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## Dysregulated coagulation associated with hypofibrinogenemia and plasma hypercoagulability: implications for identifying coagulopathic mechanisms in humans

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### SUMMARY

Identifying coagulation abnormalities in patients with combined bleeding and thrombosis history is clinically challenging. Our goal was to probe the complexity of dysregulated coagulation in humans by characterizing pathophysiologic mechanisms in a patient with both bleeding and thrombosis. The patient is a 56-year old female with a history of hematomas, poor wound healing, and thrombosis (retinal artery occlusion and transient cerebral ischemia). She had a normal activated partial thromboplastin time, prolonged thrombin and reptilase times, and decreased functional and antigenic fibrinogen levels, and was initially diagnosed with hypodysfibrinogenemia. This diagnosis was supported by DNA analysis revealing a novel *FGB* mutation (c.656A>G) predicting a Q189R mutation in the mature B $\beta$  chain that was present in the heterozygote state. However, turbidity analysis showed that purified fibrinogen polymerization and degradation were indistinguishable from normal, and B $\beta$  chain subpopulations appeared normal by two-dimensional difference in-gel electrophoresis, indicating the mutated chain was not secreted. Interestingly, plasma thrombin generation testing revealed the patient's thrombin

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### AUTHORSHIP

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### DISCLOSURES

The authors declare no competing financial interests.

generation was higher than normal and could be attributed to elevated levels of factor VIII (FVIII, 163-225%). Accordingly, in an arterial injury model, hypofibrinogenemic mice (Fgn<sup>+/-</sup>) infused with FVIII demonstrated significantly shorter vessel occlusion times than saline-infused Fgn<sup>+/-</sup> mice. Together, these data associate the complex bleeding and thrombotic presentation with combined hypofibrinogenemia plus plasma hypercoagulability. These findings suggest previous cases in which fibrinogen abnormalities have been associated with thrombosis may also be complicated by co-existing plasma hypercoagulability and illustrate the importance of “global” coagulation testing in patients with compound presentations.

## Keywords

thrombin generation; factor VIII; hypofibrinogenemia; thrombosis; bleeding

## INTRODUCTION

Identifying coagulopathies in patients with a history of both bleeding and thrombosis is clinically challenging due to complex, interacting components during blood coagulation. During coagulation, thrombin generation is triggered by exposure of the factor VIIa/tissue factor (TF) complex to blood, and augmented by intrinsic tenase [activated factors IX and VIII (FVIII)]. Low levels of certain plasma procoagulants, including factors XI, IX, VIII, and prothrombin, cause reduced thrombin generation (1, 2) and are associated with bleeding. In contrast, *in vitro*, epidemiologic, and murine studies have associated elevated levels of these procoagulant proteins with abnormally high thrombin generation (1-3) and increased risk of thrombosis (4-8).

The ultimate substrate in these reactions, fibrinogen, is a 340 kDa glycoprotein composed of two copies each of three polypeptide chains (A $\alpha$ , B $\beta$  and  $\gamma$ ) transcribed from three genes encoding the fibrinogen B $\beta$  (*FGB*), A $\alpha$  (*FGA*), and  $\gamma$  (*FGG*) chains, ordered from centromere to telomere, clustered in a region of approximately 50 kb on the long arm of human chromosome 4 at 4q28-31 (9). Mature fibrinogen A $\alpha$ , B $\beta$  and  $\gamma$  chains are composed of 610, 461, and 411 residues, respectively (10). Fibrinogen is synthesized and assembled in hepatocytes as a hexameric molecule, and secreted into the bloodstream (11). During coagulation, thrombin cleaves fibrinopeptides A and B from the N-termini of the A $\alpha$  and B $\beta$  chains, respectively, catalyzing the conversion of fibrinogen to fibrin, which polymerizes, stabilizes platelets, and localizes fibrinolytic enzymes (12). Fibrin can bind thrombin and block its activity towards protein substrates; this activity is known as “antithrombin I” (13). Fibrinogen mutations (dysfibrinogenemias) have been associated with both bleeding (14-16) and thrombosis (16-23).

Herein, we identified a novel *FGB* mutation (c.656A>G) predicting a Q189R mutation in the mature B $\beta$  chain of a patient with a history of bleeding, thrombosis, and low functional and antigenic fibrinogen levels, consistent with an initial diagnosis of hypodysfibrinogenemia. However, this diagnosis was modified in light of findings that the mutant fibrinogen chain was not present in plasma and the circulating fibrinogen molecules were functionally normal. Interestingly, the patient demonstrated high plasma thrombin generation attributable to elevated FVIII activity, suggesting the presence of co-existing hypercoagulability as a cause for the thrombosis. This hypothesis was supported by a murine model of hypofibrinogenemia (Fgn<sup>+/-</sup>) plus elevated plasma FVIII which exhibited a shorter time to carotid artery occlusion than Fgn<sup>+/-</sup> mice infused with saline. These findings show plasma hypercoagulability was not mitigated by low fibrinogen levels. Together, these data suggest complex bleeding and thrombotic presentations can involve combined coagulopathies, and

illustrate the need for comprehensive, whole plasma testing in patients with complex presentations.

## METHODS

### Proteins and Materials

Human thrombin and corn trypsin inhibitor were from Haematologic Technologies Inc. (Essex Junction, VT). Fibronectin-, plasminogen-, and von Willebrand factor-depleted fibrinogen was from Enzyme Research Laboratories (South Bend, IN). Aprotinin was from Sigma Chemical Company (St Louis, MO). Polyclonal rabbit anti-human fibrinogen antibody was from Dako Corporation (Carpinteria, CA), and goat anti-rabbit antibodies were from Calbiochem (La Jolla, CA) or Cappel (West Chester, PA). Mouse anti-human B $\beta$  chain antibody (59D8) was a kind gift from Drs. Marshall Runge and Charles Esmon, and mouse anti-human A $\alpha$  chain antibody (Y18) was a kind gift from Dr. Susan Lord. Fluorogenic thrombin substrate (Z-Gly-Gly-Arg-AMC), TF/phospholipid reagents, and thrombin calibrator ( $\alpha_2$ -macroglobulin/thrombin) were from Diagnostica Stago (Parsippany, NJ). Human FVIII was from Baxter Healthcare Corporation (Glendale, CA). FVIII-deficient plasma was from HRF (Raleigh, NC). Tissue plasminogen activator (tPA) and batroxobin were from American Diagnostica (Greenwich, CT).

### Plasma

Blood was collected through a 21-gauge butterfly needle into a syringe via a protocol approved by the University of North Carolina Institutional Review Board. The first 5 mL were discarded. The following 30 mL were drawn into a separate syringe containing sodium citrate/corn trypsin inhibitor (0.105 M/3.2% sodium citrate, pH 6.5, 18.3  $\mu$ g/mL corn trypsin inhibitor) to minimize contact activation (24). Platelet-free plasma was prepared by sequential centrifugation (150 $\times$ g for 15 minutes, 20,000 $\times$ g for 15 minutes), aliquoted, and snap-frozen in liquid nitrogen within 2 hours of blood collection, as described (3). Plasma from healthy subjects was pooled for normal pooled plasma (NPP). Plasma was defibrinated for certain experiments by incubation with batroxobin (0.5 BU/mL final, 30 minutes, 37 °C), and removing fibrin by centrifugation (1118 $\times$ g, 10 minutes, 4°C).

