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ORIGINAL ARTICLE

In vitro and *ex vivo* rescue of a nonsense mutation responsible for severe coagulation factor V deficiency

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Abstract

Background: Coagulation factor V (FV) deficiency is a rare bleeding disorder that is usually managed with fresh-frozen plasma. Patients with nonsense mutations may respond to treatment with readthrough agents.

Objectives: To investigate whether the *F5* p.Arg1161Ter mutation, causing severe FV deficiency in several patients, would be amenable to readthrough therapy.

Methods: *F5* mRNA and protein expression were evaluated in a *F5* p.Arg1161Ter-homozygous patient. Five readthrough agents with different mechanisms of action, i.e. G418, ELX-02, PTC-124, 2,6-diaminopurine (2,6-DAP), and Amlexanox, were tested in *in vitro* and *ex vivo* models of the mutation.

Results: The *F5* p.Arg1161Ter-homozygous patient showed residual *F5* mRNA and functional platelet FV, indicating detectable levels of natural readthrough. COS-1 cells transfected with the FV-Arg1161Ter cDNA expressed 0.7% FV activity compared to wild-type. Treatment with 0-500 μM G418, ELX-02, and 2,6-DAP dose-dependently increased FV activity up to 7.0-fold, 3.1-fold, and 10.8-fold, respectively, whereas PTC-124 and Amlexanox (alone or in combination) were ineffective. These findings were confirmed by thrombin generation assays in FV-depleted plasma reconstituted with conditioned media of treated cells. All compounds except ELX-02 showed some degree of cytotoxicity. *Ex vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient, which were negative at FV immunostaining, turned positive after treatment with all 5 readthrough agents. Notably, they were also able to internalize mutant FV rescued with G418 or 2,6-DAP, which would be required to maintain the crucial platelet FV pool *in vivo*.

Conclusion: These findings provide *in vitro* and *ex vivo* proof-of-principle for readthrough-mediated rescue of the *F5* p.Arg1161Ter mutation.

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KEYWORDS
bleeding, factor V deficiency, megakaryocytes, nonsense-mediated decay, nonsense mutation, translational readthrough

1 | INTRODUCTION

Coagulation factor V (FV) [1], encoded by the *F5* gene, is a liver-derived multidomain glycoprotein (A1-A2-B-A3-C1-C2) present both in plasma (80%) and in platelet α-granules (20%). Its activated form (FVa) accelerates factor Xa–catalyzed conversion of prothrombin to thrombin >1000-fold [2], making FV indispensable to life [3].

Congenital FV deficiency is a rare autosomal recessive bleeding disorder of variable severity [4], ranging from mucosal and posttraumatic bleeding to life-threatening intracranial hemorrhages [5]. The main determinant of bleeding tendency is the level of residual platelet FV, traces of which are usually sufficient to guarantee minimal hemostasis [6]. In fact, platelet FV, which originates from endocytosis and intracellular processing of plasma FV by bone marrow megakaryocytes [7], has enhanced procoagulant properties [8]. Moreover, circulating tissue factor pathway inhibitor (TFPIα), a direct inhibitor of FV activation [9] and early prothrombinase complexes [10], is constitutively low in FV deficiency [11] because FV and particularly its minor splicing isoform FV-short [12] are required to maintain TFPIα in the circulation [11]. Since no FV concentrate is clinically available [13], the treatment and prophylaxis of FV-deficient patients still rely on fresh-frozen plasma [14]. However, alternative therapeutic approaches are being explored [15,16], including molecular strategies targeting specific genetic defects [17–19].

Nonsense mutations, which introduce a premature termination codon (PTC), represent ~13% (26/199) of the FV deficiency mutational spectrum [20]. These mutations are usually considered null defects because PTCs trigger messenger RNA (mRNA) degradation by the nonsense-mediated mRNA decay (NMD) pathway [21] and/or result in truncated non-functional proteins. However, a fraction of the PTC-containing mRNA actually escapes NMD as the PTC is mistakenly translated into an amino acid, allowing the synthesis of tiny amounts of full-length protein ("translational readthrough") [22,23]. The efficiency of this rescue mechanism depends on the position, identity, and sequence context of the PTC and can be pharmacologically enhanced by small molecules known as readthrough agents [23,24]. The latter include aminoglycoside antibiotics, such as G418 (Geneticin) [25] and its synthetic analog ELX-02 [26,27], which alter the ability of the eukaryotic ribosome to decode stop codons; PTC-124 (Ataluren), which inhibits release factor–dependent termination of protein synthesis [28,29]; 2,6-diaminopurine (2,6-DAP), which makes it possible for the tryptophan transfer RNA to recognize the UGA stop codon [30,31]; and Amlexanox (AMX), which not only promotes PTC readthrough, but also inhibits the degradation of PTC-bearing mRNA by NMD [32]. The fact that PTCs are ~10-fold more susceptible to translational readthrough than natural stop codons provides a therapeutic window for the use of these molecules as a potential treatment for genetic diseases caused by nonsense mutations [33]. Although PTC suppression efficiency is typically low, coagulation factor deficiencies represent ideal targets for this therapeutic approach because even minimal increases in factor expression can significantly improve the bleeding phenotype [34–37].

In this study, we have explored the feasibility of readthrough-based therapy for FV deficiency caused by a *F5* nonsense mutation (p.Arg1161Ter) identified in several unrelated patients [38–42] (Table). To this end, we have determined residual *F5* mRNA and protein expression in a patient homozygous for *F5* p.Arg1161Ter (to assess *in vivo* NMD and natural readthrough of the mutant *F5* mRNA) and tested the effects of 5 different readthrough agents in *in vitro* and *ex vivo* models of this mutation.

2 | MATERIALS AND METHODS

2.1 | Patient characterization

2.1.1 | Blood collection and workup

The study was approved by the local institutional review board (code nr. 242/2020) and conducted according to the Helsinki Declaration. Following written informed consent, venous blood was collected in 0.109 M sodium citrate and in Tempus Blood RNA tubes (Thermo Fisher Scientific) from the FV-deficient patient, his parents and a normal control. Citrated blood was centrifuged at 1200 rpm for 15 minutes (to obtain platelet-rich plasma [PRP]) and again at 5000 rpm for 15 minutes (to obtain platelet-poor plasma [PPP]). The platelet count in PRP was adjusted to 240 000 platelets/μL using autologous PPP. A second sample of citrated blood, collected 8 years later from the same patient and a normal control, was used to prepare *ex vivo* differentiated megakaryocytes (see below), as well as PRP and PPP. As a negative control for the *ex vivo* experiments, megakaryocytes were also prepared from a previously characterized FV-deficient patient (PD-III, 0.6% FV) who is compound heterozygous for 2 missense mutations (*F5* p.Trp255Arg and p.Tyr1623Asp) [6,11,43].

