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Factor V east Texas variant causes bleeding in a three-generation family

Julie A. Peterson[1,#], **Sweta Gupta**[2,#], **Nicholas D. Martinez**[1], **Brandon Hardesty**[2], **Susan A. Maroney**[1], **Alan E. Mast**[1,3,*]

[1]Versiti, Blood Research Institute, Milwaukee, WI 53226

[2]Indiana Hemophilia & Thrombosis Center, Indianapolis, IN USA 46260

[3]Department of Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, Milwaukee, WI 53226

Abstract

Background: The factor V east Texas bleeding disorder (FVETBD) is caused by increased plasma tissue factor pathway inhibitor-α (TFPIα) concentration. The underlying cause is a variant in *F5* causing alternative splicing within exon 13 and producing FV-short, which tightly binds the C-terminus of TFPIα, prolonging its circulatory half-life.

Objectives: To diagnose a family presenting with variable bleeding and laboratory phenotypes.

Patients/Methods: Samples were obtained from 17 family members for *F5* exon 13 sequencing. Plasma/platelet TFPI and platelet FV were measured by ELISA and/or Western blot. Plasma thrombin generation potential was evaluated using calibrated automated thrombography.

Results: The FVET variant was identified in all family members with bleeding symptoms and associated with elevated plasma TFPIα (4.5- to 13.4-fold) and total TFPI (2- to 3-fold). However, TFPIα and FV-short were not elevated in platelets. TF-initiated thrombin generation in patient plasma was diminished but was restored by a monoclonal anti-TFPI antibody or factor VIIa. TFPIα localized within vascular extracellular matrix in an oral lesion biopsy from an affected family member.

Conclusions: FVETBD was diagnosed in an extended family. The variant was autosomal dominant and highly penetrant. Elevated plasma TFPIα, rather than platelet TFPIα, was likely the primary cause of bleeding. Plasma FV-short did not deplete TFPIα from extracellular matrix. *In vitro* thrombin generation was restored with an anti-TFPI antibody or factor VIIa suggesting

[*]Alan E. Mast MD PhD, Versiti Blood Research Institute, PO Box 2178, Milwaukee, WI 53201-2178, Tel: 414-937-6310, aemast@versiti.org.

Authorship details

JAP: Designed and performed experiments, analyzed data, wrote manuscript

SG: Made the initial diagnosis, enrolled ETBD patients, obtained clinical samples, wrote manuscript

NDM: Performed experiments

BH: Enrolled ETBD patients, obtained clinical samples, wrote manuscript

SAM: Performed histological analyses, wrote manuscript

AEM: Conceived and designed experiments, analyzed data, wrote manuscript

[#]Co-first authors

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effective therapies may be available. Increased awareness of, and testing for, bleeding disorders associated with *F5* exon 13 variants and elevated plasma TFPI are needed.

Keywords

TFPI; Factor V; Bleeding

Introduction

Unidentified bleeding disorders are a clinical conundrum and an intellectual challenge for hematologists. Despite first and second tier workup for common and rare bleeding disorders, many individuals remain undiagnosed, which could delay appropriate clinical management and result in significant morbidity. Hemostasis evaluation for rare disorders includes, but is not limited to, measurement of fibrinogen, factor (F)II, FV, FVII, FX, FXI, FXIII, combined FV and FVIII, plasminogen activator inhibitor-1, and α2-antiplasmin, as well as platelet disorders such as Glanzmann Thrombasthenia and Bernard Soulier Syndrome.[1] Further evaluation for other rare bleeding disorders necessitates a high index of clinical suspicion and expanding the laboratory armamentarium beyond commercially available assays.

Three *F5* variants, *F5*-east Texas,[2, 3] *F5*-Amsterdam,[4] and *F5*-Atlanta,[5] recently have been found to produce bleeding disorders. Each variant promotes an alternative splicing event within exon 13 of *F5*, which encodes the B-domain, and results in increased production of an altered form of FV called FV-short. The B-domain of FV contains over 700 amino acids. The N-terminal end (amino acids 963–1008) is enriched in basic, positively charged amino acids, while the C-terminal end (amino acids 1493–1537) is enriched in acidic, negatively charged amino acids. Interaction between these positively and negatively charged regions of the B-domain maintains FV as an inactive blood coagulation factor.[6] Removal of either the basic region, acidic region, or the entire B-domain, converts FV into active coagulation factor.[6] The alternative splicing events producing FV-short remove the amino terminal end of the B-domain, including its basic region. Thus, FV-short is an active procoagulant factor.[7, 8]

Tissue factor pathway inhibitor (TFPI) is an anticoagulant protein that dampens initial procoagulant responses through inhibition of the tissue factor-factor VIIa (TF-FVIIa) catalytic complex [9] and early forms of prothrombinase (FXa-FVa) that assemble before thrombin is generated.[10] In humans, TFPI is produced in two alternatively spliced isoforms, TFPIα and TFPIβ, which have different C-terminal regions.[11] The C-terminal region of TFPIα has homology with the basic region of the FV B-domain and tightly binds the acidic region of the FV-short B-domain.[10, 12] This binding interaction stabilizes plasma TFPIα and, consequently, plasma TFPIα is elevated 5- to 20-fold in patients with *F5* exon 13 variants.[2, 4, 5] Thus, even though FV-short is an active procoagulant factor, it paradoxically produces a bleeding disorder by increasing the plasma concentration of TFPIα. Here, we describe the finding of east Texas bleeding disorder in three generations of a family, who presented with variable bleeding and laboratory phenotypes.