### Sequencing

Sequencing was performed by standard Sanger sequencing (25) using primers covering all exons and exon/intron boundaries of *FGB*, *FGA* and *FGG*. Polymerase chain reaction was performed on genomic DNA. Sequencing was performed using BigDye V3.1 on an ABI3730xl. Analysis was conducted using Sequencher V4.9 and Mutation Surveyor V3.97. The mutation was replicated and evaluated bidirectionally. Primers and reaction conditions are available upon request.

### Fibrinogen purification

Fibrinogen was purified from the patient and normal plasmas as described (26). Briefly, plasmas were supplemented with phenylmethanesulfonyl fluoride and benzamidine (1 mM and 5 mM, final, respectively). Vitamin K-dependent proteins were removed by two adsorptions against 30% BaSO<sub>4</sub> (0.2 mL/mL plasma). Fibrinogen was precipitated twice by glycine addition (165 mg/mL plasma), end-over-end rotation (1 hour, room temperature), and centrifugation (4472 $\times$ g, 15 minutes). SDS-PAGE and western blotting demonstrated no fibrinogen in the supernatants of either the patient or control samples (data not shown), indicating all fibrinogen was precipitated. Purified fibrinogen was then re-suspended and dialyzed to 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (pH 7.4), 150 mM NaCl (HBS) containing 5 KIU/mL aprotinin.

### Fibrin polymerization and fibrinolysis

Undiluted plasma was clotted with thrombin and CaCl<sub>2</sub> (10 nM and 20 mM, final, prepared in HBS). Purified fibrinogen (0.5 mg/mL, final) was incubated for 1 minute with CaCl<sub>2</sub> (5 mM, final) and clotted with thrombin (5 nM, final). In both cases, fibrin polymerization was followed by turbidity at 405 nm in a SpectraMax Plus340 spectrophotometer (Molecular Devices, Sunnyvale, CA). The lag time was defined as the time until the initial turbidity increased by 10 mOD units.

Tissue plasminogen activator (tPA, 0.25 µg/mL, final) was added to plasma before triggering clot formation via addition of thrombin and CaCl<sub>2</sub> (10 nM and 20 mM, final), as indicated. Clot formation and lysis (turbidity increase and subsequent decrease, respectively) were followed by turbidity at 405 nm. The fibrin lysis rate was determined as the change in mOD *versus* time in the descending portion of the fibrinolysis curves.

### Fibrinogen degradation

Fibrinogen (0.3 mg/mL, final) was degraded with 6 µg/mL plasmin in the presence of 1 mM CaCl<sub>2</sub> or 5 mM EDTA at 37 °C. Aliquots were removed at 15 and 30 minutes and 4 hours, quenched with SDS-PAGE sample buffer, and immediately boiled. The zero time point was performed with fibrinogen in the absence of plasmin. Samples were stored at -80 °C until analysis by SDS-PAGE and Western blotting.

### Two-dimensional difference in-gel electrophoresis (2D-DIGE)

Protein analysis by 2-Dimensional Differential In-Gel Electrophoresis (2D-DIGE) was performed at the University of North Carolina Systems-Proteomics Center, as described (27). Briefly, control and patient fibrinogens were labeled with Cy5- and Cy3-, respectively, and the 2D SDS-PAGE standards with Cy2-. Individual images of Cy2-, Cy3- and Cy5-labeled proteins were obtained using a Typhoon 9410 scanner (Amersham, Piscataway, NJ) with excitation/emission wavelengths of 480/530 nm for Cy2-, 520/590 nm for Cy3-, and 620/680 nm for Cy5-. After imaging, gels were stained with colloidal Coomassie Blue G-250 (BioRad, Hercules, CA). Fold differences between control and patient samples were determined by Differential In-gel Analysis using DeCyder 2D software (version 7.0). Gel images were calibrated in DeCyder 2D against the pI and MW of four of the known 2D SDS-PAGE standards. The 2D gel was blotted onto polyvinylidene fluoride membrane and probed with mouse anti-human B $\beta$  chain (59D8), mouse anti-human fibrinogen A $\alpha$  chain (Y18), or rabbit polyclonal antibody against human fibrinogen. Membranes were stripped between blots with 62.5 mM Tris-HCl pH 6.7, 2% SDS, and 100 mM  $\beta$ -mercaptoethanol.

### FVIII activity measurements

Patient plasma was mixed with FVIII-deficient plasma (5% patient plasma and 95% FVIII-deficient plasma), and clotting was initiated with Kontakt reagent (33% total volume) and calcium (10 mM, final). The clot formation rate was measured in a SpectraMax Plus340 plate reader (28) and compared to a standard curve created by mixing normal pooled plasma (NPP) spiked with FVIII (to 150-300%) with FVIII-deficient plasma.

### Thrombin generation measurements

Thrombin generation in whole and defibrinated plasma was measured by calibrated automated thrombography (CAT) in reactions triggered with 1 pM TF/4 µM lipid and 5 pM TF/4 µM lipid in a Fluoroskan Ascent® fluorometer (ThermoLabsystem, Helsinki, Finland), as described (3). Thrombin generation parameters were calculated using Thrombinoscope software version 3.0.0.29 (Thrombinoscope BV, Maastricht, Netherlands).

### Measurement of fibrinogen in mice

Fibrinogen heterozygous mice ( $Fgn^{+/-}$ ) having a deletion of the  $\gamma$ -chain gene have been previously described (29). Blood was drawn from the retro-orbital plexus of  $Fgn^{+/+}$  and  $Fgn^{+/-}$  mice through heparinized capillary tubes into 30 U/mL Heparin (Sigma). Platelet-poor plasma was prepared by centrifuging blood at  $5,000\times g$  for 10 minutes. Plasma fibrinogen levels were measured by ELISA. Briefly, 96-well plates were coated with goat anti-human fibrinogen antibody (Cappel) diluted 1:500 in 20 mM phosphate pH 7.4, 150 mM NaCl (PBS) for 1 hour at room temperature, washed with PBS plus 0.1% Tween-20 (PBST), and blocked with 1% BSA in PBS. Plasmas diluted serially in PBS were added to coated plates, incubated at room temperature for two hours, and then washed with PBST. Plates were incubated with peroxidase-conjugated goat anti-human fibrinogen antibody (Cappel) diluted 1:25,000 in PBS with 1% BSA for one hour at room temperature, washed in PBST, and developed with SureBlue TMB peroxidase substrate (KPL). Absorbance was monitored at 635 nm on a SpectraMax Plus340 spectrophotometer. The rate of increase in absorbance was fit on a semi-log plot, and the level of fibrinogen in plasma from  $Fgn^{+/-}$  mice was determined by comparison to  $Fgn^{+/+}$  mice.

