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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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Declarations.

Ethics approval and consent to participate: Trauma patient group: Emergency consent was obtained from the trauma team leader who acted as the patient's legally authorised representative. Written informed consent from the patient, or next of kin, was obtained as soon after enrolment as appropriate. The study was reviewed and approved by East London Regional Ethics Committee (REC reference: 07/Q0603/29). 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.

Data Availability: The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

Contributions of Authors: NC conceived, conducted clinical and experimental work, analysed results, and wrote the manuscript. GM and JAH conducted experimental work. All authors analysed results and provided input into the writing of the manuscript and revision.

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Abstract

Trauma induced coagulopathy (TIC) 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 TIC. 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 TIC, and specifically to evaluate the effect 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 analysed. Plasma samples from a cohort of trauma patients were evaluated using TG and CLT, and results correlated to clinical parameters and FVIII, FV, APC, sTM and fibrinolytic measures. Increasing sTM concentrations in volunteer plasma led to reductions in ETP and prolongation of 50% CLT times, 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, ETP (median 1483 vs. 1681 nM/min) and longer 50% CLT times (41.9 vs. 54.0 mins). In conclusion, sTM concentrations, across trauma ranges, impact both TG and 50% CLT times, unlike APC. Despite increased circulating sTM levels, the overriding dynamic coagulation effects seen after injury are: (a) accelerated thrombin generation and (b) increased rates of fibrinolysis. We find no evidence for sTM as the major determinant of the coagulation changes seen in early TIC.

Keywords:

Traumatic coagulopathy, thrombomodulin, clot lysis, thrombin generation, activated protein C

Introduction

Trauma induced coagulopathy (TIC) is a term describing the myriad changes to coagulation which occur after injury. [1] TIC is complex and incompletely understood, affecting one quarter of injured patients and is strongly associated with increased bleeding and a 3- to 4-fold greater risk of death [1,2,3]. The thrombomodulin-protein C axis is believed to be central to the evolution of TIC [3 - 7]. Soluble thrombomodulin (sTM) levels are elevated after injury and are associated with poorer clinical outcome [7,8 9].

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

Recently, an inherited bleeding condition, caused by genetic variants within the thrombomodulin 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 (50-100-fold higher than normal); reduced thrombin generation and slower rates of fibrinolysis [12-15]. Clinically, the bleeding tendency suggests the reduction in thrombin generation 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 TIC. 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 effect of sTM and APC, on two dynamic hemostatic assays: thrombin generation and clot lysis.

Methods

Trauma patients

Adult trauma patients at Oxford University Hospitals (≥ 16 years) who met the criteria for trauma team activation were eligible. Details of the study have been published previously [16]. The study was approved by East London Regional Ethics Committee: 07/Q0603/29. Up to 20 mL blood was drawn within 20 minutes and analysed for routine coagulation, including ROTEM. Remaining whole blood was spun (3000*g*, room temperature, 20 minutes) to obtain platelet poor plasma (PPP) and stored at -80°C. Clinical data were collected on patient demographics, mechanism of injury (blunt or penetrating) and vital signs. Blood transfusion requirements were collected during the first bleeding episode.

Healthy volunteers

20mL whole blood was drawn and PPP obtained from citrated samples, as above, and stored. Ethical approval was granted by the Wales Research Ethics Committee: 20/WA/0313.

Thrombin generation

Thrombin generation (TG) was triggered with phospholipids (4 µM), thrombin fluorogenic substrate (Z-Gly-Gly-Arg-AMC) and calcium chloride (CaCl2) (Diagnostic Stago, France). In some cases, recombinant human TM (0 – 256 ng/mL) (Peprotech Inc, UK), human activated protein C (0 – 400pM) (HYPHEN Biomed, France) or murine anti-thrombomodulin antibody (1 µg/mL; ab6980, Abcam) was added. TG was measured using the calibrated automated thrombogram [17].

Clot lysis

PPP (30%), phospholipids (16 µM) (Rossix, Sweden), tPA (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, Missouri, USA); recombinant human TM (0 – 64 ng/mL); murine anti-thrombomodulin antibody (1 µg/mL) or human APC (0 – 400pM) were incorporated. Clotting was initiated with 10.6 mM CaCl2. Clot formation and lysis were monitored with Absorbance (405 nm) was measured every 60 seconds for 4hr and analysed using Shiny App software [18].

ELISA assays

Thrombomodulin, factors V and VIII, antithrombin, plasmin-antiplasmin (PAP), APC, PAI-1, thrombin anti-thrombin (TAT), tPA, fibrinogen antigen levels were quantified in PPP. Kits used: PAP (Technozym, USA); APC (2b Scientific Ltd, UK); the remaining were Abcam, UK.

Protein C activation assay

70 nM human protein C (PC), sTM (0-200 ng/mL) in PBS, 0.6 mM $MgCl_2$, 1% BSA were added to a 96-well plate. 0.1 U/ml thrombin and 3 mM $CaCl_2$ initiated PC activation and incubated at 37°C for 30 min. 1 U/ml hirudin (Sigma-Aldrich) stopped the reaction. 0.42 mg/ml BIOPHEN CS-21(66) (HYPHEN BioMed), a chromogenic substrate for APC, was added. In other experiments, APC (1.5625-100 nM) in PBS, 0.6 mM $MgCl_2$ and 1% BSA was mixed with 0.42 mg/ml BIOPHEN CS-21(66). Absorbance at 405 nm was measured every 30s for 2hr.

Data analysis

Results are represented by mean ± standard deviation (SD)/median ± interquartile range (IQR), with comparisons made using t-tests or Mann-Whitney tests, as appropriate. Normality was assessed using visual assessment of histograms and D'Agostino-Pearson omnibus test. Significance was set at $p < 0.05$. Correlations were performed using Spearman tests. Normal ranges were calculated using

samples from 20 healthy volunteers and 1.96SD of the mean or log-transformed mean [19]. Statistical analysis was performed using Graph Pad Prism version 10.1.2.

Results

Ninety-one trauma patients were included. Baseline characteristics are shown in Table 1. Twenty healthy volunteers were included (65% male, mean age 38.7 (SD 9.3) yr). sTM levels were elevated in the trauma cohort when compared to healthy volunteers (Fig. 1A, Mann Whitney, p = 0.02). Median sTM for the trauma cohort was 9.9 ng/mL (IQR: 8.2 – 11.6, range: 5.7 – 25.9 ng/mL). (Normal range: 5.85 – 11.67 ng/mL). Median APC value for the trauma cohort was 66.7 pM (IQR: 26.5 – 130.7 pM, range 3.3 – 294.6 pM); higher than in volunteers: 35.0 pM (IQR: 23.9 – 53.8 pM), (Fig. 1B. Mann Whitney, p = 0.02). We proceeded to evaluate the effects of sTM and APC elevation on dynamic coagulation assays in normal plasma using a concentration range of 0 – 64 ng/mL sTM, and 0 – 400 pM APC; encompassing sTM and APC levels found in trauma patients.