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Methods

Human subject approval

The collection and study of human blood specimens was approved by the St Vincent Institutional Review Board, Indianapolis, IN. Each patient provided informed consent.

Sample collection

Whole blood was drawn into 3.2% sodium citrate tubes and centrifuged within two hours. Platelet-poor plasma was immediately frozen at −70 °C.

Clinical Assays

Coagulation assays were performed at Mid America Clinical Labs, Indianapolis, IN. Mixing studies with normal plasma were performed when the activated partial thromboplastin time (aPTT) or prothrombin time (PT) were prolonged.

Measurement of plasma TFPI and protein S

Plasma total TFPI and TFPIα were measured by ELISA as described [13, 14] using mouse monoclonal antibodies directed against the first (MαK1), second (MαK2),[15] or third (MαK3) Kunitz domains of human TFPIα (all kindly provided by Novo Nordisk, Copenhagen Denmark). Total and free protein S antigen were measured by an ELISA kit (Diapharm, West Chester, OH) per manufacturer's protocol. Protein S is reported as percent relative to reference plasma provided by the manufacturer.

Measurement of platelet TFPI

Platelets were isolated from citrated whole blood and washed as described.[16] Platelets were lysed with 30mM CHAPS, 10 mM EDTA, 0.02 mM E64,0.1 mM DCI, 1mM PMSF at 1×10^9 platelets/mL. The lysate was centrifuged at 14,000 × g and supernatant was subjected to BCA analysis for total protein normalization and measurement of TFPIα by ELISA.

SDS–PAGE and western blot analysis

Plasma TFPI was precipitated with bovine FXa agarose.[17] SDS-PAGE was performed with 4–16% polyacrylamide gradient gels. Following western blotting, TFPIα was detected with a rabbit polyclonal antibody against the C-terminal 11 amino acids of TFPIα. Images were developed on an Amersham Imager 680 (Cytiva, Marlborough, MA). Factor V was detected with mouse monoclonal antibody 5146 (Thermo Fisher Scientific, Waltham, MA).

FV sequencing

DNA was isolated from buffy coat or buccal swab using QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany). A 2393 bp DNA fragment surrounding c.2350A>G NM_000130.5 East Texas variant was PCR-amplified with primer F1 5'catgcgtggagaatctgtgacggt3' [2] in exon 12 and reverse primer R1 5'gtgaaaggtcctcggagataaag3' in exon 13. The PCR product was gel purified and subjected

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to sequence analysis with forward primer F2 5'cagtcatggctacacggaaa3' in exon 13 and in reverse direction with primer R1.

Thrombin generation assays

Calibrated automated thrombography was performed using a Fluoroskan Ascent microplate fluorometer (Diagnostica Stago, Inc. Parsippany, NJ), as described.[10] Briefly, 40 μL platelet poor plasma was diluted 9:1 in 50 mM Hepes, 100 mM NaCl and 0.05% bovine serum albumin and incubated for 10 minutes at 37°C with 10 μL PPP reagent (Diagnostica Stago Inc.) containing a mixture of phospholipids and TF (5 pM final concentration). Reactions were initiated by addition of a mixture of fluorogenic thrombin substrate and calcium (Diagnostica Stago Inc.). For some reactions MαK2 [15] or recombinant FVIIa (rFVIIa; a gift from Novo Nordisk) were included. Data were analyzed using Thrombinoscope software (Diagnostica Stago, Inc.).

Histology

A biopsy of a chronic lesion from the lower lip buccal mucosa of one family member was obtained from the clinical pathology laboratory. Healthy human buccal mucosal tissue was obtained from the Medical College of Wisconsin Tissue Bank, fixed in 10% formaldehyde buffered at pH 7.4, and processed into four-micron sections. Tissues were stained using a rabbit polyclonal antibody against the C-terminal 11 amino acids of TFPIα, and other antibodies including, sheep anti-thrombomodulin (R&D Systems, Minneapolis, MN), 59D8, a mouse monoclonal antibody specific for the β-chain of fibrin (Millipore Sigma, Burlington, MA). Isotype-matched mouse, rabbit and sheep IgG controls were from BD Biosciences, San Jose, CA. Secondary antibodies were from Thermo Fisher Scientific or Jackson ImmunoResearch Laboratories. The Motic Easy scan imaging system (Motic 8 Inc., LTD, Hong Kong) was used to scan trichrome stained slides. Immunofluorescent images were obtained with a Nikon Eclipse Ti2 inverted microscope with a DS-Ri2 high speed color camera using either a 20X or 60x/1.45 numerical aperture oil objective (Nikon Instruments Inc., Melville, NY). Images were analyzed with the Nikon NIS-Elements software platform and processed with Imaris multi-channel microscopy software (Bitplane Inc., Concord, MA). Image formatting was performed in Photoshop CS-6 and Illustrator CS-6 (Adobe, San Jose, CA).

Results

Clinical course of the proband

The male proband initially presented at age 7 to the Indiana Hemophilia and Thrombosis Center and is presently 20 years old. His symptoms have included mucocutaneous bleeding, prolonged wound healing, post traumatic bleeding, delayed post-surgical bleeding resulting in subgaleal hemorrhage and severe anemia requiring blood transfusion. Due to an inadequate response to neurosurgical drainage and a combination of universal hemostatic agents including desmopressin acetate and antifibrinolytics, he was treated with rFVIIa with successful resolution of the subgaleal bleed.

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Bleeding history of family members

Detailed bleeding histories were obtained from members of this three-generation family with variable bleeding phenotypes present in nine family members (Supplemental Table 1 and Figure 1). The major symptom noted in several family members was post-surgical bleeding. The only abnormality in the hemostatic evaluation of the proband and some symptomatic family members was variably prolonged aPTT and PT (Supplemental Table 1). Additional clinical features of the proband and family members are presented in the online supplement.