### Carotid artery thrombosis model

Procedures were approved by the University of North Carolina Institutional Animal Care and Use Committee. Mice were anesthetized with 2-2.5% isoflurane in 2% oxygen, and the left saphenous vein was exposed under a SZX12 dissecting microscope (Olympus) and catheterized as described (30). Human FVIII or saline was administered through the catheter on a per weight basis (blood volume [mL] is 7% of body weight [g]) 5 minutes before injury. The endogenous FVIII concentration in mice (1 U/mL, 100%) (31) was raised by infusion of human FVIII to levels (total murine plus human) similar to the patient's FVIII levels. Human FVIII binds murine vWF, has comparable cofactor activity as murine FVIII, promotes coagulation after tail clipping and vessel injury in hemophilic mice, and has sufficient half-life in murine circulation for these experiments (8, 32-36). The final plasma FVIII concentration achieved in mice ( $198\pm 1\%$ ) was confirmed in separate, uninjured mice ( $n=2$ ) by clotting assays, as we have described (8).

The carotid artery thrombosis model was performed as described (30). Briefly, the right common carotid artery was exposed after midline cervical incision, dried, and treated with 10% ferric chloride ( $FeCl_3$ , 0.62 M on  $0.5\times 0.5$ -mm filter paper) for 2 minutes, and washed with warm saline. Blood flow was monitored via Doppler ultrasonic flow probe (Indus Instruments, Webster, TX). The time to occlusion (TTO) was defined as the time between  $FeCl_3$  administration and lack of flow for 1 minute. Experiments were stopped after 40 minutes if there was no stable occlusion. Blood was drawn from the inferior vena cava (IVC) into 3.2% sodium citrate and processed to platelet-poor plasma by centrifugation at  $5000\times g$  for 10 minutes.

### Statistical analysis

Data from plasma polymerization and fibrinolysis were analyzed by non-parametric statistics using the Wilcoxon test. Thrombin generation parameters were analyzed by unpaired students t test. TTO data were analyzed by log-rank test (Mantel-Cox) due to censored TTO values.  $P<0.05$  was considered statistically significant.

## RESULTS

### Patient history

A 56 year-old female presented with a lifelong history of mild to moderate bleeding including gum bleeding, spontaneous bruising, and hematoma formation. She had developed

significant hematomas following cardiac catheterization via her brachial artery, as well as a neck hematoma following injury with a kickboard while swimming. Additionally, she had poor wound healing following surgery for a broken wrist and following mastectomy for breast cancer. She also had prior thrombotic events: a left-sided central retinal artery occlusion at age 35 and an episode of transient global amnesia thought to be ischemic at age 53, although MRI and MRA were both negative. She had no menorrhagia, and underwent 3 caesarean sections and total abdominal hysterectomy with bilateral salpingo-oophorectomy without abnormal bleeding. She had no hyperlipidemia, diabetes, or coronary disease.

Laboratory testing revealed prolonged thrombin and reptilase times, low functional and antigenic fibrinogen values, and prolonged platelet function analysis on two separate occasions by PFA-100, both on and off aspirin (Table 1). The patient had elevated FVIII activity in three separate blood draws spaced 20 months apart, but normal factor XIII activity and von Willebrand factor antigen, activity, and multimers (Table 1). She tested negative for prothrombin G20210A and factor V Leiden mutations, and for antiphospholipid antibodies. The patient's father and one of her three sons exhibited similar laboratory fibrinogen abnormalities, but were asymptomatic. The patient's father died following myocardial infarction at age 65; his fibrinogen was never sequenced. The sons were unavailable for further study.

DNA sequencing was performed only in the proband, revealing a single heterozygous A>G base substitution in *FGB* at position 656, predicting a glutamine to arginine substitution at position 189 (Q219R numbering includes the signal peptide) of the mature fibrinogen B $\beta$  chain. This variant was not found in the 1000 genomes catalogue of human genetic variation (<http://www.1000genomes.org/>). No other mutations were found in the three fibrinogen genes.

### Plasma shows impaired clot formation and fibrinolysis

We first characterized the patient's plasma clotting characteristics. Consistent with prolonged thrombin and reptilase times, the formation rate and final turbidity of the patient's plasma clots were 1.4- and 1.6-fold lower than NPP ( $p < 0.0001$ ), although the lag time was similar (Figure 1A, Table 2). When fibrinolysis was induced by adding tPA at the onset of clotting, the decrease in turbidity was slower in the patient's plasma than NPP ( $-6.7 \pm 1.0$  versus  $-14.2 \pm 2.4$  mOD/min, respectively,  $p < 0.0001$ ), but reached baseline at the same time as NPP (Figure 1B).

### Fibrinogen isolated from the patient's plasma has normal composition and function

To characterize the clotting dysfunction, we then isolated fibrinogen from the patient's plasma and compared it to fibrinogen isolated from normal plasma. SDS-PAGE and western blotting demonstrated no fibrinogen in the supernatant following glycine precipitation (data not shown), indicating all fibrinogen was precipitated. Figure 2A shows purified fibrinogen chains from the patient's plasma separated by SDS-PAGE under reducing conditions compared to fibrinogen purified from NPP and a commercial fibrinogen preparation. Fibrinogen from all three sources appeared similar, with all three chains present at the expected molecular weight. These results were confirmed by Western blot, where no additional bands were seen in the patient sample (Figure 2B).

In contrast to findings in plasma clotting assays, clotting of the patient's purified fibrinogen was indistinguishable from normal fibrinogen at equal concentrations (Figure 2C, Table 2). Furthermore, when purified patient and control fibrinogens were incubated with plasmin in the presence of calcium or EDTA, the appearance and molecular weights of the degradation products were identical by Coomassie Blue staining (Figure 2D) and Western blotting (data

not shown). These findings indicate that the patient's circulating fibrinogen, while low, could be normally activated and degraded.

### **Circulating fibrinogen contains all three polypeptide chains at the expected molecular weights and charges**

Since findings with purified fibrinogen suggested the patient's circulating fibrinogen function was normal, we used 2D-DIGE to explicitly determine whether fibrinogen containing the Q189R point mutation was present in the patient's plasma. This mutation, if present, should introduce a positive charge in the fibrinogen B $\beta$  chain and consequently, shift B $\beta$  chain migration in an isoelectric field. The 2D-DIGE was performed using ampholytes with a pI range of 3-10 and 5-8 in the first dimension; results were better visualized in the pI range of 3-10. Cy5-labeled control and Cy3-labeled patient fibrinogens were mixed and sequentially separated by size and charge. Figure 3 shows fluorescence from control and patient fibrinogens individually and merged. The patient's B $\beta$  chains had pIs of 6.1, 6.3, 6.4, and 6.6 (left to right), with a mass of approximately 57 kDa, similar to control and that reported in the ExPASy database (37). Western blot analysis with anti-fibrin(ogen) antibody 59D8 (specific for the B $\beta$  chain) confirmed the spots migrating at 57 kDa corresponded to the B $\beta$  chains. The membrane was re-probed with anti-fibrinogen antibody Y18 (specific for the N-terminus of the A $\alpha$  chain), confirming that no degraded A $\alpha$  chain was present in the B $\beta$  position (data not shown). These data show that fibrinogen isolated from the patient's plasma had a similar molecular weight and charge as control fibrinogen, indicating the patient had hypo- but not dys-fibrinogenemia.

