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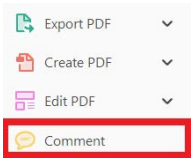

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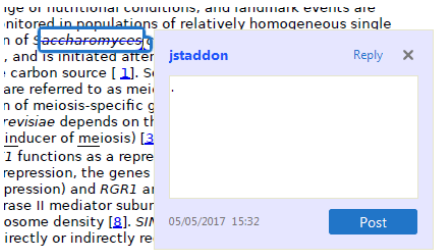

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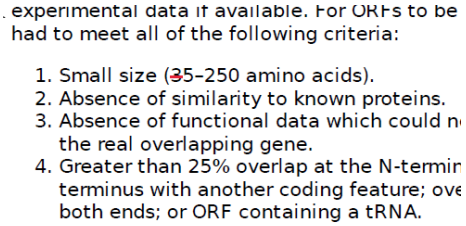

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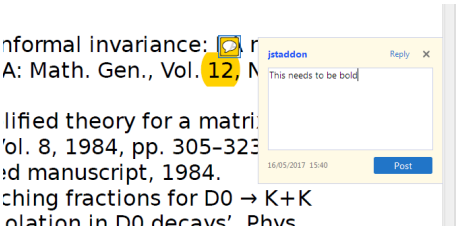

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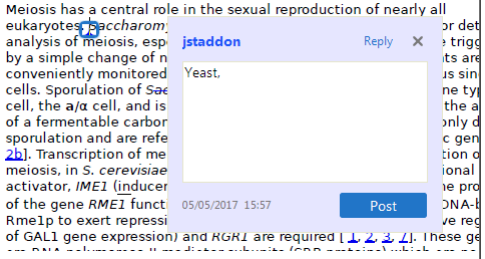

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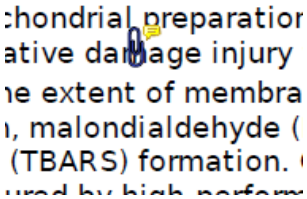

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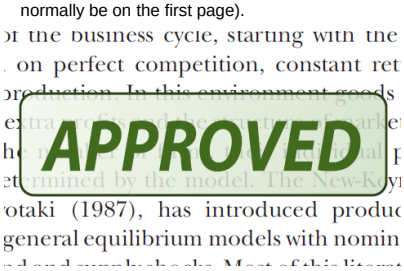

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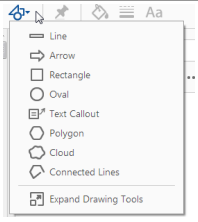

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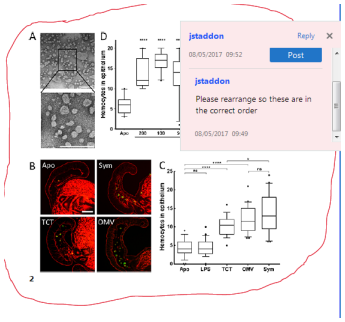

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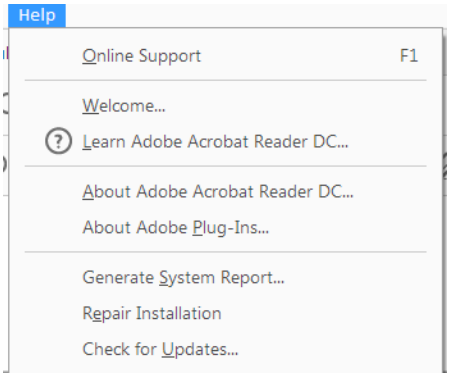

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Haemophilia WILEY

ORIGINAL ARTICLE

Missense changes in the catalytic domain of coagulation factor X account for minimal function preventing a perinatal lethal condition

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Abstract

Introduction: Inherited deficiencies in the coagulation pathway provide diversified models to investigate the molecular bases of perinatal lethality associated with null-like variants. Differently from X-linked haemophilias, homozygous/doubly heterozygous null variants in the rare autosomally inherited deficiency of factor X (FX) might be incompatible with perinatal survival.

Aim: To provide experimental evidence about the null/close-to-null FX function.

Methods: The residual secreted (ELISA) and functional (thrombin generation assays) protein levels associated with the novel nonsense (c.1382G>A; p.Trp461Ter) and missense (c.752T>C; p.Leu251Pro) variants, found in the proposita with life-threatening symptoms at birth, were characterized through recombinant (r)FX expression.

