APC. Despite increased circulating sTM levels, the overriding dynamic coagulation effects seen after injury are: (i) accelerated TG and (ii) increased rates of fibrinolysis. We found no evidence for sTM as the major determinant of the coagulation changes seen in early trauma-induced coagulopathy.

Introduction

Trauma-induced coagulopathy is a term describing the myriad changes to coagulation which occur after injury.[1] It is a complex and incompletely understood phenomenon, affecting one quarter of injured patients and is strongly associated with increased bleeding and a 3- to 4-fold greater risk of death.[1-3] The thrombomodulin (TM)-protein C axis is believed to be central to the evolution of trauma-induced coagulopathy.[3-7] Soluble thrombomodulin (sTM) levels are elevated after injury and are associated with poorer clinical outcome.[7-9]

TM plays several roles in hemostasis. In health, it is found on the endothelial surface, and binds thrombin avidly. The resultant thrombin-TM complex can activate two proteins: protein C and thrombin activatable fibrinolysis inhibitor (TAFI). When the thrombin-TM complex binds protein C presented by the endothelial protein C receptor, activated protein C (APC) is formed which can cleave and inactivate factor Va and factor VIIIa.[10] The resultant effect is a reduction in thrombin formation. Additionally, APC can bind to plasminogen activator inhibitor 1, leaving tissue plasminogen activator unopposed to promote fibrinolysis.[10] The thrombin-TM complex is also able to activate TAFI to TAFIa. TAFIa, a metallocarboxypeptidase, removes C-terminal lysines from fibrin, removing its binding sites for tissue plasminogen activator and plasminogen, thereby attenuating fibrinolysis.[11]

Recently, an inherited bleeding condition, caused by genetic variants within the TM gene, *THBD,* has been reported.[12-15] Although the variants differ, the common features include: a bleeding diathesis initiated by injury; a markedly elevated

sTM level (50- to 100-fold higher than normal); reduced thrombin generation (TG) and slower rates of fibrinolysis.[12-15] Clinically, the bleeding tendency suggests the reduction in TG outweighs the attenuated clot lysis. Taken together, these inherited conditions mirror some of the changes seen after injury (e.g., raised sTM levels, hypocoagulability, bleeding) and could add weight to the importance of the TM pathway in trauma-induced coagulopathy. The aims of this study were to explore whether sTM (at concentrations found in patients after injury) plays an important role in the early coagulopathy of trauma and specifically to evaluate the effects of sTM and APC on two dynamic hemostatic assays: TG and clot lysis.

Methods

Ethics approval and consent to participate

For the group of trauma patients, emergency consent was obtained from the trauma team leader who acted as the patient's legally authorized representative. Written informed consent from the patient, or next of kin, was obtained as soon after enrollment as appropriate. The study was reviewed and approved by East London Regional Ethics Committee (REC reference: 07/Q0603/29). For the healthy, volunteer group, written informed consent was obtained prior to sample collection. Ethical approval was granted by the Wales Research Ethics Committee (REC reference: 20/WA/0313).

Trauma patients and healthy volunteers

Adult trauma patients (≥ 16 years) at Oxford University Hospitals who met the criteria for trauma team activation were eligible. Details of the study have been published previously.[3] Up to 20 mL of blood were drawn within 20 minutes and analyzed for routine coagulation parameters, including rotational thromboelastometry (ROTEM). Remaining whole blood was spun (at 3,000 g, room temperature, for 20 min) to obtain platelet-poor plasma and stored at -80°C. Clinical data were collected on patients' demographics, mechanism of injury (blunt or penetrating) and vital signs. Blood transfusion requirements during the first bleeding episode were recorded.

Twenty milliliters of whole blood were drawn from healthy volunteers. Platelet-poor plasma was obtained from the citrated samples, as above, and stored.

Thrombin generation

TG was triggered with phospholipids (4 μM), a thrombin fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and calcium chloride ($CaCl_2$) (Diagnostic Stago, France). In some cases, recombinant human TM (0-256 ng/mL) (Peprotech Inc, UK), human APC (0-400 pM) (HYPHEN Biomed, France) or murine anti-TM antibody (1 μg/mL; ab6980, Abcam) was added. TG was measured using a calibrated automated thrombogram.[16]

Clot lysis

Platelet-poor plasma (30%), phospholipids (16 μM) (Rossix, Sweden), and tissue plasminogen activator (90 pM) (Sigma-Aldrich, USA) in 10 mM TRIS pH 7.4 0.01% Tween20 were added to 96-well flat-bottom plates. In some cases, potato tuber carboxypeptidase inhibitor (PTCI; 50 ng/mL) (Sigma-Aldrich, USA), recombinant human TM (0-64 ng/mL), murine anti-TM antibody (1 μg/mL) or human APC (0-400 pM) were incorporated. Clotting was initiated with 10.6 mM $CaCl_2$. Clot formation and lysis were monitored by light absorbance (405 nm), measured every 60 s for 4 h and analyzed using Shiny App software.[17]

Enzyme-linked immunosorbent assays

TM, factors V and VIII, antithrombin, plasmin-antiplasmin, APC, plasminogen activator inhibitor 1, thrombin-antithrombin, tissue plasminogen activator, and fibrinogen antigen levels were quantified in platelet-poor plasma. The kits used to measure plasmin-antiplasmin and APC were from Technozym, USA and 2b Scientific Ltd, UK, respectively. The other kits were from Abcam, UK.

Protein C activation assay

Human protein C (70 nM), sTM (0-200 ng/mL) in phosphate-buffered saline, 0.6 mM $MgCl_2$ and 1% bovine serum albumin were added to a 96-well plate. In order to initiate protein C activation, 0.1 U/mL thrombin and 3 mM $CaCl_2$ were added to the wells and incubated at 37°C for 30 min. The reaction was stopped with 1 U/mL hirudin (Sigma-Aldrich). A chromogenic substrate for APC, 0.42 mg/mL BIOPHEN CS-21(66) (HYPHEN BioMed), was added. In other experiments, APC (1.5625-100 nM) in phosphate-buffered saline, 0.6 mM $MgCl_2$ and 1% bovine serum albumin were mixed with 0.42 mg/mL BIOPHEN CS-21(66). Absorbance at 405 nm was measured every 30 s for 2 h.

Data analysis

Results are represented by mean ± standard deviation (SD) or median ± interquartile range (IQR), with comparisons made using *t* tests or Mann-Whitney tests, as appropriate. Normality was assessed using visual inspection of histograms and the D'Agostino-Pearson omnibus test. Statistical significance was set at $P<0.05$. Correlations were performed using Spearman tests. Normal ranges were calculated using samples from 20 healthy volunteers and 1.96 SD of the mean or log-transformed mean.[18] The statistical analysis was performed using Graph Pad Prism version 10.1.2.