### **Patient plasma demonstrates increased thrombin generation independent of hypofibrinogenemia**

The low levels of fibrinogen and prolonged platelet function test (Table 1) provided rationales for the patient's bleeding tendency. However, although thrombosis in afibrinogenemic patients may result from absence of "antithrombin I" activity (16, 38), the patient's fibrinogen level was ~50% of normal, making this explanation unlikely. To determine the mechanism for the patient's thrombotic tendency, we utilized a "global" assay to measure plasma coagulation potential. We first triggered clotting in NPP and the patient's plasma with 4  $\mu$ M phospholipid and 1 or 5 pM TF, and compared thrombin generation by CAT. Interestingly, the patient's plasma demonstrated higher thrombin generation parameters than NPP, most strikingly with 1.5-fold increased peak thrombin when reactions were triggered with 1 pM TF (Figure 4A, Table 3). When thrombin generation was induced with 5 pM TF, the patient still generated more thrombin compared to NPP, but the difference in peak thrombin was more subtle (data not shown).

We then defibrinated patient and normal plasmas and compared thrombin generation. Similar to that seen in the presence of fibrinogen, defibrinated patient plasma exhibited 1.5-fold higher peak thrombin than defibrinated NPP (Figure 4B, Table 3), indicating the difference in plasma thrombin generation was not due to the difference in fibrinogen concentration. Accordingly, adding fibrinogen to the patient plasma to reach normal levels (330 mg/dL) further increased peak thrombin 1.4-fold over that seen in patient whole plasma and 2-fold higher than that seen in whole NPP (data not shown). These findings suggested the patient's thrombosis may have resulted from a plasma hypercoagulability that was independent of the hypofibrinogenemia.

### **Increased plasma thrombin generation can be attributed to elevated plasma FVIII levels**

Given the patient's increased plasma thrombin generation peak, it was of interest that she had an elevated FVIII level in three separate blood draws spaced over 20 months (Table 1). We previously showed that FVIII increases peak thrombin generation with little to no

change in the lag time or time to peak (3). Therefore, we hypothesized that the increased thrombin generation seen in the patient's plasma was due to increased FVIII. To test this hypothesis, we spiked FVIII (to 163, 225 and 275%, final) into defibrinated NPP and measured thrombin generation. Raising FVIII to 225% produced similar (statistically indistinguishable) thrombin generation in defibrinated NPP as in defibrinated patient plasma in reactions triggered with 4  $\mu$ M phospholipid and 1 pM TF (Figure 4C, Table 3, results with 163 and 275% are not shown) or 5 pM TF (data not shown). This finding implicated elevated FVIII in the patient's increased plasma thrombin generation.

### Elevated FVIII increases thrombosis in hypofibrinogenemic mice

We previously showed that elevated FVIII is prothrombotic in mice with normal fibrinogen levels (8). To determine whether elevated FVIII could be prothrombotic in a background of low fibrinogen, we used a murine model of hypofibrinogenemia ( $Fgn^{+/-}$ ) (29), expressing  $39 \pm 2\%$  (mean  $\pm$  standard deviation, n=2) of the fibrinogen level of wild type ( $Fgn^{+/+}$ ) littermates, into which we infused factor VIII or saline, as we have described (8). Thrombosis was induced via 2-minutes application of  $FeCl_3$  to the carotid artery and the TTO was recorded by Doppler.

Consistent with our previous findings (8),  $Fgn^{+/+}$  mice infused with FVIII exhibited a shorter TTO than saline-infused  $Fgn^{+/+}$  mice. Furthermore, consistent with prior observations that  $Fgn^{+/-}$  mice have normal thrombin-induced clotting times (29), the TTO of  $Fgn^{+/-}$  mice infused with saline was statistically indistinguishable from wild type ( $Fgn^{+/+}$ ) littermates (Figure 5). Importantly,  $Fgn^{+/-}$  mice infused with FVIII (to  $\sim 200\%$  total, endogenous murine plus infused human protein), exhibited a significantly ( $p < 0.05$ ) shorter TTO than  $Fgn^{+/-}$  mice infused with saline (Figure 5). These data show that a low fibrinogen level does not mitigate the prothrombotic effect of elevated plasma FVIII.

## DISCUSSION

Diagnosis of patients with complex coagulopathies that include both bleeding and thrombosis is a significant clinical challenge. Fibrinogen abnormalities have been observed in patients with bleeding and/or thrombosis and implicated in the etiology of both coagulopathies (14-23). To our knowledge, prior studies have not included thrombin generation testing to rule out co-existing coagulopathies in these patients. In our patient, the presence of a novel heterozygous mutation in the 656 A>G position in exon 4 of *FGB* was consistent with the initial diagnosis of hypodysfibrinogenemia. However, this diagnosis was modified following our findings that the mutant fibrinogen chain was not present in plasma and the circulating fibrinogen molecules had normal functional characteristics. Further coagulation testing revealed elevated thrombin generation in the patient's plasma consistent with her elevated FVIII level, as well as a shortened time to vessel occlusion in an *in vivo* thrombosis model; these findings demonstrated the prothrombotic potential of this abnormality in spite of the low fibrinogen level. Together, these data suggest a combined etiology involving both hypofibrinogenemia and hypercoagulability.

Hypofibrinogenemia is a quantitative fibrinogen disorder characterized by reduced coagulant activity due to low antigen levels (39). Causative mutations are divided into two main classes: null mutations with no protein production, and mutations producing abnormal protein chains that are retained inside the cell because of impaired hexamer assembly or secretion (40). The most likely mechanism for the hypofibrinogenemia is a lack of secretion due to abnormal fibrinogen assembly. Of the 403 fibrinogen molecular abnormalities reported in the literature, 80 mutations are in the  $B\beta$  chain (41). Most *FGB* gene mutations that cause hypofibrinogenemia are located in the  $\beta C$  domain, some of which result in  $B\beta$  chain truncation. The present mutation, c.656 A>G ( $B\beta$  Q189R, numbering omits the signal

peptide), is located at the end of the second half of the coiled-coil  $\alpha$ -helical region, next to the second disulphide ring (42), where B $\beta$  cysteines 193 and 197 form interchain disulphide bonds, and close to the  $\beta$ C domain that encompasses residues 198-461. Studies with recombinant systems using deletion and substitution mutants indicate the coiled-coil region and inter- and intra-chain disulphide bonds are needed to complete chain assembly (11, 43, 44). To date, the g.5157T>A (p.Leu172Gln) (45) is the only other mutation reported in exon 4 of the FGB gene; this missense mutation does not affect protein secretion, but activates a cryptic acceptor splice site in exon 4 that results in B $\beta$  chain truncation. Inspection of the nucleotide sequence surrounding the present patient's mutation suggests the mutation does not create a typical splice site. Thus, the reason why protein containing a B $\beta$  Q189R mutation is not expressed is currently unknown.

