Coagulation factur V deficiency

Coagulation factor V deficiency:
From molecular diagnosis to molecular therapy

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“ Tell me and I forget,
Teach me and I may remember,
Involve me and I learn.”
(Benjamin Franklin)

Table of contents

1

General Introduction

Haemostasis

Hemostasis[1] is a physiological mechanism by which our body maintains the fluidity of the blood, heals injured blood vessels and removes blood clots after vascular integrity has been reinstated. Disturbances of hemostasis, due to genetic and/or acquired factors, can lead to thrombotic or bleeding disorders.

Hemostasis is the result of the coordinated action of the vessel wall, platelets and several plasma proteins (coagulation and fibrinolytic factors). The processes most relevant to the work described in this thesis are **primary hemostasis**, in which platelets adhere to the damaged vessel wall and aggregate to form a temporary plug, and especially **secondary hemostasis**, in which the coagulation cascade produces a fibrin network that stabilizes the platelet plug. Platelet activation and coagulation reactions overlap in time and space and are strongly inter-dependent.

Primary hemostasis

Platelets

Platelets[2] are 2-4-μm anucleated discoid cells produced in the bone marrow and released in the bloodstream, where they circulate at a concentration of $150\text{-}400 \cdot 10^9$/L with a half-life of 5-10 days. Platelets are characterized by different types of granules: α-granules store several proteins, including the coagulation-related proteins fibrinogen, von Willebrand factor (vWF), factor V, factor XIII and protein S, whereas dense bodies contain serotonin, ADP, GTP and divalent cations (*i.e.* Ca^{2+} and Mg^{2+}). Platelets also present a wide variety of membrane receptors for (among others) collagen, vWF, fibrinogen, thrombin, ADP and tromboxane A_2. These receptors mediate the interactions and initiate the signalling pathways that lead to platelet adhesion, aggregation and degranulation at the site of injury (see below).

Platelets originate in the bone marrow from megakaryocytes, large polyploid cells derived from the pluripotent hematopoietic stem cell.[3-5] Megakaryocyte differentiation is mainly driven by thrombopoietin (TPO) in conjunction with interleukin (IL)-3, IL-6 and IL-11. During this process, immature megakaryocytes undergo multiple rounds of endomitosis (DNA replication without cytoplasmic division), accompanied by cytoplasmic enlargement and a maturation process where secretory granules are formed and the plasma membrane invaginates to build an interconnected membrane network of cisternae and tubules (demarcation membrane system) within the cytoplasm. Platelets are eventually shed from pseudopodia (pro-platelets) extending from this membranous grid.

Platelet adhesion, activation and aggregation

The role of platelets in primary haemostasis has been recently reviewed.[6,7] Upon vessel wall injury, platelets interact with components of the exposed sub-endothelial extracellular matrix *via* specific receptors present on their plasma membranes. Initial binding to vWF through the glycoprotein (GP)Ib-V-IX receptor and integrin αIIbβ3 causes the platelets to tether and roll over the injured endothelium. This is followed by firm adhesion to sub-endothelial collagen, which is mainly mediated by the GPVI receptor. Bound platelets undergo a shape change, secrete the contents of their granules and expose negatively charged phospholipids (phosphatidylserine), providing an ideal surface for coagulation reactions. Thromboxane A_2 (produced by activated platelets), ADP (released from the dense bodies) and thrombin (generated by the coagulation cascade) activate additional platelets, recruiting them to the growing thrombus. This leads to the formation of a platelet aggregate in which platelets are cross-linked by fibrinogen molecules (derived from plasma and platelet α-granules) *via* integrin αIIbβ3. This temporary platelet plug rapidly seals the blood leakage.

Secondary hemostasis

Coagulation factors

Coagulation factors (each indicated by an "F" followed by a roman numeral) comprise a group of proteins mainly synthesized in the liver and circulating in plasma as inactive precursors.[8] FII (prothrombin) and FVII, FIX, FX, FXI and FXII are zymogens of serine-proteases, FXIII is a pro-transglutaminase, FV and FVIII are non-enzymatic pro-cofactors and fibrinogen is the precursor of fibrin.

FII, FVII, FIX and FX have an N-terminal domain rich in γ-carboxyglutamate (Gla) residues (Gla domain), which mediates Ca^{2+}-dependent binding to negatively charged phospholipids. The enzyme responsible for the post-translational γ-carboxylation of glutamate residues in the Gla domain requires vitamin K hydroquinone (KH_2) as a cofactor, making these proteins vitamin K-dependent.[9] Un(der)carboxylated coagulation factors are poorly secreted as well as non-functional. This forms the basis for anticoagulant therapy with vitamin K antagonists, such as warfarin and coumarin, which block vitamin K recycling from the oxidized to the reduced (active) state.

Coagulation cascade

The coagulation cascade[10,11] is the process by which coagulation factors are sequentially activated, eventually leading to the conversion of fibrinogen to fibrin.[12] Most coagulation reactions take place at the surface of negatively charged phospholipids[13] and some enzymes assemble with their cofactors in membrane-bound macromolecular complexes, such as the intrinsic tenase complex (FIXa-FVIIIa, which activates FX) and the prothrombinase complex (FXa-FVa, which generates thrombin, the most important coagulation enzyme).

Since each factor can activate several molecules of the following factor in the cascade, the procoagulant signal is considerably amplified along the cascade.

