Brief Report

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THROMBOSIS AND HEMOSTASIS

Altered fibrinolysis in autosomal dominant thrombomodulin-associated coagulopathy

Kate Burley,[1] Claire S. Whyte,[2] Sarah K. Westbury,[1] Mary Walker,[3] Kathleen E. Stirrups,[4,5] Ernest Turro,[4-6] NIHR BioResource,[5] Oliver G. Chapman,[7] Christopher Reilly-Stitt,[3] Nicola J. Mutch,[2] and Andrew D. Mumford[1]

[1]School of Clinical Sciences, University of Bristol, Bristol, United Kingdom; [2]Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom; [3]Department of Haematology, University Hospitals Bristol National Health Service Foundation Trust, Bristol, United Kingdom; [4]Department of Haematology, University of Cambridge, Cambridge Biomedical Campus, Cambridge, United Kingdom; [5]National Institute for Health Research BioResource – Rare Diseases, Cambridge University Hospitals, Cambridge Biomedical Campus, Cambridge, United Kingdom; [6]Medical Research Council Biostatistics Unit, Cambridge Institute of Public Health, Cambridge Biomedical Campus, Cambridge, United Kingdom; and [7]Department of Haematology, University Hospitals Coventry and Warwickshire National Health Service Trust, Coventry, United Kingdom

Key Points

- TM-AC is a heritable bleeding disorder in which high plasma TM levels reduce thrombin generation.
- High plasma TM levels also delay clot lysis by enhancing TM/thrombin-mediated activation of TAFI.

Thrombomodulin-associated coagulopathy (TM-AC) is a newly recognized dominant bleeding disorder in which a p.Cys537Stop variant in the thrombomodulin (TM) gene *THBD*, results in high plasma TM levels and protein C-mediated suppression of thrombin generation. Thrombin in complex with TM also activates thrombin-activatable fibrinolysis inhibitor (TAFI). However, the effect of the high plasma TM on fibrinolysis in TM-AC is unknown. Plasma from TM-AC cases and high-TM model control samples spiked with recombinant soluble TM showed reduced tissue factor–induced thrombin generation. Lysis of plasma clots from TM-AC cases was significantly delayed compared with controls but was completely restored when TM/thrombin-mediated TAFI activation was inhibited. Clots formed in blood from TM-AC cases had the same viscoelastic strength as controls but also showed a TAFI-dependent delay in fibrinolysis. Delayed fibrinolysis was reproduced in high-TM model plasma and blood samples. Partial restoration of thrombin generation with recombinant activated factor VII or activated prothrombin complex concentrate did not alter the delayed fibrinolysis in high-TM model blood. Our finding of a previously unrecognized fibrinolytic phenotype indicates that bleeding in TM-AC has a complex pathogenesis and highlights the pivotal role of TM as a regulator of hemostasis. (*Blood.* 2016;128(14):1879-1883)

Introduction

Thrombomodulin (TM) is a type-1 transmembrane glycoprotein encoded by *THBD* and expressed at high levels on vascular endothelial cells.[1] TM binds thrombin with high affinity and changes its substrate specificity to favor protein C, which when activated to activated protein C (APC) downregulates further thrombin generation by inactivating factors Va and VIIIa.[2,3] Consistent with this downregulatory role, reduced endothelial TM expression has been associated with disseminated intravascular coagulation[4] and venous thrombosis.[5] TM has also been linked to the pathogenesis of a dominant bleeding disorder caused by a *THBD* variant predicting a p.Cys537Stop substitution in the TM transmembrane domain. This results in high plasma TM levels, consistent with shedding of TM extracellular domain from the endothelium.[6,7] It has been proposed that abnormal bleeding in this disorder, hereafter termed TM-associated coagulopathy (TM-AC), results from enhanced TM-mediated APC generation and suppression of further thrombin generation.[6,7]

In addition to mediating APC generation, TM in complex with thrombin is also a potent activator of thrombin-activatable fibrinolysis inhibitor (TAFI).[8] Activated TAFI (TAFIa) cleaves carboxyl-terminal lysine residues from the fibrin surface thereby reducing binding of plasminogen and tissue plasminogen activator (tPA) and downregulating fibrinolysis.[9,10] Here we describe the impact of high plasma TM levels on TAFI-mediated clot lysis in an unreported pedigree with TM-AC and reveal a previously unrecognized fibrinolytic component of the TM-AC phenotype.

