Natural and engineered carboxy-terminal variants: decreased secretion and gain-of-function result in asymptomatic coagulation factor VII deficiency

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ABSTRACT

We report 2 asymptomatic homozygotes for the nonsense p.R462X mutation affecting the carboxy-terminus of coagulation factor VII (FVII, 466 aminoacids). FVII levels of 3-5% and 2.7±0.4% were found in prothrombin time-based and activated factor X (FXa) generation assays with human thromboplastins. Noticeably, FVII antigen levels were barely detectable (0.7±0.2%) which suggested a gain-of-function effect. This effect was more pronounced with bovine thromboplastin (4.8±0.9%) and disappeared with rabbit thromboplastin (0.7±0.2%). This suggests that the mutation influences tissue factor/FVII interactions.

Whereas the recombinant rFVII-462X variant confirmed an increase in specific activity (~400%), a panel of nonsense (p.P466X, p.F465X, p.P464X, p.A463X) and missense (p.R462A, p.R462Q, p.R462W) mutations of the FVII carboxy-terminus resulted in reduced secretion but normal specific activity.

These data provide evidence for counteracting pleiotropic effects of the p.R462X mutation, which explains the asymptomatic FVII deficiency, and contributes to our understanding of the role of the highly variable carboxy-terminus of coagulation serine proteases.

Key words: FVII, mutation, asymptomatic, carboxy-terminal.

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Introduction

The congenital deficiency of coagulation factor VII (FVII) (OMIM: 227500) is a rare hemorrhagic disorder with an autosomal recessive inheritance pattern.[1] Clarification of the relationship between FVII coagulant activity levels in plasma and bleeding diathesis is greatly helped by our understanding of the molecular mechanisms through which *F7* gene mutations[2-3] modulate FVII levels.

Nonsense mutations, by inducing mRNA degradation[4] and/or premature termination of translation and synthesis of truncated proteins, are often responsible for very severe forms of human diseases. As expected from the crucial role of FVII in coagulation,[5] very few homozygous patients present this type of mutation and these are associated with undetectable FVII levels and severe[6-8] or moderate[9] bleeding.

Coagulation serine proteases share extensive homology[10] but are highly variable in their carboxy-terminal region, the functional role of which has still not been established.

In this study, we characterized the p.R462X nonsense mutation and provided evidence for its pleiotropic effects, *i.e.* reduced secretion and increased specific activity, thus explaining the asymptomatic phenotype in patients. Furthermore, a panel of recombinant carboxy-terminal variants contributed to our understanding of the role of this highly variable region in coagulation serine proteases.

Design and Methods

Patients

The proposita (PFVII-R462X) is a 12-year old girl who had been diagnosed for FVII deficiency during a routine coagulation screening. The patient presented with a prolonged prothrombin time (8%) and decreased FVII coagulant activity (3 and 5% of normal in 2 samples taken one year apart); all other coagulation factors functioned normally. She had no history of any bleeding and continues to be asymptomatic. *F7* gene sequencing[11] identified the c.1384C>T transition (GenBank #NM_000131.3) in a homozygous condition. This

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results in the p.R462X nonsense mutation. The unrelated parents carried the mutation and showed half-reduced FVII levels.

Screening for *F7* gene mutations identified the homozygous p.R462X mutation in an unrelated asymptomatic subject from France (male, 50 years old) who was diagnosed for FVII deficiency at 49 years of age during pre-operative screening. His FVII activity and antigen levels were 5% and less than 1% of normal values, respectively, and he has no history of bleeding.

The research was part of a more extensive study on FVII deficient patients which was approved by the Review Board of the University of Palermo (the original institution of GM). Furthermore, the authors obtained informed consent from the proposita and her parents to conduct the study.

FVII activity and protein assays

FVII coagulant (FVIIc) levels were assessed by standard prothrombin time (PT) based assays using RecombiPlasTin 2G (HemosIL©, Instrumentation Laboratory, Lexington, MA, USA).

Calibrated automated thrombin activity was measured according to Hemker *et al.*;[12] TF was used at 5 pM concentration.