It is challenging to relate thrombosis to fibrinogen abnormalities (23). Although thrombosis in afibrinogenemic patients has been attributed to the absence of "antithrombin I" activity (16, 38), the present patient's fibrinogen level was ~50% of normal, making this explanation unlikely. The patient did not carry the prothrombin G20210A or factor V Leiden mutations or antiphospholipid antibodies; however, the thrombotic events may be at least partially attributable to elevated FVIII levels, a common hypercoagulable state associated with increased thrombosis risk (4-8). We previously showed that elevated FVIII increases the thrombin generation peak height, with highest sensitivity in reactions utilizing a low TF trigger (3). Our current data are consistent with these findings; the patient's plasma demonstrated a higher thrombin peak than seen in NPP, and when defibrinated NPP was supplemented with FVIII, similar thrombin peak values were obtained as in the patient's defibrinated plasma (Figure 4). Importantly, we were able to recapitulate the patient's situation in an *in vivo* model of hypofibrinogenemia (Fgn<sup>+/-</sup> mice) (29) by raising FVIII to the level present in the patient's plasma and inducing thrombosis in the carotid artery (Figure 5). The prothrombotic effect of elevated FVIII in Fgn<sup>+/-</sup> mice is consistent with our prior findings in wild type mice with elevated FVIII and a normal fibrinogen level (8). Together, these data suggest a pathogenic role for elevated FVIII in this patient.

It is of interest that Fgn<sup>+/-</sup> mice expressing ~40% fibrinogen levels had a similar TTO as Fgn<sup>+/+</sup> mice (Figure 5). Indeed, neither the  $\gamma$  chain-deleted Fgn<sup>+/-</sup> mice used in our study, nor hemizygous A $\alpha$ <sup>+/-</sup> mice expressing 75% of normal fibrinogen levels, demonstrate a hemorrhagic phenotype or abnormal thrombin times (29, 46). The lack of bleeding in mice with low fibrinogen levels differs from humans, who exhibit prolonged thrombin times and mild to moderate bleeding complications with low fibrinogen.(47-51) Thus, although our study demonstrates the utility of animal models for examining pathogenic mechanisms in humans, the difference in phenotype in humans and mice with low fibrinogen levels suggests additional modifiers influence clotting in these species.

Although our experiments identified likely mechanisms to explain both the bleeding and thrombotic events, additional pathogenic mechanisms may also be present. Virchow's Triad suggests roles for abnormal blood composition, vascular wall function, and blood flow in the pathogenesis of thrombosis (52). Localization of the patient's thrombosis to the retinal artery suggests vascular bed-specific pathology also contributes to the phenotype. Currently, experimental means to investigate the contributions of the vessels and local shear *in vivo* are not feasible. The relative contribution of the patient's decreased platelet function to the bleeding events is unknown. This abnormality may reflect the decreased fibrinogen levels or stem from an alternate mechanism; we were unable to assess this aspect in the murine model. It is also unknown whether the patient's low fibrinogen and high FVIII levels are related. Fibrinogen is synthesized in the liver, whereas FVIII is synthesized in the liver and endothelium (53, 54). Both fibrinogen and FVIII levels increase during an acute phase response. Hepatotoxicity related to failure to secrete the mutant fibrinogen chain may induce

higher FVIII expression; however, the patient's alanine transaminase and aspartate aminotransferase levels were normal, suggesting normal liver function.

In conclusion, we present a novel *FGB* mutation, c.656 A>G, that predicts a p.Q189R change, but is not secreted. This new hypofibrinogenemia case was confounded by a hypercoagulable state related to elevated FVIII levels that increased plasma thrombin generation and accelerated the time to vessel occlusion in a murine thrombosis model. To our knowledge, this is the first characterization of combined congenital hypofibrinogenemia and plasma hypercoagulability in a patient with bleeding and thrombosis. Thrombin generation testing is not usually conducted in patients with suspected fibrinogen abnormalities. Our findings suggest previous cases in which fibrinogen abnormalities have been associated with thrombosis may also be complicated by co-existing hypercoagulability, and therefore, warrant re-investigation.

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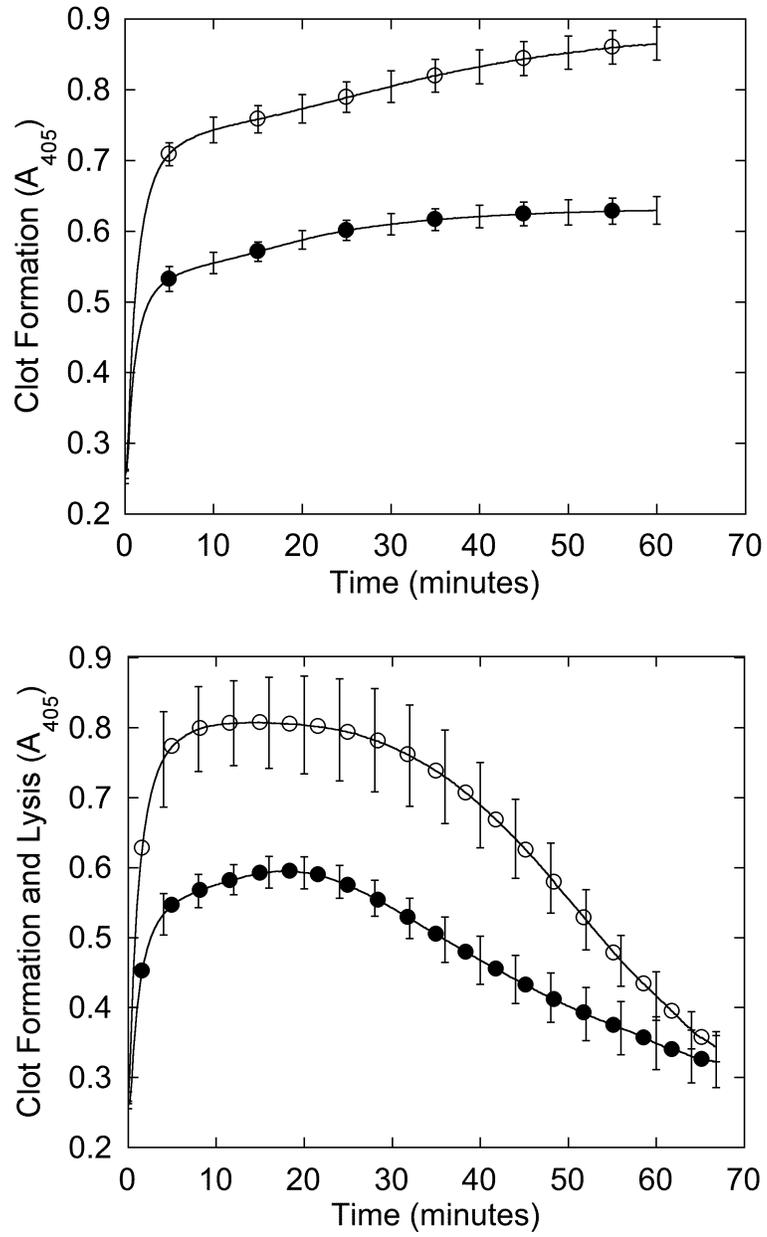
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**1. What is known on this topic**