Study design

Study cases and coagulation studies

The study cases were enrolled to the National Institute for Health Research (NIHR) BioResources – Rare Diseases (UK REC 13/EE/0325) after informed written consent, in accordance with the Declaration of Helsinki. Plasma TM concentration was measured by ELISA (Abcam, Cambridge, United Kingdom). Calibrated automated thrombography was performed on platelet poor plasma with the PPP LOW reagent (1 pM tissue factor and a Fluoroscan fluorimeter (Thermo-Fisher, Basingstoke, United Kingdom).[11] Experiments were also

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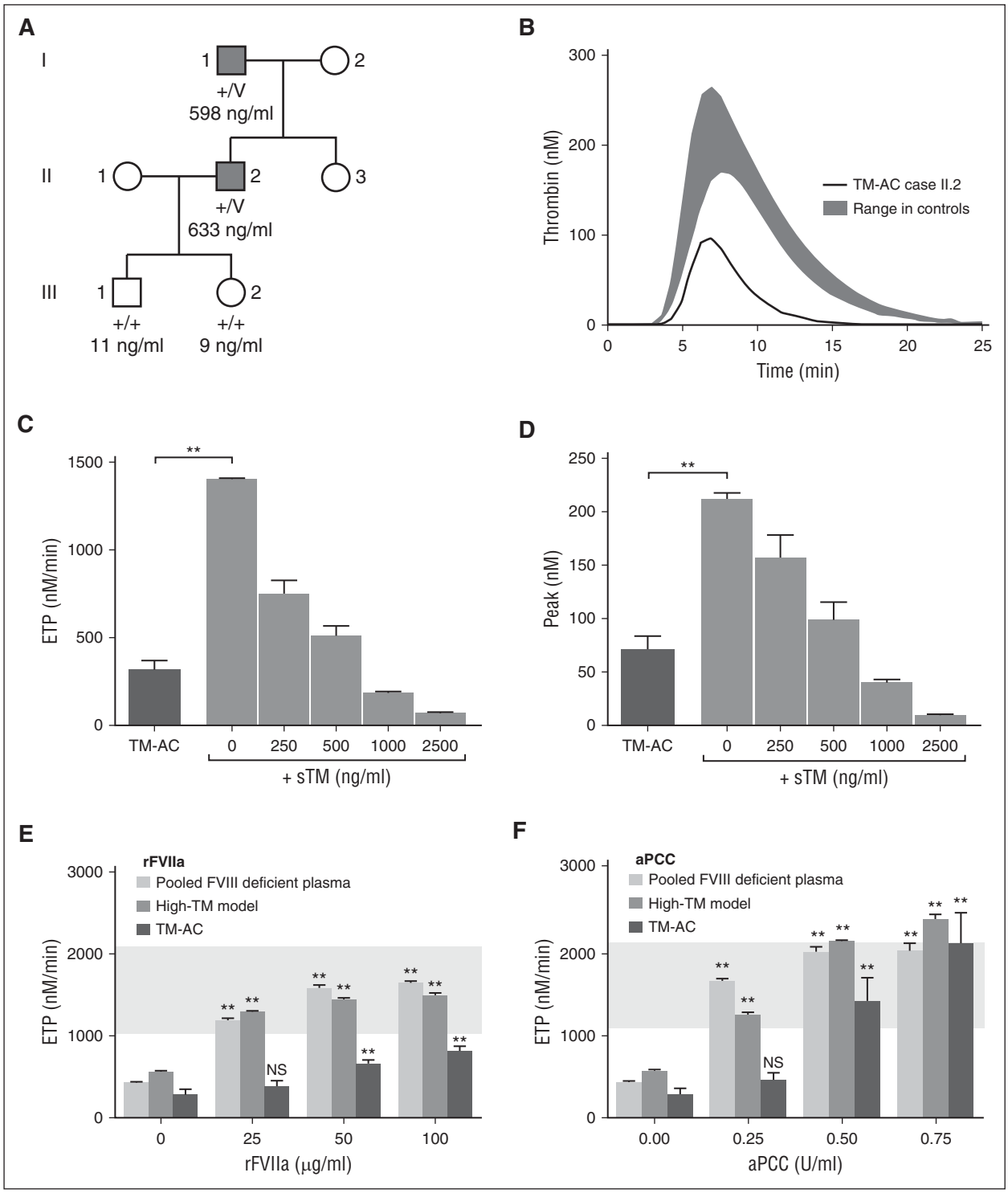