Generation of activated factor X (FXa) either in the patient's plasma or with recombinant FVII variants was evaluated essentially as previously described.[13] As coagulation trigger, we used either Innovin (Dade® Innovin®, Siemens Healthcare, Marburg, Germany) or RecombiPlasTin 2G (HemosIL©, Instrumentation Laboratory, Lexington, MA, USA), containing recombinant human TF, or the Pro-IL-Complex or the PT-Fibrinogen HS PLUS (HemosIL©) reagents, containing bovine or rabbit brain thromboplastins, respectively. Inhibition by TFPI was assessed as previously described.[14]

FVII antigen (FVIIag) levels in plasma or in conditioned medium were measured by ELISA (Affinity Biologicals© Inc., Canada). In our study, the detection limit was 1 ng/mL (0.2% of pooled normal plasma, PNP).

Western blotting was carried out essentially as previously reported[15] by exploiting the sheep polyclonal anti-human FVII HRP-conjugated antibody (Pierce, Thermo Scientific, Rockford, IL, USA).

Serial dilution of PNP or of rFVII-wt in FVII-deficient plasma (George King, Bio-Medical Inc., USA) was used to optimize all the assays for very low FVII levels. As a reference for studies in plasma, we used the PNP and expressed results as percentage to facilitate comparison of data across assays.

Expression vectors, cell culture and transfection

The mutations (underlined) were introduced into the human FVII cDNA cloned into the pCDNA3 vector[13] by using the QuickChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following forward primers: 5'GGAGTCCTCCTGTGAGCCCCATTTCGCTAG3' (p.R462X), 5'-GGAGTCCTCCTGCGATAGCCATTTCCCTAG-3' (p.A463X), 5'CCTCCTGCGAGCCTAGTTTCCCTAGCCCAGC3' (p.R464X), 5'CTCCTGCGAGCCCCATGACCCTAGCCCAGCAGC3' (p.R465X), 5'CTGCGAGCCCCATTTTAGTAGCCCAGCAGCCC3' (p.R466X), 5'GAGTCCT CCTGGCAGCCCCATTTCCCTAG3' (p.R462A), 5'GAGTCCTCCTGCAAGCCCCATTTCCCTAG3' (p.R462Q), 5'-GAGTCCTCCTGTGGGCCCCATTTCCCTAG-3' (p.R462W). Reverse primers were perfectly complementary to the forward ones. Direct sequencing validated the vectors.

Baby hamster kidney (BHK) cells were cultured and transiently transfected in serum-free medium (Opti-MEM®, GIBCO®, Life Technologies, Carlsbad, CA, USA) as described.[13]

The recombinant FVII (rFVII) in media were concentrated through the Amicon Ultra centrifugal filter devices (cut-off 30 KDa, Millipore, Carrigtwohill, Country Cork, Ireland).[11]

Results and Discussion

The homozygous p.R462X nonsense mutation, predicting a FVII molecule with a truncated carboxy-terminus, was found in 2 unrelated subjects with no history of bleeding and showing appreciable and comparable FVIIc levels in plasma (3-5%). The similarity of these observations in 2 homozygotes strongly suggests that the mutation is responsible for mild coagulation and clinical features. The contribution of this mutation to FVII levels *in vivo* could not be ascertained in the previously identified heterozygous or doubly heterozygous subjects.[16-17]

Functional studies were performed to identify the coagulation phenotype in the proposita's (PFVII-R462X) plasma aimed at monitoring, upon extrinsic trigger, the generation of thrombin and of FXa. In these assays, we intentionally included a plasma sample from a patient homozygous for the p.A354V-p.P464Hfs† mutation (PFVII-A354V-P464fs†) predicted to be responsible for a FVII molecule with an elongated carboxy-terminal region. Homozygotes for this mutation showed low antigen and very low activity levels.[2]

As an informative variable to interpret the effects of FVII deficiency on thrombin generation, we considered the lag time, since among the other parameters (endogenous thrombin potential, time to peak) this showed a better correlation with FVII levels (Figure 1A, inset). In this analysis, activity in PFVII-R462X and PFVII-A354V-P464fs† plasma samples was 1.5±0.1% and 0.25% of PNP, respectively (Figure 1A).

The FXa generation activity in the PFVII-R462X plasma upon triggering coagulation with Innovin was clearly measurable (1.8±0.5% of PNP) whereas that in PFVII-A354V-P464fs† plasma was barely detectable (0.2±0.1%) (Figure 1B).