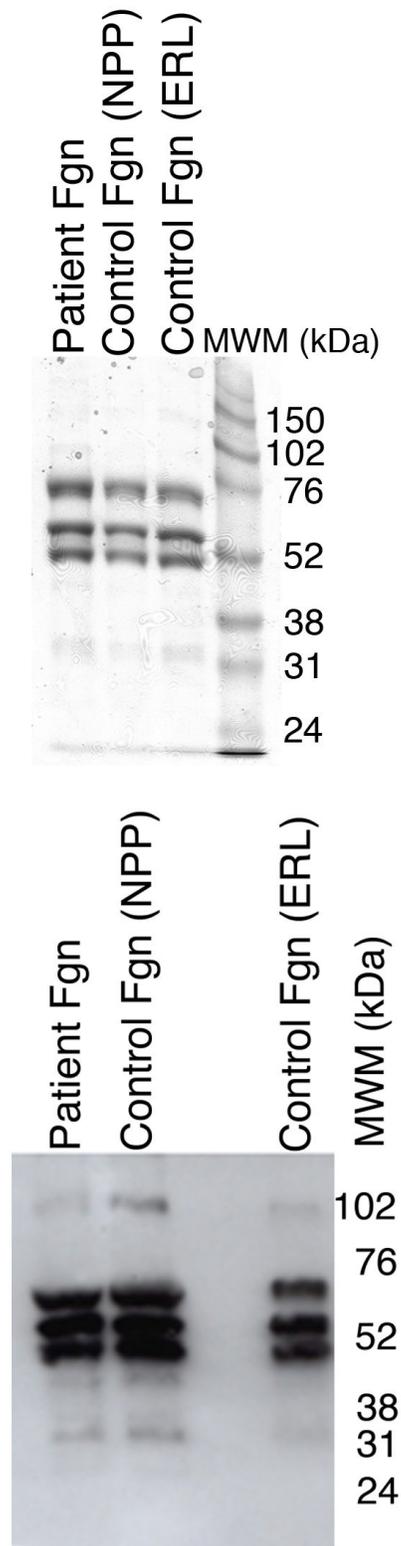
- Identifying coagulation abnormalities in patients with combined bleeding and thrombosis history is clinically challenging.
- Abnormal levels or function of fibrinogen have been associated with bleeding and/or thrombosis.
- Elevated factor VIII is a risk factor for thrombosis.

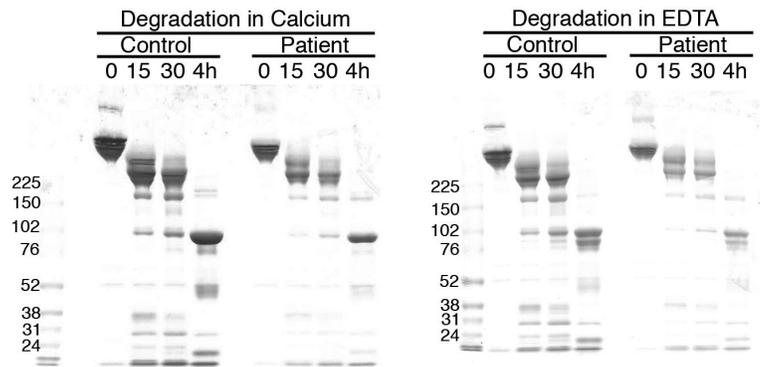
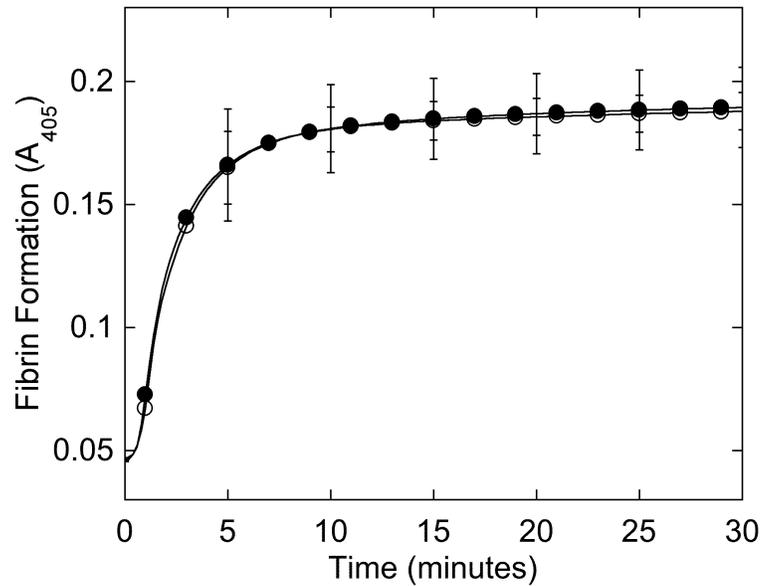
**2. What this paper adds**

- Fibrinogen containing a novel *FGB* mutation – c.656A>G predicting a Q189R mutation in the mature B $\beta$  chain – does not circulate in plasma.
- Low levels of fibrinogen (hypofibrinogenemia) do not protect against plasma hypercoagulability.
- Global plasma testing may be necessary to understand complex coagulopathies.



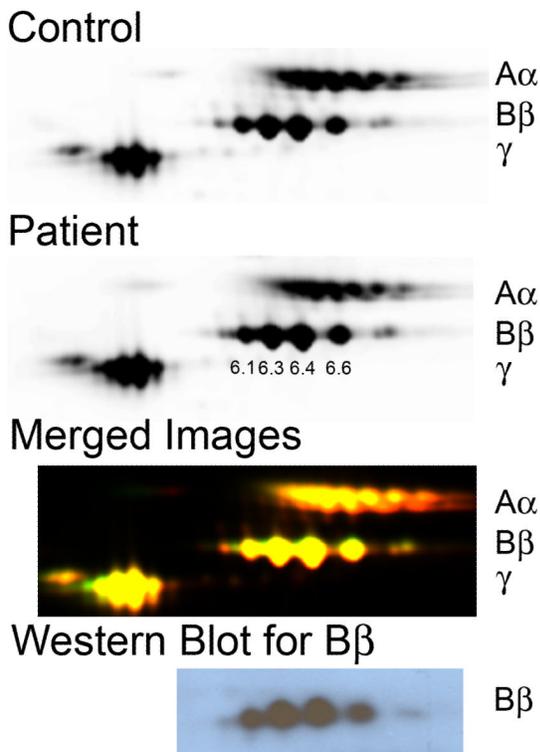
**Figure 1. Plasma demonstrates impaired clot formation and fibrinolysis**  
 (A-B) Plasma was clotted with CaCl<sub>2</sub> and thrombin (20 mM and 10 nM, final, respectively) in the absence (A) and presence (B) of 0.25 μg/mL tPA. Data shown are representative of three separate reactions performed in triplicate at room temperature and followed by turbidity at 405 nm. Symbols are: NPP (○) and patient plasma (●).