Results: The rFX-461Ter showed very low secretion and undetectable function. Expression and function of the predicted readthrough-deriving missense variants (rFX-461Tyr, rFX-461Gln) were also severely impaired. These unfavourable features, due to nucleotide and protein sequence constraints, precluded functional readthrough over the 461 stop codon. Differently, the poorly secreted rFX-251Pro variant displayed residual function that was characterized by anti-TFPI aptamer-based amplification or selective inhibition of activated FX function by fondaparinux in plasma and found to be reduced by approximately three orders of magnitude. Similarly to the rFX-251Pro, a group of catalytic domain missense variants cause poorly secreted molecules with modest function in FX-deficient patients with life-threatening symptoms.

Conclusions: Our data, contributing to the knowledge of the very severe FX deficiency forms, support life-saving requirement of trace FX function, clearly exemplified by the dysfunctional but not completely inactive rFX-251Pro variant that, albeit with severely reduced function, is compatible with a residual activity ensuring minimal haemostasis and permitting perinatal survival.

KEYWORDS
factor X deficiency, missense variants, nonsense variants, null variants, recombinant proteins

Ferrarese and Baroni are contributed equally.

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1 | INTRODUCTION

Definition of the molecular bases of perinatal/neonatal lethality of human mutations would contribute to counteract the impact of the most clinically severe conditions and favour precocious prophylactic or therapeutic intervention. The residual expression, albeit at very low level, might also influence the immunological tolerance in relation to therapies since null conditions are major candidates in triggering immune complications.[1]

The coagulation pathway represents a suitable model for this investigation because of (a) several well-characterized genes and proteins working in a common cascade-shaped pathway, (b) huge number of variants described in the X-linked members of the pathway (Haemophilia A, MIM# 306700; Haemophilia B, MIM# 306900) (www.factorix.org; http://www.factorviii-db.org/) and (c) ample differences in the effects of null variants affecting specific genes depending on the role of the encoded protein within the cascade, in addition to the type of inheritance (autosomal/X-linked).[2]

In this scenario, the relatively frequent and well-known haemophilia A or B do not permit to investigate natal or perinatal survival/lethality because null variants, frequent in factor VIII (FVIII) and factor IX (FIX) mutational patterns, are associated with defective clot stabilization but are clearly compatible with coagulation initiation.[3] Differently, the complete deficiency of factor VII (FVII; 1:500.000; MIM# 227500)[4] or factor X (FX; 1:1.000.000; MIM# 227600),[5] both rare autosomal recessive conditions, might be virtually incompatible with life, as suggested by *F7* or *F10* knockout mouse models.[6,7]

Human coagulation FX is a vitamin K-dependent serine protease synthesized in the liver and circulating in plasma as a disulphide-linked zymogen composed of a light and a heavy chain. Proteolytic cleavage at the Arg234-Ile235 (chymotrypsin numbering, c15-c16) peptide bond in the amino-terminal region of the heavy chain converts zymogen FX to the active protease (FXa),[8] which in turn exerts a central role as the key thrombin activator. Thus, catalytically incompetent FX molecules resulting from *F10* gene (MIM# 613872; http://www.umd.be/F10/) variants could have a profound impact on the clinical severity of the associated FX deficiency.[9] Clinical observations and the pivotal FX function in coagulation might imply that subtle amounts of FX are required to ensure haemostasis. However, the hypothesis that minimal FX amounts are essential for survival at birth in humans needs experimental validation. Further, findings obtained for this serine protease could represent a paradigmatic model for contributing to the knowledge on the molecular bases of deficiencies affecting the high number of members belonging to the chymotrypsin-like family, whose complex biochemical interactions and functions cover a wide range of biological pathways.[10]

Here, to provide experimental evidence about the null/close-to-null FX function, we deeply investigated at the protein and activity levels the residual FX expression produced by variants detected in a neonate affected by life-threatening intracranial bleeding, which characterizes the severest clinical phenotypes. By detailing the variant-specific contribution to the resulting molecular defect, we found that the FX amino acid change substantially impacts on secretion but partially maintains the hydrophobic character of the affected region, as well as amino acid interactions that are highly conserved in the chymotrypsin-like family members. This is compatible with a residual activity that, albeit reduced by approximately three orders of magnitude, ensures minimal haemostasis.

2 | MATERIALS AND METHODS

2.1 | Nomenclature

Variants found in the patient are reported according to the Human Genome Variation Society (HGVS) nomenclature, with nucleotide numbering of F10 sequence (reference NM_000504.3) starting at the ATG translation initiation codon.[11]

2.2 | Patient

Parents of the young proposita gave informed consent to conduct the study, which was in accordance with the Helsinki Declaration.