In spite of having significantly different activities, the FVII antigen levels in plasma from PFVII-R462X (0.7±0.2% of PNP) and PFVII-A354V-P464fs† (0.8%) were similarly reduced. This observation was further reinforced by Western blotting (Figure 1C, inset) that revealed only tiny amounts of FVII in PFVII-R462X and PFVII-A354V-P464fs† samples, with estimated levels below 1% of PNP. In order to understand this mechanism, we performed additional FXa generation assays on the proposita's plasma with different sources of TF, and particularly RecombiPlasTin 2G, the recombinant human TF used in PT-based assays, and bovine or rabbit brain thromboplastins (Figure 1C). The FXa generation activity with RecombiPlasTin 2G (2.7±0.4% of PNP; >3 times higher specific activity) was higher than that observed with Innovin, and therefore reflected the FVIIc levels better. Interestingly, the highest activity was detected with bovine TF (4.8±0.9%) whereas the increased activity was no longer detectable with rabbit brain TF (0.7±0.2%).

Since the carboxyl-terminal residues of FVII might be involved in recognition by inhibitors, as demonstrated for FXa,[18] we tested FVII inhibition in plasma by adding 2-4 U/mL Tissue Factor Pathway Inhibitor. However, we failed to demonstrate any difference between the proposita's plasma and PNP (*data not shown*). These results indicate the presence of traces of a FVII variant with higher than normal activity in PFVII-R462X's plasma.

The decreased amount of circulating protein could theoretically be attributed either to reduced FVII mRNA levels, through the nonsense mediated decay (NMD) mecha-