The effects of soluble TM and APC on dynamic coagulation assays in healthy volunteers

Thrombin generation

TG was optimised for trauma conditions to maximise sensitivity for relevant sTM values, and the Microparticle Reagent (4 µM phospholipid alone), was chosen as the trigger, as the use of tissue factor (TF) masked the effects of the sTM concentrations required for these experiments. (Table S1).

sTM effects: Increasing sTM concentrations in volunteer plasma led to reductions in peak height and ETP, and shortening of time to start tail, in a dose dependent manner (Fig. 2A-B, Table S2). Previous work from our group has shown that this effect can be reversed by adding an antibody against APC [15]. *APC effects:* No significant differences were seen across rising concentrations of added APC for four of the measured TG parameters (lagtime, ETP, peak height, time to peak) (Fig. 2C-D, 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 (1nM APC and above) a significant reduction of ETP and peak height, with prolongation of lag time could be elicited (data not shown).

This suggests that the sTM in 'trauma levels' is sufficient to generate enough APC to reduce thrombin generation, 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 (Fig 3). These show that sTM, in similar concentrations (e.g. 25 ng/mL) to that found in trauma patients, can sufficiently activate PC to cleave the chromogenic substrate, but a similar effect is only seen at 25nM APC.

Clot lysis

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

sTM effects: There was a stepwise increase in 50% CLT (Fig. 2E-F) with increasing sTM (0-16 ng/mL, 1-way 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 (Fig. S1). *APC effects:* There was no change to 50% CLT (Fig. 2G-H) with increasing trauma level APC concentrations. These results suggest that sTM attenuates clot lysis times through its action on TAFI but that APC does not affect lysis at trauma concentrations.

Trauma cohort characteristics

Average age of the cohort was 43 years with 77% participants being male (table 1). Median injury severity score (ISS) was 10, and 28 (31%) of the cohort had severe injury, defined as an ISS >15. All but three suffered blunt injury (n = 88, 96.7%) and nine had isolated traumatic brain injury (TBI) (9.9%). Almost half (45%) received tranexamic acid (TXA) prior to admission and blood sample draw.

TXA dosing was 1g intravenous bolus followed by 1g intravenous infusion over 8 hours. Prior to admission, few participants received PRBC (n = 4, 4.4%) or FFP (n = 2, 2.2%).

Thirteen participants (14.2%) had TIC defined by an EXTEM CA5 less than 40mm [20]. Median Clauss fibrinogen was 2.7 g/L, and median D-dimer was 8,688 ng/mL. APC levels were higher in the trauma cohort compared to volunteers; 66.7 pM (IQR: 26.5 – 130.7 pM) vs. 35.0 pM (IQR: 23.9 – 53.8 pM), respectively (Mann Whitney, p = 0.02). Coagulation parameters (Table 3) show changes consistent with TIC, notably significant fibrinolytic activity with very high D-dimer and PAP levels. Factor VIII levels were elevated at 2.91 IU/mL (p < 0.0001). Factor V and AT levels were no different to volunteers, at 0.72 IU/mL and 0.97 iu/mL, suggesting both a lack of a significant APC effect or evidence of DIC, respectively. Twelve participants required transfusion within the first 12 hours of injury (13.2%) and represent the cohort 'trauma bleeding', the remaining cohort having minimal transfusion requirements (Table 2).

Thrombomodulin and APC levels in the trauma cohort

sTM values broadly rose with increasing clinical measures of shock, e.g. falling SBP, rising HR and worsening base excess (BE), but statistical correlation was not found to be significant. ISS did not correlate with sTM levels, although broadly ISS values rose as sTM rose. The sTM values for the 'trauma bleeding' cohort did not differ from the non-bleeding cohort (Mann Whitney, p = 0.81). sTM admission levels did not correlate with factor V or VIII levels, PAP levels, or more global clotting assays such as the INR, APTT 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 'bleeding trauma' cohort did not differ from the non-bleeding cohort (Mann Whitney, p = 0.44). Admission APC levels did not correlate with admission factors V or VIII. There was no association between 50% CLT and admission APC values.

Thrombin generation in the trauma cohort

When compared to volunteers, there were differences in all TG parameters except ETP (Fig. 4). Trauma patients had significantly shorter lag times: 6.6 (IQR: 7.3 – 11.3) vs. 12.6 (IQR: 11.1 – 14.8) mins; greater peak height:282.9 (IQR: 216.2 – 377.2) vs. 212.9 (IQR: 181.3 – 264.2) nM; shorter times to peak: 11.78 (IQR: 9.56 – 14.4) vs. 16.95 (IQR: 15.1 – 18.8) mins; and shorter times to start tail: 31.3 (IQR: 28.9 – 34.2) vs. 36.8 (IQR: 33.4 – 40.2) mins. All differences were significant: $p < 0.0001$. Despite these changes, there was no difference in overall ETP: ACIT patients: 1,506 (IQR: 1,361 – 1,740) vs. volunteers: 1,491 (IQR: 1,383 – 1,727) nM/min; $p = 0.19$ (Fig. 4A - E). TAT results confirmed this finding, with a median TAT of 1193 ng/mL (IQR: 940.4 - 1872) vs. 1353 (IQR: 865.4 - 1999) for trauma and volunteers respectively, $p = 0.68$, Mann Whitney. The 'trauma bleeding' cohort had no overall difference in ETP, but had significantly shorter times to lag, peak and start tail (Fig. 4F).

The interactions of sTM and APC values and TG were further explored. There were no differences in lagtime, 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 lowest (median ETP: 1483 nM/min (IQR: 1235 – 1554) (n=21), and highest sTM groups (1681 nM/min (IQR: 1432 – 1917) ($n = 21$), 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, $p = 0.06$) (Fig. 5A). Separating the trauma cohort according to admission APC tertiles, no differences were seen in ETP values (all comparisons NS) (Fig. 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 Ab (1 µg/mL)(Fig. S2). In both groups, anti-TM Ab 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 Ab led to a rise in ETP ($p = 0.04$) in healthy volunteer plasma, data not shown). This suggests 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, due to 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. The two groups (those with TXA, n = 40, and those with no TXA, n = 51) were different with regards their injury severity, with median ISS of 14.5 (IQR: 9 – 26) vs. 10.5 (5 – 17), TXA vs. no TXA, respectively. CLT were significantly shorter in the non-TXA trauma group when compared to volunteers: 46.7 (IQR: 42.0 – 56.0) vs. 53.3 (IQR: 49.2 – 595) mins (p = 0.009) (Fig. 4G). Notably, the 'trauma bleeding' group had faster 50% CLT: 38.0 (IQR: 31.6 – 38.9) mins; shorter than the non-bleeding group 46.7 mins (IQR: 42.0 – 56.0) (p = 0.007) and volunteers, 53.3 mins (IQR: 49.2 – 59.5)(p = <0.0001).

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

Discussion

This study evaluates 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 at 'trauma' levels led to progressively slower and reduced quantities of TG, as well as slower rates of clot lysis, as predicted [21, 22]. 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 [6], respectively. Low concentrations of sTM have previously been reported to not affect TG parameters [23], however, our experimental TG assay excluded tissue factor, thereby maximising 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 show 'trauma sTM concentrations' lead to robust protein C activation, but adding APC alone requires much higher concentrations (nM range) than those found in trauma to detect recordable catalytic activity. Another group similarly showed that at least 10nM APC was required to reduce TG and fibrin polymerisation [24,25].