2.3 | Creation of expression vectors for recombinant FX variants

Expression vectors for recombinant FX (rFX) nonsense and missense variants were created by site-directed mutagenesis (QuickChange® II XL Site-Directed Mutagenesis Kit, Agilent Technologies) of the human FX cDNA cloned in the pCMV4 expression vector.[11]

The forward oligonucleotides $^{5'}$GTCCCTGGCAGGCCCCGCT CATCAATGAGG$^{3'}$ (pFX-251Pro), $^{5'}$CGCCTTCCTCAAGTAGATCGACA GGTCCATG$^{3'}$ (pFX-461Ter), $^{5'}$CCGCCTTCC TCAAGTACATCGACAG GTCCATG$^{3'}$ (pFX-461Tyr) and $^{5'}$CCGCCTTCCTCAAGCAGATC GACA GGTCCATG$^{3'}$ (pFX-462Gln) were used. The modified nucleotides (underlined) and triplets (italics) are indicated. Reverse oligonucleotides were complementary to the forward ones.

2.4 | Expression of rFX variants

Expression studies were carried out by transient transfection of human embryonic kidney (HEK) 293 cells by using the Lipofectamine 2000 reagent (Life Technologies) in serum-free medium (Opti-MEM, Gibco, Life Technologies) supplemented with 5 μg/mL vitamin K (Konakion, 10 mg/mL).[12] Transfected cells were treated with 100 μg/mL G4128 (SIGMA-Aldrich) as optimized previously.[13] Media and cell lysates, prepared in RIPA buffer (Thermo Fisher Scientific), were collected 24 hours post-transfection.

2.5 | Evaluation of rFX protein levels

Protein levels of rFX variants in media or cell lysates were evaluated by ELISA with polyclonal anti-human FX antibodies (Cedarlane), with known concentrations of plasma-derived FX (Haematologic

Technologies Inc.) as the reference curve, as described.[12] Cell lysates were normalized for total protein content.

2.6 | Functional assays in plasma system with fluorogenic substrate and FX inhibitors

Functional assays evaluating the thrombin generation activity of rFX variants were adapted from those previously described.[14] FX-deficient plasma (George King Bio-Medical.) was supplemented with rFX-containing medium, and activity was monitored over time at 37°C on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific) after addition of the extrinsic activator Innovin (Siemens Healthcare) and a thrombin-specific fluorogenic substrate (250 μmol/L, Thrombin Substrate III, EMD Biosciences Inc; 360 nm excitation, 455 nm emission). Inhibition of FX function was obtained by addition of an anti-tissue factor pathway inhibitor (TFPI) aptamer (1 μM) [15] or Fondaparinux (400 nM).[16] Wild-type rFX (rFX-wt) or medium from cells transfected with the empty vector (mock) were used as controls.

2.7 | Data analysis

Data were analysed by the statistic software GraphPad Prism 5. Bell-shaped curves and the specific parameter lag time were obtained by extrapolating the first derivative of relative fluorescence units (RFU) as a function of time (seconds).[17] Statistical differences were evaluated by the *t* test.

3 | RESULTS

3.1 | Identification of FX variants and expression studies with recombinant proteins

The proposita presented at birth with major bleeding symptoms and evidence of cerebral micro-bleeds. Sequencing of all *F10* exons, exon/intron boundaries, 3′UTR and 5′UTR regions revealed compound heterozygosity for the novel nucleotide changes c.752T>C (p.Leu251Pro; inherited from the father) in exon 7 and c.1382G>A (p.Trp461Ter; inherited from the mother) in exon 8 (nomenclature according to numbering starting at codon +1). Routine laboratory assays on plasma from the proposita revealed residual FX coagulant activity below 1% of normal, and thus a severe coagulation phenotype, which requires continuous substitutive prophylaxis with therapeutic FX-containing products.[18] FX parameters in parents suggested their heterozygous condition, confirmed by genotyping (data not shown). Since standard laboratory assays are not able to distinguish values below 1%, and thus do not permit a reliable evaluation of very low FX levels, and plasma from the patient contains therapeutic FX molecules with confounding effects on the assay, we characterized the proposita FX variants at the expression and functional level in eukaryotic cellular models.