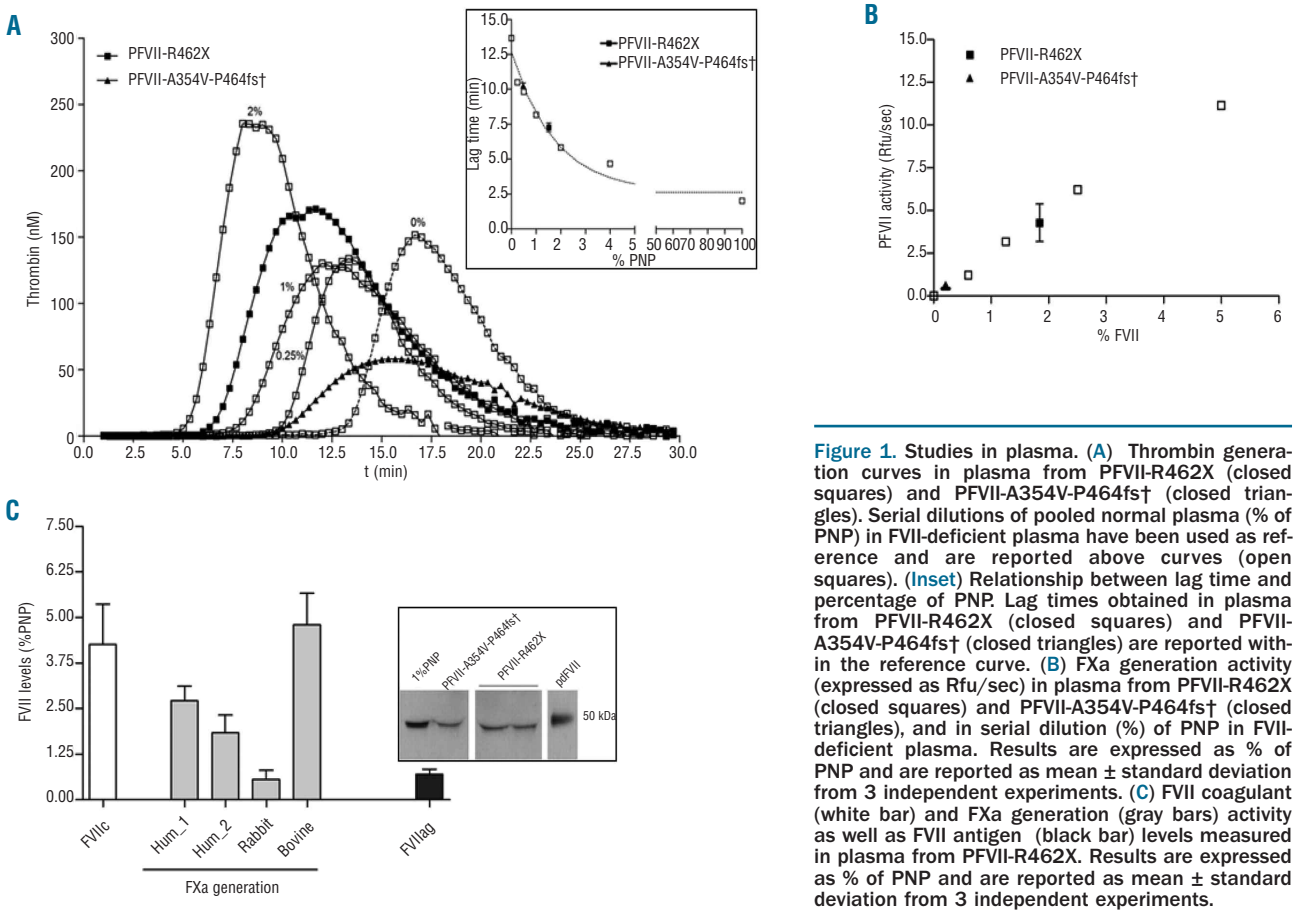

Figure 1. Studies in plasma. (A) Thrombin generation curves in plasma from PFVII-R462X (closed squares) and PFVII-A354V-P464fs† (closed triangles). Serial dilutions of pooled normal plasma (% of PNP) in FVII-deficient plasma have been used as reference and are reported above curves (open squares). (Inset) Relationship between lag time and percentage of PNP. Lag times obtained in plasma from PFVII-R462X (closed squares) and PFVII-A354V-P464fs† (closed triangles) are reported within the reference curve. (B) FXa generation activity (expressed as Rfu/sec) in plasma from PFVII-R462X (closed squares) and PFVII-A354V-P464fs† (closed triangles), and in serial dilution (%) of PNP in FVII-deficient plasma. Results are expressed as % of PNP and are reported as mean ± standard deviation from 3 independent experiments. (C) FVII coagulant (white bar) and FXa generation (gray bars) activity as well as FVII antigen (black bar) levels measured in plasma from PFVII-R462X. Results are expressed as % of PNP and are reported as mean ± standard deviation from 3 independent experiments. Hum_1 and Hum_2 are referred to RecombiPlasTin 2G and Innovin, respectively. (Inset) Western blotting analysis of FVII molecules in plasma from PFVII-R462X (2 independent samples) and PFVII-A354V-P464fs†. Diluted PNP (1%) and plasma-derived FVII (pdFVII, 50 ng/mL) have been used as controls. The Supersignal® West Femto reagent (Thermo Scientific, Rockford, IL, USA) was exploited for detection. The films were exposed overnight.

nism,[4] and/or impaired protein biosynthesis. However, the introduction of a premature nonsense triplet in proximity of the natural termination signal (position 467) in the last exon makes the occurrence of NMD very unlikely, thus supporting the hypothesis of a major impact at the post-translational level. To investigate this, the p.R462X mutation was inserted into the human FVII cDNA (Figure 2A) and expressed in BHK cells. While the FVII antigen levels of rFVII-wt in conditioned medium were 120±18 ng/mL, those of the rFVII-462X were virtually undetectable, thus matching the *in vivo* findings. On the other hand, the rFVII-462X levels in cell lysates (72±4.9%) were roughly comparable to those of rFVII-wt, thus excluding a preferential intracellular accumulation.

Interestingly, when centrifugal devices were used to concentrate the conditioned media, the rFVII-462X protein was detected both by ELISA (2.5±0.2 ng/mL) and Western blotting (Figure 2B, inset) which led us to evaluate its activity. Functional assays were conducted in plasma systems with human TF (Innovin) and by exploiting the FXa fluorogenic substrate to better mimic physiological conditions and to guarantee high sensitivity to very low activity levels.[6,13] Compared to the activity (9.5±1.8 Rfu/sec/nM FVII) of a similar concentration of rFVII-wt, that of the rFVII-462X appeared to be approximately four times higher (36.3±8 Rfu/sec/nM FVII; 382%) (Figure 2B and C). Overlapping results were obtained with RecombPlasTin 2G (440%) and bovine thromboplastin (400%), whereas there was no appreciable activity with rabbit thromboplastin.