In our trauma cohort, as expected, the sTM levels were higher than 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 other reports [24,25]. We found 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 patient 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 do 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 more rapid, and greater peak thrombin in the injured cohort. These differences may be explained by the higher FVIII levels in the trauma cohort

and a relative reduction in AT and fibrinogen [21,22]. Of perhaps more interest, is the possibility of greater tissue factor (TF) in the trauma samples. During optimisation experiments, our data (Table S3) demonstrate that increasing concentrations of TF in volunteer plasma led to shorter lag times, greater ETP and peak height at the same sTM concentrations. TF-rich extracellular vesicles are known to be increased after significant injury [31], and the trauma samples in this study were processed in a manner that will 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, that was reversible with PTCI and anti-TM antibody, confirming the effect to be via TAFI activation. In the trauma patients, higher admission sTM was 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 clot 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 FVa and FVIIIa) and thereby reducing TAFI activation, or, by directly forming a complex with, and inhibiting, PAI-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 T-TM activation of TAFI or via inhibition of PAI-1 [22, 24]. 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 three hours of injury [32] and requires further investigation.

Our study has limitations. The cohort of trauma patients we have included is small. The presence of TXA in a large proportion of the samples reduces the strength of the CLT data. The data we report evaluates the effects of sTM and APC in plasma and does not look at the influence of cell surface proteins or how membrane bound TM might differ to sTM [35]. All our experiments used PPP and do not include the effects that platelets, or indeed red cells, may exert [37,38]. The sTM levels we report include all detected TM fragments. sTM is cleaved from the endothelial cell surface by

metalloproteinases after injury and different length sTM fragments confer different hemostatic activities [36]. Delineating the variability of sTM fragments between patients was beyond the scope of this work. The experimental set up in these experiments aimed to optimise the effects of low sTM (e.g. up to 16 ng/mL), and this led us to avoid the experimental use of TF and thrombin. The TM/T and TM/APC axes are complex and influenced by thrombin concentrations, making these results applicable in our experimental set up and may not reflect the physiological generation of thrombin via TF activation pathways.

Conclusion

Our results confirm that increasing sTM concentrations, when spiked in plasma, lead to lower ETP, and longer clot lysis, across trauma sTM ranges. Trauma APC ranges do not impact these dynamic tests. Despite increased circulating sTM levels, the overriding coagulation changes seen after injury are: (a) rapid bursts of thrombin generation and (b) increased rates of fibrinolysis. Important changes therefore are 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 TM axis on coagulation after injury in the presence of endothelial cells, and under flow conditions, will increase our understanding of these complex pathways.

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Table 1. Baseline characteristics, values at hospital admission.

	Trauma patients n = 91
Age, mean (SD)	43.8 (20)
ISS, median	10 (IQR: 4 – 18, range 0 - 43)
Male, n (%)	70 (77%)
Blunt injury, n (%)	88 (96.7%)
Isolated TBI, n (%)	9 (9.9%)
GCS, median	15 (IQR: 14 – 15, range 3 – 15)
Time from injury to ED, minutes	87 (SD 21, range 19 – 120)
SBP, mmHg, mean (SD)	139 (SD 25, range 70 - 203)
HR, bpm, mean (SD)	87 (SD 21; range 51 - 143)
In receipt of TXA pre-admission, n (%)	41 (45%)
Base excess, mEq/mol, median (IQR)	0.6 (-2.1 – 2.0)
Pre-hospital	
Crystalloid, mL, median	0 (IQR: 0 – 0, range 0 - 1000)
PRBC, units, median	0 (IQR: 0 – 0, range: 0 - 3)
FFP, units, median	0 (IQR: 0 – 0, range: 0 - 2)
Bloods at admission	
Hb, 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 greater than 1.2, n (%)	2 (2.2%)
Clauss Fg, g/L, median (IQR)	2.7 (2.1 – 3.1)
D-dimer, ng/mL, median (IQR)	8688 (2199 – 19400)
ROTEM	
EXTEM CA5, mm, mean (SD)	44 (8.5)
EXTEM CA5 less than 40mm, n (%)	13 (14.2%)
EXTEM ML, mean (SD)	10.5 (4.8)
FIBTEM CA5, mm, mean (SD)	13 (4.9)

Key: APTT – activated partial thromboplastin time; CA5 – clot amplitude at 5 minutes; ED – emergency department; FFP – fresh frozen plasma; Fg – fibrinogen; GCS – Glasgow Coma Score; Hb – haemoglobin; HR – heart rate; INR – international normalised ratio; ISS – injury severity score; ML – maximal lysis; PRBC – packed red blood cells; PT – prothrombin time; SBP – systolic blood pressure; TBI – traumatic brain injury; TXA – tranexamic acid.

Table 2. Clinical outcomes for trauma cohort.

	Trauma patients (n = 91)
PRBC units, 0 – 24 hr, median (IQR, range)	0 (0 – 0, 0 – 19)
FFP units, 0 – 24 hr, median (IQR, range)	0 (0 – 0, 0 – 14)
Platelet pools, 0 – 24 hr, median (IQR, range)	0 (0 – 0, 0 – 2)
Cryoprecipitate pools, 0 – 24 hr, median (IQR, range)	0 (0 – 0, 0 – 6)
In receipt of transfusion within 12 hours, n (%)	12 (13%)
Length of stay, days, median (IQR)	7 (3 – 16.5)
Died by 28 day, n (%)	1 (1.2%)

Key: FFP – fresh frozen plasma; PRBC – packed red blood cells.

Table 3. Extended coagulation test results.

Coagulation factor	Complete ACIT Cohort (n = 91)	Healthy volunteers (n = 20)	P value
Soluble thrombomodulin (ng/mL)	9.9 (IQR: 8.2 – 11.6)	9.2 (IQR: 7.6 – 10.2)	0.02
Fibrinogen antigen (g/L)	2.4 (IQR: 2.4 – 3.5)	3.3 (IQR: 2.6 – 4.4)	0.05
FV (IU/mL)	0.72 (IQR: 0.58 – 0.93)	0.91 (IQR: 0.66 – 1.14)	0.09
FVIII (IU/mL)	2.91 (IQR: 2.06 – 4.66)	1.13 (IQR: 0.97 – 1.35)	<0.0001
PAP (ng/mL)	2983 (IQR: 1000 – 9143)	684.5 (IQR: 442.5 – 1200)	<0.0001
PAI-1 (ng/mL)	3.62 (IQR: 2.42 – 5.92)	2.45 (IQR: 1.52 – 3.36)	0.004
tPA (ng/mL)	2.16 (IQR: 1.26 – 3.50)	0.78 (IQR: 0.42 – 2.5)	0.008
APC (pM)	66.7 (IQR: 26.5 – 130.7)	35.0 (IQR: 23.9 – 53.8)	0.02
Antithrombin (IU/mL)	0.97 (IQR: 0.85 – 1.07)	1.04 (IQR: 0.92 – 1.18)	0.11

All results for the trauma cohort are from blood drawn at time of admission to hospital.