Identification of the FV east Texas variant.

The proband had a normal hemostatic workup for common and rare bleeding disorders including repeated VWD panels, testing for deficiency of plasminogen activator inhibitor-1 (PAI-1), α2 antiplasmin, FXIII and the variability in PT and aPTT assays. Mixing studies with normal plasma corrected the aPTT and PT suggesting a coagulation factor deficiency, but all were within reference intervals, as was platelet function testing for Glanzmann Thrombasthenia and Bernard Soulier Syndrome, raising suspicion for a rare condition caused by a blood clotting protein not measured in standard assays, such as the east Texas bleeding disorder.

Sequence analysis of the proband identified heterozygosity inF5(NM_000130.5):c.2350A>G corresponding to the east Texas variant (Table 1). This variant produces a FV-short transcript containing an in-frame deletion of 702 amino acids that omits the basic region of the FV B domain while retaining the acidic region that tightly binds the basic region of the TFPIα C terminus.[2] Additional sequencing revealed the variant in 10 family members (Figure 1). Remarkably, the proband's grandfather transmitted the variant to all four of his sons (Figure 1), akin to flipping heads four times in a row. All family members with the FV east Texas variant reported bleeding symptoms except for the proband's cousins (IV:5 and IV:10), who are both children. Samples from two spouses of blood relatives were tested and found to lack the FV east Texas variant and have normal TFPI levels, serving as shipping and sample handling controls (Table 1).

Plasma TFPI was elevated in the proband and family members with bleeding symptoms.

TFPIα and total TFPI were measured by ELISA in plasma from family members with and without bleeding symptoms (Table 1). Ratios of TFPIα to a healthy pool were determined using a single plasma sample from each subject. TFPIα was 11.8-fold higher in plasma collected from the proband compared to a pooled plasma from 13 healthy individuals. TFPIα and total TFPI levels were elevated in multiple family members across three generations who had mild to severe bleeding symptoms (Supplemental Table 1, Table 1). *F5* exon 13 sequencing in other family members revealed an exact correlation between the FV east Texas allele and elevated plasma TFPI levels (Table 1). Western blot of plasma samples showed substantially more TFPIα in the family members with the variant, confirming the ELISA findings (Figure 2A).

Platelet TFPIα was not elevated in two subjects with the FV east Texas variant.

Platelet TFPIα from two family members with the east Texas variant was measured by ELISA after washing and lysis. Platelet TFPIα concentrations from both subjects were

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similar to those of three healthy individuals (Table 1). Notably, FV-short was not elevated in washed platelet lysates from two family members with the east Texas variant (Figure 2B), but it was abundant in east Texas plasma.

Elevated TFPIα levels resulted in reduced thrombin generation.

Plasma from subject III:1 carrying the FV east Texas variant was evaluated in TF-initiated thrombin generation assays. The plasma exhibited greatly reduced thrombin generation (Figure 3) compared to pooled plasma. Inhibition of plasma TFPI activity by addition of a high-affinity monoclonal antibody directed against the second TFPI Kunitz domain restored thrombin generation (Figure 3A) in a dose dependent manner. Addition of rFVIIa also dose-dependently improved thrombin generation (Figure 3B).

Protein S levels were not affected in family members with the FV east Texas variant.

Total and free plasma protein S levels were measured in 15 family members (Table 1). They were within normal reference intervals for patients and controls.

Buccal mucosa imaging

In our pedigree, three affected individuals have reported delayed wound healing with one male suffering from recurrent oral lesions of unknown etiology. We had to opportunity to examine a tissue biopsy of a lesion from this individual. H&E staining revealed chronic inflammation with hemorrhage (Figures 4A and 4B). We recently found that the primary source of heparin-releasable TFPIα is the extracellular matrix (ECM) surrounding blood vessels.[14] It is not known how FV-short affects the distribution of TFPIα between plasma and the ECM. The availability of this tissue allowed us to examine the location of TFPIα within and surrounding the vasculature of a patient with east Texas Bleeding Disorder. Immunofluorescent studies identified TFPIα within the vascular ECM of the lesion where it can be clearly seen beneath endothelial cells stained for thrombomodulin (Figures 5B–D). Healthy buccal mucosa is shown for comparison (Figures 5F–H). Immunofluorescence is not a quantitative technique, but these findings indicate that a high concentration of plasma FV-short does not totally deplete TFPIα from the ECM surrounding the vasculature. Additional studies were performed to further characterize the lesion (Supplemental Figure I). Trichrome staining again shows hemorrhage and large amounts of inflammatory cells within the lesion (Supplemental Figures IA and IB) Fibrin deposits were present within the hemorrhage but not within other areas of the lesion (Supplemental Figure IC). Picrosirius red staining identified collagen within the inflamed area of the lesion. Healthy buccal mucosal tissue is shown for comparison (Supplemental Figures IE and IF).