2.1.2 | Thrombin generation

Thrombin generation in PRP and PPP was measured using the calibrated automated thrombogram method, as described previously [6].

2.1.3 | FV levels

FV activity in plasma and activated washed platelets was quantified using a prothrombinase-based assay, as described previously [6]. FV

TABLE Overview of *F5* c.3481C>T (p.Arg1161Ter) mutation carriers.

Patient code in reference	Age	Sex	Geographical origin/ethnicity	Zygosity for c.3481C>T	Other *F5* mutation(s)	FV:C (%)	Bleeding symptoms	Reference
Proband A	16 y	Female	Southern Italy	Homozygous	FV Leiden (in *cis*)	<1	Bleeding after tooth extraction, post-traumatic hematoma, menorrhagia	van Wijk et al. 2001 [38]
Proband B[a]	7 y	Male	Southern Italy	Homozygous	FV Leiden (in *cis*)	<1	Asymptomatic	van Wijk et al. 2001 [38]
Case 4	36 y	Male	China	Homozygous	-	2	Right knee hemarthrosis since the age of 3 y Occasional gum or nose bleeding, joint bleeding	Cao et al. 2008 [39]
Patient 19	Not reported	Female	Germany	Homozygous	-	1	Hematoma	Delev et al. 2009 [40]
Patient 20	Not reported	Female	Germany	Homozygous	-	1	Hematoma	Delevet al. 2009 [40]
P8	Unknown	Unknown	Iran	Homozygous	-	1	Not available	Paraboschi et al. 2020 [42] Prof R. Asselta, personal communication
Patient[b]	25 y	Male	Korea	Compound heterozygous	c.6027_6032delGAACAG	4	Bleeding after tooth extraction	Song et al. 2009 [41]
P2	Unknown	Unknown	Iran	Compound heterozygous	c.3924_3927delTCAG	<1	Not available	Paraboschi et al. 2020 [42] Prof R. Asselta, personal communication
P43	Unknown	Male	Italy	Heterozygous	-	50	Asymptomatic	Paraboschi et al. 2020 [42] Prof R. Asselta, personal communication
-	Unknown	Male	African/African American	Heterozygous	-	Unknown	Unknown	gnomAD, August 2023
-	Unknown	Male	European (non-Finnish)	Heterozygous	-	Unknown	Unknown	gnomAD, August 2023

FV, factor V.

[a] This patient and the patient described in the present report are the same person (Prof P. M. Mannucci, personal communication).

[b] Proband of a family with inherited factor V deficiency. The *F5* c.3481C>T (p.Arg1161Ter) mutation was also present in his monozygotic twin and in 1 sister [41].

antigen levels were determined with an in-house enzyme-linked immunosorbent assay (ELISA) [18].

2.1.4 | TFPIα levels

Plasma TFPIα levels were determined using an in-house ELISA [44].

2.1.5 | Genetic analysis

Genomic DNA was isolated from buffy coats using the QIAamp DNA Blood Mini kit (QIAGEN). All exons and splicing junctions of the patient's *F5* gene were amplified and Sanger-sequenced, essentially as previously described [45].

2.1.6 | *F5* mRNA analysis

Whole blood RNA was isolated using the Tempus Spin RNA Isolation kit (Thermo Fisher Scientific) and quantified with a NanoDrop 2000 (Thermo Fisher Scientific). Total RNA was reverse-transcribed with a *F5*-specific primer using the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). The *F5* region surrounding the nonsense mutation was amplified using a forward primer located in exon 13 and a reverse primer located in exon 14 (to prevent co-amplification of genomic DNA), followed by a nested polymerase chain reaction (PCR) within exon 13 and direct sequencing. Sequencing peaks were quantified using QSVanalyzer [46]. In a parallel experiment, total RNA was reverse-transcribed with random primers, and cDNA fragments corresponding to *F5* exons 5-6 and 18-20 were quantified by real-time quantitative PCR (RT-qPCR) on a Roche LightCycler 480 using *GAPDH* as the housekeeping gene. All primer sequences are available on request.

2.2 | *In vitro* model

2.2.1 | FV expression constructs

The pMT2/V construct, containing the wild-type *F5* cDNA, was used as a template for site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis kit; Agilent Technologies) to introduce the FV-Arg1161Ter mutation, alone and in combination with FV-Arg534Gln (FV Leiden) [47].

2.2.2 | Cell transfection and treatment

COS-1 cells were maintained at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) with low glucose (Biowest), supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin. For each experiment, cells were seeded in 6-well plates at ~70% confluency and transiently transfected with 2 μg *F5* cDNA using Lipofectamine 2000 (Invitrogen) in Opti-MEM (Gibco). Transfected cells were treated with 0-500 μM G418 (Sigma-Aldrich), ELX-02 (MedChemExpress), PTC-124 (Cayman Chemicals), 2,6-DAP (Sigma-Aldrich), or AMX (Sigma-Aldrich) in DMEM. After 48 hours, conditioned media were harvested for FV activity measurements.

2.2.3 | FV measurements in cell media

Conditioned media diluted 1:40 (for wild-type FV) or 1:5 (for mutant FV) were assayed for FV activity in a prothrombinase-based assay, as described previously [11]. FV activity was also evaluated by measuring thrombin generation in 80 μL FV-depleted plasma (Siemens Healthcare) reconstituted with 25 μL conditioned medium. Thrombin generation was determined by the Calibrated Automated Thrombogram (CAT) method after initiation of coagulation with 20 pM tissue factor (TF) and 30 μM phospholipid vesicles (dioleoyl-phosphatidylserine/dioleoyl-phosphatidylcholine/dioleoyl-phosphatidylethanolamine in a molar ratio of 20/60/20) in the presence of 40 μg/mL thermostable inhibitor of contact activation.

2.2.4 | Cell viability assay

COS-1, Huh-7, and HepG2 cells seeded in 96-well plates were treated with 0-500 μM G418, ELX-02, PTC-124, 2,6-DAP or AMX for 48 hours. Cell viability was determined using the CyQUANT XTT Cell Viability Assay (Invitrogen) according to the manufacturer's instructions.

2.3 | *Ex vivo* model

2.3.1 | Preparation of *ex vivo* differentiated megakaryocytes

Hematopoietic progenitor cells were enriched from citrated blood of the *F5* p.Arg1161Ter-homozygous patient and controls and differentiated to megakaryocytes as described before [17,43] with minor modifications. Briefly, peripheral blood mononuclear cells isolated by Histopaque-1077 (Sigma-Aldrich) density gradient centrifugation were resuspended in serum-free StemSpan-ACF medium (StemCell Technologies) supplemented with 2 mM L-glutamine, 1% insulin-transferrin-selenium (Invitrogen), 10 ng/mL Stem Cell Factor, 50 ng/mL thrombopoietin, 10 ng/mL interleukin-3, and 20 ng/mL interleukin-6 (all from PeproTech). Cells were seeded in 24-well plates containing glass coverslips coated with gelatine (Sigma-Aldrich) and maintained in an incubator at 37°C and 5% CO_2 for 4 days before switching to serum-free Iscove's Modified Dulbecco's Medium (Euroclone) supplemented with 2 mM L-glutamine, 1% insulin-transferrin-selenium, 50 ng/mL thrombopoietin, and 10 ng/mL interleukin-3. This model, which has been extensively characterized earlier [43], produces megakaryocyte-like cells that can both express and internalize FV.