Key: APC – activated protein C; F – factor; PAI-1 – plasminogen activator inhibitor-1; PAP – plasminogen anti-plasmin; tPA – tissue plasminogen activator.

Figure legends.

Figure 1. Soluble thrombomodulin and activated protein C levels.

Key. APC – activated protein C

Figure 2. Effect of increasing soluble thrombomodulin and APC concentrations on thrombin generation and clot lysis in healthy volunteers.

Legend.
Healthy volunteer plasma with increasing concentrations of sTM and APC. A – D: Thrombin generation performed using microparticle reagent (4mM phospholipid, Stago). A. Representative curves of thrombin generation: added sTM ranging 0 – 64 ng/mL. The curves show the amalgamated mean results of all 20 healthy volunteer thrombin generation results (in triplicate) when all 60 results were averaged. B. Mean and 95% CI, ETP values with sTM (n = 20, in triplicate). C. Representative curves of thrombin generation: added APC ranging 0 – 400 pM. The curves show the amalgamated mean results of all 20 healthy volunteer thrombin generation results (in triplicate) when all 60 results were averaged. D. Mean and 95% CI, ETP values with APC. E – H: Clot lysis performed using 90pM t-PA. E. Normalised mean representative curves: added sTM, 0 – 64 ng/mL. F. Mean and 95% CI, 50% clot lysis times (n = 20, in triplicate). G. Normalised mean representative clot lysis curves: added APC, 0 – 400 pM. The curves show the amalgamated mean results of all 20 healthy volunteer clot lysis results (in triplicate) when all 60 results were averaged. F. Mean and 95% CI, 50% clot lysis times (n = 20, in triplicate). Dotted lines denote normal range.

Key: APC – activated protein C; TM – thrombomodulin.

Figure 3. Effect of sTM concentrations on substrate cleavage, compared directly to APC concentrations.

Legend.
Figures 3A and 3B show rates of cleavage of a chromogenic substrate sensitive to APC, according to rising concentrations of sTM or APC, respectively. 3A. sTM at 25 ng/mL (the upper end of the range seen circulating after injury) results in absorbance of 0.1 at 60 minutes. S3B. An equivalent absorbance is seen with 25 nM APC – which far exceeds the upper end of the 'APC trauma range' of 295 pM.

Key: APC – activated protein C; sTM – soluble thrombomodulin.

Figure 4. Thrombin generation and clot lysis in trauma participants and healthy volunteers.

Legend.
A – F. Thrombin generation parameters. Trauma patients requiring early transfusion: 'ACIT bleeding', n = 12 (green), and those not bleeding: 'ACIT', n = 79 (blue), healthy volunteers, n = 20 (red). A: lagtime; B: Endogenous thrombin potential (ETP); 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. ACIT bleeding (n = 4); ACIT non-bleeding (n = 46), healthy volunteers (n = 20). G. Amalgamated normalised mean data, clot lysis curves. H. 50% clot lysis times. Dotted lines denote normal range.

Figure 5. ETP and clot lysis values in trauma patients, according to admission sTM and APC levels.

Legend. A/B. Trauma cohort represented according to sTM tertiles. C/D. Trauma cohort represented according to APC tertiles. (n = 91 for ETP (tertiles low to high: sTM, n = 22, 49, 22 and APC, n = 20, 44, 26), n = 50 for clot lysis (tertiles low to high: sTM, n = 9, 26, 15 and APC, n = 15, 22, 13).

Key: ETP – endogenous thrombin potential; sTM – soluble thrombomodulin.

Figure 1.

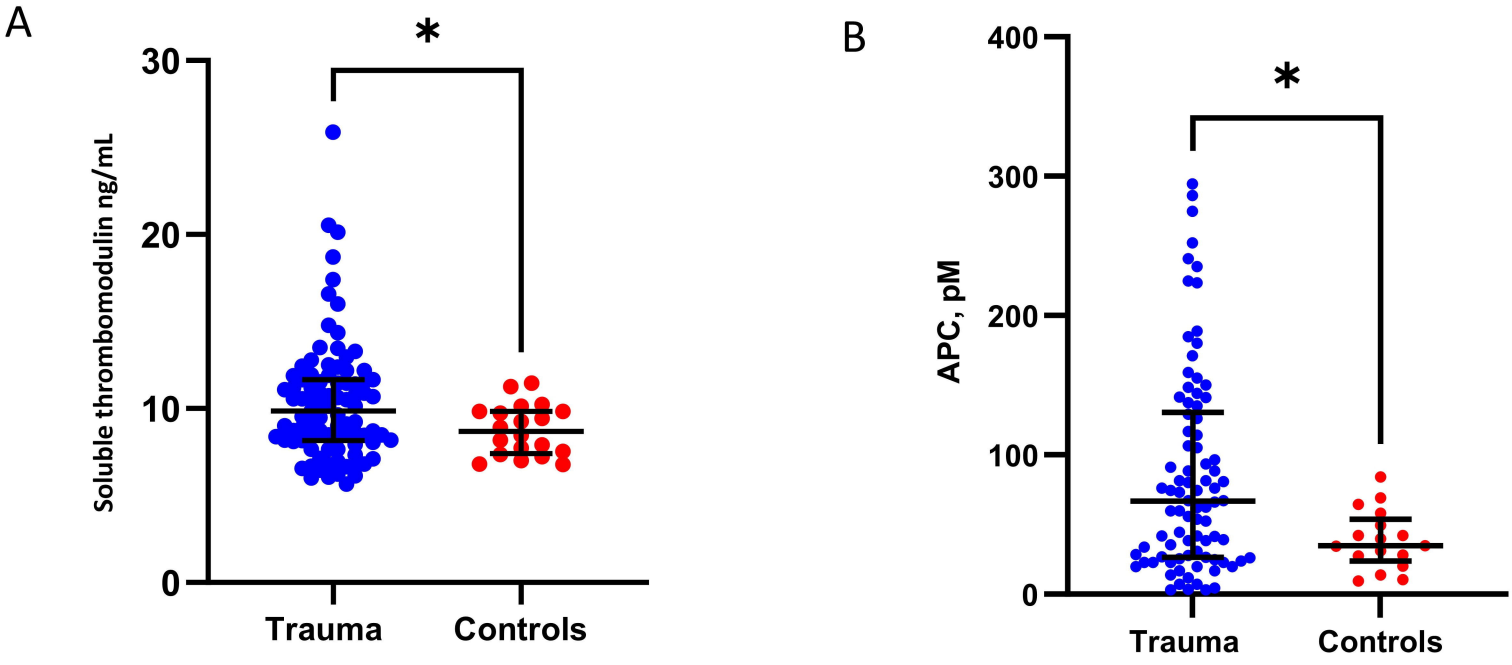

A
*
30
20
10
0
Soluble thrombomodulin ng/mL
Trauma
Controls
B
*
400
300
200
100
0
APC, pM
Trauma
Controls

Figure 2.