We mimicked the p.Leu251Pro and p.Trp461Ter variants by introducing the corresponding nucleotide changes (Figure 1A) into the FX cDNA and performing expression studies in transiently transfected HEK293 cells. Secreted protein levels of the rFX-461Ter and rFX-251Pro variants were 1.3 ± 0.1% and 0.6 ± 0.2% of rFX-wt, respectively (Figure 2). FX antigen levels in cell lysates for the rFX-461Ter (42 ± 4.3%) and rFX-251Pro (50 ± 5.2%) were lower than those of cells expressing rFX-wt, thus suggesting potentially unstable proteins with very low secretion profiles.

Although these protein levels prevent the purification and biochemical characterization of variants, the availability of very sensitive assays offered us the possibility to assess very low FX activity and thus to distinguish between null and non-null function.

3.2 | Impact of translational readthrough on the rFX-461Ter variant

Nonsense variants usually result in truncated proteins devoid of residual function. However, at very low frequency (10^{-4}), traces of full-length proteins might stem from spontaneous suppression of the premature stop codon (PTC) mediated by translational readthrough,[19] which is driven by PTC misrecognition and incorporation of a restricted panel of amino acids.[20] Importantly, translational readthrough, by producing full-length proteins with wild-type features or potentially harbouring missense changes,[21,22] may differentially contribute to the patient phenotype as we observed for rare homozygous nonsense variants in FVII deficiency.[13]

Trp461 is conserved among the homologous factors X, VII, IX and protein C (Figure 1A) as well as in chymotrypsin.[23] Spontaneous suppression of the p.Trp461Ter (TAG) nonsense variant, which might explain the trace levels of secreted FX, predicts the insertion of glutamine or tyrosine,[20] which led us to express the rFX-461Tyr (TAC codon) and rFX-461Gln (CAG codon) missense variants (Figure 1A). Substitution of the original tryptophan by tyrosine or glutamine resulted in low (3.6 ± 1.2% of rFX-wt) or very low (1.0 ± 0.3%) secreted protein levels, respectively (Figure 2). To investigate the functional properties of these poorly secreted molecules, we performed very sensitive fluorogenic functional assays evaluating the generation of the central player thrombin in FX-deficient plasma systems supplemented with rFX-containing medium.

We detected a very low thrombin generation capacity (Figure 3A), much lower than expected by the measured secreted protein levels. This activity was slightly higher than that measured with medium collected from HEK293 cells transfected with the empty vector (mock), which represents the baseline activity provided by the commercial FX-deficient plasma instrumental to this experimental system (Figure 3A). These data indicate that the poorly secreted rFX-461Tyr and rFX-461Gln missense variants, potentially deriving from translational readthrough of the p.Trp461Ter, are highly dysfunctional and characterized by an extremely reduced activity.

Context-dependent effects, namely the type of stop codon and the downstream nucleotide,[24] dictate the quantitative protein output arising from translational readthrough. In particular, the TAG-A context resulting from the c.1382G>A change (p.Trp461Ter; Figure 1A) is predicted to have a low degree of spontaneous suppression (0.9%)[24]