Taken together, the similarity between the *in vivo* (Figure 1C) and *in vitro* (Figure 2C, inset) findings supports the hypothesis of an association of the p.R462X mutation with traces of circulating FVII molecules with increased specific activity and gain-of-function features. This helps explain the asymptomatic phenotype observed in homozygous patients. It also throws light on a novel form of FVII deficiency and provides information as to the original features of this highly heterogeneous disease. In contrast, the p.R457X nonsense mutation in factor IX, lying five codons upstream of the natural stop codon as the p.R462X, was found to be associated to severe hemophilia B (FIXc <1% and FVIIag <1%).[19]

The detrimental impact of the p.R462X mutation on secreted FVII levels is explained by the synthesis of truncated molecules lacking five residues at the carboxy-terminal end. This region has been demonstrated to be essential for secretion of other coagulation serine protease family members such as factor IX[20] and protein C (PC),[21] whose carboxy-terminal variants displayed normal specific activity.

Although a recent study[17] on FVII reported a similar impact on protein secretion of sequential deletions in the

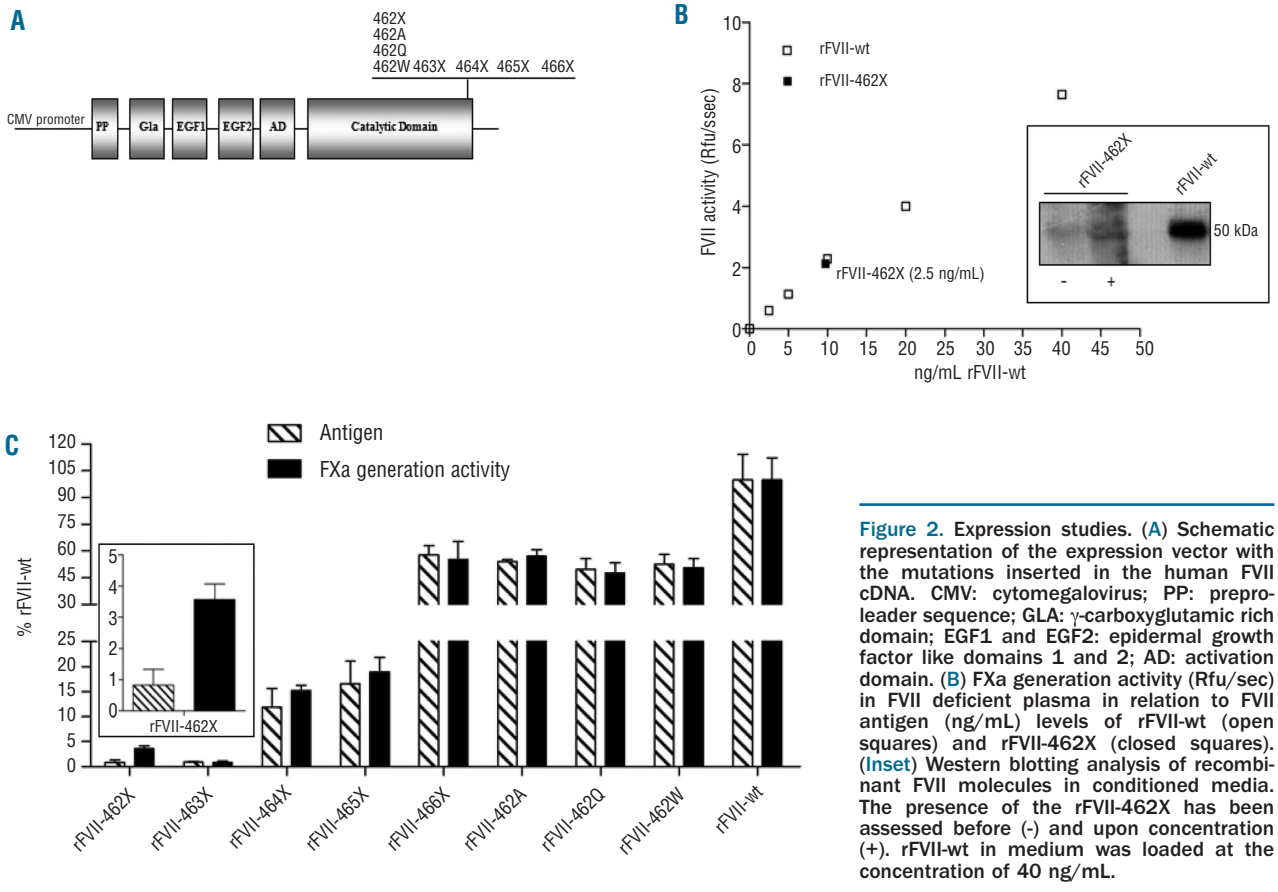

Figure 2. Expression studies. (A) Schematic representation of the expression vector with the mutations inserted in the human FVII cDNA. CMV: cytomegalovirus; PP: prepro-leader sequence; GLA: γ-carboxyglutamic rich domain; EGF1 and EGF2: epidermal growth factor like domains 1 and 2; AD: activation domain. (B) FXa generation activity (Rfu/sec) in FVII deficient plasma in relation to FVII antigen (ng/mL) levels of rFVII-wt (open squares) and rFVII-462X (closed squares). (Inset) Western blotting analysis of recombinant FVII molecules in conditioned media. The presence of the rFVII-462X has been assessed before (-) and upon concentration (+). rFVII-wt in medium was loaded at the concentration of 40 ng/mL. (C) Antigen (striped bars) and FXa generation activity (black bars) levels of the rFVII-wt and of the nonsense (rFVII-462X, rFVII-463X, rFVII-464X, rFVII-465X, rFVII-466X) and missense (rFVII-462A, rFVII-462Q, rFVII-462W) variants in conditioned medium. Results are expressed as % of rFVII-wt and are reported as mean ± standard deviation from 3 independent experiments.

carboxy-terminus up to the 462 position, their functional consequences are unknown. Therefore, we expressed the rFVII-466X, rFVII-465X and rFVII-464X variants (Figure 2A) that resulted in secreted FVII molecules with progressively reduced antigen levels but virtually normal activity either in functional assays with Innovin (Figure 2C) or RecombiPlasTin 2G (*data not shown*). Although expressed at extremely low levels, the rFVII-463X showed normal specific activity.