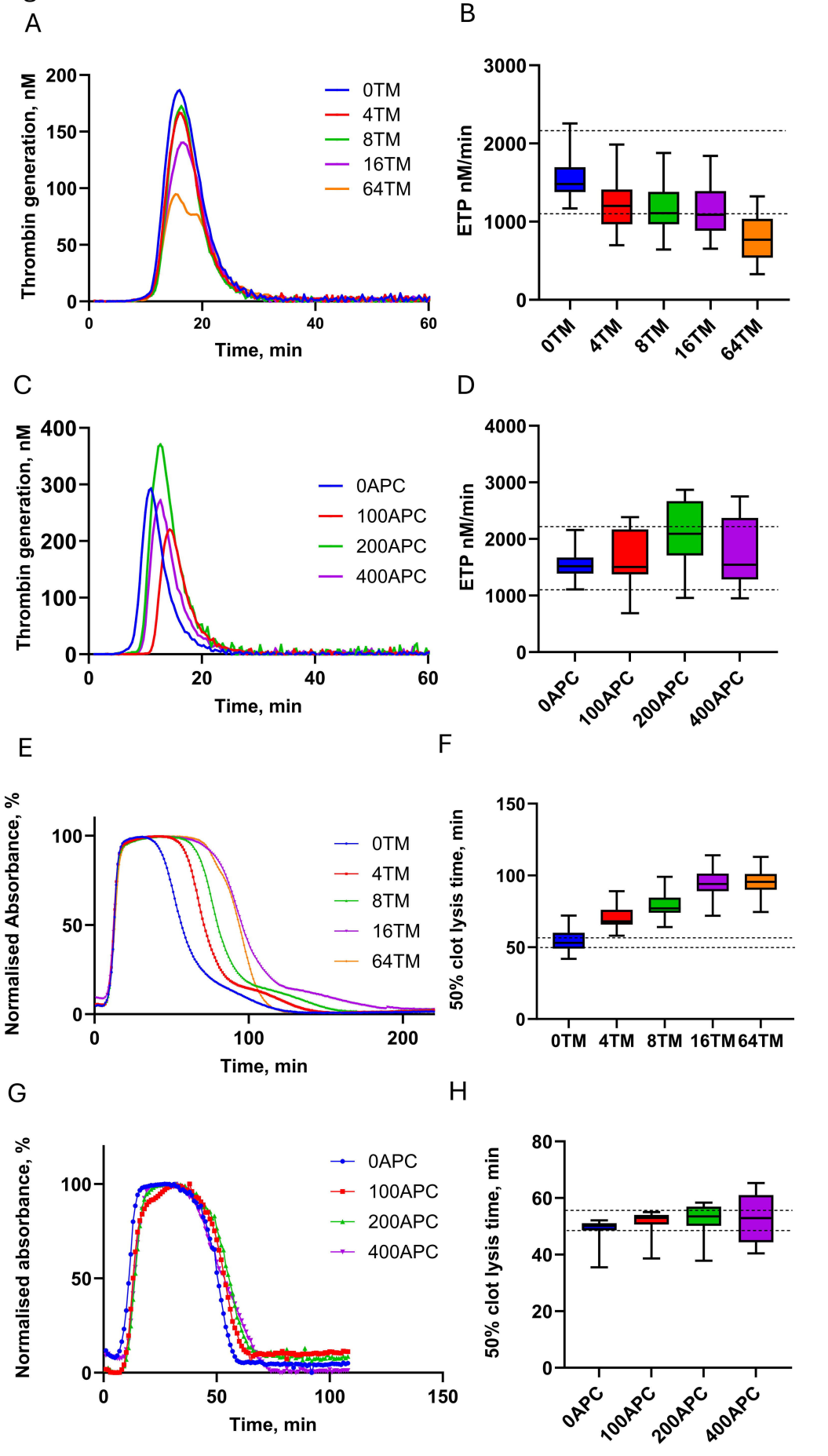
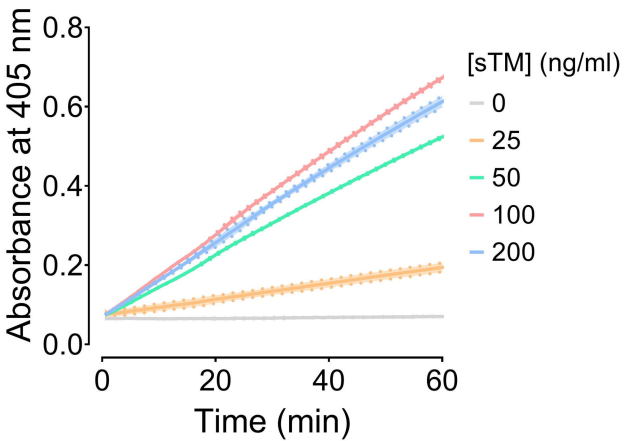

A
Thrombin generation, nM
200
150
100
50
0
0
20
40
60
Time, min
0TM
4TM
8TM
16TM
64TM
B
ETP nM/min
3000
2000
1000
0
0TM
4TM
8TM
16TM
64TM
C
Thrombin generation, nM
400
300
200
100
0
0
20
40
60
Time, min
0APC
100APC
200APC
400APC
D
ETP nM/min
4000
3000
2000
1000
0
0APC
100APC
200APC
400APC
E
Normalised Absorbance, %
100
50
0
0
100
200
Time, min
0TM
4TM
8TM
16TM
64TM
F
50% clot lysis time, min
150
100
50
0
0TM
4TM
8TM
16TM
64TM
G
Normalised absorbance, %
100
50
0
0
50
100
150
Time, min
0APC
100APC
200APC
400APC
H
50% clot lysis time, min
80
60
40
20
0
0APC
100APC
200APC
400APC

Figure 3.

A


Absorbance at 405 nm
0.0
0.2
0.4
0.6
0.8
0
20
40
60
Time (min)
[sTM] (ng/ml)
0
25
50
100
200

B

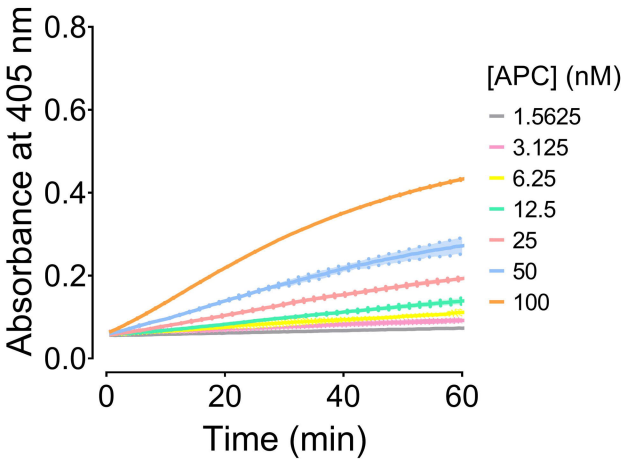

Absorbance at 405 nm
0.0
0.2
0.4
0.6
0.8
0
20
40
60
Time (min)
[APC] (nM)
1.5625
3.125
6.25
12.5
25
50
100

Figure 4.