Discussion

The FV east Texas bleeding disorder[2, 3] was identified in three generations of a family with previously undiagnosed bleeding after more than a decade of evaluation at the Indiana Hemophilia and Thrombosis Center. The proband’s grandfather, father, three uncles, brother, and four cousins had the associated *F5* variant. All family members with bleeding symptoms had the *F5* variant and elevated plasma TFPIα concentration indicating high penetrance of the variant. Two cousins, who were less than 10 years old, have the variant but have

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not experienced bleeding symptoms. The diagnosis has provided considerable relief for family members, as they now know the cause of their bleeding and receive appropriately targeted treatments for bleeding episodes. The main hemostatic product utilized is rFVIIa at 90–200 μg/kg and an as needed antifibrinolytic agent, tranexamic acid or aminocaproic acid, with dosing based on the nature of bleed or surgery. Risk of thrombosis and cost of medication is weighed in the decision-making process. Select minor bleeding episodes and surgical interventions have been successfully managed with antifibrinolytics alone. The original *F5*-east Texas pedigree was treated with FEIBA at 10–20 units/kg (communication with Dr Miguel Escobar), while *F5*-Amsterdam patients were treated with prothrombin complex concentrate, fresh frozen plasma (FFP) and antifibrinolytic agents.[4] The wide range of hemostatic products used to date underscores the need for development of standard guidelines for management as new FV-short variants are uncovered, and affected families are newly diagnosed.

Diagnosis of these bleeding disorders is not straightforward. Our cohort of patients had a similar bleeding profile to the originally described east Texas pedigree and the *F5*-Amsterdam patients with variable severity of mucocutaneous, post-surgical, and posttraumatic bleeding, which is similar to that observed in many common bleeding disorders.[1–5] A characteristic coagulation screening test abnormality was not present, but they had occasional prolongation in the aPTT or PT tests with normal levels of all coagulation factors. Dominant transmission of the bleeding disorder through the family aroused suspicion followed by laboratory testing which identified greatly elevated plasma TFPIα. The diagnosis was confirmed by sequencing of the *F5* B-domain that identified the exon 13 variant associated with east Texas bleeding disorder.

The biochemical underpinnings of these bleeding disorders are complex, but interesting, and inform about treatment strategies. The serine protease FXa combines with its cofactor FVa to form prothrombinase, the catalytic complex that generates thrombin to form a blood clot. TFPIα inhibits early forms of prothrombinase that assemble with forms of FVa that retain the acidic region of the B-domain, including FV-short as well as FVa produced through limited proteolysis by FXa and forms of FVa released from platelet α-granules.[10] Binding of the TFPIα C-terminal basic region to the FV B-domain acidic region dampens prothrombinase activity by maintaining FV in the inactive cofactor conformation and impeding further cleavage of the B-domain by thrombin.[8, 18] The inhibitory reaction also involves binding of the second Kunitz domain to TFPIα to the active site of FXa, and an interaction between Leu252-Thr255 of TFPIα and the heavy chain of FVa.[19] The interaction with the heavy chain of FV is weakened when prothrombinase is assembled with FV Leiden.[20] Thus, alterations in the interactions between FV and TFPIα contribute to hypercoagulable conditions as well as bleeding disorders.

TFPIα is also present in platelets.[21, 22] Platelet TFPIα does not localize to α-granules where FV is located, but instead appears to be in the cytoplasm.[22] The plasma concentrations of FV and TFPIα correlate[16, 23] and increases in plasma FV-short greatly increase plasma TFPIα.[2, 4, 5] However, platelet TFPIα and FV concentrations do not correlate, which is consistent with their different locations within the platelet.[16, 23] Here, we found that platelet TFPIα in two individuals with the FV east Texas variant

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was normal, indicating that FV-short does not alter platelet TFPIα, and implicating elevated plasma TFPIα as the cause of the bleeding disorder. Human platelet FV is adsorbed from plasma.[24] We were unable to detect FV-short in the platelets of patients with the FV east Texas variant indicating that the FV-short-TFPIα complex is not absorbed from plasma into platelets. This finding suggests that TFPIα binding to FV-short alters FV uptake by platelets, or alternatively, the structure of the FV-short B-domain may alter its uptake. Further, since essentially all plasma TFPIα is bound to FV-short, these data are consistent with platelet TFPIα being produced by megakaryocytes.[22]

The elevated TFPI in plasma from patients with *F5*-east Texas greatly diminished thrombin generation in TF-initiated CAT assays. Thrombin generation was restored by addition of rFVIIa to the plasma, which is consistent with its therapeutic efficacy in these patients. A high-affinity monoclonal antibody directed against the second Kunitz domain of TFPI also restored thrombin generation.[15] These findings are consistent with previous studies showing restoration of thrombin generation with polyclonal anti-TFPI antibodies or a mixture of anti-TFPI monoclonal antibodies.[2, 4] The restoration of thrombin generation with this single monoclonal antibody is consistent with a central role for the binding of the second Kunitz domain to the active site of FXa in the TFPIα anticoagulant activity producing bleeding in these patients, which may be mediated by its role in the inhibition of TF-FVIIa[9] or prothrombinase.[19] In this regard, there are several humanized anti-TFPI monoclonal antibodies directed against the second Kunitz domain presently in clinical trials for hemophilia as a non- factor therapy for prophylaxis.[25–29] It remains to be determined if these may hold promise as a prophylactic agent for severe spontaneous bleeding episodes in some patients with FV-short.[5]

Interestingly in our pedigree, three affected individuals have reported delayed wound healing with one suffering from recurrent angular cheilitis that did not heal properly and persisted for over a year. Similar recurrent lesions were reported by a patient from the original east Texas pedigree (communication with Dr. Miguel Escobar). It is yet to be determined if there is an underlying association of these lesions with the *F5* variant. However, the extracellular matrix (ECM) surrounding blood vessels is the major reservoir for the pool of heparin-releasable TFPIα.[14] It is tempting to speculate that the poor wound healing in these patients may be caused in part by altered amounts of TFPIα in the ECM. As a first step to examine this, a biopsy specimen from a mucosal lesion in one family member was examined. This lesion had chronic inflammation with blood vessels containing TFPIα in surrounding ECM. Thus, it does not appear that FV-short sequesters TFPIα from the vascular ECM into plasma. Further studies with additional samples from affected family members are needed to further define the etiology of these lesions.