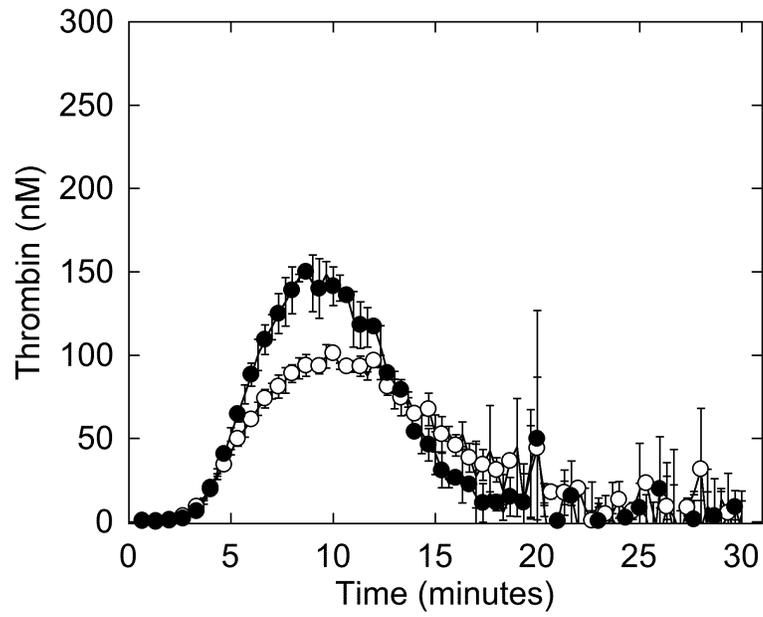
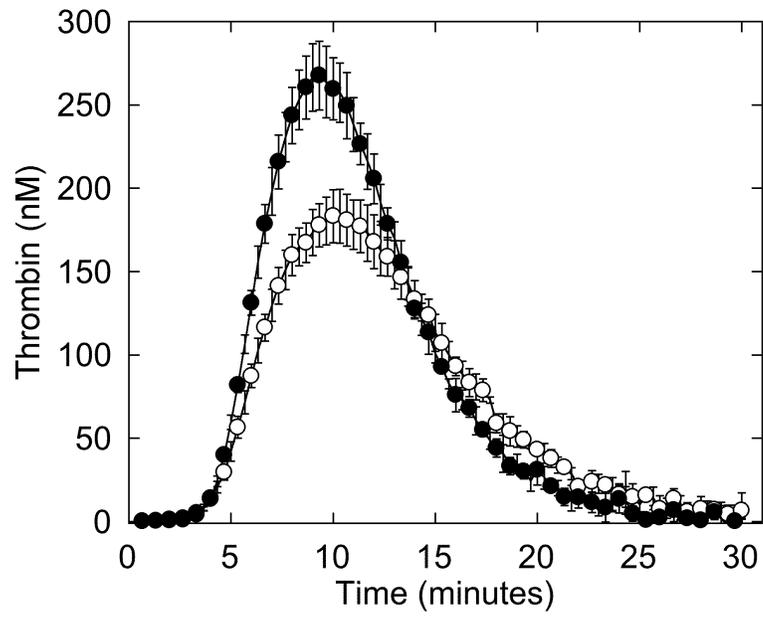
**Figure 2. Purified fibrinogen contains all three polypeptide chains at the expected molecular weights and has normal clotting function**

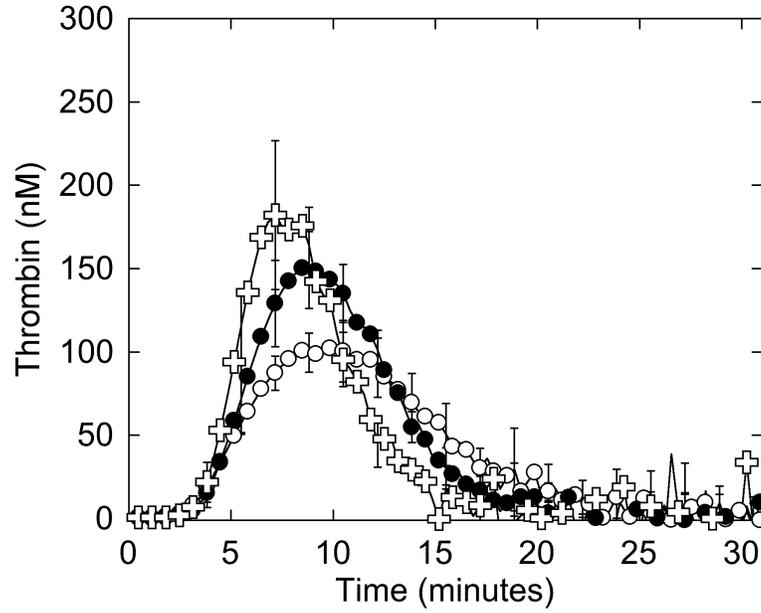
A-B) Fibrinogen was precipitated from plasma, and equivalent amounts (10  $\mu$ g) were separated by 10% SDS-PAGE under reducing conditions. A) Coomassie Brilliant Blue R250-stained gel. B) Western blot using rabbit anti-human fibrinogen polyclonal antibody. Lanes are: (P) Patient fibrinogen, (N) Fibrinogen precipitated from NPP in-house, and (C) Commercial fibrinogen. Molecular weight markers are indicated. C) Purified fibrinogen (0.5 mg/mL in HBS) was pre-incubated with  $\text{CaCl}_2$  (5 mM, final) for 1 minute, and clotted by adding thrombin (5 nM, final). Panel C shows mean and standard deviation from three separate reactions performed in triplicate at room temperature and followed by turbidity at 405 nm. Symbols are: NPP (○) and patient plasma (●); note that NPP and patient fibrinogen polymerization curves are identical. (D) Purified fibrinogen was incubated with plasmin in the presence of 1 mM  $\text{CaCl}_2$  or 5 mM EDTA (final, as indicated) at 37°C. Timed aliquots were quenched with SDS-PAGE loading dye, boiled, separated by non-reducing 4-12% SDS-PAGE, and stained by Coomassie Brilliant Blue R250.



**Figure 3. No abnormal fibrinogen is detected in the patient plasma by 2D-DIGE**

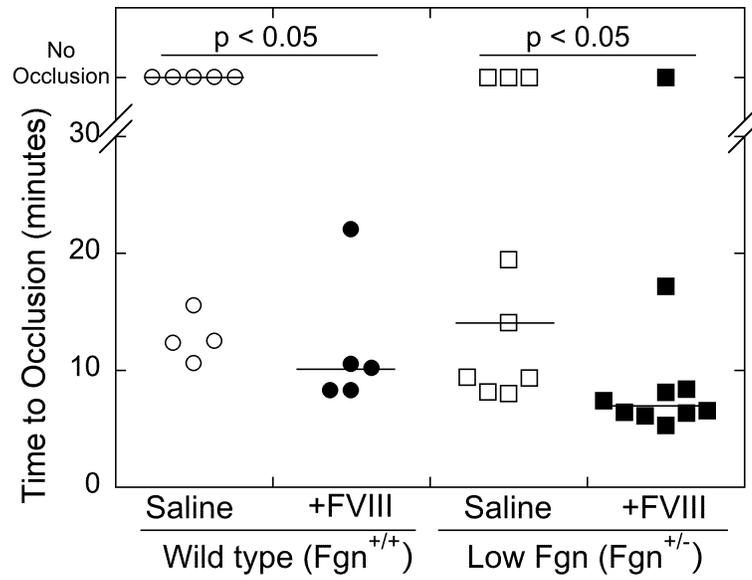
Control and patient samples and SDS-PAGE standards were labeled with Cy5-, Cy3-, and Cy2-, respectively, and separated by 2D-DIGE. Individual images of labeled proteins were obtained using a Typhoon 9410 scanner and compared using DeCyder 2D software (v7.0). Top to bottom: Control and patient samples individually, superimposed images of control and patient, and western blot of the 2D-DIGE using anti-human B $\beta$  chain antibody (59D8). The B $\beta$  chain pIs are indicated in the patient image.