Figure 1. Reduced thrombin generation in the study cases and response to rFVIIa and aPCC. (A) Pedigree of the study cases with traumatic bleeding indicated by the solid symbols and no abnormal bleeding by open symbols. Genotyped cases are indicated as +/V for the heterozygous *THBDN* p.Cys537Stop variant and +/+ for reference sequence. Plasma TM concentrations are indicated beneath each pedigree symbol. (B) A representative thrombin generation curve from case II.2 in recalcified plasma collected into 0.106 mM trisodium citrate, following activation with 1 pM tissue factor. The gray shading represents the limits of thrombin generation curves observed in 20 healthy controls. (C-D) Plasma ETP peak thrombin concentration from cases I.1 and II.1 (n = 10) with TM-AC and in plasma from 20 healthy controls in the absence or presence of recombinant C-terminal truncated human TM (sTM; 250-2500 ng/mL; high-TM model plasma). (E-F) ETP in plasma from cases I.1 and II.1 and in high-TM model plasma containing 500 ng/mL sTM and either 25 to 100 μg/mL rFVIIa or 0.25 to 0.75 U/mL aPCC. Statistical comparisons are between ETP values in the presence of the stated concentration of bypassing agent vs ETP in the corresponding sample with no added bypassing agent. The gray boxes indicate the range of ETP observed in 20 healthy control plasmas without addition of sTM. Data are means ± SEM; $^{**}P \leq .01$. NS, not significant.