Screening for elevated plasma TFPIα and variants that produce FV-short should be added to testing protocols for rare bleeding disorders (see Supplemental Figure II for algorithm). The limitation to this algorithm is need for commercial availability for TFPI assays and *F5* B-domain sequencing to enable hematologists to diagnose these less well-known bleeding disorders. The reported cases appear to be just the tip of the iceberg. The creation of an international database or a registry is needed to better understand disease symptoms and define effective treatments.

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Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Conflict of Interest and Disclosure Statement

AEM receives research funding from Novo Nordisk and has received honoraria for serving on Novo Nordisk and Vega Therapeutics Advisory Boards. The other authors have declared that no conflict of interest exists.

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Essentials

- Several bleeding disorders associated with factor V (FV) exon 13 variants have been identified.
- FV east Texas (FVET) bleeding disorder was identified in three generations of an Indiana family.
- All family members with bleeding had the FVET variant and elevated plasma TFPIα.
- Platelet TFPIα was normal suggesting elevated plasma TFPIα was the primary cause of bleeding.

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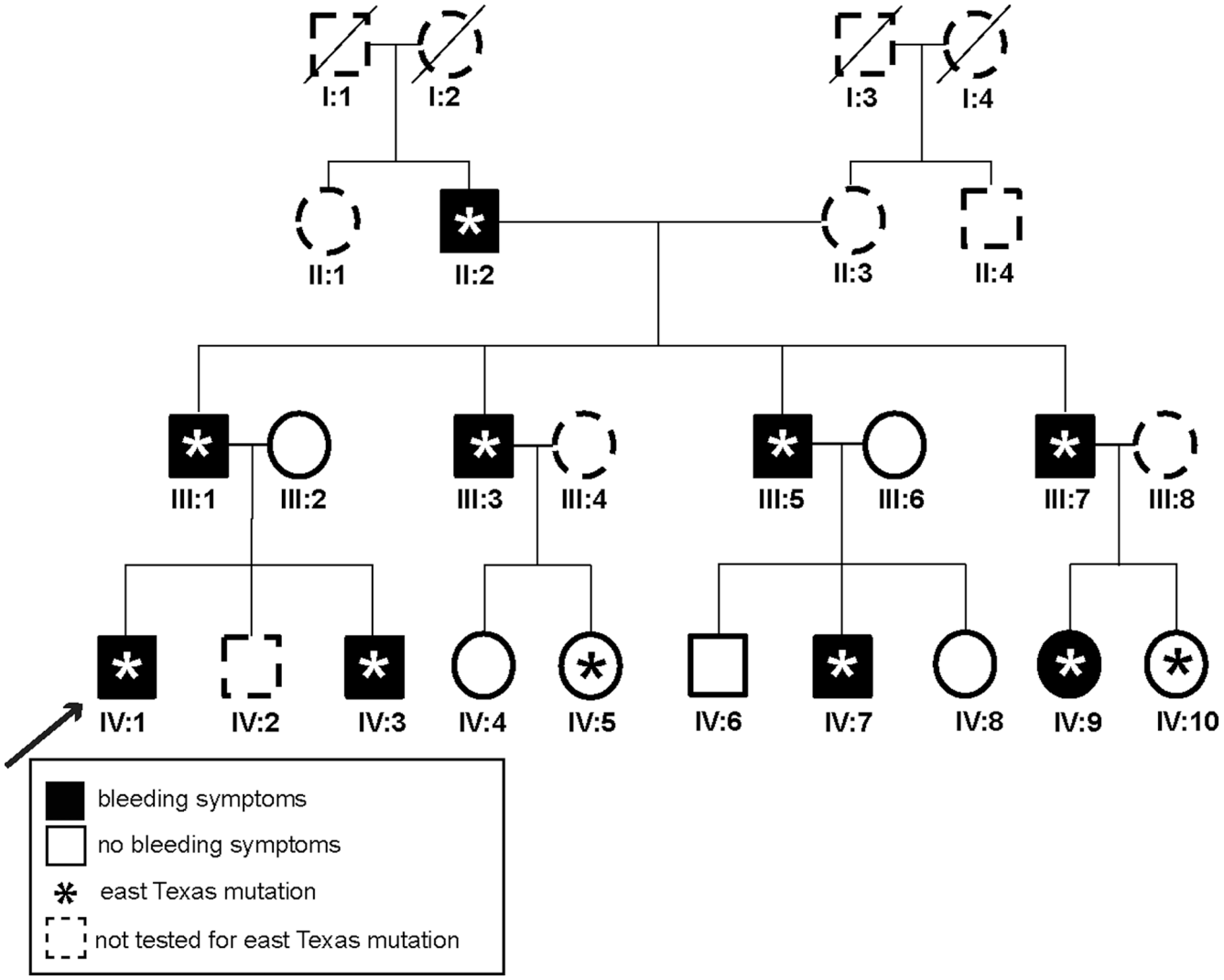

Figure 1. Family pedigree showed inheritance of the FV east Texas variant.

All individuals with the east Texas variant reported bleeding symptoms except for IV:5 and IV:10 who are children. *F5* sequencing of a buccal swab from IV:10 identified the FV east Texas variant, but a blood sample was not obtained for plasma TFPI measurement. The arrow indicates the proband.