Results

Ninety-one trauma patients were included. Their baseline characteristics are shown in Table 1. Twenty healthy volunteers were included (65% male, mean age 38.7 [SD=9.3] years). sTM levels were higher in the trauma cohort than

Table 1. Baseline characteristics of the trauma patients and values at hospital admission.

Characteristic	Trauma patients N=91
Age in years, mean (SD)	43.8 (20)
Injury severity score, median (IQR) [range]	10 (4-18) [0-43]
Male, N (%)	70 (77)
Blunt injury, N (%)	88 (96.7)
Isolated TBI, N (%)	9 (9.9)
GCS, median (IQR) [range]	15 (14-15) [3-15]
Time from injury to ED in min, mean (SD) [range]	87 (21) [19-120]
SBP, mmHg, mean (SD) [range]	139 (25) [70-203]
HR, bpm, mean (SD) [range]	87 (21) [51-143]
In receipt of TXA pre-admission, N (%)	41 (45)
Base excess, mEq/mol, median (IQR)	0.6 (-2.1 to 2.0)
Pre-hospital support	
Crystalloid, mL, median (IQR) [range]	0 (0-0) [0-1,000]
PRBC, units, median (IQR) [range]	0 (0-0) [0-3)
FFP, units, median (IQR) [range]	0 (0-0) [0-2)
Blood tests at admission	
Hemoglobin, g/L, mean (SD)	144 (14)
Platelet count, x10^9/L, mean (SD)	242 (69)
APTT, secs, median (IQR)	25 (22.2-28)
INR, ratio, median (IQR)	1.0 (0.9-1.0)
INR >1.2, N (%)	2 (2.2)
Clauss Fg, g/L, median (IQR)	2.7 (2.1-3.1)
D-dimer, ng/mL, median (IQR)	8,688 (2,199-19,400)
ROTEM	
EXTEM CA5, mm, mean (SD)	44 (8.5)
EXTEM CA5 <40 mm, N (%)	13 (14.2)
EXTEM ML, mean (SD)	10.5 (4.8)
FIBTEM CA5, mm, mean (SD)	13 (4.9)

SD: standard deviation; IQR: interquartile range; TBI: traumatic brain injury; GCS: Glasgow Coma Score; ED: emergency department; SBP: systolic blood pressure; HR: heart rate; TXA: tranexamic acid; PRBC: packed red blood cells; FFP: fresh-frozen plasma; APTT: activated partial thromboplastin time; INR: international normalized ratio; Fg: fibrinogen; ROTEM: rotational elastometry; CA5: clot amplitude at 5 minutes; ML: maximal lysis.

in healthy volunteers (Mann-Whitney, *P*=0.02) (Figure 1A). The median sTM in the trauma cohort was 9.9 ng/mL (8.2-11.6; range, 5.7-25.9 ng/mL) (normal range, 5.85-11.67 ng/mL). The median APC value in the trauma cohort was 66.7 pM (26.5-130.7 pM; range, 3.3-294.6 pM), which was higher than that in the healthy volunteers: 35.0 pM (23.9-53.8 pM) (Mann-Whitney, *P*=0.02) (Figure 1B). We then proceeded to evaluate the effects of the raised levels of sTM and APC on dynamic coagulation assays in normal plasma using concentration ranges of 0-64 ng/mL sTM and 0-400 pM APC, which encompass sTM and APC levels found in trauma patients.

The effects of soluble thrombomodulin and activated protein C on dynamic coagulation assays in healthy volunteers

Thrombin generation

TG was optimized for trauma conditions to maximize sensitivity for relevant sTM values. Microparticle reagent (4 µM phospholipid alone) was chosen as the trigger, as the use of tissue factor masked the effects of the sTM concentrations required for these experiments (*Online Supplementary Table S1*).

Effects of sTM: increasing sTM concentrations in volunteer plasma led to reductions in peak height and endogenous thrombin potential (ETP), and shortening of time to start tail, in a dose-dependent manner (Figure 2A, B; *Online Supplementary Table S2*). Previous work from our group showed that this effect can be reversed by adding an antibody against APC.[15]

Effects of APC: no significant differences were seen across rising concentrations of added APC for four of the measured TG parameters (lag time, ETP, peak height, time to peak) (Figure 2C, D; *Online Supplementary Table S3*). All APC concentrations led to prolongation of time to start tail, when compared to no APC, but there was no dose response thereafter. At higher concentrations (1 nM APC and above), significant reductions of ETP and peak height, with prolongation of lag time could be elicited (*data not shown*). This suggests that sTM at 'trauma levels' is sufficient to generate enough APC to reduce TG, but that the plasma APC 'trauma levels' are not sufficient to have the same effect. To explore this further, we compared the rates of cleavage of a chromogenic substrate sensitive to APC, using either increasing sTM or known concentrations of APC (Figure 3). These show that sTM, at similar concentrations (e.g., 25 ng/mL) to those found in trauma patients, can sufficiently activate protein C to cleave the chromogenic substrate, but a similar effect is only seen at 25 nM APC.

Clot lysis

Clot lysis was performed using 90 pmol of tissue plasminogen activator without added thrombin, to mirror the activation of clotting by phospholipid alone (i.e., no tissue factor) within the TG experiments.

Effects of sTM: there was a stepwise increase in 50% clot lysis time (CLT) (Figure 2E, F) with increasing sTM (0-16 ng/mL, 1-way analysis of variance [ANOVA], *P*<0.0001). There was no further change in 50% CLT between 16-64 ng/mL sTM (*P*=0.43). The effect of sTM was confirmed to be via TAFI activation, as shortening of CLT was elicited with addition of PTCI and/or anti-TM antibody (*Online Supplementary Figure S1*).