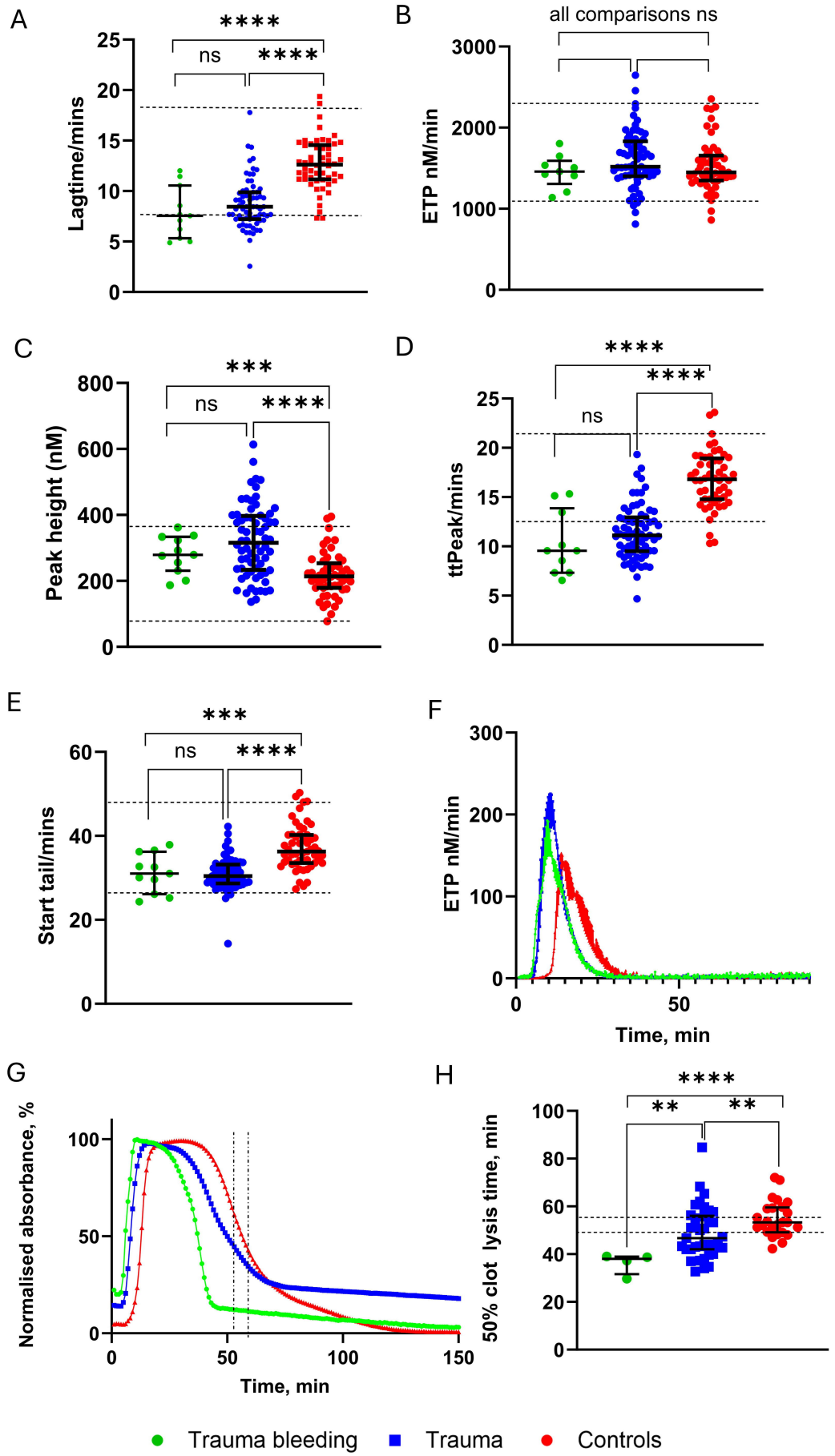
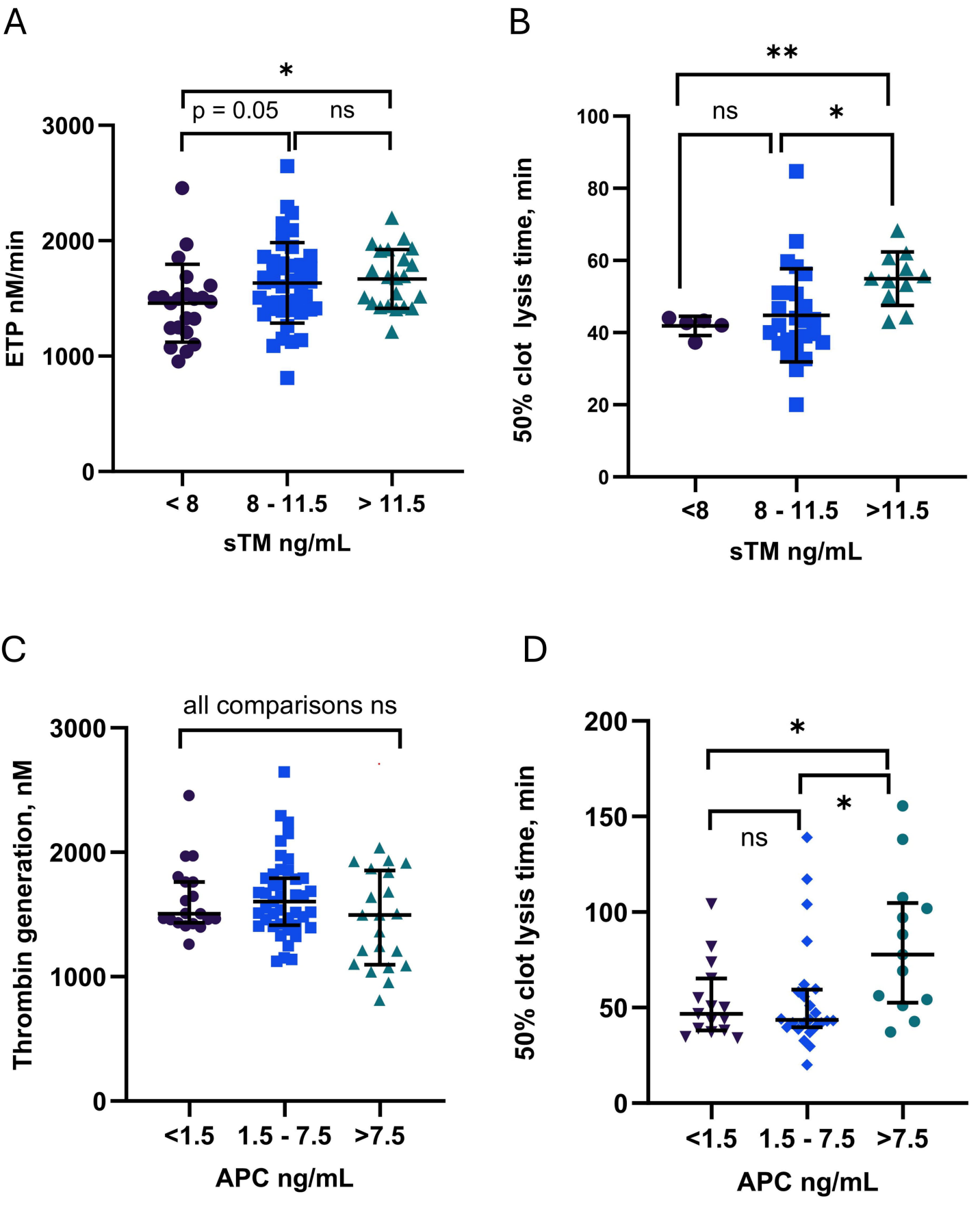