The classical coagulation model[14] comprises two pathways dovetailing in a common pathway at the level of the prothrombinase complex (Figure 1). The extrinsic pathway is initiated by tissue factor (TF), whereas the intrinsic pathway starts with the activation of FXII by "foreign surfaces", like silica, denatured proteins and RNA. This dual model is still reflected in the clotting tests used to diagnose coagulation defects in the clinical laboratory: the prothrombin time (PT) probes the extrinsic pathway, while the activated partial thromboplastin time (APTT) probes the intrinsic pathway.

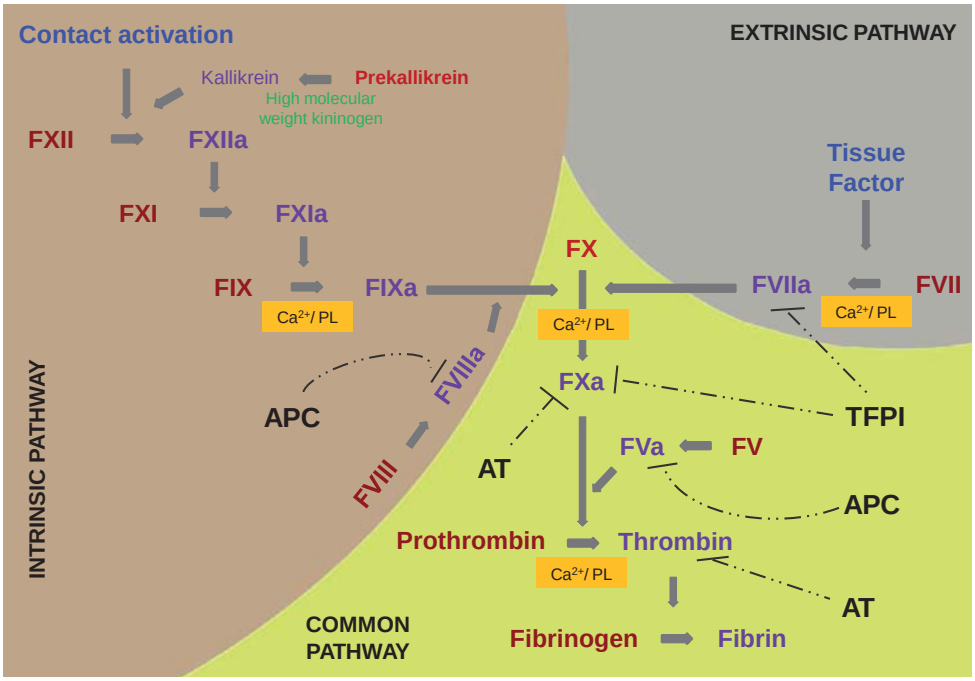

Figure 1. Coagulation cascade and its regulation.
The scheme illustrates the intrinsic pathway (brown) and extrinsic pathway (grey) of coagulation, converging on the common pathway (green) at the level of FXa. Coagulation factors are indicated in red (inactive precursors) and purple (activated enzymes and cofactors). Coagulation inhibitors are shown in black. PL, phospholipids.

According to the more recent cell-based model of coagulation,[14,15] coagulation is initiated by TF at the surface of TF-bearing cells. Binding of TF to circulating FVIIa activates small amounts of FIX and FX. In turn, FIXa and FXa associate with their respective cofactors FVIIIa and FVa (activated by FXa) on the surface of activated platelets to form the tenase and prothrombinase complexes, generating trace amounts of thrombin (initiation phase). The latter are insufficient to generate fibrin, but enough to amplify the coagulation signal via feedback activation of platelets (*via* protease-activated receptors PAR-1 and PAR-4), FXI and the procofactors FV and FVIII (propagation phase). This leads to a tremendous burst of thrombin, which cleaves off fibrinopeptides A and B from fibrinogen molecules, giving rise to fibrin monomers. The latter spontaneously polymerize to form fibrin fibers, which are eventually cross-linked by FXIIIa (activated by thrombin) to form the fibrin network that stabilizes the clot (stabilization phase).

Regulation of coagulation

Several inhibitors acting at all levels of the coagulation cascade limit coagulation reactions to the site of injury (Figure 1).

Tissue factor pathway inhibitor (TFPI)[16] is a kunitz-type protease inhibitor mainly synthesized by endothelial cells. The human *TFPI* gene produces two main splicing isoforms: TFPIα (containing an acidic N-terminus, three kunitz domains and a basic C-terminus) and TFPIβ (in which the last kunitz domain and the basic C-terminus are replaced by a GPI anchor). Both isoforms are associated with the endothelial surface, but TFPIα is also present in platelets and in plasma (plasma concentration ~2.5 nM). However, most plasma TFPIα is C-terminally truncated and bound to lipoproteins, only 10% (0.25 nM) being in the fully active free full-length conformation. TFPIα and TFPIβ down-regulate the

initiation of coagulation by inhibiting both the TF/FVIIa complex (*via* their kunitz-1 domain) and FXa (*via* their kunitz-2 domain).[17] The anticoagulant activity of full-length TFPIα is stimulated by its cofactor protein S,[18] which binds to the kunitz-3 domain and helps localize TFPIα to the membrane surface.[19] Moreover, the C-terminus of TFPIα contains a binding site for FV,[20] which has been proposed to mediate inhibition of prothrombinase by TFPIα.[21]

Protein C[22,23] is a vitamin K-dependent zymogen produced in the liver and circulating in plasma at a concentration of ~65 nM. Protein C is activated by thrombin bound to the endothelial transmembrane protein thrombomodulin, a reaction which is enhanced by the endothelial protein C receptor. