**TITLE PAGE**

**Activated Protein C Drives the Hyperfibrinolysis of Acute Traumatic Coagulopathy**

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**ABSTRACT**

***Background:*** Major trauma is a leading cause of morbidity and mortality worldwide with hemorrhage accounting for 40% of deaths. Acute Traumatic Coagulopathy (ATC) exacerbates bleeding but controversy remains over the degree to which inhibition of procoagulant pathways (anticoagulation), fibrinogen loss and fibrinolysis drive the pathological process. Through a combination of experimental study in a murine model of trauma hemorrhage (TH) and human observation our objective was to determine the predominant pathophysiology of ATC.

***Methods:*** First, a prospective cohort study of 300 trauma patients admitted to a single level 1 trauma center with blood samples collected on arrival. Second, a murine model of ATC with suppressed Protein C activation via genetic mutation of thrombomodulin. In both studies analysis for coagulation screen, aPC levels and ROTEM were performed.

***Results:*** In patients with ATC we have demonstrated elevated aPC levels with profound fibrinolytic activity and early depletion of fibrinogen. Procoagulant pathways were only minimally inhibited with preservation of capacity to generate thrombin. Compared to Factor V and VIII, proteases which do not undergo aPC-mediated cleavage were reduced but maintained within normal levels. In transgenic mice with reduced capacity to activate PC, both fibrinolysis and fibrinogen depletion were significantly attenuated. Other recognised drivers of coagulopathy were associated with less significant perturbations of coagulation.

***Conclusions:*** APC **associated** fibrinolysis and fibrinogenolysis, rather than inhibition of procoagulant pathways, predominate in ATC. In combination these findings suggest a central role for the Protein C pathway in ATC, and provide new translational opportunities for management of major trauma hemorrhage.

## INTRODUCTION

Trauma is a major global public health issue causing nearly six million deaths worldwide each year.[1] Hemorrhage is responsible for approximately half of these deaths and is the leading cause of preventable death after injury.[2,3] Bleeding is exacerbated by Acute Traumatic Coagulopathy (ATC) which is present immediately on hospital admission in up to 25% of severely injured patients.[4-6] ATC is associated with a four-fold increase in mortality, more severe organ injury and higher transfusion requirements.[5-7] Resuscitation strategies targeted at ATC have shown potential to dramatically reduce death from injury, possibly mediated by early reversal of coagulopathy.[8] However, controversy exists over the precise mechanism of ATC and the balance between inhibition of procoagulant pathways (anticoagulation or consumption), fibrinogen loss and fibrinolysis.[9-13] Failure to define the pathophysiology of ATC has prevented identification of the optimal therapeutic intervention for early traumatic coagulopathy and remains an important research priority for trauma care.

ATC is a clinical syndrome[14] evident soon after injury[4,5,15], characterised predominantly by functional reductions in clot generation and clot strength with only minor prolongations in clotting times[16]. Biomarker[7,17,18] and animal studies[19] have highlighted the pathogenic contributions of shock and tissue injury in ATC[17,20] suggesting an endogenous process resulting in systemic anticoagulation, hyperfibrinolysis[7,10,21,22] and fibrinogen depletion[23,24]. It is postulated that thrombin is switched from a pro- to anticoagulation function with diversion from fibrin generation towards increased production of activated PC (aPC)[7,17,25] and subsequent cleavage of Factors Va and VIIIa. In excess aPC consumes plasminogen activator inhibitor-1 (PAI-1) and releases fibrinolysis from inhibitory control with consequent rise in tissue plasminogen activator (tPA) and plasmin[7,26]. Tissue injury itself is associated with increased thrombin generation[27], release of tPA from the endothelium and activation of the fibrinolytic system[28]. Some hypothesise these early derangements after trauma haemorrhage represent Disseminated Intravascular Coagulation (DIC) with a fibrinolytic phenotype[13] and propose the early fibrinogen loss is a direct result of fibrinogenolysis[10]. With a lack of mechanistic confirmation, the interplay between thrombin generation, anticoagulation, fibrinogen availability and hyperfibrinolysis in ATC remains unclear. Shock and tissue injury both independently promote fibrinolysis but in combination, through the release of aPC, could in theory provide the stimulus for massive systemic fibrinolytic activity and potentially unify the apparent opposing theories of ATC.

The overall objective of this combined prospective human cohort study of trauma patients and experimental model of trauma hemorrhage, was to determine the role of aPC in ATC and specifically quantify the balance between inhibition of procoagulant pathways (anticoagulation), fibrinogen loss and fibrinolysis. This study builds on our previous evaluation of the downstream elements of fibrinolysis in ATC[21], with emphasis on mechanistic study rather than clinical outcome, in a detailed examination of functional coagulation and biomarkers of both procoagulant and fibrinolytic pathways. The first aim was to functionally characterise ATC with measurement of aPC levels in trauma patients to determine the relationship between aPC, procoagulant factors, thrombin generation and fibrinolysis. Second, we wished to characterise the relationship of plasma aPC levels with markers and mediators of fibrinolytic activation in humans. Third, we aimed to mechanistically confirm the role of aPC in a murine model of trauma hemorrhage to understand the effects of genetic modulation of the PC pathway on clot formation, fibrinolysis and fibrinogenolysis. Finally, we report outcomes for trauma patients with ATC stratified by aPC levels soon after injury.

## METHODS

### Human study design

Single center, prospective, cohort study of trauma patients presenting directly to a level 1 trauma center (January 2007 - June 2009). The study is part of the Activation of Coagulation and Inflammation in Trauma (ACIT) research programme. We have previously published data from the same cohort on functional measures of coagulation[16] and fibrinolysis[21]. ACIT is approved by the UK National Research Ethics Committee (East London and the City, London, UK). Patient consent was in accordance with the Mental Capacity Act (England; 2005) for inclusion of incapacitated patients into emergency medical research using a Professional Consultee (independent physician).

*Patient Sampling and Data Collection*

All adult trauma patients (>15 years) meeting local criteria for full trauma team activation were eligible for enrolment and recruited when research personnel present (08:00-22:00 daily). ACIT inclusion/exclusion criteria, data collection, blood sampling, assays and outcome measures have been previously reported[21]. A 20-mL research sample of blood was drawn from either the femoral vein or antecubital fossa, and the standard trauma laboratory tests were performed within 20 min of arrival in the ED. Blood for ROTEM analysis was drawn into a 2.7-mL citrated vacutainer (0.109 M buffered sodium citrate, 3.2%; Becton Dickinson, Plymouth, UK). Samples for PT determination were collected in 4.5-mL glass vacutainers (0.109 M buffered sodium citrate, 3.2%; Becton Dickinson), 9:1 (v/v). The sample for hemostatic assays and thrombin generation was placed in a citrated tube, and spun down within 2 h of blood draw. The sample was first spun at 1750g for 10 min; the supernatant was then extracted, and respun at 1750g for a further 10 min. The extracted plasma was stored in aliquots at -80°C. Arterial blood analysis for base deficit (BD) was performed simultaneously with the research sample collection. Assays for coagulation factors were complete in 99-100% of patients. Plasma samples for biomarkers requiring ELISA analysis were incomplete in 3% of cases except for aPC which could not be quantified in 7% of patients.