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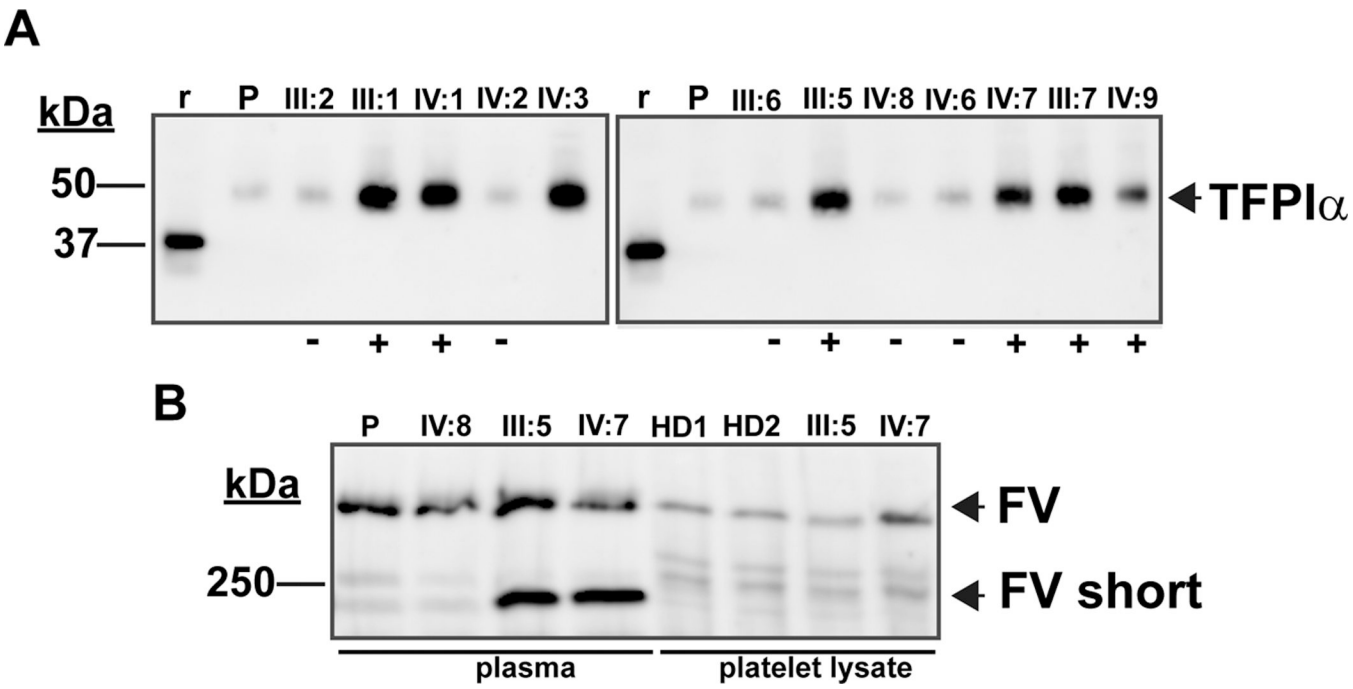

Figure 2. Elevated levels of plasma TFPIα and FV short were identified in plasma but not platelets from affected family members.

A) Elevated levels of TFPIα were detected in proband (IV:1) and select family members with bleeding symptoms. Purified recombinant TFPIα expressed in *E. coli* (r) and plasma TFPI precipitated with FXa immobilized agarose beads from pooled plasma (P) and indicated family members were detected by Western blot analysis with polyclonal anti-TFPIα. Presence or absence of the FVET variant is indicated below the blot with a + or −. **B)** Full-length FV in pooled plasma (P) and various family members were detected by Western blot analysis with monoclonal anti-FV. FV-short was detected only in family members with the east Texas variant. The blotting pattern of FV in platelet lysate was similar for two family members with the east Texas variant (IV:7, III:5) and two healthy donors (HD1, HD2) without elevated amounts of FV-short.

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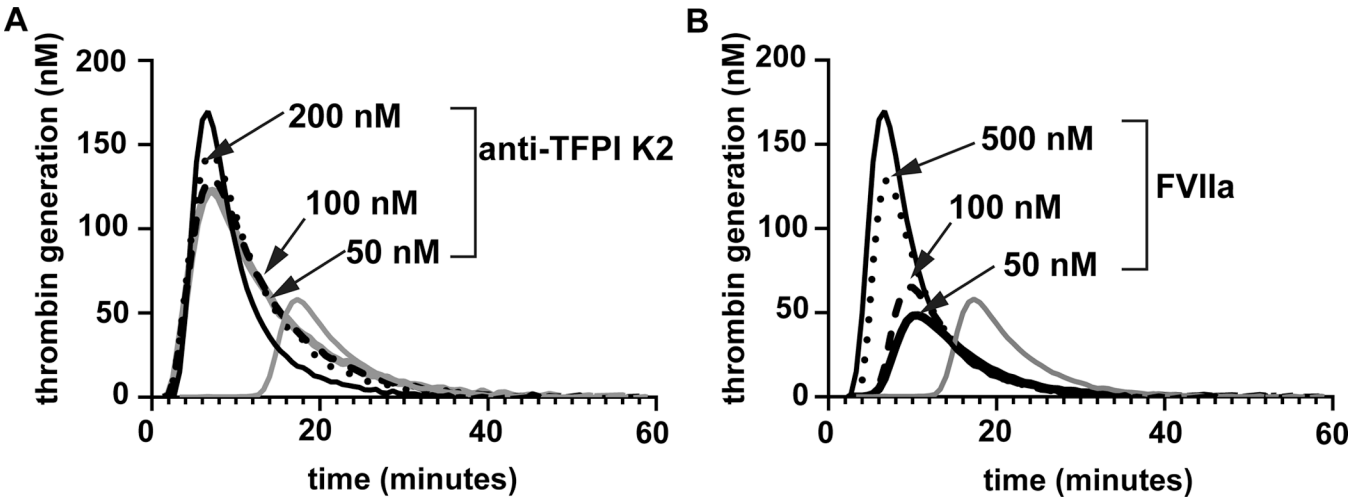

Figure 3. Thrombin generation in east Texas plasma was inhibited by elevated TFPIα levels.