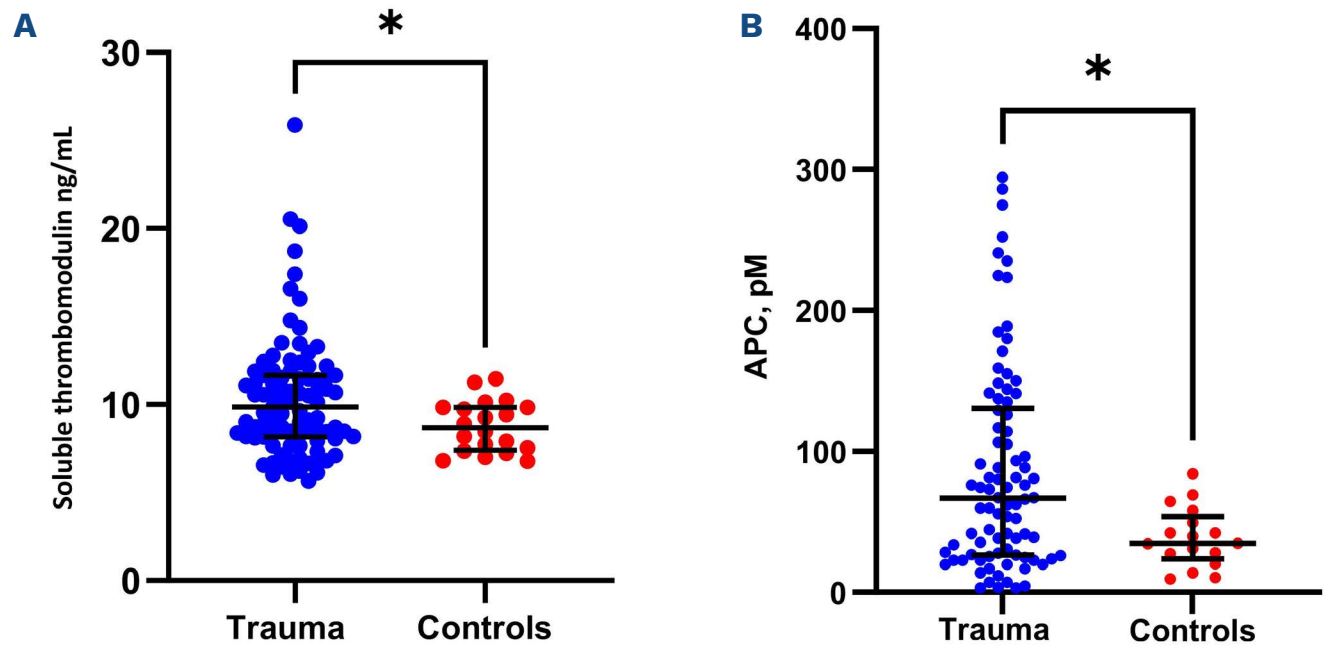


Figure 1. Soluble thrombomodulin and activated protein C levels in the trauma cohort and healthy volunteers. *P<0.05. APC: activated protein C.

Effects of APC: there was no change in 50% CLT (Figure 2G, H) with increasing trauma level APC concentrations. These results suggest that sTM attenuates CLT through its action on TAFI but that APC does not affect lysis at trauma concentrations.

Trauma cohort characteristics

The average age of the trauma cohort of patients was 43 years with 77% of the participants being male (Table 1). The median injury severity score was 10, and 28 (31%) of the cohort had severe injury, defined as an injury severity score >15. All but three had suffered a blunt injury (N=88, 96.7%) and nine had isolated traumatic brain injury (9.9%). Almost half (45%) received tranexamic acid (TXA) prior to admission and blood sample withdrawal. The TXA dosing was 1 g intravenous bolus followed by 1 g intravenous infusion over 8 h. Prior to admission, a few participants received packed red blood cells (N=4, 4.4%) or fresh-frozen plasma (N=2, 2.2%).

Thirteen participants (14.2%) had trauma-induced coagulopathy defined by an EXTEM CA5 less than 40 mm.[19] The median Clauss fibrinogen level was 2.7 g/L while the median D-dimer concentration was 8,688 ng/mL. APC levels were higher in the trauma cohort than in the volunteers, being 66.7 pM (26.5-130.7 pM) *versus* 35.0 pM (23.9-53.8 pM), respectively (Mann-Whitney, P=0.02). Coagulation parameters (Table 3) showed changes consistent with trauma-induced coagulopathy, notably significant fibrinolytic activity with very high D-dimer and plasmin-antiplasmin levels. Factor VIII levels were elevated at 2.91 IU/mL (P<0.0001). Factor V and antithrombin levels in the trauma cohort were no different from those in the volunteers, at 0.72 IU/mL and 0.97 IU/mL, respectively, suggesting both a lack of a significant effect of APC or evidence of disseminated intravascular coagulation, respectively. Twelve participants required transfusion within the first 12 hours after injury (13.2%) and represent the 'trauma bleeding' cohort, the other cohort had minimal transfusion requirements (Table 2).

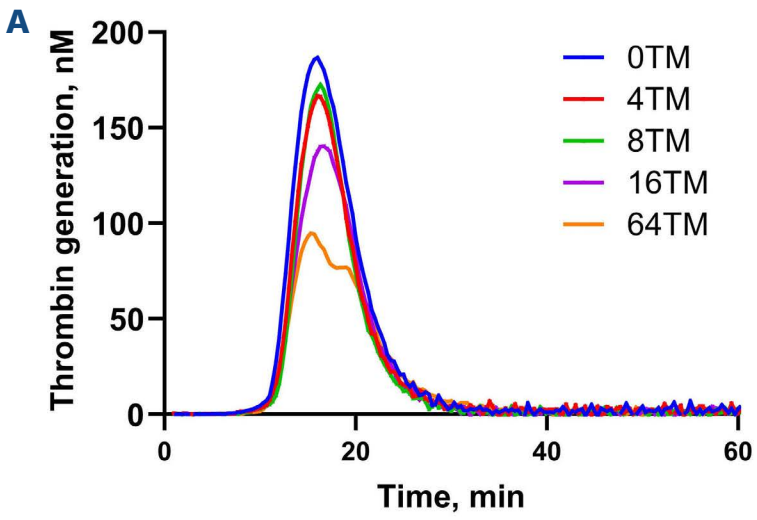
Thrombomodulin and activated protein C levels in the trauma cohort

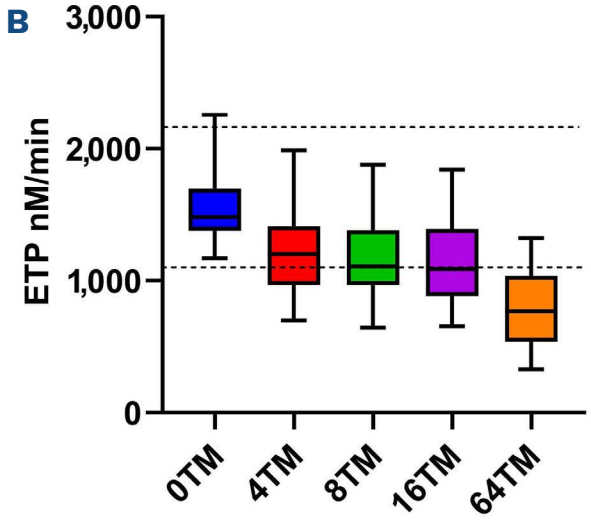
sTM values broadly rose with increasing clinical measures of shock, e.g. falling systolic blood pressure, rising heart rate and worsening base excess, but correlations were not found to be statistically significant. The injury severity score did not correlate with sTM levels, although broadly the score values rose as sTM levels rose. The sTM values for the 'trauma bleeding' cohort did not differ from those of the non-bleeding cohort (Mann-Whitney, P=0.81). sTM admission levels did not correlate with factor V or VIII levels, plasmin-antiplasmin levels, or more global clotting assays such as the International Normalized Ratio, activated partial thromboplastin time or the CA5 EXTEM ROTEM values (*data not shown*).