2.3.2 | Treatment with readthrough agents

Starting from day 12, readthrough agents were added to the culture medium at every medium change (twice in 7 days) before retrieving the glass coverslips for immunofluorescence analysis. Based on titrations of each readthrough agent in control megakaryocyte cultures, where toxicity was evaluated by visual inspection of the cells under the microscope, the patient's megakaryocytes were treated with 100-500 μM G418, 500-1000 μM ELX-02, 100-250 μM PTC-124, 50-250 μM 2,6-DAP, 50-100 μM AMX or 0.7% dimethyl sulfoxide (DMSO, vehicle of PTC-124, 2,6-DAP and AMX) as a negative control.

2.3.3 | FV uptake experiments

To test whether FV produced by readthrough of the p.Arg1161Ter mutation could be internalized by the patient's megakaryocytes, 0.33 nM wild-type FV (positive control) or mutant FV rescued with G418 or 2,6-DAP was added to the culture medium at day 12 and left for 4 days before immunofluorescence analysis. Conditioned media of COS-1 cells expressing the different FV variants were used as a source of FV after concentration with Amicon Ultra-4 Centrifugal Filter Units (Millipore) with a 50-kDa cutoff.

2.3.4 | Immunostaining and fluorescence microscopy

Glass coverslips were processed essentially as previously described [17,43]. Briefly, cells were fixed with 2% paraformaldehyde and permeabilized with 0.5% Triton X-100. FV was visualized with a mouse monoclonal antibody against the human FV light chain (AHV-5108, Haematologic Technologies) and a fluorescein isothiocyanate (FITC)–labeled goat anti-mouse immunoglobulin G (Sigma-Aldrich) secondary antibody. Cell nuclei were stained with Draq5 (Abcam). Slides were mounted with Mowiol antifade solution (Sigma-Aldrich) and observed under a Leica DMI6000CS fluorescence microscope (Leica Microsystems CMS) using a 20×/0.40 dry objective or a 63×/1.40 oil immersion objective. Images were acquired with a DFC365FX camera and analyzed using the Leica Application Suite (LAS-AF) 3.1.1 software (Leica Microsystems).

3 | RESULTS

3.1 | Patient characterization

The patient is a 29-year-old Italian man with undetectable plasma FV (FV:Ag <1%, FV:C <1%) and a moderate bleeding tendency. He first came to clinical attention at the age of 3 years for a traumatic hemorrhage of the lip, but according to his mother, he has been suffering from easy bruising since early infancy. His bleeding history includes recurrent knee hemarthroses from the age of 9 years, an episode of rectal bleeding at the age of 20 years, and a right thigh muscle hematoma following a sport trauma at the age of 21 years. He is treated on demand with fresh-frozen plasma or with tranexamic acid for minor bleeding episodes. Both blood samples used in this study were collected at least 2 months after the last plasma transfusion. As we found out during the preparation of this manuscript, this patient has been reported before as a child [38] (Prof. P. M. Mannucci, personal communication).

Both parents have partial FV deficiency (Figure 1A). The patient's father developed deep vein thrombosis and massive pulmonary embolism at the age of 32 years after a long car drive, followed by multiple episodes of superficial thrombophlebitis despite oral anticoagulation in the therapeutic range. Thrombophilia screening revealed marked activated protein C (APC) resistance in the ProC Global assay (normalized APC sensitivity ratio 0.42, normal range ≥0.80) as well as heterozygosity for the *F2* 20210G>A mutation [48]. After taking vitamin K antagonists for 18 years, he is currently on lifelong treatment with apixaban (2.5 mg twice daily). The patient's mother is asymptomatic.

Upon *F5* gene sequencing, the patient proved homozygous for the c.3481C>T mutation in exon 13 [38–42], which introduces a premature stop codon (TGA) in the B domain (p.Arg1161Ter) (Figure 1B). Moreover, he was homozygous for the FV Leiden mutation [47], which, however, was not expressed due to linkage with the nonsense mutation on both alleles. Accordingly, the patient's parents were heterozygous for both mutations and shared the same *F5* haplotype, suggesting identity by descent. In addition, the patient's father carried the Met2148Thr variant (rs9332701) on the other *F5* allele. This variant has been associated with mildly decreased FV levels [49] and may explain the father's lower FV levels compared to those of the mother.

Whole blood RNA analysis by RT-qPCR indicated that the expression of *F5* mRNA was markedly reduced in the patient compared to a normal control (relative expression 0.21 and 0.38 for *F5* exons 5-6 and 18-20, respectively), whereas both parents had intermediate levels (Figure 1C). This suggested that the PTC-containing mRNA may be subject to NMD degradation, which was confirmed by direct sequencing of the region surrounding the nonsense mutation in the heterozygous parents (Figure 1B, bottom). However, the mutant *F5* mRNA was only partially degraded, and the homozygous patient showed ~30% residual *F5* mRNA (Figure 1B, C).

To assess the patient's overall hemostatic capacity, thrombin generation was measured in PPP and PRP. Thrombin generation in PPP (Figure 2A) was triggered with 1-50 pM TF and 30 μM phospholipids. Control PPP showed progressively shorter lag times and higher peaks at increasing TF concentrations, whereas the patient's PPP did not generate thrombin at any TF concentration, in line with his undetectable plasma FV. Thrombin generation in the mother's PPP (50% FV) was similar to that of control PPP, whereas thrombin generation in the father's PPP (38% FV) was somewhat delayed and decreased, reflecting his ongoing anticoagulant treatment with (at that time) vitamin K antagonists.