Samples were analysed at the conclusion of the study with an automated analyser (Sysmex CA-CS2100i System, Siemens AG, Germany) to measure coagulation Factor activity [normal range]: II [78-117], V [66-114], VII [50-150] VIII [52-153], IX [58-138], X [50-150], XI [50-150], XIII [70-140], von Willebrand Factor (vWF) [50-160], Protein C (PC) [75-134], Antithrombin (AT) [80-130]. Enzyme linked immunosorbant assays were used to quantify tissue plasminogen activator (tPA; Asserachrom tPA, Diagnostica Stago, France) (normal range 2–12 ng/mL); prothrombin fragments 1+2 (PF 1+2; Enzygnost F1+2 monoclonal, Siemens Healthcare Diagnostics, Germany) (normal range 69-229 pmol/L); plasmin-α2-antiplasmin complex (Plasmin-Antiplasmin; DRG Plasmin-Antiplasmin micro, Germany) (normal range 120-700 µmol/L); aPC (EIA, Surgical Research Laboratory, University of California, San Francisco General Hospital, USA [1-3ng/ml]). PT and Clauss fibrinogen were processed by the central hospital laboratory along with a standard full blood count (FBC). Prothrombin ratio (PTr) was calculated as observed PT divided by mean normal PT for the reagent used.

ATC was defined using ROTEM (EXTEM) – Amplitude (of clot) 5 minutes (A5) after Clotting Time (CT) based an earlier study demonstrating that low CA5 and Maximum Clot Firmness (MCF) were the viscoelastic hallmarks of ATC[16]. Additional EXTEM parameters reported were EXTEM assays, Clot Formation Time (CFT), Alpha angle and Maximum Clot Firmness (MCF). Prolongation of PT is associated with coagulopathy clinically and PTr >1.2 was used for comparison between human and animal studies.[16,20] Hypoperfusion was defined as base deficit (BD) >6mEq/l[29,30] FVIII:vWF ratio calculated to reflect the differential inhibition by aPC. Blood for aPC analysis was collected into protease inhibitors (P100 - Becton Dickinson, Plymouth, UK) and measured using ELISA[31]. Protein C circulates in plasma at 70 nM as the zymogen of the anticoagulant serine protease, activated protein C (aPC), which averages 40 pM (~ 2.3 ng/mL +/- 0.2ng/ml) in normal plasma[32]. Kaiser et al[33] reported in vitro inhibition of clotting at 10 ng/ml aPC and for convenience we divided groups into four with the aPC <3ng/ml representing normal range. Thrombin generation was measured via calibrated automated thrombogram (CAT) using standard protocol[34] in duplicate in a Fluoroskan Ascent® reader (thermo Labsystems, Helsinki, Finland; filters 390nm excitation and 460 nm emission). Thrombin generation curves and the area-under-the-curve (endogenous thrombin potential, ETP) were calculated using Thrombinoscope™ software (Thrombinoscope BV, Maastricht, Netherlands).

*Outcome measures*

Patients were followed until hospital discharge or death. For mortality analysis patients surviving to hospital discharge were assumed to still be alive. Outcome measures were recorded for 28-day ventilator-free days, blood transfusion requirements in the first 12 hours and length of critical care and overall hospital stay.

**Animal study design**

To mechanistically verify the clinical findings in humans we analysed coagulation profiles of experimental models of trauma hemorrhage. Wild type (*WT*) mice and transgenic thrombomodulin knock-in (*TMKI*) mice with reduced capacity to activate PC were compared. In addition we evaluated ATC in homozygous mice for Factor V Leiden who are resistant to aPC mediated cleavage. A novel murine model for ATC was developed based on the experimental procedure previously reported in a rat model of trauma hemorrhage[20] . All mice were cared for in accordance with the UK Home Office Guidance in the Operation of the Animals (Scientific Procedures) Act 1986. General anaesthesia was induced and maintained with spontaneous respiration of isoflurane carried in medical air. Animals were placed supine on a heated anaesthesia platform and body temperature was maintained at 37 ± 1 °C by means of a rectal probe attached to a homeothermic blanket (Harvard Apparatus). The left carotid artery and external jugular vein of all animals were catheterised with polyethylene tubing (Portex) connected to a pressure transducer (Capto SP 844, AD instruments) and syringe pump (PHD 22/2000, Harvard Apparatus). Fluid was continuously infused at a rate of 50 µl per hour through the carotid catheter with supplementary flushes as required in order to maintain patency.

Mice in the trauma haemorrhage group received a 2-cm paramedian laparotomy, closed in one layer with 7-mm surgical clips (Harvard Apparatus) and bilateral mid-shaft closed tibia and fibula fractures. The experimental period commenced immediately after traumatic injury or 7 min after completion of carotid catheterization (the mean time taken to complete traumatization). Animals were then bled from an average mean arterial pressure of 91 mmHg (MAP) to a target of 25–30 mmHg over a period of 60 min and this pressure was maintained by further withdrawals as necessary.

Study randomisation and blinding was ensured by using a predefined alternated assignment of animals to experimental groups and the use of identification codes with respect to the *WT* and the *TMKI* groups throughout the experimental intervention and data analysis (both with a C57/B6 phenotype). For FVL studies the genetic background was revealed at the end of the study.

*Factor V Leiden*

During the experimental period the genetic background was unknown. V Leiden mice (Strain B6.129S2-F5tm2Dgi/J, Jackson Laboratory, Bar Harbor, Main, USA) are knock-out in a C57BL/6 background, expressing factor VR504Q, resistant to activated protein C proteolysis. When performing the data analysis the background was revealed: 14 mice were *WT*, 15 were V Leiden heterozygous (HT) and 15 were homozygous (mutant -MT).

*Transgenic inhibition of aPC*

Homozygote TMPro 'knock-in' (*TMKI*) mice with thousand-fold reduced capacity to activate PC (Blood Research Institute of Wisconsin, Milwaukee, USA) bred from C57/B6 mice after targeted mutagenesis with a single amino acid substitution (Glu404Pro) for the thrombomodulin receptor. 26 *TMKI* mice underwent laparotomy and trauma hemorrhage to MAP 25–30 mmHg for 60 min (*TMKI* TH). The same protocol was performed in 26 experimental controls (C57/B6 TH). A further mice of each genotype were anaesthetised and monitored for 60 min to act as sham controls (anaesthestised/monitored): *TMKI* sham (n=12) and C57/B6 sham (n=12).