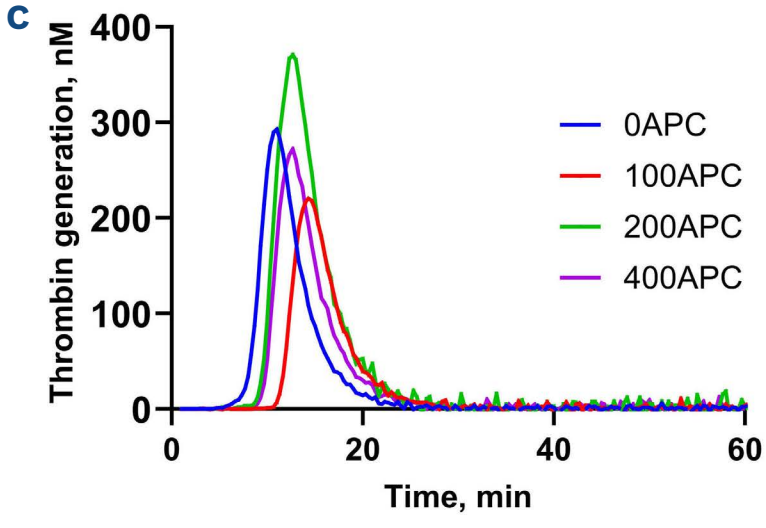
APC values did not correlate with admission sTM levels (P=0.44) and did not change with clinical parameters of shock or injury severity. The APC values for the trauma bleeding cohort did not differ from those of the non-bleeding cohort (Mann-Whitney, P=0.44). Admission APC levels did not correlate with admission concentrations of factor V or VIII. There was no association between 50% CLT and admission APC values.

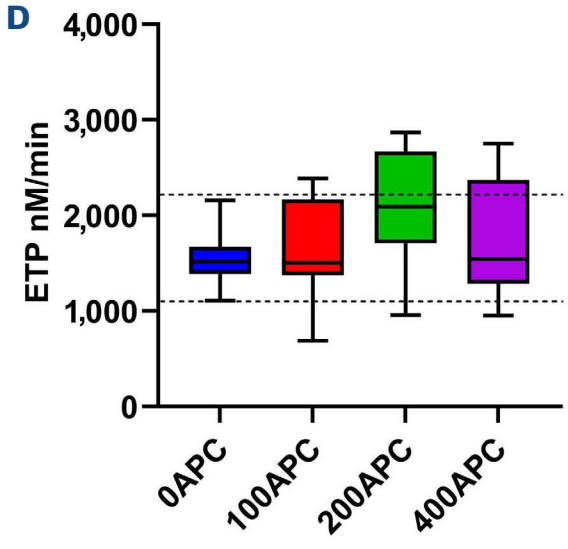
Thrombin generation in the trauma cohort

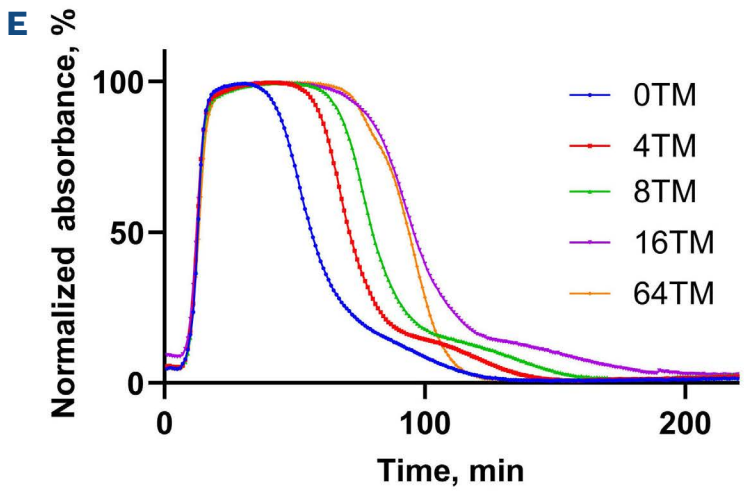
There were differences in all TG parameters, except ETP, between trauma patients and healthy volunteers (Figure 4). Trauma patients had significantly shorter lag times: 6.6 (7.3-11.3) *versus* 12.6 (11.1-14.8) min; greater peak height: 282.9 (216.2-377.2) *versus* 212.9 (181.3-264.2) nM; shorter times to peak: 11.78 (9.56-14.4) *versus* 16.95 (15.1-18.8) min; and shorter times to start tail: 31.3 (28.9-34.2) *versus* 36.8 (33.4-40.2) min. All differences were statistically signifi-