A
****
ns
****
Lagtime/mins
25
20
15
10
5
0
B
all comparisons ns
ETP nM/min
3000
2000
1000
0
C
***
ns
****
Peak height (nM)
800
600
400
200
0
D
****
****
ns
ttPeak/mins
25
20
15
10
5
0
E
***
ns
****
Start tail/mins
60
40
20
0
F
ETP nM/min
300
200
100
0
0
50
Time, min
G
Normalised absorbance, %
100
50
0
0
50
100
150
Time, min
H
****
**
**
50% clot lysis time, min
100
80
60
40
20
0
Trauma bleeding
Trauma
Controls

Figure 5.


A
*
p = 0.05
ns
3000
2000
1000
0
ETP nM/min
< 8
8 - 11.5
> 11.5
sTM ng/mL
B
**
ns
*
100
80
60
40
20
0
50% clot lysis time, min
<8
8 - 11.5
>11.5
sTM ng/mL
C
all comparisons ns
3000
2000
1000
0
Thrombin generation, nM
<1.5
1.5 - 7.5
>7.5
APC ng/mL
D
*
*
ns
200
150
100
50
0
50% clot lysis time, min
<1.5
1.5 - 7.5
>7.5
APC ng/mL

Supplementary Data.

Table S1. Interaction of added Tissue Factor and soluble thrombomodulin in healthy volunteers.

	Innovin* (dilution)	Concentration of added soluble thrombomodulin (ng/mL)			
		0 TM	4TM	8TM	16 TM
Lagtime (mins)	**Neat**	7.2 (0.27)	7.1 (0.17)	7.1 (0.16)	7.2 (0.27)
	1 in 2	8.7 (0.5)	7.3 (0.17)	8.7 (0.31)	5.3 (0.16)
	1 in 4	8.8 (0.17)	9 (0.41)	8.3 (0.28)	8.9 (0.14)
	No innovin	13.1 (0.6)	15.6 (1.52)	13.2 (1.21)	14.7 (2.01)
ETP (nM/min)	**Neat**	2588 (22.7)	2550 (71.8)	2401 (44.9)	2321 (84.8)
	1 in 2	2555 (14.6)	1793 (76.3)	1931 (105.9)	2034 (156.8)
	1 in 4	2388 (54.4)	2276 (39.3)	2371 (103.9)	2274 (84.6)
	No innovin	982 (29.5)	874 (156.3)	939 (127.2)	614 (43.1)
Peak height (nM)	**Neat**	194 (3.99)	191 (0.69)	191 (1.81)	195 (3.75)
	1 in 2	172 (2.2)	161 (8.48)	180 (3.91)	253 (23.2)
	1 in 4	156 (3.3)	181 (21.4)	176 (7.4)	176 (4.4)
	No innovin	73 (10.6)	84 (19.5)	78 (5.3)	58 (3.3)
Time to Peak (mins)	**Neat**	13.3 (0.47)	13.5 (0.17)	13.5 (0.16)	13.0 (0.31)
	1 in 2	19.2 (0.57)	14.4 (0.17)	17.5 (0.16)	9.8 (0.31)
	1 in 4	17.0 (0.5)	16.5 (1.17)	15.5 (0.17)	16.3 (0)
	No innovin	20.2 (0.67)	22.2 (1.36)	18.9 (1.0)	58.2 (1.0)
Start tail (mins)	**Neat**	40.6 (0.79)	40.4 (0.33)	41 (0.27)	38.8 (0.83)
	1 in 2	47.4 (1.5)	37.6 (0.17)	43.6 (0.94)	29.1 (2.75)
	1 in 4	44.8 (0.33)	43.9 (1.5)	44.1 (0.5)	41.3 (0.17)
	No innovin	48.0 (1.28)	46.0 (3.2)	41.0 (0.98)	43.2 (1.12)

Serial dilutions were performed on Innovin®(Dade®), with a starting dilution of 1 in 17,000 (denoted as 'neat' concentration of Innovin in the table, determined to be a concentration of 1pM TF).
Data are mean (n = 20) and standard deviation.

Key: ETP – endogenous thrombin potential.

Table S2. Effect of soluble TM concentrations on thrombin generation in healthy volunteers.

	Concentration of added soluble thrombomodulin (ng/mL)					
	0 TM	**4 TM**	**8 TM**	**16 TM**	**64 TM**	**One way ANOVA p value**
Lagtime (min)	12.5 (2.5)	12.8 (2.7)	13.2 (2.9)	13.1 (2.5)	13.1 (2.9)	NS
ETP (nM/min)	1583 (307)	1237 (384)	1189 (325)	1150 (372)	788 (322)	p = <0.0001
Peak height (nM)	229.3 (69.7)	187.6 (71.1)	184.8 (67.1)	187.1 (68.2)	147.8 (67.1)	p = <0.0001
ttPeak (min)	16.3 (2.8)	16.6 (3.3)	17.0 (3.4)	16.6 (2.8)	16.1 (3.0)	NS
Start tail (min)	36.4 (4.4)	36.1 (4.7)	36.3 (4.8)	35.5 (3.9)	34.1 (3.9)	p = <0.0001

Data are mean (n = 20, in triplicate) and standard deviation.

Key: ETP – endogenous thrombin potential; TM – thrombomodulin.

Table S3. Effect of APC concentrations on thrombin generation in healthy volunteers.