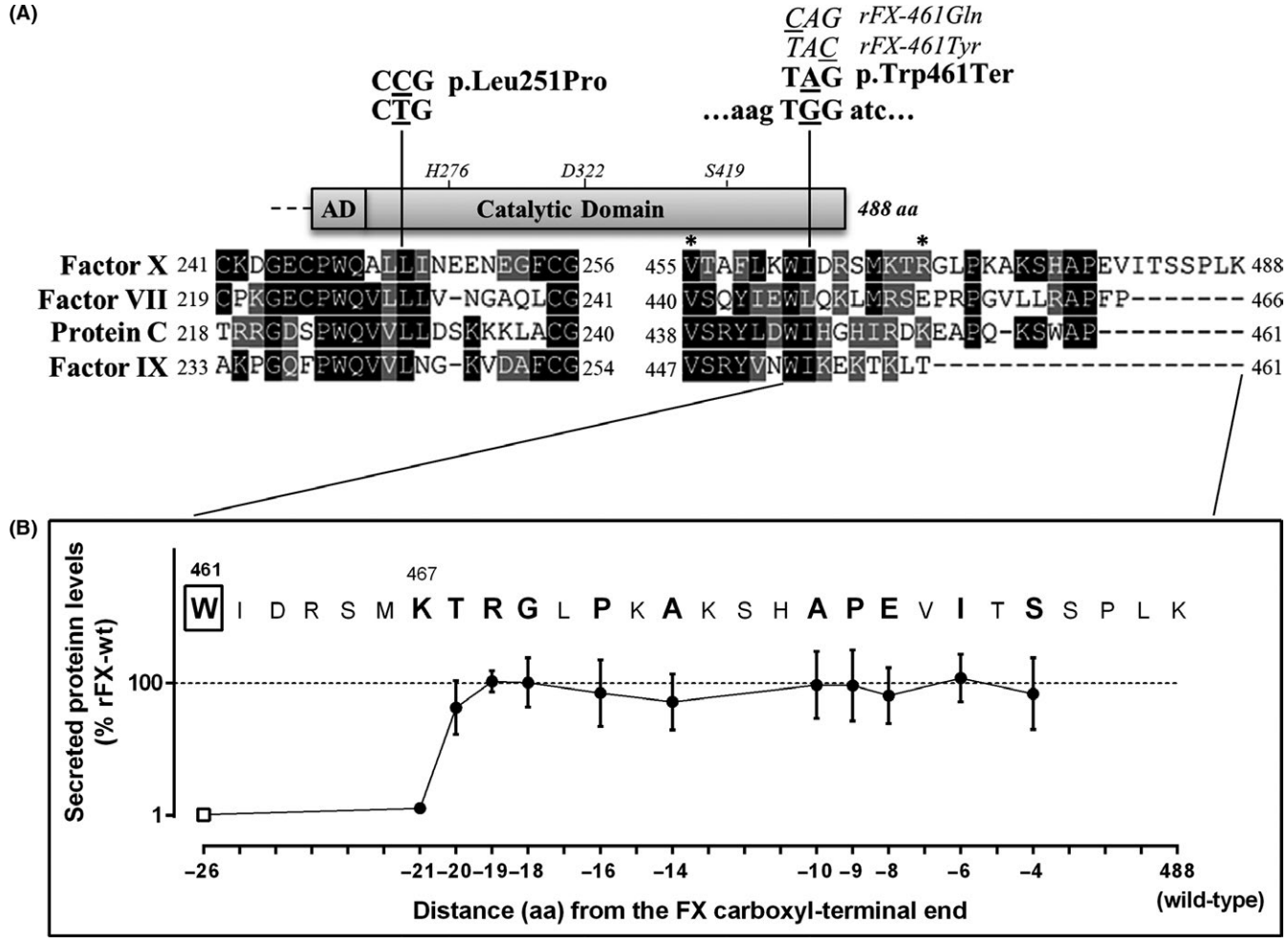

FIGURE 1 Investigated recombinant variants. (A) FX variants found in the proposita (c.752T>C, p.Leu251Pro; c.1382G>A, p.Trp461Ter) are shown on top of wild-type FX sequence (NM_000504.3). The missense variants predicted to arise from translational readthrough (rFX-461Gln, TAC codon; rFX-461Tyr, CAG codon) are indicated (italic) on top of the TAG stop codon. Underlined letters indicate nucleotide changes introduced to obtain the corresponding protein variants. Alignment of the highly homologous factor X (NP_000495.1), factor VII (NP_000122.1), protein C (NP_000303.1) and factor IX (NP_000124.1) is shown below the partial FX schematic representation, also showing the catalytic triad residues. Asterisks delimitate the region involved in interactions with prothrombin. Numbers on left and right of aligned sequences indicate the first and the final aligned residues (B) Secreted protein levels of rFX variants with progressive deletion of the carboxyl-terminal region. Truncated variants were efficiently secreted after removal of up to 21 residues. The primary sequence (single-letter code) of the carboxyl-terminal tract of FX is indicated on top, with residues at the new carboxyl-terminus highlighted in bold. The investigated W461 residue is boxed. Secreted protein levels are shown as % of those of rFX-wt (dotted line, set to 100%) as a function of the distance from the last residue. Data on the rFX-461Ter variant (white square) indicate a role of this residue for both secretion and function (see main text)

that can be magnified by drug induction.[13,21,22] Based on these premises, we challenged the rFX-461Ter by treatment of transfected cells with the potent readthrough inducer G418. This resulted in secreted protein levels (1.2 ± 0.2% of rFX-wt) overlapping with those of untreated rFX-461Ter-expressing cells (Figure 2). Importantly, thrombin generation capacity with medium from rFX-461Ter-expressing cells, either untreated or treated with G418, was undistinguishable from that of medium over untransfected cells (Figure 3B).

Overall, these data indicate that translational readthrough is hampered by constraints both at mRNA (unfavourable sequence context) and protein (detrimental amino acid substitutions) levels impairing expression of even minimal full-length and functional FX from the mother-inherited p.Trp461Ter nonsense variant. These findings prompted us to detail the impact of the father-inherited p.Leu251Pro amino acid substitution.