Thrombin generation was initiated by 5 pM TF in pooled plasma (black solid line) and subject III:1, who carries the FV east Texas variant (gray line). Addition of monoclonal anti-TFPI-K2 at concentrations ≥50 nM to east Texas plasma restored the lag time for thrombin generation to that of pooled plasma. Addition of rFVIIa reduced the lag time in a concentration dependent manner.

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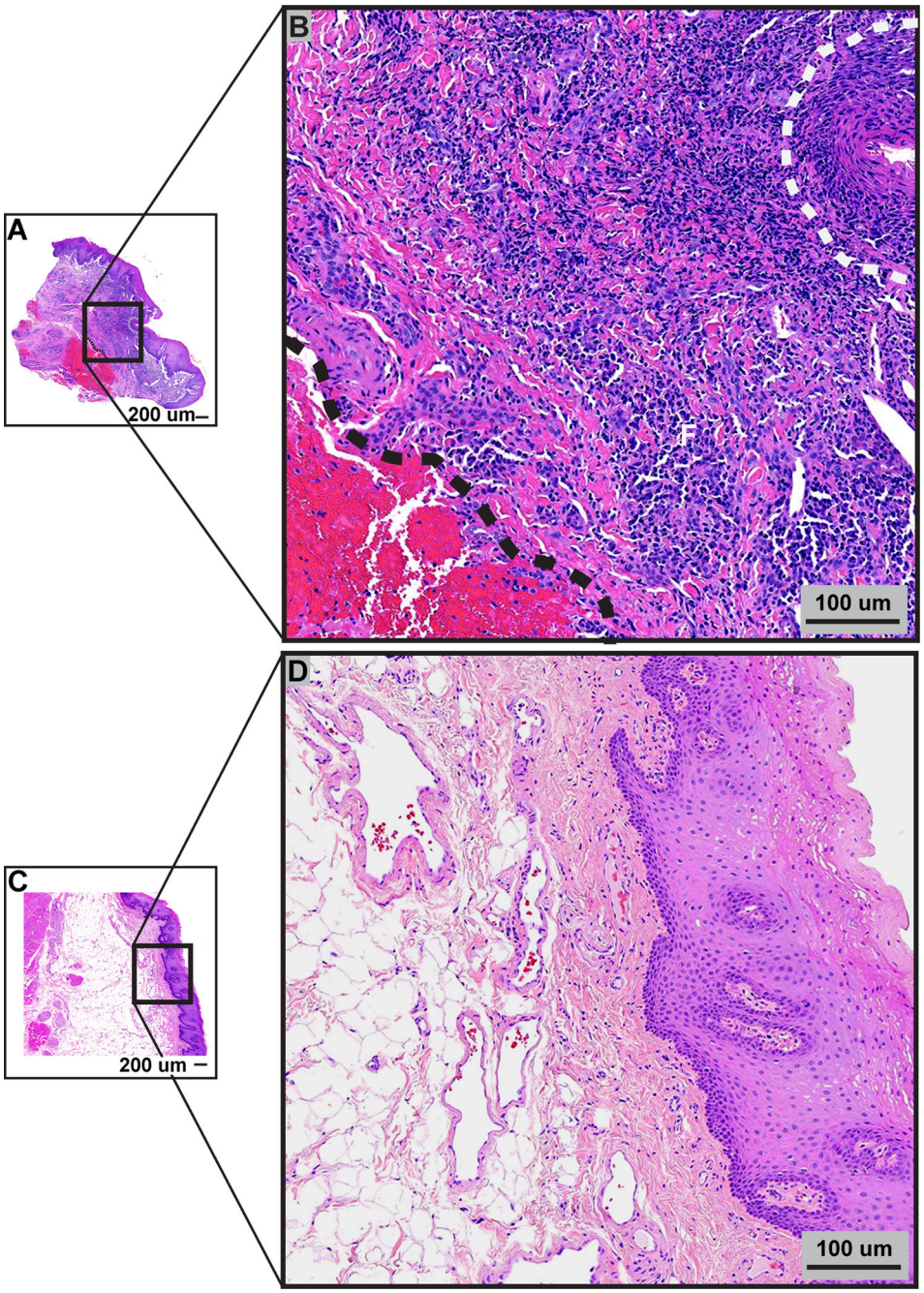

Figure 4. Biopsy of chronic oral ulcer from family member with east Texas Bleeding Disorder.
A and B) Tissue from chronic oral ulcer stained with H&E. **C and D)** Healthy buccal mucosal tissue The boxes in **A** and **C** depict the enlarged regions in **B** and **D**. The lesion in **B** contains hemorrhage (lower left corner, demarked with black dashed line) adjacent to chronically inflamed tissue evident by abundance of nuclear staining throughout the tissue. In **D**, an intact mucosal surface on the right with normal subcutaneous tissue on the left is shown for comparison.