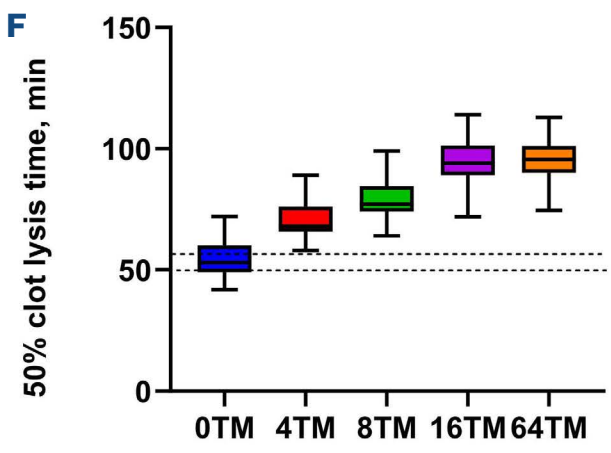


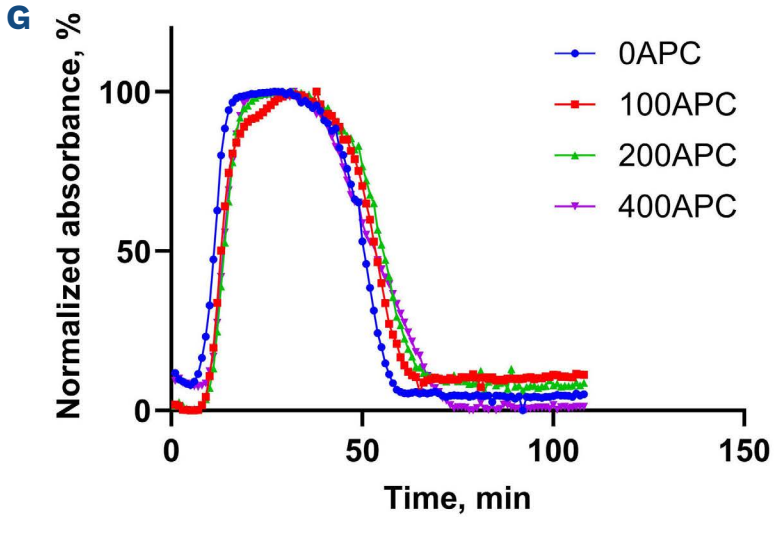












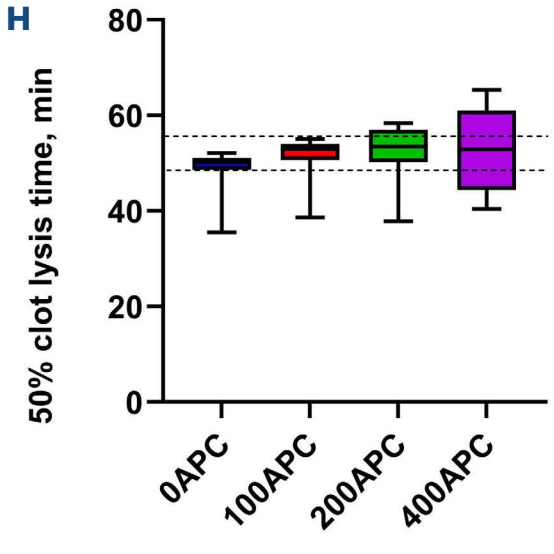


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Figure 2. Effects of increasing soluble thrombomodulin and activated protein C concentrations on thrombin generation and clot lysis in healthy volunteers. Increasing concentrations of soluble thrombomodulin (sTM) and activated protein C (APC) were added to plasma from healthy volunteers. (A-D) Thrombin generation performed using a microparticle reagent (4 mM phospholipid, Stago). (A) Representative curves of thrombin generation with the addition of sTM at concentrations ranging from 0-64 ng/mL. The curves show the amalgamated mean values for all 20 healthy volunteers of the thrombin generation tests (performed in triplicate) when all 60 results were averaged. (B) Means and 95% confidence intervals of the endogenous thrombin potential (ETP) values with the addition of increasing concentrations of sTM (N=20, in triplicate). (C) Representative curves of thrombin generation with the addition of APC at concentrations ranging from 0-400 pM. The curves show the amalgamated mean values for all 20 healthy volunteers of the thrombin generation tests (performed in triplicate) when all 60 results were averaged. (D) Means and 95% confidence intervals of the ETP values with the addition of increasing concentrations of APC. (E-H) Clot lysis performed using 90 pM tissue plasminogen activator. (E) Normalized mean representative curves with sTM added in concentrations in the range from 0-64 ng/mL. (F) Means and 95% confidence intervals for 50% clot lysis times (N=20, in triplicate). (G) Normalized mean representative clot lysis curves with APC added at concentrations of 0-400 pM. The curves show the amalgamated mean values for all 20 healthy volunteer clot lysis tests (performed in triplicate) when all 60 results were averaged. (H) Means and 95% confidence intervals of the 50% clot lysis times (N=20, in triplicate). Dotted lines denote the normal range.

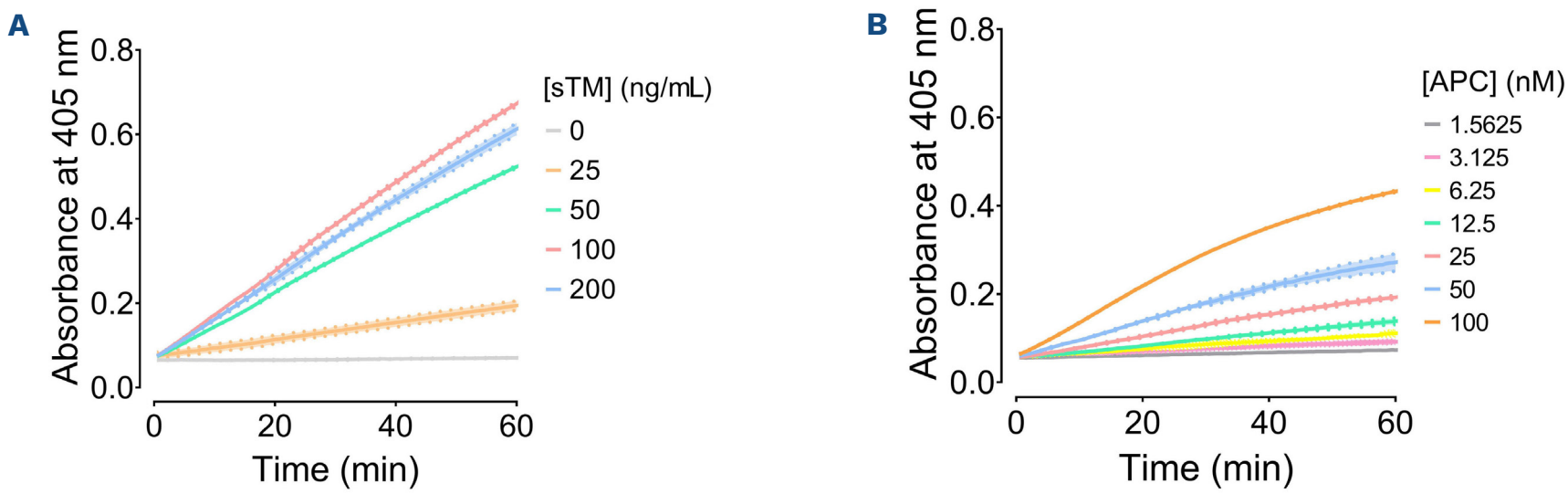

Figure 3. Effect of soluble thrombomodulin concentrations, compared directly to activated protein C concentrations, on substrate cleavage. (A, B) Rates of cleavage of a chromogenic substrate sensitive to activated protein C (APC), according to rising concentrations of soluble thrombomodulin (sTM) or APC, respectively. (A) sTM at 25 ng/mL (the upper end of the range seen circulating after injury) results in absorbance of 0.1 at 60 min. (B) An equivalent absorbance is seen with 25 nM APC, which far exceeds the upper end of the range of APC concentrations following trauma (295 pM).

cant (P<0.0001) (Figure 4A, C-E). Despite these changes, there was no difference in overall ETP: trauma patients: 1,506 (1,361-1,740) *versus* volunteers: 1,491 (1,383-1,727) nM/min; P=0.19 (Figure 4B). The thrombin anti-thrombin results confirmed this finding, with a median thrombin anti-thrombin concentration of 1,193 (940.4-1,872) *versus* 1,353 (865.4-1,999) ng/mL for trauma patients and volunteers, respectively (Mann-Whitney, P=0.68). The 'trauma bleeding' cohort had no overall difference in ETP, but had significantly shorter times to lag, peak and start tail (Figure 4F).