3.3 | Functional contribution of the rFX-251Pro variant to trace functional levels

Missense variants, which are a major cause of coagulation factor disorders, may exert pleiotropic effects at different levels, including splicing, folding/intracellular trafficking or secretion/function.[25,26]

The rFX-251Pro variant, secreted at very low levels in medium (Figure 2), revealed a low thrombin generation capacity that was clearly distinguishable from that of the FX-deficient plasma baseline (Figure 3C), thus suggesting a residual function.

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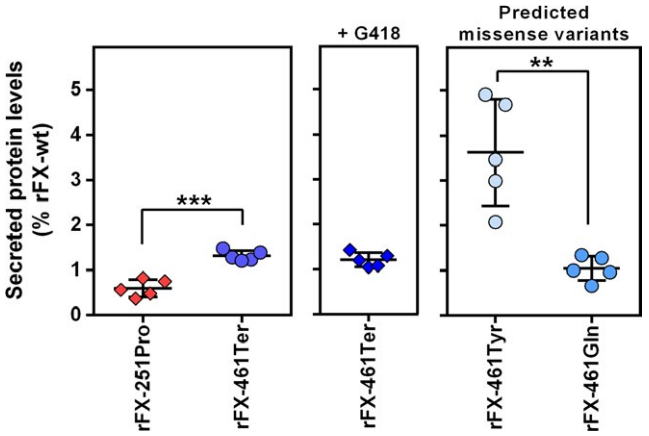

6 FIGURE 2 Expression studies with recombinant FX variants. Secreted protein levels, measured by polyclonal ELISA, of rFX variants. Left panel, natural variants. Central panel, induction (+G418) of translational readthrough of the TAG stop codon. Right panel, predicted missense variants resulting from translational readthrough of the TAG stop codon. Results are reported as % of rFX-wt (mean ± standard deviation from five independent experiments). **, $P = 0.007$; ***, $P = 0.0002$

Demonstration of the ability of the rFX-251Pro to sustain thrombin generation would be of utmost importance to address the specific contribution of this FX variant to the bleeding phenotype of the proposita. To this purpose, we optimized and carried out complementary assays by selective amplification (i) or inhibition (ii) of FX function.

(i) The FX function was amplified by inhibition of TFPI, the major inhibitor in the tissue factor-initiated coagulation, which counteracts both activated FX (FXa) generation and FXa activity. By using an anti-TFPI aptamer,[15] thrombin generation triggered by the rFX-251Pro was potentiated. This was indicated by a clearly shortened (~2 minutes) lag time (Figure 3D), which represents a key time parameter dependent from the FX concentration [13] and defines the efficiency of thrombin generation. The observed amplification of thrombin generation supports the contribution of the rFX-251Pro variant.
(ii) We took advantage of fondaparinux,[16] which acts by accelerating the action of antithrombin that leads to the specific and dose-dependent inhibition of FXa (Figure 3D, inset). The lag time of thrombin generation by the rFX-251Pro was prolonged by fondaparinux (Figure 3D), which strongly supported the FX-dependent contribution of the variant.

Overall, these reciprocal results point towards a residual function of the p.Leu251Pro variant associated with the generation of minute amounts of thrombin sustaining minimal haemostasis.

4 | DISCUSSION

Here, we report functional insights into variants associated with coagulation and clinical phenotypes defining life-threatening FX deficiency. Through complementary approaches, we dissected the variant-specific contribution underlying the severe molecular defect in a virtually unique example of a close-to-null condition in FX deficiency, shaped by the nonsense (c.1382G>A; p.Trp461Ter) and missense (c.752T>C; p.Leu251Pro) variants with a differential impact on the observed life-threatening phenotype.

Our experimental elements neither support haemostatic impact of truncated FX molecules nor of translational readthrough and point towards null features of the p.Trp461Ter. Accordingly, previous observations [12] support that only truncation of the FX carboxyl-terminal region downstream of Lys467 is compatible with FX secretion (Figure 1B). Moreover, the Trp461 residue resides in the amino acid stretch (455-469) involved in interactions with prothrombin (Figure 1A),[28] which further supports that protein truncation at this position suppresses FX activity.

Multiple experimental approaches point towards the p.Leu251Pro-specific contribution to the residual FX function. While the combination of impaired biosynthesis/secretion with dysfunctional features of the rFX-251Pro led to a reduction of activity by approximately three orders of magnitude, a residual function was detected, which would sustain minimal haemostasis. In spite of the life-threatening symptoms requiring continuous prophylaxis, the trace FX function would prevent a lethal phenotype, thus permitting perinatal survival. In addition, we are also aware that the possibility of maternal transfer [29] of small amounts of functional FX could have further contributed to embryonic survival.