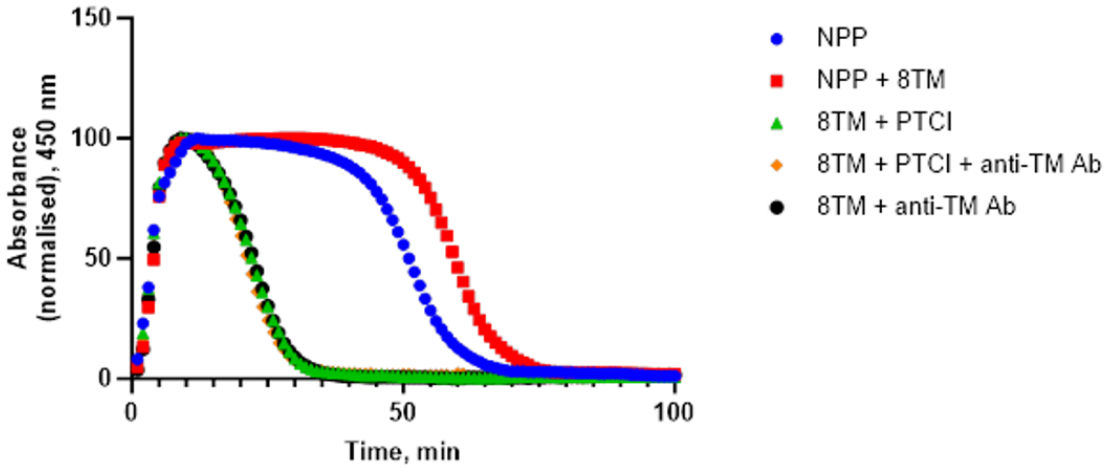
	Concentration of added activated protein C (pM)					
	0 APC	**50 APC**	**100 APC**	**200 APC**	**400 APC**	**One way ANOVA p value**
Lagtime (min)	12.95 (2.5)	14.2 (2.2)	13.59 (2.9)	14.13 (4.8)	13.38 (2.8)	NS
ETP (nM/min)	1583 (307)	1931 (939.5)	1658 (499.2)	2096 (542.5)	1748 (597.6)	NS
Peak height (nM)	229 (69.7)	254 (114.5)	224 (103.7)	300 (129.0)	240 (120.6)	0.005
ttPeak (min)	16.3 (2.8)	18.6 (3.0)	17.9 (3.7)	18.3 (6.0)	17.7 (3.7)	<0.0001
Start tail (min)	36.4 (4.4)	54.4 (5.1)	52.8 (6.2)	50.9 (6.0)	53.3 (5.9)	<0.0001

Data are mean (n = 20) and standard deviation.

Key: APC – activated protein C; ETP – endogenous thrombin potential.

Supplementary Figure 1.

Effects of PTCI and anti-thrombomodulin antibody on clot lysis.

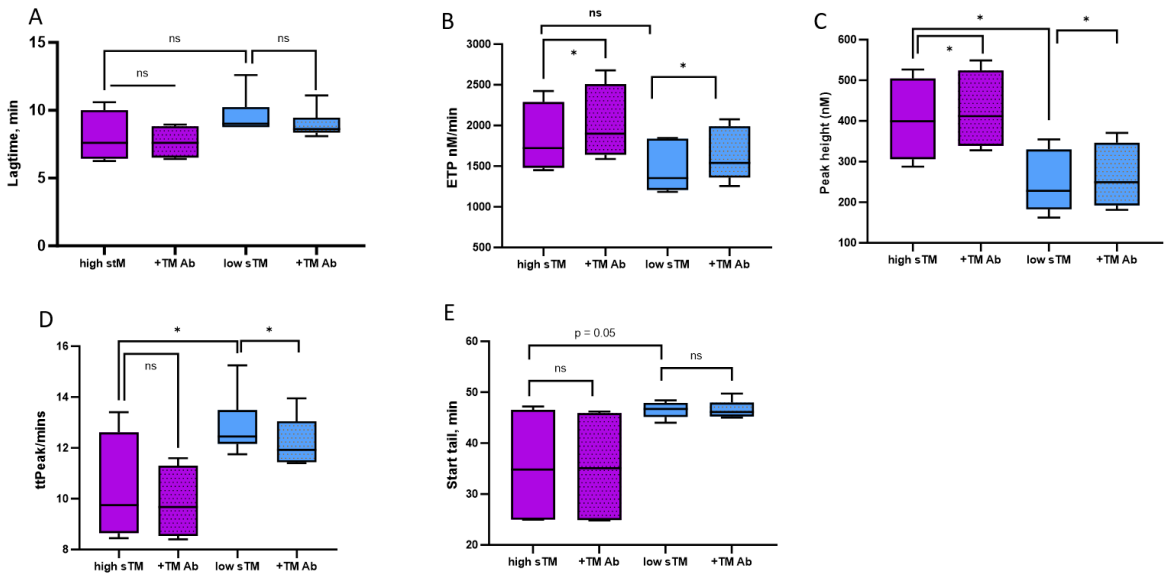


Data show curves of mean absorbance, detailing the effects of: 8ng/mL sTM, 50 ng/mL PTCI, 1 mcg/mL anti-TM antibody using normal pooled plasma on clot lysis. (Curves show the amalgamated mean data from each experimental condition, n = 3). Here the figure shows data only relating to an experimental condition using added thrombomodulin at 8ng/mL concentration. Similar effects across the 4 – 16 ng/mL sTM range were seen (data not shown).

Key. PTCI – potato tuber carboxypeptidase inhibitor; sTM – soluble thrombomodulin

Supplementary Figure 2.

Thrombin generation according to high sTM and low sTM trauma groups, with anti-TM antibody.

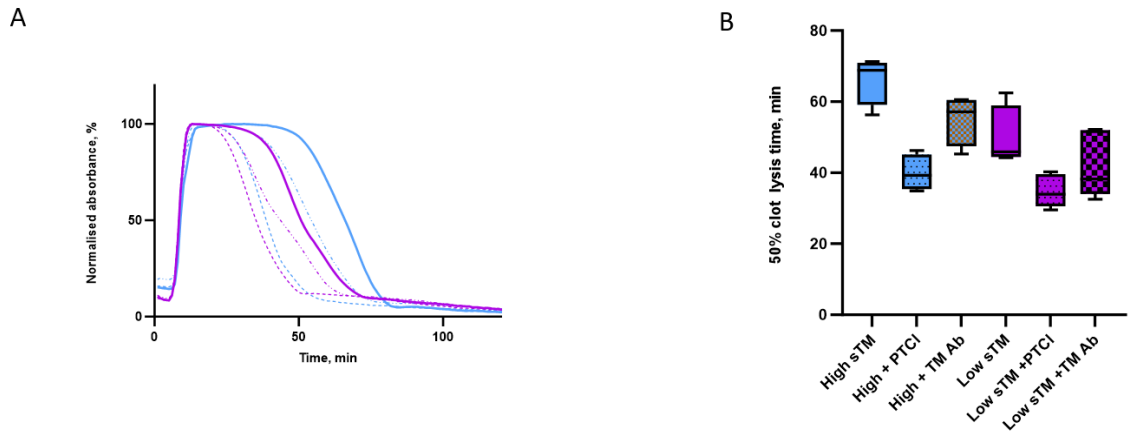


Thrombin generation parameters, comparing trauma patients with high sTM (purple) and low sTM (blue) levels, with and without anti-TM antibody, 1mcg/mL.
A: lagtime; B: Endogenous thrombin potential (ETP); C: Peak height; D: time to peak height; E: time to the start tail.

Key: ETP – endogenous thrombin potential; TM – thrombomodulin.

Supplementary Figure 3.

Clot lysis according to high sTM and low sTM trauma groups, with added PTCI or anti-TM antibody.



A. Data show amalgamated mean, normalised clot lysis curves for the trauma patients with the highest (n = 4) and lowest (n = 5) circulating sTM values. B. Data show median (IQR) 50% clot lysis times for those with the highest and lowest sTM values. Within groups, there was no significant differences between each group. The high sTM group had, on average, significantly longer times to 50% clot lysis (p = 0.03).

Key: purple lines – low sTM, blue lines – high sTM. Solid line: plasma alone, dotted line: plasma + 50 ng/mL PTCI, dot-dashed line: plasma + 1mcg/mL anti EGF5/6 TM antibody.