*Sampling technique & analysis*

50µl blood/saline aspirated from the carotid catheter of all animals and discarded. 200µl aspirated into 1 ml syringe pre-filled with 20µl 3.2% sodium citrate (Sigma, UK) to achieve 1:9 concentration. Lactate measured (Accutrend, Roche) and the remainder centrifuged at 3500g for 15 minutes. Plasma supernatants were aspirated and immediately frozen in liquid nitrogen before storage at -80°C. Citrated blood analysed using pediatric

ROTEM cups and ELISA for D-dimer (USCN Life Sciences Inc, Wuhan, China) and fibrinogen (Genway Biotech, San Diego, CA).

***Statistical analysis***

Patients with ATC were compared with those presenting without ATC for clinical characteristics and coagulation profiles. The relationship between aPC, functional coagulation (ROTEM) and key coagulation factors was then evaluated. Next key biomarkers of the fibrinolytic pathway were examined to determine their relationship to aPC and known control mechanisms e.g. PAI-1, tPA. Finally, associations between admission aPC level and clinical outcomes were explored. In the experimental models the effect of genetic modulation of the PC pathway was determined through analysis of aPC and functional parameters of coagulation (ROTEM). Finally the role of the Protein C pathway in the murine model and impact of impaired thrombomodulin generation on fibrinolysis, fibrinogen and mortality was examined.

The human study was based on the existing available data from a convenience sample. For the experimental models, sample size was calculated with statistical power of 90% and significance level $\alpha = 0.05$ to detect a 25% difference in mortality from a baseline mortality rate of 20% in a murine model of trauma haemorrhage[35] and 20% difference in ROTEM parameters based on a previous animal study in ATC[20]. Normal quantile plots were used to test for Normal distribution. Parametric data is expressed as mean ±95% confidence intervals. All non-parametric data is presented as median (interquartile range, IQR) and analysed using a two tailed Mann Whitney U test. Univariate linear regression was performed using best-fit model for calculation of $R^2$ values. Two-group analysis was performed using a two-tailed, unequal variance Student's *t* test. Multi-group analysis was performed using one-way ANOVA with Dunnett's post hoc correction or two-way ANOVA with Bonferroni's post test between subjects for determining interaction with elevated levels of aPC. $\chi^2$ test was used for dichotomous data analysis. Survival analysis in the murine model was performed by deriving Kaplan-Meier plots and curves compared using the log rank test (Mantel-Haenszel method. Significance level was set at $p<0.05$ and all statistical analysis was performed using GraphPad Prism version 5.00 for Windows, GraphPad Software, San Diego California USA.

## RESULTS

There were 325 patients enrolled into the human study over the 19-month period. ROTEM sample analysis was incomplete in three patients, consent was not obtained in 15 cases and there were seven retrospective exclusions leaving 300 patients available for analysis. Median age was 33 (23-48) years, 82% were male and 21% presented following penetrating trauma. Patients had sustained significant trauma with median ISS 10 (4-25) and 41% were severely injured (ISS>15). Average time from injury to blood sampling was 86 (69-112) minutes, 9 (5-14) minutes from arrival in ED. On arrival 15% of patients were severely shocked (BD >6 mEq/L), 17% coagulopathic by ROTEM definition (A5 ≤35mm) and 9% with PTr >1.2 (median PT 11.2 [10.8-11.9] seconds). Minimal intravenous fluid was administered prior to baseline sample collection was 0 (0-400) ml and no patient received artificial colloid or vasoactive agents prior to venepuncture. Overall mortality was 8%. We retrospectively analysed the deaths of 25/26 patients from electronic archives – seven patients died from exsanguination, six from multiple organ dysfunction (MODS) or sepsis and 12 from traumatic brain injury. Mean admission aPC levels in survivors were 1.8 +/-0.6 ng/ml. Patients that died early (<24 hours) from haemorrhage had significantly higher aPC levels on admission compared to those who died after 24hrs from MODS or sepsis, early haemorrhagic deaths: aPC 30ng/ml vs MODS/sepsis deaths: aPC 8ng/ml ($p<0.05$).

### Human study (mechanistic investigation)

In this study, patients with ATC (A5 ≤35mm) had aPC levels over five times higher than those with normal coagulation (Table 1). Elevated aPC was associated with a significant concentration-dependent decrease in ROTEM parameters of clot strength (Figures 1A and 1B) and prolongation of PTr (aPC <3ng/ml: PTr 1.0 [1.0-1.0] vs aPC >9ng/ml: PTr 1.3 [1.2-1.4], $p<0.001$). ATC has been shown to be associated with early PC depletion[7] and we now demonstrate that a reduction in PC activity is associated with high aPC (aPC ≤3ng/ml: PC activity 91% vs. aPC >9ng/ml: PC activity 65%, $p<0.001$). Activated PC was elevated only in the presence of shock (BD>6 mmol/l), (BD >6mmol: aPC: 9 (5-13) ng/ml vs. BD <3mmol/l: 1 (0-1) ng/ml, $p<0.001$).

Activated PC is known to inactivate coagulation Factors V and VIII and can result in systemic anticoagulation. Factor V has the greatest reduction in activity in ATC (average 32% reduction, Table 1). High aPC levels in trauma patients were associated with a 50% reduction in FV activity (Figure 1C). 85% of patients with FV

<50% had A5 ≤35mm ($p<0.001$) and progressive loss of FV was associated with a concentration-dependent reduction of ROTEM clot strength (see Supplemental Digital Content 1A – figure showing association of falling Factor V level with reduced A5). Similarly, the FVIII:vWF ratio was significantly reduced as aPC levels increased suggesting a relative inhibition of FVIIIa (Figure 1D). However, preservation of Factor V activity did not fully protect against coagulopathy in trauma. Reduced Factor V is not sufficient to inhibit procoagulant pathways as endogenous thrombin potential (ETP) is maintained regardless of aPC concentration (Figure 1E). Consequently, in trauma patients aPC is not associated with any significant increase in CT (Figure 1F) and prolongation in PTr was only observed when 3x normal (>9 ng/ml). This suggests that the reduced FV/VIII levels associated with aPC cannot fully explain the observed functional coagulation changes in ATC. Together these data suggest that systemic aPC-induced anticoagulation is not the primary mechanism in ATC.

Levels of procoagulant factors which are not targets of aPC mediated cleavage were preserved in ATC. FII, FVII, FIX and FX activity were all maintained at >80% (see Supplemental Digital Content 1B – figure of aPC levels and FII, FVII, FIX and FX activity) and thrombin generation was in fact twofold higher in the ATC cohort (Table 1). Widespread consumption of procoagulant factors and reduced thrombin generating capacity does not appear to be a major cause of ATC. Procoagulant factor activity (sampled in pH buffered assays) was minimally affected by the degree of metabolic acidosis with levels preserved above 80% at BD>6 mmol/l (see Supplemental Digital Content 1C – figure of coagulation Factor levels stratified by BD). Hemodilution with intravenous fluids produced minimal effects on coagulation and low haematocrit was not associated with any significant changes in clot strength (see Supplemental Digital Content 1D – figure showing no change in A5 with respect to changing hematocrit). In patients receiving >1000ml fluid before sampling, procoagulant factors were all maintained above 80% with relative preservation of fibrinogen levels (0ml: Fibrinogen 2.17g/dl vs. >1000ml: Fibrinogen 1.82g/dL, $p<0.05$) (see Supplemental Digital Content 2 – table of coagulation Factor levels stratified by amount of fluid administered). Patients given intravenous fluids had only minor changes in clotting parameters and haemodilution in isolation does not appear to be an important mechanism for ATC.