These findings prompted us to compare structural and functional insights of FX variants associated with intracranial bleeding (Table S1). The analysis reveals that the majority of variants are missense changes, with a few exceptions.[30,31] Interestingly, amino acid substitutions can be grouped in the glutamic acid-rich (GLA) (i) and catalytic (ii) domains.

(i) Well-detectable FX protein levels (4%-19% of normal) were found in plasma of patients affected by the GLA domain p.Glu69Lys and p.Phe71Ser missense changes, thus suggesting that they are tolerated in terms of biosynthesis/secretion. The p.Glu54Gly and p.Glu69Lys variants directly involve two (out of 11) γ-carboxylated glutamic acid residues.[8] However, substitution of one of these residues does not support by itself a severe defect, as we observed for the poorly symptomatic p.Glu59Ala.[33] Pregnancy-associated deficiency of vitamin K, the essential enzymatic cofactor,[34] could substantially decrease carboxylation of the altered GLA domain, thus suppressing FX membrane binding and biological properties.[8] These variants offer a paradigmatic example of combination of inherited deficiency of the enzyme and acquired deficiency of the cofactor producing potentially lethal perinatal conditions.[35]
(ii) With one exception (p.Ser419Asn, catalytic serine c195), all missense changes found in the catalytic domain (Figure S1) produce a very severe deficiency through mechanisms well exemplified by the p.Leu251Pro.

Leu251 (c33) is located in a conserved hydrophobic region, in a classic β bulge structure, opposite to Phe259 (c41) and Cys260 (c42), which

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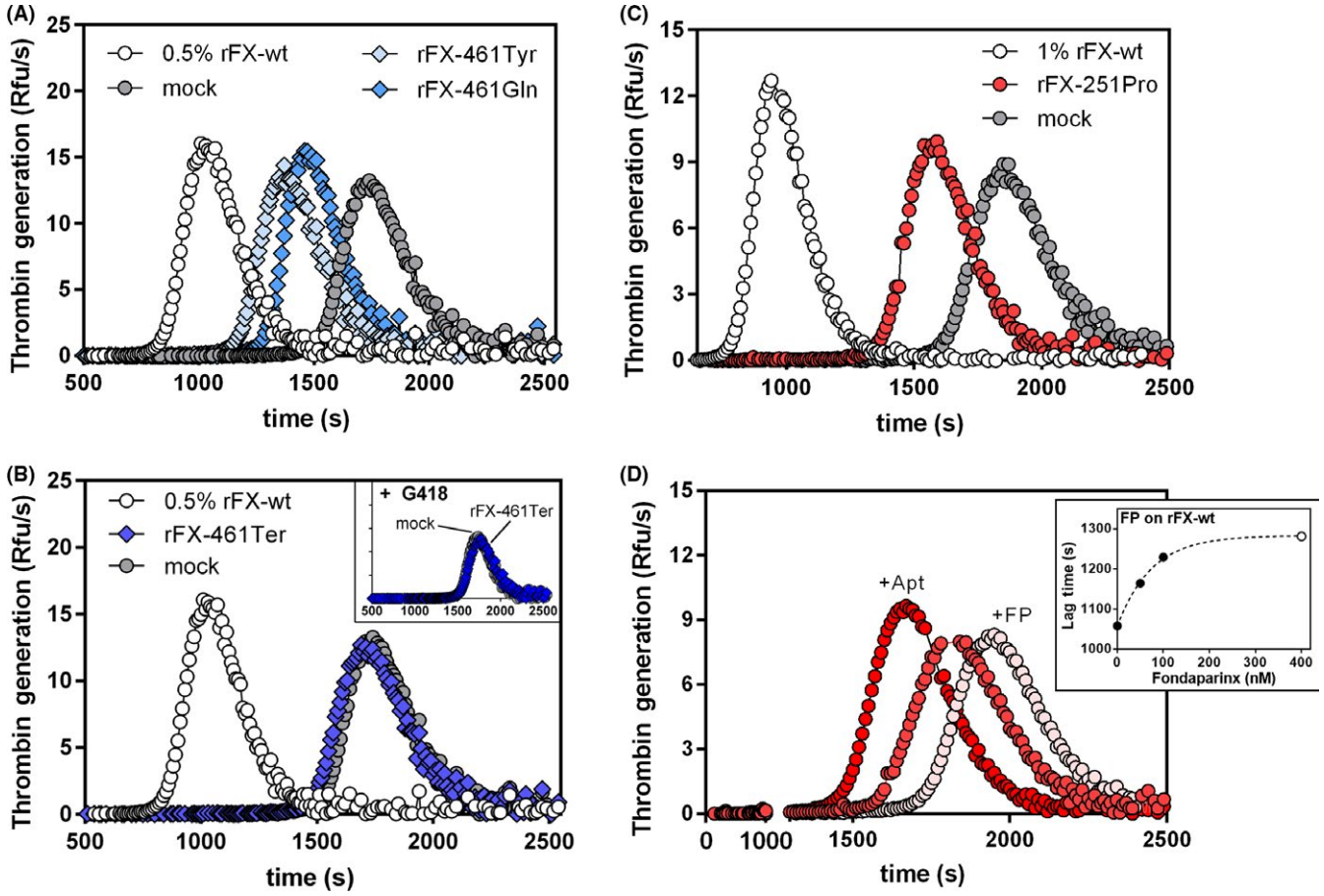