The interactions of sTM and APC values and TG were explored further. There were no differences in lag time, peak height, time to peak or start tail within the trauma cohort, when TG parameters were divided according to tertiles of sTM. There was an increase in ETP between the patients with sTM in the lowest tertile (median ETP: 1,483 nM/min [1,235-1,554], N=21), and those with sTM levels in the highest tertile (median ETP; 1,681 nM/min [1,432-1,917], N=21), with a greater ETP seen with higher sTM values (P=0.02) and a trend towards an incremental increase in ETP with rising sTM values (1-way ANOVA, P=0.06) (Figure 5A). Separating the trauma cohort according to admission APC tertiles, no differences were seen in ETP values (P=not statistically significant for all comparisons) (Figure 5B).

The trauma patients with the lowest (N=10, mean sTM 6.3 ng/mL; range, 5.7-6.7 ng/mL) and highest (N=10, mean sTM 17.8 ng/mL; range, 13.5-25.9 ng/mL) sTM values were compared, with and without anti-TM antibody (1 μg/mL) (*Online Supplementary Figure S2*). In both groups, anti-TM antibody increased ETP and peak height. The antibody did not lead to convergence of ETP results in the high and low groups, with ETP remaining greater in the 'high sTM' cohort. Anti-TM antibody led to a rise in ETP (P=0.04) in healthy volunteer plasma (*data not shown*). These data suggest that the differences in TG between the sTM tertiles are not due to differences in sTM concentration.

Clot lysis in the trauma cohort

Many of the trauma samples were not evaluable because of the presence of TXA (trauma not-bleeding, N=46; trauma bleeding, N=4). In the plasma samples that were taken from patients who had received TXA, clot lysis was not evaluable, as lysis did not occur to any significant degree.

Table 2. Clinical outcomes for the trauma cohort.

	Trauma patients N=91
PRBC units within 0-24 h, median (IQR) [range]	0 (0-0) [0-19]
FFP units within 0-24 h, median (range)	0 (0-0) [0-14]
Platelet pools within 0-24 h, median (range)	0 (0-0) [0-2]
Cryoprecipitate pools within 0-24 h, median (range)	0 (0-0) [0-6]
In receipt of transfusion within 12 h, N (%)	12 (13)
Length of stay in days, median (IQR)	7 (3-16.5)
Died within 28 days, N (%)	1 (1.2)

PRBC: packed red blood cells; IQR: interquartile range; FFP: fresh-frozen plasma.

The two groups (those who received TXA, N=40, and those who did not, N=51) were different with regards to injury severity, with median injury severity scores of 14.5 (9-26) *versus* 10.5 (5-17) for TXA *versus* no TXA, respectively. CLT were significantly shorter in the non-TXA trauma group than in volunteers: 46.7 (42.0-56.0) *versus* 53.3 (49.2-595) min (P=0.009) (Figure 4G). Notably, the 'trauma bleeding' group had a shorter 50% CLT, 38.0 (31.6-38.9) min than that of the non-bleeding group 46.7 (42.0-56.0) min (P=0.007) and volunteers, 53.3 (49.2-59.5) min (P<0.0001).

The interactions of admission sTM and APC on clot lysis in the trauma cohort were explored (Figure 5). There was a stepwise increase in 50% CLT with higher sTM levels across the tertiles: 41.9 (SD=2.6) min; 44.0 (SD=12) min; and 54.0 (SD=7.0) min. These results are in keeping with the data for the volunteers, in whom increasing concentrations of sTM led to prolongation of CLT. At the highest APC tertile, 50% CLT were prolonged compared to those in the two other groups (Mann Whitney, P=0.01, both comparisons). Adding PTCI or anti-TM antibody to trauma plasma led to similar effects whether there were high or low concentrations of sTM present, with high sTM values on average shortening by 39% and 17% with PTCI and anti-TM antibody and by 30% and 17% in the low sTM cohort, respectively (*Online Supplementary Figure S3*). By fully inhibiting TAFIa, PTCI removed the effect of sTM on CLT.

Discussion

This study evaluated the effects of sTM and APC on two dynamic assays, TG and clot lysis, in healthy volunteers and a trauma cohort. Spiking the plasma of volunteers showed that increasing sTM concentrations to 'trauma' levels led to progressively slower and reduced quantities of TG, as well as slower rates of clot lysis, as predicted.[20,21] These effects were reversed by anti-TM antibody (TG and CLT) and clot lysis was additionally reversed by PTCI, confirming the likely effectors to be APC and TAFIa,[15] respectively. Low concentrations of sTM have previously been reported to not affect TG parameters,[22] however, our experimental TG assay excluded tissue factor, thereby maximizing the assay's sensitivity to sTM.

Rising APC concentrations, at 'trauma' levels, did not lead to a reduction in TG, contrary to our expectations. This suggests that in this *in vitro* plasma system, adding in sTM generates a higher concentration of APC than is circulating after injury. Our experimental data further support this idea, as we showed that 'trauma sTM concentrations' led to robust protein C activation, but when APC alone was added, much higher concentrations (nM range) than those found in trauma were required to detect recordable catalytic activity. Another group similarly showed that at least 10 nM APC was required to reduce TG and fibrin polymerization.[23] In our trauma cohort, as expected, the sTM levels were

Table 3. Extended coagulation test results.

Coagulation factor, median (IQR)	Complete trauma cohort, N=91	Healthy volunteers N=20	*P*
Soluble thrombomodulin, ng/mL	9.9 (8.2-11.6)	9.2 (7.6-10.2)	0.02
Fibrinogen antigen, g/L	2.4 (2.4-3.5)	3.3 (2.6-4.4)	0.05
Factor V, IU/mL	0.72 (0.58-0.93)	0.91 (0.66-1.14)	0.09
Factor VIII, IU/mL	2.91 (2.06-4.66)	1.13 (0.97-1.35)	<0.0001
Plasmin-antiplasmin, ng/mL	2,983 (1,000-9,143)	684.5 (442.5-1,200)	<0.0001
PAI-1, ng/mL	3.62 (2.42-5.92)	2.45 (1.52-3.36)	0.004
tPA, ng/mL	2.16 (1.26-3.50)	0.78 (0.42-2.5)	0.008
Activated protein C, pM	66.7 (26.5-130.7)	35.0 (23.9-53.8)	0.02
Antithrombin, IU/mL	0.97 (0.85-1.07)	1.04 (0.92-1.18)	0.11

All results for the trauma cohort are from blood drawn at time of admission to hospital. PAI-1: plasminogen activator inhibitor-1; tPA: tissue plasminogen activator.