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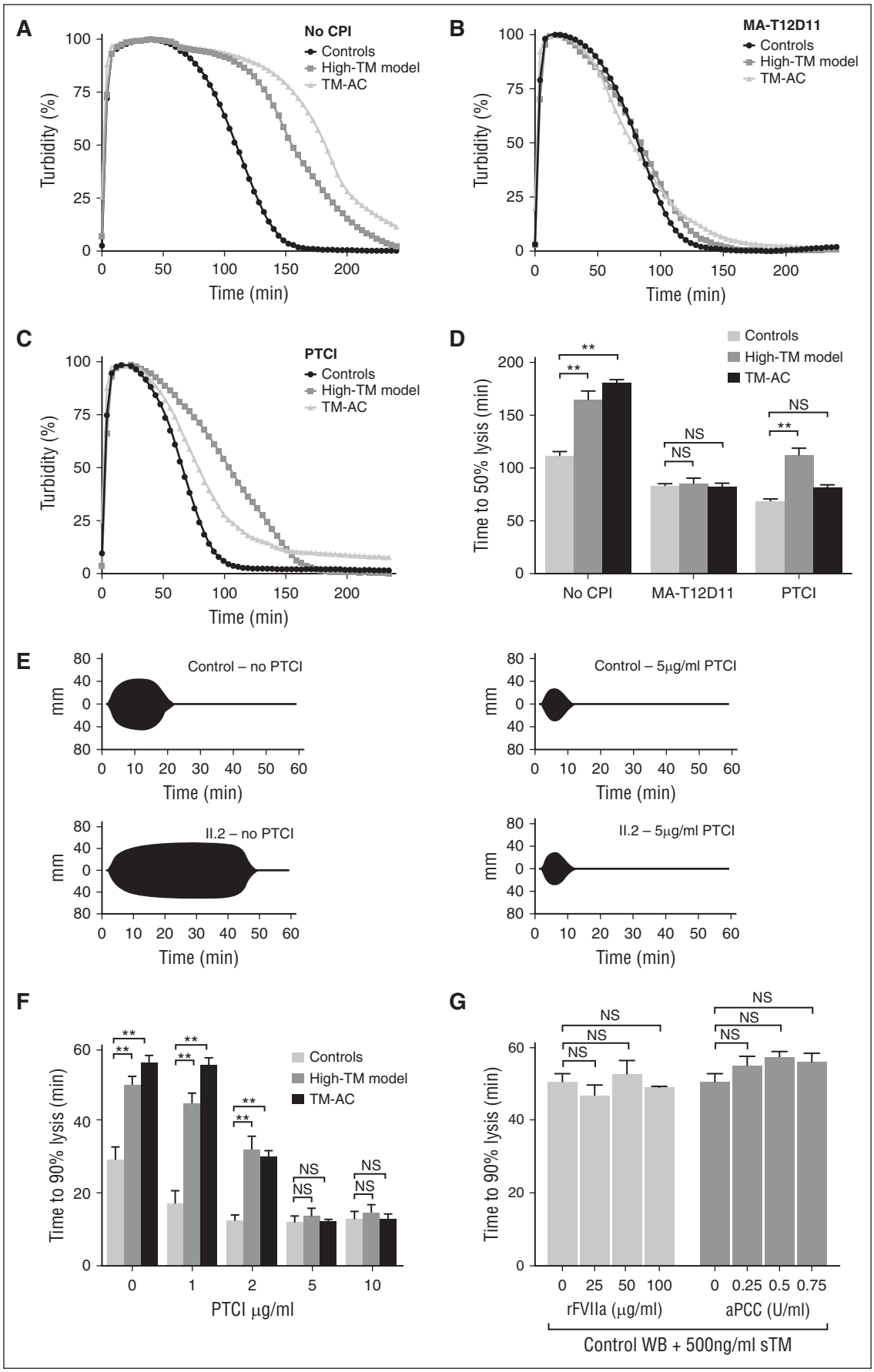

Figure 2.

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performed in high-TM model blood or plasma using samples from healthy controls spiked with 250 to 2500 ng/mL recombinant C-terminal truncated TM (Peprotech, Hamburg, Germany). *THBD* was analyzed by whole genome sequencing through the BRIDGE–bleeding and platelet disorders project[12] and the ThromboGenomics high-throughput platform.[13]

Plasma fibrinolysis

Clots were formed in recalcified, diluted plasma with 0.1 U/mL thrombin (Sigma-Aldrich, Poole, United Kingdom) as previously reported[14] in the presence of 300 pM recombinant tPA (Genentech, San Francisco, CA) and 16 μM phospholipids (Rossix, Molndal, Sweden). Clots were formed in the absence or presence of 500 ng/mL TM, 25 μg/mL potato tuber carboxypeptidase inhibitor ([PTCI] a TAFIa inhibitor; Sigma-Aldrich) or 65 μg/mL MA-T12D11 (a monoclonal antibody inhibitor of thrombin/TM-mediated TAFI activation[15]). Turbidity was monitored every minute at 405 nm.

Whole blood fibrinolysis

Clots were formed in recalcified whole blood samples containing 210 pM tPA (Genentech) using the EXTEM reagent and monitored by rotational thromboelastometry (ROTEM; Tem International GmbH, Munchen, Germany), in the presence or absence of 1 to 10 μg/mL PTCI, 25 to 100 μg/mL recombinant activated FVII (rFVIIa; NovoNordisk, Bagsværd, Denmark) or 0.25 to 0.75 U/mL activated prothrombin complex concentrate (aPCC; Baxalta, Bannockburn, IL). Data are expressed as means ± standard error of the mean (SEM) and were analyzed by 2-way analysis of variance with Dunnett's post hoc test.

Results and discussion

The study cases were males aged 59 and 34 years (I.1 and II.2; Figure 1A) with a lifelong propensity for muscle and joint bleeding after minor trauma. Treatment of bleeding with plasma or factor IX infusions was ineffective. However, single 90 μg/kg infusions of rFVIIa (NovoNordisk) were usually sufficient to resolve bleeding. Analysis of plasma by calibrated automated thrombography showed that compared with healthy controls, the cases had reduced endogenous thrombin potential (ETP; 316.3 ± 51.5 nM/min [n = 10] vs 1584.9 ± 56.2 nM/min in controls [n = 20]; $P < .01$) and reduced peak thrombin concentration (71.7 ± 12.5 nM [n = 10] vs 273.7 ± 19.6 nM in controls [n = 20]; $P < .01$; Figure 1B-C). Cases and controls showed similar lag time and time to peak thrombin. The plasma TM concentration in the cases was 640.7 ± 21.2 ng/mL (n = 4; reference interval 2.9-7.6 ng/mL). No other abnormalities were observed in coagulation factor or anticoagulant protein levels (supplemental Table 1; available on the *Blood* Web site).

Consistent with this phenotype, both cases had heterozygous c.1611C>A transversions in the major *THBD* transcript NM_000361.2, which segregated in the pedigree with abnormal bleeding. This predicted p.Cys537Stop in the TM transmembrane domain, an identical variant to the 2 previously reported TM-AC pedigrees (Figure 1A).[6,7] Addition of sTM to control plasma caused dose-dependent reductions in ETP and peak thrombin, which with 500 ng/mL sTM were similar to the cases (Figure 1C-D). This indicated a causal relationship between high plasma TM levels in the TM-AC cases and reduced thrombin generation. Addition of the bypassing agents rFVIIa or aPCC, which enhance thrombin generation in other coagulopathies, increased ETP in high-TM model plasma, similar to that in pooled FVIII-deficient control plasma samples ($P < .01$ for ETP with 2-100 μg/mL rFVIIa or 0.25-0.75 U/mL aPCC vs ETP with no bypassing agent). Restoration of ETP was less in the TM-AC plasmas ($P < .01$ for ETP with 50-100 μg/mL rFVIIa or 0.5-0.75 U/mL aPCC vs ETP with no bypassing agent; Figure 1E-F).

Fibrinolysis phenotype

Lysis of TM-AC plasma clots by tPA was significantly slower than controls (time to 50% lysis 175.1 ± 3.4 min [n = 6] vs 103.1 ± 3.9 min in controls [n = 3]; $P < .01$) and was reproduced in high-TM model plasma (time to 50% lysis 164.1 ± 8.6 min [n = 3]; $P < .01$; Figure 2A,D). Addition of MA-T12D11, which specifically inhibits thrombin/TM-mediated TAFI activation,[15] completely abrogated delayed fibrinolysis in the TM-AC and high-TM model plasma samples (Figure 2B,D). Inclusion of the TAFIa inhibitor PTCI (25 μg/mL) also reduced the delay in fibrinolysis in TM-AC plasma, although did not completely reduce the 50% lysis time in the high-TM model to control levels (Figure 2C-D).

Clots formed in TM-AC and high-TM model blood samples showed the same maximum clot firmness as controls (Figure 2E; supplemental Table 1), indicating no differences in initial clot viscoelastic strength. However, compared with controls, the time to 90% lysis was significantly delayed in TM-AC blood (time to 90% lysis 56.5 ± 2.3 min [n = 7] vs 29.5 ± 3.5 min in controls [n = 3]; $P < .01$) and in high-TM model blood (50.4 ± 2.0 [n = 3]; $P < .01$; Figure 2E-F), indicating delayed fibrinolysis. Addition of 1 to 10 μg/mL PTCI caused a dose-dependent reduction in the delay in fibrinolysis in TM-AC and high-TM model blood samples (Figure 2E-F), reproducing the effects in plasma. Addition of 25 to 100 ng/mL rFVIIa or 0.25 to 0.75 U/mL aPCC, which partially restored thrombin generation in TM-AC and high-TM model samples, did not alter the delayed fibrinolysis in high TM-model blood (Figure 2G).

We confirm that TM-AC is associated with reduced thrombin generation and reveal that delayed fibrinolysis is a component of the TM-AC phenotype using 2 experimental models. We show further that both phenotypes are a consequence of high plasma TM levels and that the delay in fibrinolysis is TAFIa mediated, in line with the established role of TM/thrombin in TAFI activation.[9,10] It is significant that in the presence of high TM levels, enhanced TAFI activation occurred despite reduced thrombin generation and that partial restoration of thrombin generation with rFVIIa or aPCC did not alter the delayed fibrinolysis. This suggests that in TM-AC, TM rather than thrombin is the main determinant of TAFI activation. Our findings highlight

Figure 2. Plasma and whole blood fibrinolysis. (A-C) Turbidity measured in triplicate at 405 nm in plasma from TM-AC cases I.1 and II.1 (n = 6), high-TM model plasma containing 500 ng/mL sTM (n = 3), or control plasma without added sTM (n = 3) diluted 30:70 in TBST buffer [10 mM tris(hydroxymethyl)aminomethane, 140 nM NaCl, 0.01% Tween-20, pH7.4] containing 16 mM phospholipids. Clotting was initiated with 0.1 U/mL thrombin and 10.6 mM $CaCl_2$ in the presence of 300 pM recombinant human tissue plasminogen activator. The curves represent mean turbidity with no carboxypeptidase inhibitor (no CPI), with 65 μg/mL MA-T12D11 or 25 μg/mL PTCI. (D) Time to 50% clot lysis in the TM-AC (n = 6), high-TM model (n = 3), and control (n = 3) plasma samples in the absence or presence of 65 μg/mL MA-T12D11 or 25 μg/mL PTCI. (E) Representative ROTEM viscoelastometry traces following clot formation with the EXTEM reagent in whole blood from a healthy control and from TM-AC case II.2. Traces are shown in the absence and presence of 5 μg/mL PTCI. (F) Time to 90% clot lysis in TM-AC (n = 6), high-TM model (n = 3), and healthy control (n = 20) blood in the absence or presence of 1 to 10 μg/mL PTCI. (G) Time to 90% clot lysis in high-TM model blood samples in the absence or presence of 25 to 100 μg/mL rFVIIa or 0.25 to 0.75 U/mL aPCC (n = 3). Data are means ± SEM; $^{**}P \leq .01$. NS, not significant.

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the multifactorial role of TM in the regulation of hemostasis and have important implications for the treatment of bleeding in TM-AC.

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Authorship

Contribution: K.B. performed experiments and cowrote the manuscript; C.S.W., M.W., and C.R.-S. performed experiments; S.K.W. and O.G.C. enrolled the study cases and provided the phenotype descriptions; K.E.S. and E.T. designed and performed genetic analyses; N.J.M. designed experiments and cowrote the manuscript; and A.D.M. oversaw the study, designed experiments, and cowrote the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

A complete list of the members of NIHR BioResource appears in the online appendix.

Correspondence: Andrew D. Mumford, School of Clinical Sciences, University of Bristol, Level 7 Bristol Royal Infirmary, Bristol BS2 8HW, United Kingdom; e-mail: a.mumford@bristol.ac.uk.

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