Average fibrinogen levels were significantly lower in the ATC cohort (A5 ≤35mm) (1.35 [1.19-1.51] g/L vs. A5 >35mm: 2.23 [1.53-2.93] g/L, $p<0.05$) and this 40% reduction in fibrinogen levels was a greater reduction than any other procoagulant factor (Table 1). Despite very high thrombin generation, fibrinogen levels were

maintained above critical levels while aPC was normal, suggesting that consumption is not the primary mechanism of fibrinogen loss (Figure 2A). High levels of aPC were associated with low fibrinogen levels (Figure 2B) and reduced functional fibrinogen activity (FIBTEM) (Figure 2C). There was a trend towards fibrinogen reduction to critical levels (<1.5g/dL) in association with high thrombin generation, only in the presence of elevated aPC, p=0.08 (Figure 2A). Currently it is not possible to accurately quantify fibrinogenolysis independent of fibrinolysis, however Plasmin-Antiplasmin levels were inversely correlated with fibrinogen ($R^2$ =0.24, p<0.01). In patients with low fibrinogen (<1.5g/L), Plasmin-Antiplasmin levels were 15 times the upper limit of normal and significantly higher than patients with normal fibrinogen (Fibrinogen <1.5g/L: Plasmin-Antiplasmin 10739 μg/L vs. Fibrinogen >1.5g/L: Plasmin-Antiplasmin 2734 μg/L, p<0.01).

High aPC was associated with increased fibrinolysis as indicated by elevated Plasmin-Antiplasmin levels (aPC ≤3ng/ml: Plasmin-Antiplasmin 2221 ±392μg/L vs aPC >9ng/ml: Plasmin-Antiplasmin 18955 ±3861μg/L, p<0.001). This tPA dependent process was completely inhibited at normal aPC levels but was massively activated when aPC was high (Figure 2D). Mechanistically aPC is known to consume PAI-1 when in excess, and there was a trend towards reduced PAI-1 in trauma patients with high aPC (>9ng/ml) (Figure 2E). Fibrinolysis is known to co-activate with thrombin generation through its direct induction of tPA release. However we were clearly able to show that, as with fibrinogenolysis, thrombin generation associated fibrinolytic activity (D dimer production) in ATC is significantly higher in the presence of elevated aPC levels (Figure 2F).

**Human study (outcomes)**

Patients with ATC had increased mortality: PTr>1.2 (52%) and A5≤35mm (26%). Consistent with our previous study[7], high aPC on admission was strongly associated with increased all-cause mortality (see Supplemental Digital Content 3A – figure). Patients with aPC >9 ng/ml had a mortality of 68% compared to 2% for those with normal aPC levels (p<0.001). There was a concentration-dependent relationship between aPC and transfusion requirements for both PRBC and FFP (see Supplemental Digital Content 3B – figure). Patients requiring a massive transfusion (10+ PRBC units) had significantly higher aPC levels compared with those receiving PRBC <10 units: aPC 20 (15-25) ng/ml vs. 3 (3-3) ng/ml (p<0.001). High aPC was associated with

longer hospital median length of stay (aPC >9ng/ml: 30 days vs. aPC ≤3ng/ml: 5 days, p<0.001). Patients with elevated aPC on admission required significantly more days in ICU (see Supplemental Digital Content 3C – figure) and had fewer ventilator-free days at 28 days (aPC >9ng/ml: 2 days vs. aPC ≤3ng/ml: 5 days, p<0.001). Increased aPC is associated with excess mortality and morbidity in ATC.

**Murine studies**

Trauma hemorrhage was associated with significantly higher aPC levels in *WT* mice compared to Sham animals: 10.3 (7.8-12.7) ng/ml vs. 1.5 (1.2-1.8) ng/ml (p<0.001) (Figure 3A). *TMKI* mice had a minor increase in aPC levels and were relatively protected from ATC after trauma hemorrhage for 60 minutes. In comparison to *WT,* clot strength (MCF) was well preserved (Figure 3B and Supplemental Digital Content 4 - table) with significantly less marked prolongations of PT and APTT (Supplemental Digital Content 4 - table). Homozygous mice for Factor V Leiden when subjected to TH had similar prolongations in CT (Figure 3C) and clot generation (Figure S3) compared with *WT animals*. In support of the findings in humans, there was only a minor prolongation in CT (20%) in *WT* mice and no change in *TMKI* animals subjected to trauma hemorrhage (Figure 3D). As with *WT* animals, Factor V Leiden mice after trauma hemorrhage had significant delays in clot formation (CFT) and reductions in clot strength, albeit to a slightly lesser extent than *WT* (see Supplemental Digital Content 5 - figures). In this experimental model a reduction in endogenous capacity to activate PC was protective against ATC in mice whilst preserved Factor V function did not fully prevent ATC.

Confirming the observation seen in humans of apparent aPC-related fibrinolytic activity, *WT* mice after 60 minutes of trauma hemorrhage exhibited a three-fold increase in D-Dimer levels which was completely abolished in the *TMKI* variants (Figure 4A). In the murine model, fibrinogen was observed to fall by over a third after trauma hemorrhage in *WT* mice (T0: 1.86g/L vs. T60: 1.25g/L, p<0.05) but *TMKI* animals were protected from this fibrinogen depletion with only a non-significant reduction in fibrinogen after trauma hemorrhage (Figure 4B). Mortality rates in *TMKI* mice were almost half that of *WT* mice subjected to TH (*WT*: 42.3% vs *TMKI*: 23.1%, p<0.05) with longer median survival times (Figure 4C).

**DISCUSSION**

In both clinical and experimental studies we have demonstrated that aPC may be a unifying link in the current opposing hypotheses of early traumatic coagulopathy. Building on previously published data from our human cohort study[21], this work completes a detailed evaluation of the procoagulant and fibrinolytic systems in ATC, highlighting a potential central mechanism for aPC with mechanistic confirmation in an experimental model of trauma haemorrhage. Patients with ATC had elevated aPC levels, found fibrinolytic activity and early depletion of fibrinogen yet with only minimal inhibition of procoagulant pathways (factor loss) and preserved thrombin generation. Fibrinogen was significantly reduced in association with elevated levels of aPC with no evidence to support a systemic consumptive process to explain this early depletion. ATC, in particular fibrinolysis and fibrinogen depletion, were both significantly attenuated following trauma hemorrhage in transgenic mice with reduced capacity to activate PC. These results add further evidence to earlier work highlighting the importance of the PC pathway[7,18,35-37] and in a murine model provide mechanistic confirmation of aPC with respect to fibrinogen loss and fibrinolysis in ATC.