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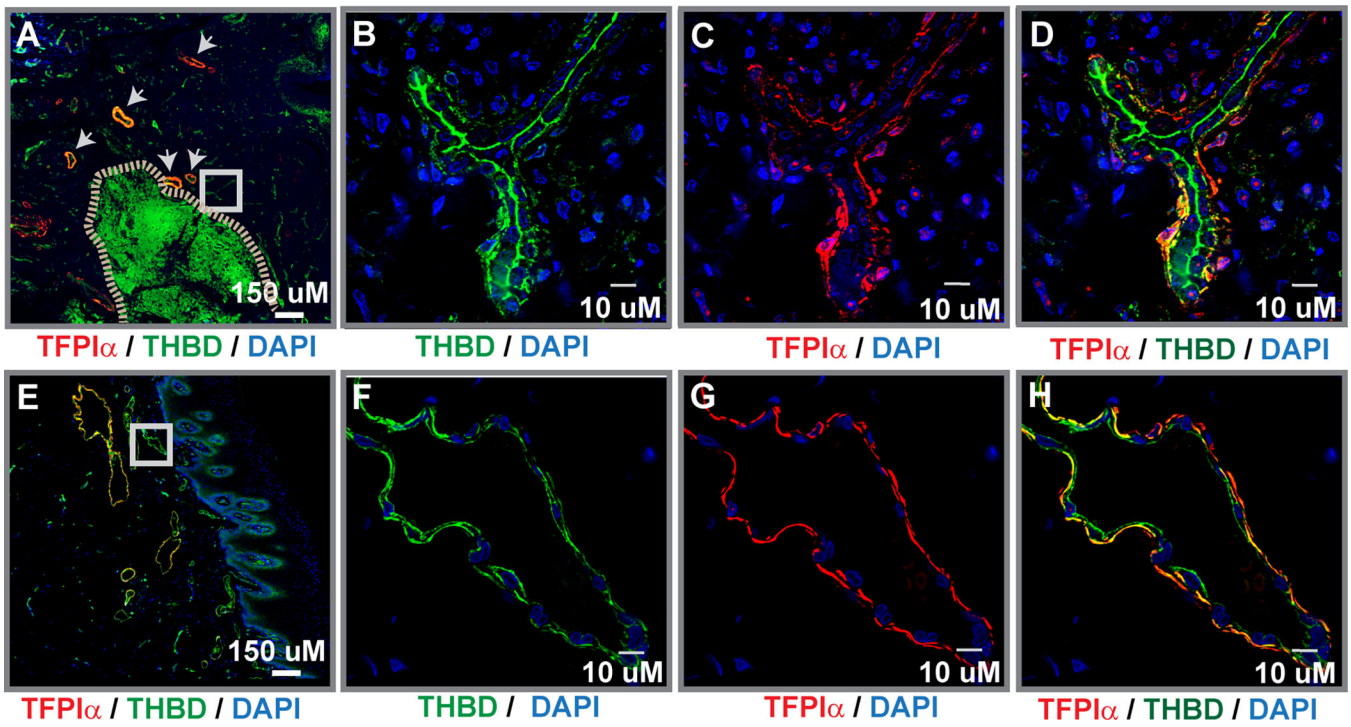

Figure 5. TFPIα localized within ECM surrounding buccal mucosal vessels.

(A) Serial sections (5 microns) from chronic buccal lesion from IV:7 was stained with polyclonal antibodies against TFPIα C-terminus (TFPIα, red), thrombomodulin (THBD, green) and DAPI stained nuclei (blue). The hemorrhage (green) is outlined with a white dotted line. White arrowheads indicate arterioles that stained brightly for TFPIα and THBD. The venuole in the white box is enlarged in panels **B-D**. **(B)** THBD and DAPI. **(C)** TFPIα and DAPI. **(D)** Overlay of THBD and TFPIα. The venuole was surrounded by inflammatory cells and had disrupted architecture of the vascular wall. TFPIα was in the subendothelial ECM. **(E)** Serial sections (5 microns) from similarly stained healthy human buccal mucosal tissue The venuole in the white box is enlarged in panels **F-H**. **(F)** THBD and DAPI. **(G)** TFPIα and DAPI. **(H)** Overlay of THBD and TFPIα. The venuole was well organized with THBD on the endothelial surface and TFPIα within the subendothelial ECM.

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Table 1:

TFPI levels and sequence analysis of proband and family members

Subject	east Texas variant	Plasma TFPIα (subject/ healthy pool)	Plasma total TFPI (subject/ healthy pool)	Platelet TFPIα subject/healthy pool	Total Protein S (%subject/ref plasma)	Free Protein S (%subject/ref plasma)
IV:1 (proband)	yes	11.8	2.3	Not tested	105	138
II:2	yes	6.0	2.7	Not tested	92	83
III:1	yes	7.2	2.3	Not tested	85	91
III:2	no	1.6	1.1	Not tested	Not tested	Not tested
III:3	yes	4.5	2.4	Not tested	92	109
III:5	yes	13.4	2.6	1.0	88	86
III:6	no	1.8	0.8	Not tested	84	90
III:7	yes	5.1	2.8	Not tested	91	109
IV:2	Not tested	1.0	0.5	Not tested	82	65
IV:3	yes	7.2	3.0	Not tested	96	113
IV:4	no	1.0	0.9	Not tested	82	73
IV:5	yes	5.2	2.6	Not tested	87	56
IV:6	no	1.5	0.9	Not tested	97	103
IV:7	yes	7.0	2.3	1.0	78	66
IV:8	no	0.7	0.9	Not tested	87	73
IV:9	yes	5.4	2.9	Not tested	89	104
IV:10	yes	Not tested	Not tested	Not tested	Not tested	Not tested