Thrombin generation in PRP, initiated with 1-10 pM TF and 20 μg/mL of collagen, showed similar trends (Figure 2B). However,

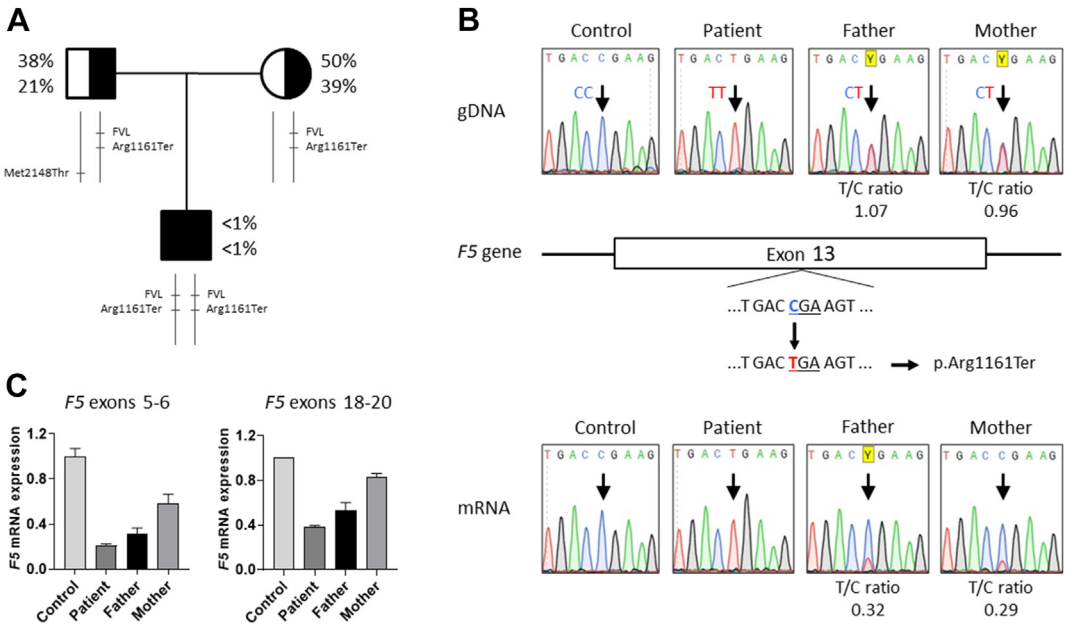

FIGURE 1 Genetic analysis of the patient and his parents. (A) Pedigree of the affected family. (Half)-filled symbols indicate (partial) FV deficiency. FV activity and antigen levels are indicated next to each family member. The bars below each family member represent the 2 *F5* alleles with the respective genetic variants (FVL, factor V Leiden). (B) Sequencing chromatograms of the portion of *F5* exon 13 containing the c.3481C>T mutation in the genomic DNA (gDNA) (top) and mRNA/cDNA (bottom) of the patient, his parents, and a normal control. In both parents, that are heterozygous for the *F5* c.3481C>T mutation, the ratio between the peaks corresponding to the mutant (T) and wild-type (C) alleles is close to 1 at the gDNA level but markedly reduced at the mRNA level, indicating that ~70% of the mutant *F5* mRNA is degraded by nonsense-mediated decay. The *F5* c.3481C>T mutation introduces a TGA premature stop codon (p.Arg1161Ter). (C) Quantification of *F5* mRNA by RT-qPCR of *F5* exons 5-6 and 18-20 from whole blood RNA of the patient and his parents relative to a normal control.

thrombin generation in the patient's PRP was measurable, albeit markedly delayed, and decreased compared to that of control PRP (lag time, 6 minutes vs 2 minutes; peak height 19 nM vs 104 nM at 10 pM TF). This indicated the presence of residual FV in the patient's platelets, which was confirmed by thrombin generation measurements in an independent plasma sample collected 8 years later (Supplementary Figure S1) as well as by direct measurement of the patient's platelet FV level (3.5% of the normal control).

Plasma TFPIα levels were decreased in the patient (37% and 40% in the 2 plasma samples) and, to a lesser extent, in his parents (father 74%, mother 51%). Since the *F5* Arg1161Ter mutation is eliminated from the FV-short mRNA by alternative splicing and should therefore not affect the expression of FV-short, the patient's low TFPIα level suggests that not only FV-short but also full-length FV may contribute to stabilizing TFPIα in the circulation, in line with earlier TFPIα immunoprecipitation experiments where both FV isoforms were retrieved in the immunoprecipitate [12].

3.2 | Effects of readthrough agents in an *in vitro* model of *F5* p.Arg1161Ter

COS-1 cells transfected with the FV-Arg1161Ter cDNA were treated with increasing concentrations (0-500 μM) of 5 different readthrough agents, and FV activity in conditioned media was determined using a prothrombinase-based assay. No FV activity was detectable in the media of non-transfected cells, whereas the mutant construct expressed 0.73% ± 0.05% FV activity of the wild-type construct (n = 5), mimicking the patient's FV deficiency. Treatment with G418, ELX-02 and 2,6-DAP resulted in a dose-dependent increase in secreted FV activity, whereas PTC-124 and AMX were ineffective (Figure 3A). Compared to untreated cells, G418 increased FV activity up to 7.0 ± 0.9 times, ELX-02 up to 3.1 ± 0.6 times and 2,6-DAP up to 10.8 ± 2.4 times at the highest treatment concentration. These effects could be attributed to the translational readthrough of the mutant *F5* mRNA because treatment of non-transfected COS-1 cells with 500 μM of each readthrough compound did not result in any detectable prothrombinase activity in the conditioned media, excluding that these molecules stimulate the secretion of FV from the cells or express FVa-like activity themselves.

To check whether the FV produced by translational readthrough of the mutant *F5* mRNA would also function in a more physiological plasma setting, thrombin generation was measured in FV-depleted plasma reconstituted with the conditioned media of untreated and treated cells (Figure 3B). Untreated FV-Arg1161Ter media supported only minimal thrombin generation (peak height 3.1 ± 0.4 nM; n = 5) compared to FV wild-type media (peak height 277 nM). However, following treatment with increasing concentrations of G418, ELX-02 or 2,6-DAP, FV-Arg1161Ter media supported progressively higher thrombin generation, with maximal peak heights of 22.2 nM (G418),

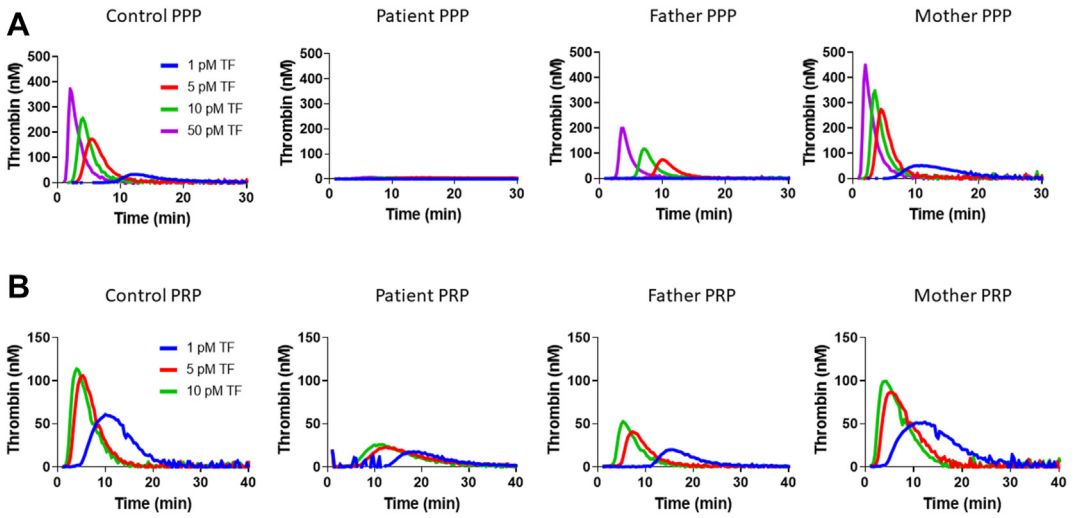

FIGURE 2 Plasma phenotyping of the patient and his parents. Thrombin generation was measured in platelet-poor plasma (PPP) (A) and platelet-rich plasma (PRP) (B) of the *F5* p.Arg1161Ter-homozygous patient, his parents, and a normal control. Thrombin generation in PPP was initiated with 1-50 pM tissue factor (TF) and 30 μM phospholipid vesicles; thrombin generation in PRP was initiated with 1-10 pM TF and 20 μg/mL collagen.

8.2 nM (ELX-02) and 36.2 nM (2,6-DAP). In contrast, no increase in thrombin generation was observed with media of cells treated with PTC-124 or AMX.

Since AMX mainly targets NMD, whereas G418 and PTC-124 have primarily readthrough activity, cells transfected with FV-Arg1161Ter were also treated with combinations of AMX and G418 or PTC-124. However, no synergistic effects were observed between these compounds (Supplementary Figure S2).

To verify that the FV Leiden mutation does not interfere with PTC readthrough at codon 1161, we compared FV expression by COS-1 cells transfected with FV-Arg1161Ter (single mutant) or with FV-Arg1161Ter in combination with FV Leiden (double mutant) before

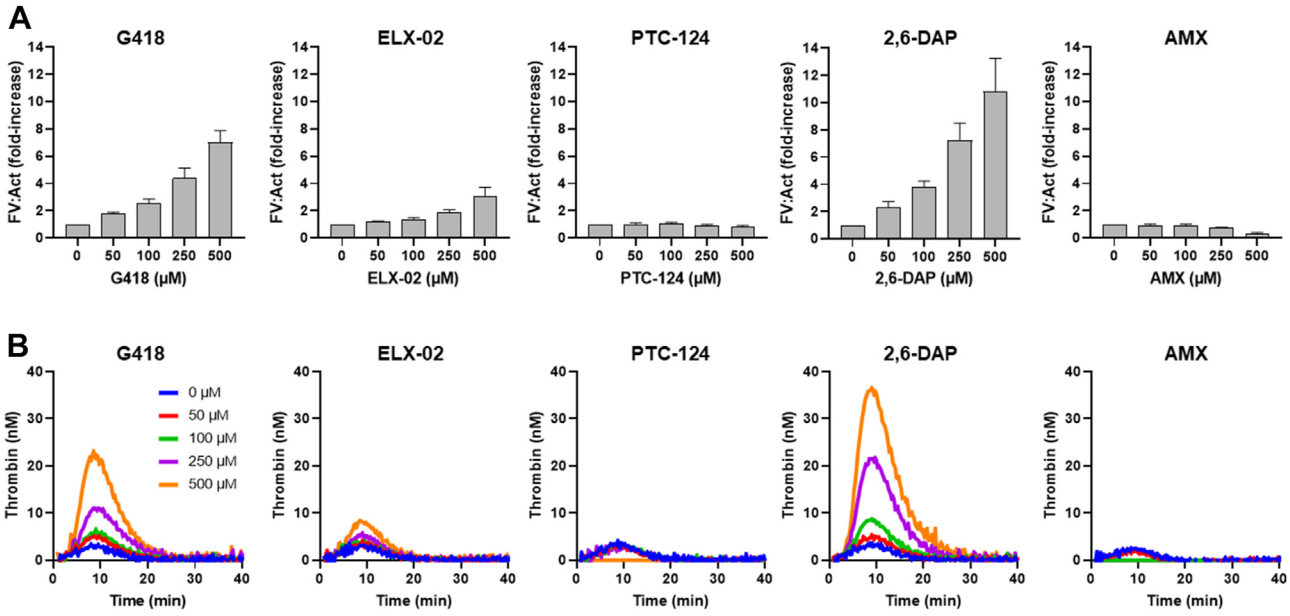

FIGURE 3 *In vitro* rescue of the *F5* p.Arg1161Ter mutation. COS-1 cells were transiently transfected with FV-Arg1161Ter cDNA and treated with 0-500 μM G418, ELX-02, PTC-124, 2,6-DAP or AMX for 48 hours. (A) FV activity in conditioned media was measured with a prothrombinase-based assay, normalized to the basal FV level in the media of untreated cells, and plotted as a function of treatment concentration. Each bar represents the mean and standard deviation of 3 biological replicates. (B) Thrombin generation in FV-depleted plasma reconstituted with conditioned media of untreated and treated cells. Thrombin generation was initiated with 20 pM tissue factor and 30 μM phospholipid vesicles.

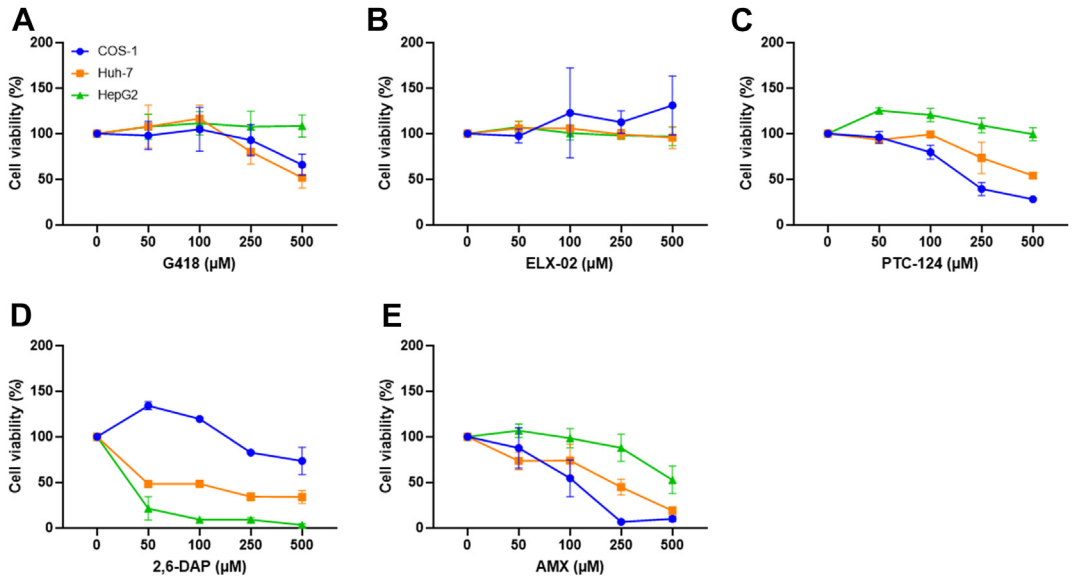

FIGURE 4 *In vitro* cytotoxicity of the readthrough compounds. COS-1, Huh-7, and HepG2 cells were treated with 0-500 μM G418, ELX-02, PTC-124, 2,6-DAP or AMX for 48 hours, and cell viability was assessed with an XTT-based assay. Results are expressed as percentage of the basal cell viability in the absence of treatment. Each bar represents the mean and standard deviation of 3 biological replicates (2 for ELX-02).

and after treatment with G418, 2,6-DAP or ELX-02. The single and double mutants expressed comparable FV activity in the media of untreated cells and achieved similar levels of correction after treatment (Supplementary Figure S3).