In the early stages after injury, generation of aPC appears to be of greater importance than other classical mediators of traumatic coagulopathy (haemodilution, acidosis, clotting factor consumption). Dilution of plasma alters the dynamics of thrombin generation by relative reductions in both pro and anti-coagulant factors.[38]. Paradoxically dilution actually increases thrombin generation until plasma proteins are reduced below 40% of normal.[27] Consistent with this observation we have shown that relatively small volumes of intravenous fluids had some minimal effect on clot amplitude. However *in vitro* measurements of clot strength in the presence of hemodilution may not be true representation of clot integrity *in vivo*. An increased proportion of plasma in the ROTEM cup produces an apparent increase in clot strength although hemodilution will simultaneously reduce the platelet count and clot strength. Platelet counts were well preserved above 150 despite hemodilution but aPC levels were significantly higher in patients receiving large amounts of crystalloids. The effects of dilution may therefore be confounded by the degree of shock, which we have shown is associated with increased aPC production. APC was associated with selective depletion of Factors V and VIII but no reduction in the capacity to generate thrombin and hence with only minimal effects on PTr. These findings are consistent with a recent study demonstrating no difference in endogenous thrombin potential between severely injured patients and healthy controls.[39] In FVL mice, resistant to aPC-mediated cleavage of V, ATC was still observed following

trauma hemorrhage, therefore inhibition of procoagulant pathways (anticoagulation) cannot fully account for functional changes of haemostasis after major trauma. Thrombin generation and platelet count in patients with ATC were normal with no clear evidence of widespread consumption of clotting factors. This is supported by Johansson and colleagues who in a study of 80 severely injured and shocked trauma patients were unable to identify any overt disseminated intravascular coagulation according to ISTH criteria.[11] ATC is defined predominantly by diminished clot strength but preservation of procoagulant pathways and thrombin generation.

Procoagulant factors are maintained at near normal levels and are unlikely to be responsible for the observed prolongation of PT or reductions in A5 and MCF. The mechanism for diminished clot strength and increased clotting times must therefore involve fibrinogen, consistent with other recent investigations of alternative mechanisms for early traumatic coagulopathy.[10,40,41] We have previously shown that fibrinogen reduction is a principle component of ATC [16,24] with both retrospective data[42,43] and a pilot randomised controlled trial of cryoprecipitate[44] demonstrating an association between early replacement and improved outcome. Fibrinogen levels were reduced significantly in the human study to levels below transfusion guidelines for replacement (<1.5g/dL).[45] In the experimental model, *TMKI* mice were protected against significant fibrinogen depletion in contrast to *WT*, suggesting an aPC related process is responsible for early loss. Currently, the critical level for haemostatic function is unknown and potential mechanisms for loss of fibrinogen remain to be elucidated[24,45] although indirect evidence for fibrinogenolysis has been demonstrated in alternative models of TH.[10] In this study activation of the fibrinolytic system was marked and occurred in association with elevated aPC and appeared to be a tPA related process with consumption of PAI-1. Genetic modulation of the PC pathway was protective in the *TMKI* with significant attenuation of fibrinolysis following TH. Plasmin can directly induce fibrinogenolysis[46] and together these novel findings suggest production of aPC early after trauma hemorrhage, may release both fibrinolysis and fibrinogenolysis from inhibitory control to give rise to systemic clot lysis and direct breakdown of fibrinogen.

High levels of aPC on admission were associated with increased mortality, longer hospital stay and increased transfusion requirements. 70% of patients that died early did so from exsanguination and had significantly higher aPC levels on admission compared to those who died later from MODS or sepsis. APC levels in these patients dying from MODS were three times normal suggesting early activation of PC, with subacute depletion

may be a risk factor for later sepsis and organ failure[17]. Low protein C levels have been reported several hours or days after injury[36,37,47] and patients who develop ventilator acquired pneumonia have persistently low plasma levels of PC in the immediate period after trauma.[36] PC depletion after sepsis is well reported[48,49] with thrombotic microvascular organ injury[50] and may contribute directly to cell dysfunction and death.[51] In trauma early depletion of PC would result in a procoagulant state with insufficient cytoprotective mechanisms and theoretically would predispose to septic complications and organ injury, as reported by Cohen and colleagues.[36]

There are several limitations to this study. First, the endothelium and thrombocytes are fundamental to both coagulation and inflammation with the platelet membrane of central importance to clot assembly. We did not measure platelet function in this study and our results are therefore based on plasma protein levels. At present the role of platelets in ATC pathophysiology remains unknown. In light of the results from the PROPPR study and potential outcome benefits of early platelet transfusion, endogenous function and aggregation capacity of transfused platelets should be a key focus of research. Second, our definition of ATC (A5 $\leq$35mm) is based on earlier work which functionally characterised it by reduced clot strength rather than prolonged clotting times[16]. Patients meeting this definition will therefore more likely have lower fibrinogen levels and possibly reduced platelet function and/or count. Third, we were unable to measure activated FV and FVIII to assess direct effects of aPC. However, increased loss of FVa and FVIIIa by aPC mediated cleavage will produce reciprocal reductions in FV and FVIII through alterations in the pharmacokinetics of the enzymatic reaction. Fourth, it is not possible to quantify separately fibrinogenolysis secondary to lysis by plasmin or cleavage by thrombin and therefore we are only able to show by process of elimination the likely mechanism of fibrinogen depletion. The coagulation response to prolonged shock, continued hemorrhage and the effects of transfusion form part of an on-going study. In the early phase of the ACIT study which to date has recruited over 2500 patients internationally, we did not capture mode of death although this is now embedded in the study protocol. Finally, the human cohort study was completed over five years ago during which time the fields of trauma induced coagulopathy and transfusion science have advanced greatly. The robust and early blood sampling in this study does however ensure validity of the study objectives to examine the early endogenous changes in coagulation.

The findings of this study have important clinical implications. There are currently no therapeutic options for ATC beyond procoagulant administration i.e. plasma, which is known to be poorly effective at correcting

coagulopathy[52,53] and the anti-fibrinolytic tranexamic acid[54]. But if the predominant pathophysiologic model converges on activation of PC, this may suggest a potential disadvantage to early administration of plasma which provides a source of plasminogen and protein C, both substrates for fibrinolytic pathways. However this assumption must be balanced against the role of plasmin inhibitors e.g. alpha 2 anti-plasmin, contained within plasma and their beneficial effects in ATC. We have previously reported preliminary results which demonstrate that early high dose FFP elicits a variable response in the coagulation system[41]. If aPC related-fibrinolysis and fibrinogen depletion are the primary problems then augmenting thrombin generation may further drive PC activation with negative effects on clot function and exacerbate bleeding. Although plasma contains important inhibitors of plasmin i.e. alpha 2 antiplasmin, resuscitation with products which lack plasminogen and protein C i.e. cryoprecipitate or fibrinogen concentrate, require further study as they may have a more favourable therapeutic profile than those which contain Protein C (plasma and prothrombin complex concentrates).

In summary ATC is defined predominantly by increased fibrinolytic activity and rapid depletion of fibrinogen. We have now shown that the Protein C pathway provides a mechanistic link between the two main theories of early trauma coagulopathy – 'Disseminated Intravascular Coagulation with fibrinolytic phenotype' [13] and primary aPC driven fibrinolysis (and possible fibrinogenolysis)[7]. Patients with raised aPC had a dose-dependent reduction in clot strength and evidence of increased fibrinolytic activity yet normal thrombin generation. The mechanistic confirmation of fibrinolysis as a central component of ATC supports clinical evidence from cohort studies[55-57] and randomised controlled trials[54] that early empiric administration of an antifibrinolytic improves outcomes in trauma. Fibrinogen was depleted early through a non-consumptive (systemic) process, theoretically by fibrinogenolysis through the direct action of plasmin. Elevated aPC was associated with worse outcomes and increased transfusions following major trauma. A unifying hypothesis defined by aPC generation and widespread fibrinolytic activation provides new translational opportunities for treating this important global disease. Effective reversal of ATC may require novel treatment strategies directed at ameliorating activation of PC and subsequent depletion of PC stores with targeted replenishment of fibrinogen.

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**FIGURE LEGENDS**

**Figure 1**

**HUMAN STUDY – activated Protein C (aPC) is associated with concentration dependent functional changes in coagulation but preservation of thrombin generation:** ROTEM is EXTEM unless specified. ANOVA with Dunnett's post hoc correction. **(A)** Elevated aPC in trauma patients are associated with diminished A5 *p<0.05 vs. normal aPC (≤3 ng/ml). **(B)** Maximum Clot Firmness falls in the presence of high aPC in trauma patients *p<0.05 vs. normal aPC (≤3 ng/ml). **(C)** Factor V (FV) falls in association with increasing aPC in trauma patients *p<0.05 vs. normal (aPC ≤3 ng/ml). **(D)** Inverse relationship between von Willebran Factor (vWF): Factor VIII (FVIII) ratio and elevated aPC indicative of selective inhibition of FVIII activity in humans following severe injury *p<0.05 vs. normal (aPC ≤3 ng/ml). **(E)** Endogenous Thrombin Potential (ETP) remains unchanged in trauma patients regardless of Factor V level. **(F)** Only minimal (non-significant) prolongation of Clotting Time (CT) is observed with high levels of aPC in trauma patients.

**Figure 2**

**HUMAN STUDY -** Fibrinogen loss and fibrinolysis are only observed in association with elevated activated Protein C (aPC) levels: One-way ANOVA **with Dunnett's post hoc correction** or two-way ANOVA between subjects (A, D, F) **with Bonferroni post-tests**. **(A) At high aPC levels of Prothrombin Fragment 1+2 (PF1+2) generation (thrombin production), there is a trend toward reduced fibrinogen in the presence of elevated aPC (p=0.08) (B)** Fibrinogen is inversely related to rising aPC in trauma patients *p<0.05 vs. aPC ≤3ng/ml. **(C)** In trauma patients FIBTEM falls in association in rising aPC *p<0.05 vs. aPC ≤3ng/ml. **(D) High levels of aPC (>9ng/ml) have a significant interaction (p<0.001) on tissue Plasminogen Activator (tPA) associated Plasmin-Antiplasmin (PAP) generation in trauma. *p<0.05, PAP ug/L: aPC ≤3 ng/ml vs. aPC >9ng/ml. (E)** High aPC (aPC>9 ng/ml) was associated with lower Plasminogen Activator Inhibitor -1 (PAI-1) in the presence of shock (BD>4) compared to aPC ≤3 ng/ml but this did not achieve significance (p=0.10). **(F)** Increased thrombin generation (PF 1+2) is associated with elevated D-dimers (fibrinolysis) with significantly greater fibrinolytic activity in the presence of high levels of aPC **(interaction of aPC >9ng/ml, p<0.05) *p<0.05, PF 1+2 ng/ml: aPC ≤3 ng/ml vs. aPC>9ng/ml.**

**Figure 3**

**MURINE STUDIES – activated Protein C (aPC) rise after trauma hemorrhage is attenuated in Factor V Leiden (FVL) animals but preservation of Factor V function does not fully prevent ATC: (A)** Trauma Hemorrhage (TH) in Wild Type (*WT*) mice is associated with a significant rise in aPC (*WT* Time 0 [T0]: 1.5ng/ml vs *WT* Time 60 mins [T60]: 10.3 ng/ml, *p<0.05) but attenuated in *TMKI* animals. **(B)** Attenuation of Acute Traumatic Coagulopathy with normal Maximum Clot Firmness (MCF) in transgenic mice subject to TH *p<0.05 vs. *WT* T0. **(C)** Clotting Time (CT) is prolonged after TH in *WT* but not thrombomodulin knock-in (*TMKI*) mice *p<0.05 *WT* T0 vs. *WT* T60 although baseline CT were significantly different between *WT* and *TMKI* at T0 (18 sec vs 22 sec, p<0.05). (D) CT is prolonged in both *WT* and FVL mice subjected to TH *p<0.05, T0 vs. T60 (*WT* and FVL).

**Figure 4**

**MURINE STUDIES – activated Protein C (aPC) is central to fibrinolysis in Acute Traumatic Coagulopathy: (A)** Attenuation of fibrinolytic activity in thrombomodulin knock-in (*TMKI*) mice subjected to Trauma Hemorrhage (TH) *p<0.05 (*WT*: Time 0 [T0] vs. *WT*: Time 60 mins [T60]) (*WT*: T60 vs *TMKI*: T60). **(B)** In *WT* mice after TH, fibrinogen fell significantly but *TMKI* animals were protected from this fibrinogen depletion *p<0.05 (*WT*: T0 vs. *WT*: T60) and (*WT*: T60 vs *TMKI*: T60). **(C)** Improved median survival times after TH in *TMKI* mice vs. *WT* *p<0.05.

**Table 1 Physiological characteristics and coagulation profile of patients**

| | Non-ATC | ATC | % difference |
|---|---|---|---|
| **N** | 250 | 50 | - |
| **Median ISS** | 9 (4-22) | 23 (10-34)* | |
| **Temperature (°C)** | 35.2 ± 0.2 | 35.9 ± 0.5* | 2% |
| **Lactate (mmol/L)** | 2.4 ± 0.3 | 4.4 ± 1.2* | 83% |
| **BD mmol/L** | 1.5 ± 0.5 | 5.2 ± 1.6* | -252% |
| | | | |
| **Platelets** | 249 ± 8 | 195 ± 19* | 22% |
| **PT median (secs)** | 11.2 (10.7-11.8) | 12.1 (11.5-13.8)* | 7% |
| **CT (secs)** | 65 ± 3 | 78 ± 9* | 17% |
| **CFT (secs)** | 87 ± 2 | 173 ± 19* | 50% |
| **Alpha angle** | 73 ± 0 | 60 ± 2* | -18% |
| **A5 (mm)** | 45 ± 1 | 29 ± 2* | -36% |
| **MCF (mm)** | 61 ± 1 | 48 ± 2* | -21% |
| | | | |
| **activated Protein C (ng/ml)** | 2 ± 1 | 11 ± 4* | 427% |
| **Protein C** | 90 ± 2 | 70 ± 6* | -22% |
| **Antithrombin** | 96 ± 2 | 81 ± 5* | -16% |
| **Plasmin-Antiplasmin (µg/L)** | 3477 ± 649 | 10103 ± 3018* | 66% |
| **D dimer** | 20073 ± 4622 | 70019 ± 24756* | 71% |
| **tPA (ng/ml)** | 11 (10-12) | 18 (10-26) | 40% |
| **Fibrinogen (g/dL)** | 2.2 ± 0.1 | 1.4 ± 0.2* | -39% |
| **II** | 98 ± 2 | 79 ± 5* | -19% |
| **PF 1+2 (pmol/L)** | 1371 ± 197 | 4231 ± 1313* | 68% |
| **V** | 103 ± 3 | 70 ± 10* | -32% |
| **VII** | 102 ± 4 | 88 ± 7* | -14% |
| **VIII** | 289 ± 16 | 267 ± 44* | -8% |
| **VIII:vWF ratio** | 1.3 ± 0.1 | 1.1 ± 0.2* | -15% |
| **IX** | 120 ± 3 | 97 ± 8* | -19% |
| **X** | 102 ± 2 | 98 ± 6* | -19% |
| **XI** | 111 ± 3 | 99 ± 9* | -18% |
| **XIII** | 104 ± 3 | 100 ± 7* | -25% |

**ROTEM defined Acute Traumatic Coagulopathy (ATC, A5 ≤35mm) vs. non-ATC (A5 >35mm). EXTEM: Clotting Time (CT), Clot Formation Time (CFT), Alpha angle, Amplitude (of clot) at 5 minutes (A5) and Maximum Clot Firmness (MCF). Factors levels are iu/dL unless specified. Prothrombin Fragments 1+2 (PF 1+2), Von Willebrand Factor (vWF). Values are mean ± 95% confidence interval or median (IQR). Student t test for parametric comparison or Mann Whitney U test for non-parametric data. *p<0.05**

FIGURE 1 (HUMAN)

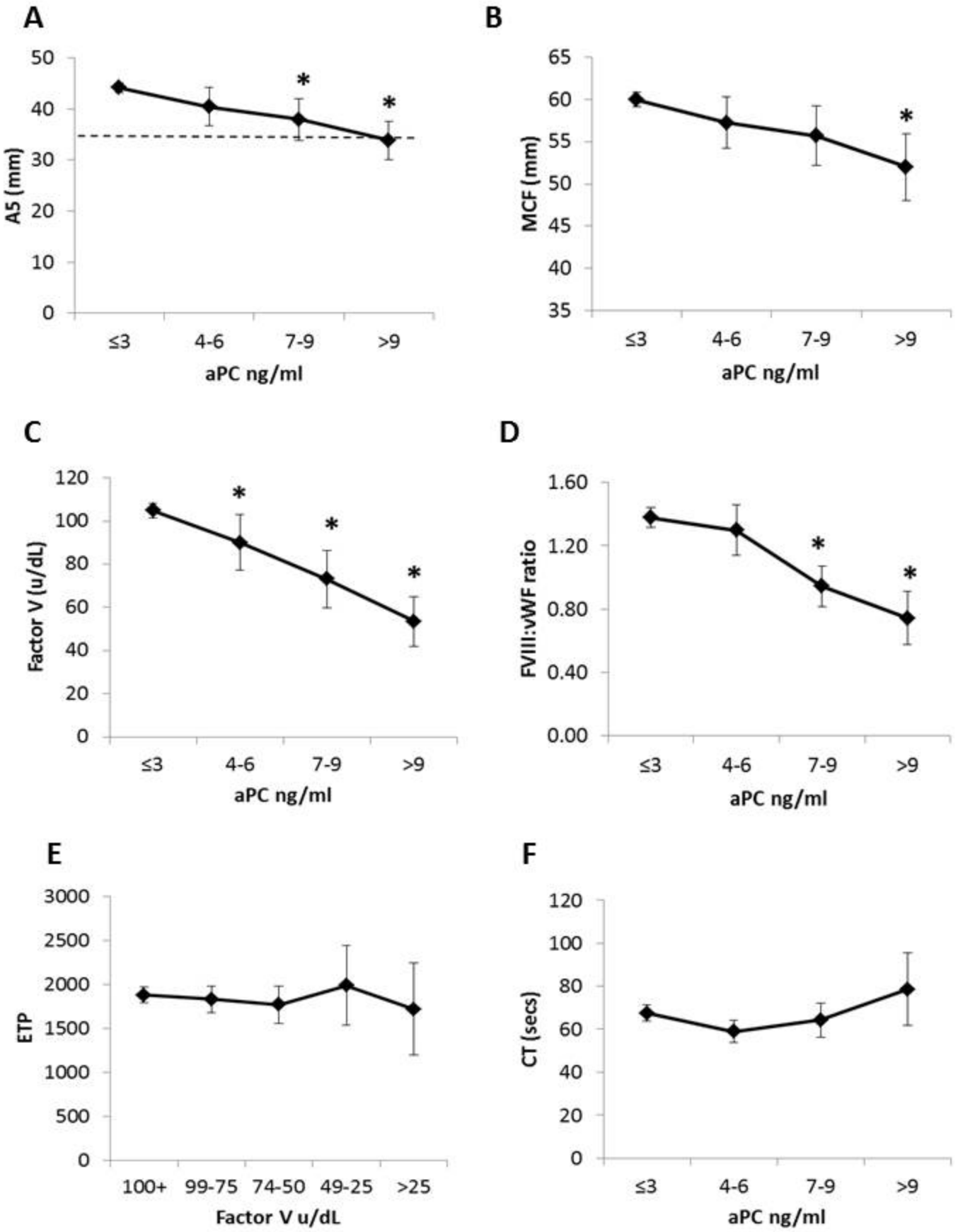

FIGURE 2 (HUMAN)