**Figure 4. Whole and defibrinated patient plasmas demonstrate abnormal thrombin generation that can be recapitulated by raising the FVIII level in NPP**

Thrombin generation was initiated by adding 4  $\mu\text{M}$  phospholipid and 1 pM TF to whole (A) or defibrinated (B, C) plasmas. CAT detects higher thrombin generation in the presence of fibrinogen than in its absence because fibrin-bound thrombin is protected from inhibition by antithrombin III but can still cleave fluorogenic substrate (3, 56, 57). A, B) Symbols are: NPP (○), patient plasma (●). C) Thrombin generation was initiated by adding 4  $\mu\text{M}$  phospholipid and 1 pM TF to defibrinated NPP (○), defibrinated patient plasma (●), and defibrinated NPP plus FVIII (†, 225%, final). Data show mean and standard deviations from experiments performed in triplicate in three independent assays for whole plasmas, and in triplicate in two to five independent assays for defibrinated plasmas.



**Figure 5. Elevated FVIII shortens the TTO after FeCl<sub>3</sub> injury to the carotid artery of Fgn<sup>+/-</sup> mice**  
 Fgn<sup>+/+</sup> and Fgn<sup>+/-</sup> mice were infused with saline or FVIII to 198±1% of normal. Thrombosis was induced by application of 10% FeCl<sub>3</sub> to the carotid artery for 2 minutes, and the TTO was determined by Doppler. In vessels that did not occlude, the TTO was censored at 40 minutes. Each point represents a separate mouse. Lines show median values.

**Table 1**  
**Summary of screening tests**

| Coagulation Tests                              | Normal range     | Patient <sup>a</sup>   |
|--|------------------|------------------------|
| aPTT (sec)                                     | 25.2 – 36.2      | 29.6, 31.9             |
| Prothrombin Time (sec)                         | 10.1 – 12.6      | 11.9, 12.2             |
| Thrombin Time (sec)                            | 13.4 – 18.0      | 18.2, 22.6             |
| Reptilase Time (sec)                           | 15.0 – 19.2      | 21.3                   |
| Functional fibrinogen (Clauss, mg/dL)          | 208 – 409        | 162                    |
| Fibrinogen antigen (mg/dL)                     | 150 – 375        | 148                    |
| Functional fibrinogen (clot weight(55), mg/dL) | 330 <sup>b</sup> | 170                    |
| von Willebrand factor antigen (% of normal)    | 56 – 223         | 147                    |
| von Willebrand factor activity (% of normal)   | 50 – 204         | 140                    |
| FVIII activity in blood draw 1 (% of normal)   | 54 – 161         | 163                    |
| FVIII activity in blood draw 2 (% of normal)   | 100 <sup>c</sup> | 225                    |
| FVIII activity in blood draw 3 (% of normal)   | 100 <sup>c</sup> | 225                    |
| Antithrombin III (% of normal)                 | 88 – 133         | 97                     |
| PFA-100: collagen and epinephrine (sec)        | 84 – 178         | >300, 132 <sup>d</sup> |
| PFA-100: collagen and ADP (sec)                | 60 – 107         | 119, 119 <sup>d</sup>  |
| <b>Chemistry Tests</b>                         |                  |                        |
| alanine transaminase (U/L)                     | 15 – 48          | 24, 25, 22             |
| aspartate aminotransferase (U/L)               | 14 – 38          | 25, 21, 27             |
| Cholesterol (mg/dL)                            | 100 – 199        | 220, 251, 192          |
| Triglycerides (mg/dL)                          | 1 – 149          | 55, 85, 54             |

<sup>a</sup>Multiple values are from separate blood draws

<sup>b</sup>Fibrinogen concentration in NPP

<sup>c</sup>FVIII activity in NPP

<sup>d</sup>First PFA-100 was on aspirin, second was off aspirin for 2 weeks

**Table 2**  
**Summary of plasma and purified fibrin polymerization parameters**

|   | <b>NPP</b> | <b>Patient</b> |
|---|------------|----------------|
| <b>Plasma Clotting Parameters</b> <sup>a</sup>              |            |                |
| Onset (min) <sup>b</sup>                                    | 0.2 ± 0.1  | 0.3 ± 0.1      |
| Rate (Vmax, mOD/min)  | 292 ± 52   | 202 ± 35 *     |
| Turbidity change (mOD)                                      | 612 ± 19   | 374 ± 18 *     |
| <b>Purified Fibrinogen Clotting Parameters</b> <sup>a</sup> |            |                |
| Onset (min) <sup>b</sup>                                    | 0.9 ± 0.2  | 0.9 ± 0.4      |
| Rate (Vmax, mOD/min)  | 67 ± 20    | 70 ± 22        |
| Turbidity change (mOD)                                      | 142 ± 8    | 143 ± 16       |

<sup>a</sup>Mean ± standard deviation from three independent experiments from two separate blood draws, performed in triplicate

<sup>b</sup>Time until turbidity increased 10 mOD units

\*p < 0.0001 versus NPP

**Table 3**  
**Thrombin generation parameters in whole and defibrinated plasmas**

|                           | Whole Plasma <sup>a</sup> |             | Defibrinated Plasma <sup>b</sup> |            |                |
|---------------------------|---------------------------|-------------|----------------------------------|------------|----------------|
|                           | NPP                       | Patient     | NPP                              | Patient    | NPP plus FVIII |
| Lag Time (min)            | 4.4 ± 0.2                 | 4.5 ± 0.2   | 3.7 ± 0.4                        | 4.0 ± 0.4  | 3.8 ± 0.6      |
| Time to peak (min)        | 10.2 ± 0.2                | 9.4 ± 0.2 * | 10.4 ± 2.0                       | 9.0 ± 0.3  | 7.7 ± 0.5      |
| Peak thrombin (nM)        | 183 ± 16                  | 267 ± 20 *  | 107 ± 27                         | 158 ± 14 # | 181 ± 45 #     |
| ETP <sup>c</sup> (nM.min) | 2052 ± 141                | 2343 ± 162  | 1066 ± 216                       | 1244 ± 74  | 1146 ± 119     |

Experiments were triggered with 1 pM TF and 4 μM phospholipids.

<sup>a</sup>Mean ± standard deviation from three independent experiments from two separate blood draws, performed in triplicate

<sup>b</sup>Mean ± standard deviation from two to five independent experiments performed in triplicate

<sup>c</sup>ETP, endogenous thrombin potential

\* p<0.05 versus whole NPP

# p<0.01 versus defibrinated NPP