The effects of increasing concentrations (0-500 μM) of each readthrough agent on cell viability were evaluated in COS-1 cells (used for the *in vitro* model) as well as in the liver cell lines Huh-7 and HepG2 (hepatocytes being the natural site of FV biosynthesis). While ELX-02 was extremely well tolerated up to the highest concentration, all other compounds showed variable degrees of cytotoxicity in the different cell types (Figure 4).

3.3 | Effects of readthrough agents in an *ex vivo* model of *F5* p.Arg1161Ter

The rescuing efficacy of the different readthrough agents was also tested in *ex vivo* differentiated megakaryocytes [17,43] of the *F5* p.Arg1161Ter-homozygous patient. To this end, hematopoietic progenitor cells were cultured in the presence of thrombopoietin and interleukin-3 for 11 days to promote megakaryocyte differentiation, treated with readthrough agents (or not) for the next 7 days and analyzed by immunofluorescence. Treatment concentrations were adjusted according to preliminary titrations of each readthrough agent in control megakaryocyte cultures to determine the maximum concentration that could be tolerated without excessive toxicity. FV was visualized using a monoclonal antibody directed against the light chain (ie, the C-terminal portion) of FV to ensure exclusive detection of the full-length readthrough product. Based on this immunofluorescence staining, megakaryocytes from a normal control showed abundant intracellular expression of FV (Figure 5A), whereas no FV could be detected in the untreated patient's megakaryocytes (Figure 5B). All 5 readthrough agents restored FV expression in the patient's megakaryocytes (Figure 5C–G, Supplementary Figure S4A–G), whereas 0.7% DMSO (vehicle) was completely ineffective (Supplementary Figure S4B). As a control, the same treatments were performed on megakaryocytes of a different FV-deficient patient (PD-III) [6,11,43] who is compound heterozygous for missense mutations and should therefore not respond to readthrough agents. As expected, no rescue of FV expression was observed in this case (Supplementary Figure S5), confirming the specificity of the readthrough agents for nonsense mutations and excluding that they induce aspecific green fluorescence in the treated cells.

When untreated patient's megakaryocytes were cultured in the presence of FV variants produced in COS-1 cells, they were able to internalize not only wild-type FV but also FV obtained by rescuing the *F5* p.Arg1161Ter mutation with G418 (which mostly incorporates the natural amino acid Arg at the PTC site) [50] or 2,6-DAP (which incorporates Trp [30]) (Figure 6, Supplementary Figure S6). The same was true for megakaryocytes of patient PD-III (Supplementary Figure S7). This indicates that the positive FV immunostaining truly reflects FV internalization rather than endogenous FV expression induced by traces of readthrough agents present in the concentrated media that were used as a source of G418-rescued or 2,6-DAP–rescued mutant FV.

4 | DISCUSSION

In contrast to all other coagulation factor deficiencies, for which concentrates and/or recombinant preparations of the missing factor

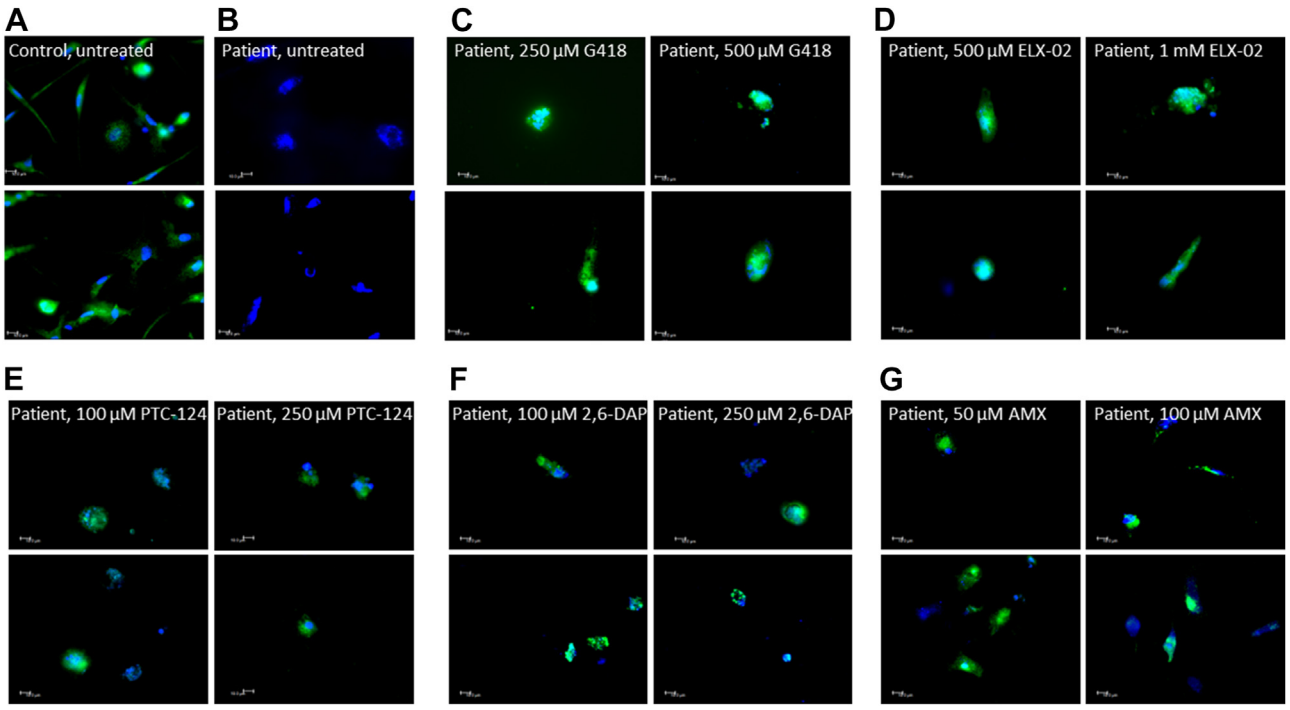

FIGURE 5 Effect of readthrough agents on FV expression in *ex vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient. Hematopoietic progenitor cells isolated from peripheral blood of a normal control and the *F5* p.Arg1161Ter-homozygous patient were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were treated with readthrough compounds for 7 days and then processed for immunofluorescence analysis. Cell nuclei were stained with Draq5 (blue). FV was visualized using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), which recognizes only full-length FV molecules, followed by a FITC–labeled goat anti-mouse immunoglobulin G antibody (green). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 63×/1.40 oil immersion objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analyzed using the LAS-AF 3.1.1 software. The different panels show 2 representative microscope fields for each of the following conditions: (A) untreated control megakaryocytes, (B) untreated patient's megakaryocytes, and patient's megakaryocytes treated with the indicated concentrations of (C) G418, (D) ELX-02, (E) PTC-124, (F) 2,6-DAP, and (G) AMX. Scale bar: 10 µm.