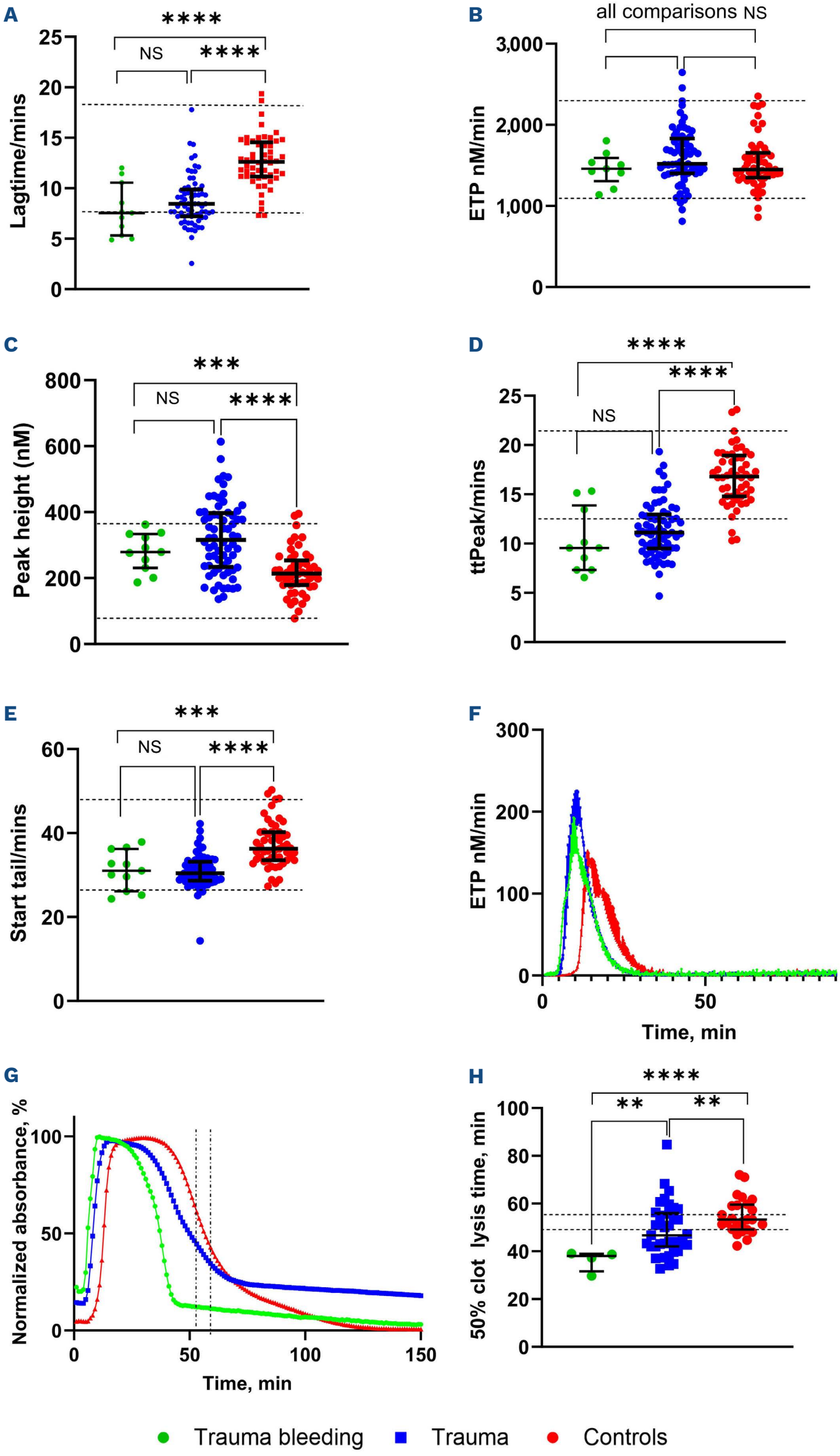

Figure 4. Thrombin generation and clot lysis in trauma participants and healthy volunteers. (A-F) Thrombin generation parameters. Trauma patients requiring early transfusion: 'Trauma bleeding', N=12 (green), and those not bleeding: 'Trauma', N=79 (blue), healthy volunteers, N=20 (red). (A) Lagtime. (B) Endogenous thrombin potential. (C) Peak height. (D) Time to peak height. (E) Time to the start tail. (F) Amalgamated mean thrombin generation curves for each cohort. (G, H) Clot lysis results. Trauma bleeding (N=4); trauma non-bleeding (N=46), healthy volunteers (N=20). (G) Amalgamated normalized mean data, clot lysis curves. (H) 50% clot lysis times. Dotted lines denote normal range. NS: not statistically significant; $^{**}P<0.01$, $^{***}P<0.001$; $^{****}P<0.0001$. ETP: endogenous thrombin potential.