FIGURE 3 Functional characterization of recombinant FX variants. Thrombin generation assays in commercially available FX-deficient plasma on (A) missense variants arising from translational readthrough over the TAG stop codon, (B) rFX-461Ter variant before (main panel) and after (inset) G418 treatment of transfected cells, (C) activity of the rFX-251Pro missense variant, and D) rFX-251Pro variant alone or in the presence of 1 μM anti-TFPI aptamer (+Apt) or 400 nM Fondaparinux (+FP). Dilutions of rFX-wt mimicking very low FX levels (0.5%, 1%) or medium from HEK293 cells transfected with the empty vector (mock), representing the baseline activity provided by the commercial FX-deficient plasma, were used as controls. These panels are representative of three independent experiments. Bell-shaped curves of thrombin generation were obtained by extrapolating the first derivative of fluorescence emission (RFU, Relative Fluorescence Units) as a function of time (seconds)

are highly conserved within chymotrypsin-like family members.[23] Interestingly, β bulges would accommodate structural changes without totally disrupting the β sheet.[36] Further, the side chain of Leu251 contributes to the hydrophobic character of a pocket positioned directly in front of the S1 pocket, of special interest as coagulation protease substrates and inhibitors commonly bind this region.[37] Substitution by proline is expected to alter protein native structure, potentially interfering but not suppressing protein folding, secretion and activity by preserving the hydrophobic nature of the highly conserved region.

The p.Gly244Arg, p.Gly420Arg and p.Cys404Arg (Table S1), similarly to the p.Leu251Pro but differently from the GLA domain mutants, lead to very low circulating FX levels. In particular, Gly244 (c25), located in the small Cys241-Cys246 disulphide-bridged loop, results in an altered trafficking towards secretion.[25] Gly420 (c196) is fully conserved in FX, FIX, FVII and protein C, even among different species,[38] as well as in the serine protease family.[23] It participates to the c43-c196 contact with a key role for the correct structure and folding of the FXa catalytic domain. Last, the p.Cys404Arg variant disrupts the highly conserved intra-chain Cys390-Cys404 (c168-c182) disulfide bridge in the FX heavy chain.[39] It remains to be investigated how these profound structural changes would alter but not suppress protein folding, secretion and activity, and thus be compatible, as the rFX-251Pro, with a dysfunctional but not completely inactive FX requiring substitutive therapy immediately after birth.

5 | CONCLUSIONS

The essential role for perinatal survival of a minimal FX function, supported by our experimental approaches, is in line with the mutational pattern of FX deficiency that, even limited, does not report *F10* homozygous nonsense changes or extended deletions. The life-saving requirement for trace levels of FX clearly differentiates the mutational pattern of FX deficiency from those of the well-characterized haemophilia A and B, and parallels that of the rare deficiency of FVII, essential for triggering the whole cascade as FX is for the mandatory generation of thrombin.

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M.F. created all recombinant plasmids and performed expression studies; M.B. optimized and performed thrombin generation assays; P.D.V., I.S. and A.P. extracted DNA from patient blood and found FX variants; A.D.A. visited the patient and revised the manuscript. A.B. and F.B. conceived the study and designed research; A.B., F.B. and M.P analysed and interpreted data and wrote the manuscript. All authors revised and approved the final version of the manuscript.

CONFLICT OF INTEREST

The authors have no interests which might be perceived as posing a conflict or bias.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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