are available, FV-deficient patients are still dependent on fresh-frozen plasma for their treatment and prophylaxis. Although effective, (repeated) plasma administration is burdensome and not without risks [14], calling for alternative therapeutic strategies for FV deficiency [15–17,19]. In this study, we show that the *F5* p.Arg1161Ter nonsense mutation is amenable to pharmacological readthrough in *in vitro* and *ex vivo* models. To our knowledge, this is the first study on readthrough therapy of FV deficiency.

While most *F5* mutations are private, the *F5* p.Arg1161Ter mutation has been reported in at least 9 unrelated FV-deficient patients of Caucasian or Asian descent [38–42] and in 2 subjects of the gnomAD database (https://gnomad.broadinstitute.org, accessed in August 2023), 1 of whom was of African ancestry (Table). Its incidence and widespread geographical distribution suggest that this C>T transition at a CpG dinucleotide has occurred independently more than once.

The outcome of a nonsense mutation depends on the extent of mRNA degradation by NMD and on the susceptibility of the PTC to translational readthrough. As observed earlier [38] and confirmed here, the *F5* p.Arg1161Ter mRNA is only partially degraded by NMD, possibly because the PTC is located in the middle of an extremely large exon (2821 bp), which decreases NMD efficiency [51]. Moreover, the stop codon introduced by the p.Arg1161Ter mutation (UGA) is the most permissive to translational readthrough, even if followed by a less favorable A [24,52]. Accordingly, the absence of life-threatening bleeding manifestations in all *F5* p.Arg1161Ter homozygotes described so far (Table) indicates that this nonsense mutation allows some full-length FV expression by low-frequency natural readthrough of the PTC. While quickly cleared from plasma, these FV traces accumulate in the platelet α-granules (as demonstrated by the measurable platelet FV and thrombin generation in our patient's PRP) and can support minimal hemostasis [6,53], especially in combination with low plasma TFPIα levels [11].

These favorable characteristics predicted that the *F5* p.Arg1161Ter mutation could respond to treatment with molecules that enhance translational readthrough and/or suppress NMD. To verify this, we screened 5 readthrough agents with different mechanisms of action in COS-1 cells transfected with FV-Arg1161Ter cDNA (*in vitro* model) and in *ex vivo* differentiated megakaryocytes of a *F5* p.Arg1161Ter-homozygous patient (*ex vivo* model).

The *in vitro* model allowed quantitative assessment of the efficacy and cytotoxicity of the different readthrough agents, revealing 3 active compounds. G418 increased secreted FV activity up to 7 times, in line

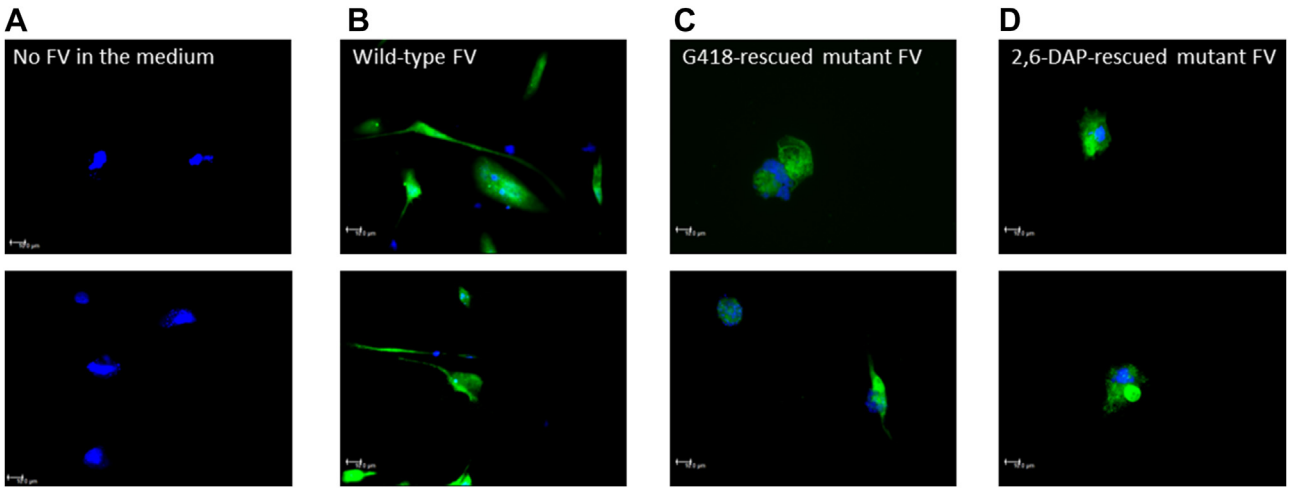

FIGURE 6 Uptake of rescued mutant FV by *ex vivo* differentiated megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient. Hematopoietic progenitor cells isolated from peripheral blood of the *F5* p.Arg1161Ter-homozygous patient were cultured in the presence of thrombopoietin and interleukin-3 to induce megakaryocyte differentiation. Starting from day 12, cells were either (A) left unexposed or exposed for 4 days to (B) 0.33-nM wild-type FV, (C) 0.33 nM mutant FV rescued with G418, or (D) 0.33 nM mutant FV rescued with 2,6-DAP in the culture medium before being processed for immunofluorescence analysis. Cell nuclei were stained with Draq5 (blue). Internalized FV was visualized using a mouse monoclonal antibody against the light chain of human FV (AHV-5108), followed by a FITC–labeled goat anti-mouse immunoglobulin G antibody (green). Glass slides were observed under a Leica DMI6000CS fluorescence microscope using a 63×/1.40 oil immersion objective. Images (overlay of blue and green fluorescence channels) were acquired with a DFC365FX camera and analyzed using the LAS-AF 3.1.1 software. Two representative microscope fields are shown for each condition. Scale bar: 10 μm.

with similar studies on other coagulation factor deficiencies [34–36]. Unfortunately, the clinical use of this compound is limited by its ototoxicity and nephrotoxicity [54,55]. The synthetic aminoglycoside analog ELX-02, which is currently in phase 2 clinical trials for other genetic diseases [26,27], was somewhat less effective, increasing FV activity up to ~3 times. Interestingly, ELX-02 did not show any *in vitro* cytotoxicity up to a concentration of 500 μM, which is in line with its promising *in vivo* safety profile [26]. The most potent compound was 2,6-DAP, which increased FV activity up to ~11 times but also showed considerable toxicity in 2 liver cell lines. However, no toxicity was observed in other studies, including *in vivo* mouse models [30,31].

The FV produced by readthrough of the p.Arg1161Ter mutation was fully functional not only in a prothrombinase assay with purified components but also in a plasma-based thrombin generation assay. This indicates that the replacement of Arg1161 by a different amino acid as a result of the readthrough process is structurally and functionally well tolerated, consistent with the B domain being largely unstructured and removed upon FV activation. This may also explain the remarkable density of nonsense mutations in the B domain of FV compared to other domains [4] because natural readthrough of B-domain PTCs would be more likely to result in a viable protein, as recently proposed for the B domain of factor VIII [56]. Accordingly, other nonsense mutations in the FV B domain might also respond to pharmacological induction of translational readthrough, potentially extending the relevance of our study to a larger group of FV-deficient patients. However, each mutation should be evaluated individually [37], as readthrough success critically depends on the type of stop codon and its sequence context [24].

PTC-124 and AMX (alone or in combination) did not rescue the p.Arg1161Ter mutation *in vitro* and even decreased FV activity at the highest treatment concentrations, probably due to progressive cell death. In the case of AMX, which is primarily an NMD inhibitor [32], the observed toxicity may reflect the importance of NMD as a physiological protection mechanism against truncated and potentially harmful proteins [51]. In general, the toxicity of readthrough agents may derive from imperfect discrimination between PTCs and natural stop codons. Accordingly, major research efforts are ongoing to identify new active compounds with reduced toxicity [57–60].

Although megakaryocytes are not the natural source of FV *in vivo* [7,8], they can synthesize FV in culture [43,61,62], providing a model to test the readthrough agents directly on patients' cells *ex vivo*. Interestingly, all 5 readthrough agents effectively restored FV expression in cultured megakaryocytes of our *F5* p.Arg1161Ter-homozygous patient, as qualitatively assessed by immunofluorescence staining. Despite the absence of a clear dose-response, the specificity of the observed effects is supported by 1) the use of a monoclonal antibody against the FV light chain (recognizing only the full-length readthrough product), 2) the ability of the readthrough agents to restore FV expression in megakaryocytes of the *F5* p.Arg1161Ter-homozygous patient but not in those of a patient with missense mutations; and 3) the lack of effect of 0.7% DMSO (vehicle). Unfortunately, due to the low number of cells and technical difficulties, we were unable to quantify FV expression in treated megakaryocyte lysates by ELISA and/or functional assays. The discrepancy between the *in vitro* model, where PTC-124 and AMX did not rescue mutant FV expression, and the *ex vivo* model, where all readthrough

agents were effective, might be explained, at least in part, by the absence of introns in the *F5* cDNA used to transfect COS-1 cells. In fact, NMD (the main target of AMX [32]) and premature termination of translation (targeted by PTC-124 [29]) are interdependent [52] and tightly coupled to pre-mRNA splicing [21], which is bypassed in the absence of introns. In this respect, the *ex vivo* model is much more physiological than the *in vitro* model and could be further improved by using induced pluripotent stem cell–derived patient's hepatocytes, which are the natural site of FV biosynthesis and would, therefore, optimally mimic the transcriptional and posttranscriptional regulation of *F5* gene expression as it occurs *in vivo*. The use of physiologically relevant test models has been recently emphasized as a key factor in bridging the translational gap of readthrough agents [63].

For readthrough-based therapy of FV deficiency to be successful *in vivo*, the little FV produced by PTC readthrough in hepatocytes and secreted in plasma should be internalized by bone marrow megakaryocytes to build and maintain the protective platelet FV pool [6,53]. The ability of *ex vivo* differentiated megakaryocytes to endocytose exogenous FV [43] allowed us to verify that mutant FV rescued with G418 and 2,6-DAP can be efficiently internalized, even at the very low concentration of 0.33 nM, corresponding to a plasma FV level of ~1.5%. This is consistent with FV uptake by megakaryocytes being mediated by the light chain without involvement of the B domain [64].

The FV Leiden mutation [47], present in our patient but absent in most *F5* p.Arg1161Ter carriers, did not interfere with readthrough-mediated rescue of the p.Arg1161Ter mutation *in vitro*. *In vivo*, the procoagulant properties of FV Leiden would help the patient to achieve adequate hemostasis at very low FV, but expression of slightly higher levels of FV Leiden in the setting of low TFPIα levels (37-40%) could expose him to a risk of thrombosis, as recently observed in a patient with very low levels (3%) of another APC-resistant FV variant (FV Besançon) [65]. This may be even more relevant to our patient in view of his family history of thrombosis. In fact, although his father's thrombotic events may be ascribed to a combination of high prothrombin (due to the *F2* 20210G>A mutation) and decreased FV and TFPIα levels, all of which are known to contribute to APC resistance [66], it cannot be excluded that an unknown thrombophilic defect segregates in the family.

In conclusion, we have obtained *in vitro* and *ex vivo* proof-of-principle for readthrough-mediated rescue of functional FV from the *F5* p.Arg1161Ter nonsense mutation. The extent to which this approach could be applicable to other *F5* nonsense mutations, particularly outside of the B domain, remains to be determined. Moreover, the *in vivo* efficacy as well as the short- and long-term safety of this form of therapy need to be addressed in future studies.

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AUTHOR CONTRIBUTIONS

A.M.T. performed the genetic analysis on the family and the *in vitro* model experiments, assisted in the *ex vivo* experiments, analyzed data, and drafted the manuscript. C.M.R. and S.T. performed the *ex vivo* experiments and analyzed data. M.L.S., M.C., and A.C. were in charge of the patient, recorded clinical history, and arranged blood sampling. B.L., D.G., and F.B. carried out preliminary studies on the family. P.S., E. Cam, and C.B. arranged informed consent, blood sampling, and workup for the *ex vivo* model. T.M.H. provided funding and supervision. E. Cas designed, coordinated, and supervised the study, performed plasma phenotyping, analyzed data, and wrote the manuscript. All authors critically revised the manuscript and approved its final version.

DECLARATION OF COMPETING INTERESTS

T.M.H. is cofounder and shareholder of Coagulation Profile BV as well as coinventor on the thermostable inhibitor of contact activation patent WO2013028069A1. The other authors declare no conflicts of interest.

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SUPPLEMENTARY MATERIAL

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