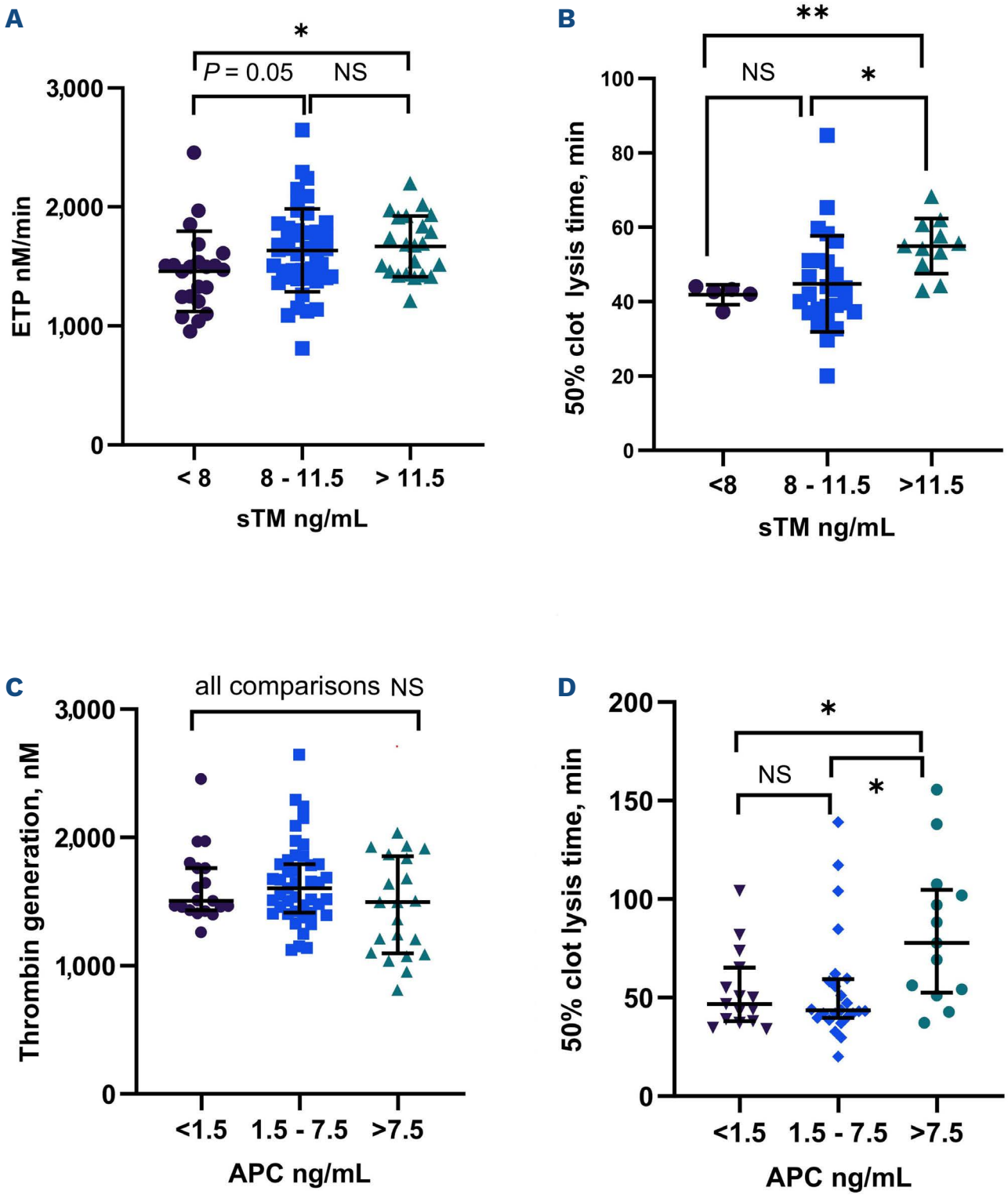

Figure 5. Endogenous thrombin potential and clot lysis values in trauma patients, according to levels of soluble thrombomodulin and activated protein C on admission to hospital. (A, B) Endogenous thrombin potential (ETP) values (A) and 50% clot lysis times (B) in the trauma cohort divided according to tertiles of soluble thrombomodulin (sTM) concentration. (C, D) Thrombin generation (C) and 50% clot lysis times (D) in the trauma cohort divided according to tertiles of activated protein C concentration. N=91 for ETP (tertiles from low to high: sTM, N=22, 49, 22 and APC, N=20, 44, 26). N=50 for clot lysis (tertiles from low to high: sTM, N=9, 26, 15 and APC, N=15, 22, 13). NS: not statistically significant; *P<0.05, **P<0.01.

higher than in volunteers and broadly rose as shock and injury severity parameters worsened. These same associations were not seen with APC, which was unexpected. Despite this, APC levels were much higher in the patients and were in line with those in other reports.[5,23] We found that higher admission sTM values in patients were associated with greater, rather than reduced, ETP. This is at odds with our spiking data. We were, however, able to show that anti-TM antibody increased ETP in individual patients' samples, but notably, it did not cause convergence of the TG parameters in the high and low sTM groups.

Although the expected TG changes, if sTM and or APC were strong effectors of overall TG following injury, were not seen, our data did show a weak effect of 'trauma sTM' in reducing TG, which is reversible with anti-TM inhibition. Contrary to this, circulating 'trauma APC' levels had no impact on TG, in line with our spiking data. Taken together this draws out the differential effects of circulating plasma APC levels compared with the effects of sTM-generated APC. Overall, the trauma and volunteer groups had similar TG capacity, as measured by ETP. All other TG parameters were markedly different; most notably, there was more rapid, and greater peak thrombin in the injured cohort. These differences may be explained by the higher factor VIII levels in the

trauma cohort and a relative reduction in antithrombin and fibrinogen.[20,24] Of perhaps more interest is the possibility of higher levels of tissue factor in the trauma samples. During optimization experiments, our data (*Online Supplementary Table S3*) demonstrated that increasing concentrations of tissue factor in volunteer plasma led to shorter lag times, greater ETP and greater peak height at the same sTM concentrations. Tissue factor-rich extracellular vesicles are known to be increased after significant injury,[25] and the trauma samples in this study were processed in a manner that would have retained extracellular vesicles. This requires further evaluation.

Our data examining CLT were more predictable. Spiking volunteer plasma with increasing sTM led to prolongation of 50% CLT, which was reversible with PTCI and anti-TM antibody, confirming the effect to be via TAFI activation. In the trauma patients, higher admission sTM levels were associated with longer CLT and inhibition of sTM in a subset of samples led to predictable shortening of CLT. Again, APC at 'trauma' concentrations did not alter CLT when spiked into volunteer plasma. APC might be expected to affect clot lysis in one of two ways: either indirectly, by reducing TG (via cleavage of factor Va and factor VIIIa) and thereby reducing TAFI activation, or by directly forming a complex with, and inhibiting, plasminogen activator inhibitor 1. Either way, CLT would be predicted to shorten, and our experiments did not show this effect. Other groups have also failed to demonstrate that sTM causes hyperfibrinolysis by reduction of thrombin-TM activation of TAFI or via inhibition of plasminogen activator inhibitor 1.[21,22] Our data align with their results, and support the findings that sTM primarily attenuates, rather than promotes, clot lysis. This is clinically relevant, given the poorer outcomes in trauma patients in receipt of TXA after 3 h of injury[26] and requires further investigation.

Our study has limitations. The cohort of trauma patients we studied was small. The presence of TXA in a large proportion of the samples reduces the strength of the CLT data. The data we report refer to the effects of sTM and APC in plasma and do not reflect the influence of cell surface proteins or how membrane-bound TM might differ from sTM.[27] All our experiments used platelet-poor plasma and did not include the effects that platelets, or indeed red cells, may exert.[28,29] The sTM levels we report include all detected TM fragments. sTM is cleaved from the endothelial cell surface by metalloproteinases after injury and sTM fragments of different lengths confer different hemostatic activities.[30] Delineating the variability of sTM fragments between patients was beyond the scope of this work. The experimental set up in our experiments aimed to optimize the effects of low sTM concentrations (e.g., up to 16 ng/mL), and this led us to avoid the experimental use of tissue factor and thrombin. The TM-thrombin and TM-APC axes are complex and influenced by thrombin concentrations, meaning that the findings in our experimental set up may not reflect the physiological generation of thrombin via tissue factor activation pathways. In conclusion, our results confirm that increasing sTM concentrations, when spiked in plasma, lead to lower ETP, and longer clot lysis, across ranges of sTM concentrations present in trauma. Trauma APC ranges do not affect these dynamic tests. Despite increased circulating sTM levels, the overriding coagulation changes seen after injury are: (i) rapid bursts of TG and (ii) increased rates of fibrinolysis. Important changes are, therefore, evident in TG and clot lysis after injury but can be explained at best only in part by elevated sTM and APC levels. Further evaluation of the impact of the TM axis on coagulation after injury in the presence of endothelial cells, and under flow conditions, will increase our understanding of these complex pathways.

Disclosures

No conflicts of interest to disclose.

Contributions

NSC conceived and conducted clinical and experimental work, analyzed results, and wrote the manuscript. GBM and JA-H conducted experimental work. All authors analyzed results and provided input into the writing of the manuscript and revision.

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Data-sharing statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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