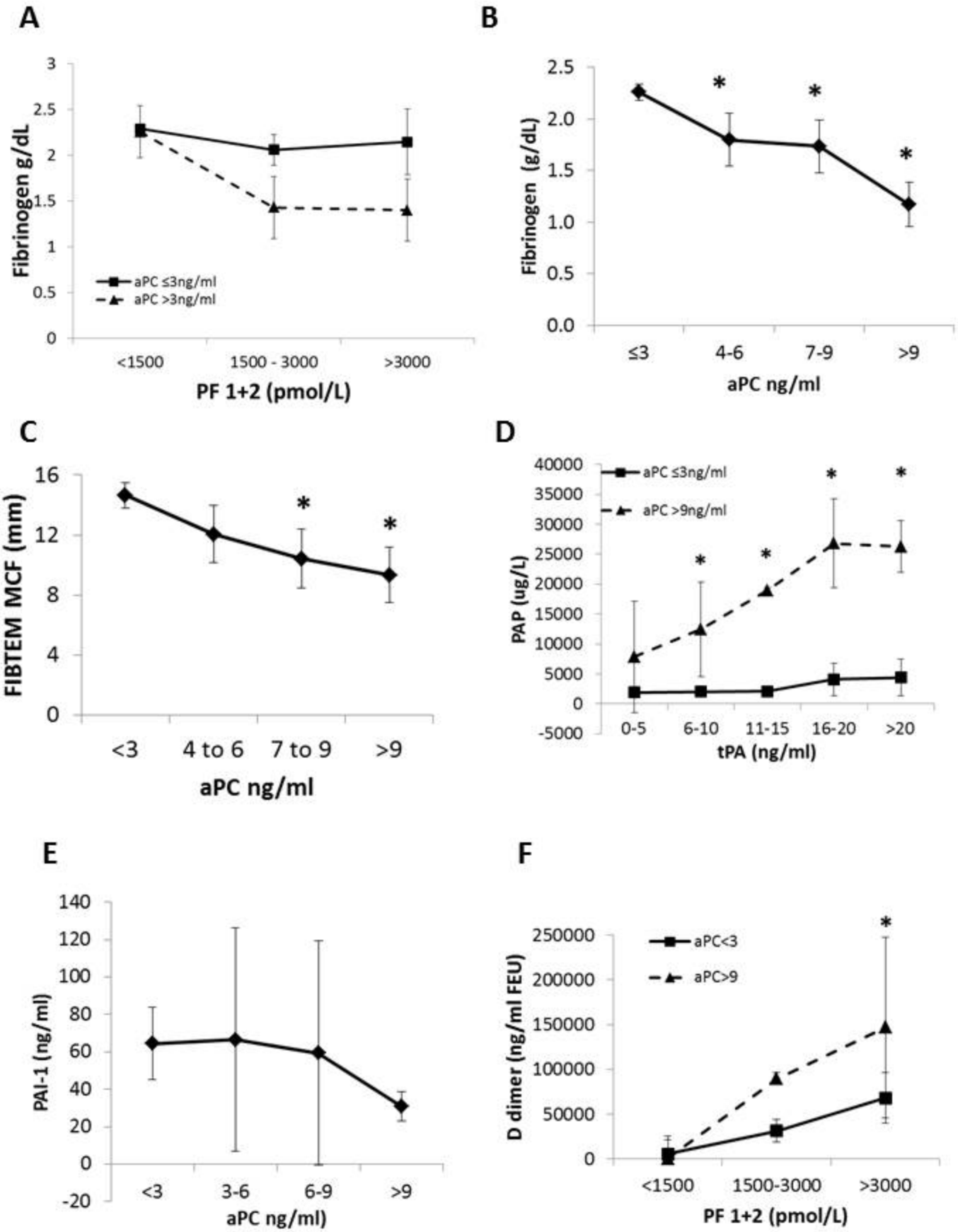

**Figure 3 (MURINE)**

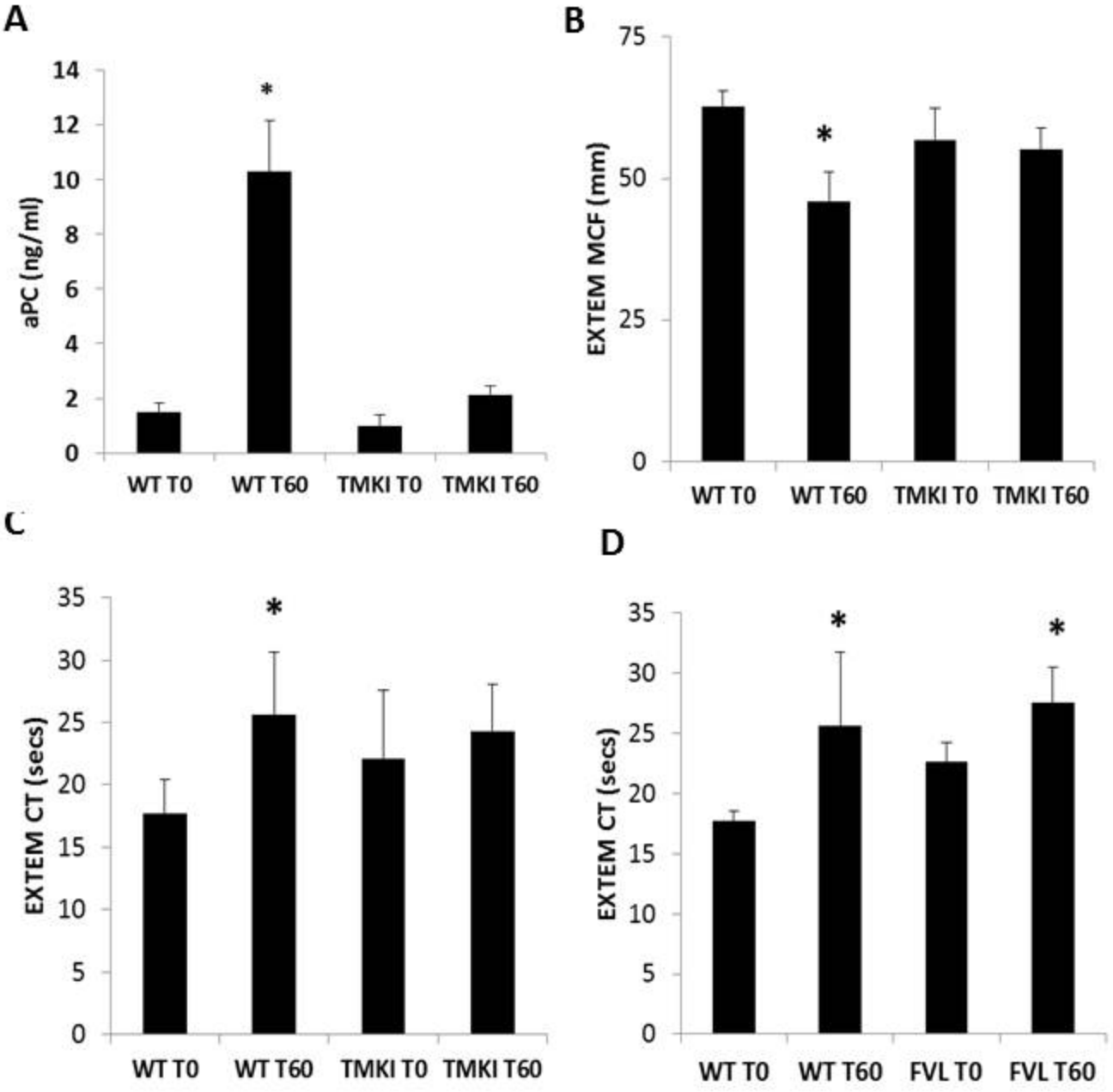

**Figure 4 (MURINE)**