The increased activity observed in the presence of the p.R462X mutation could be due to the truncated molecule, but also to traces of a full-length FVII variant resulting from ribosome readthrough, a process known to occur at very low (10^{-3}-10^{-4}) rates.[22] Under this process, insertion at the nonsense position of aminoacids other than the natural one could alter biosynthesis, produce dysfunctional FVII variants (as we reported for the p.K376X and p.W424X mutations[7]) or elicit gain-of-function effects, which have so far not been documented after readthrough.

We explored this hypothesis by investigating the effects on full-length FVII biosynthesis and function of the replacement of R462 with glutamine or tryptophan, aminoacids frequently inserted by readthrough, or alanine, a residue extensively exploited to assess functional role of single residues[23] (Figure 2A). Expression experiments showed that the secreted rFVII-462A, rFVII-462Q and rFVII-462W protein (53.9±1.18%, 49.9±5.9% and 52.7±5.4% of rFVII-wt) and activity (57.2±3.6%, 47.7±5.9% and 50.7±5.1%) levels were similarly reduced (Figure 2C), implying normal specific activity. It is worth noting that the nearby p.L460A change in the carboxy-terminal region of FVII did not alter its binding to tissue factor or activity.[23] These observations, albeit not excluding an unpredictable role of other aminoacid changes at the 462 position, do not confirm that missense FVII variants potentially arising from readthrough play a major role, and point toward particular features of the truncated FVII-462X molecule. The differential activity observed with various TF forms suggests an impact on TF-binding properties and allosteric conformational changes leading to the catalytically competent FVIIa form. In contrast to the well characterized FVII Padua variant[24] which exhibits reduced or normal specific activity with human or bovine thromboplastins, the FVII-462X molecule displays gain-of-function features. However, the very low protein levels secreted in medium mean that detailed information about their biochemical characterization cannot be obtained.

These novel and counteracting consequences on protein biosynthesis and function highlight the pleiotropic effects of natural mutations that, in addition to shaping patients' phenotype, could have contributed to the evolution and divergence of coagulation serine proteases, particularly in their highly variable carboxy-terminus.

Authorship and Disclosures

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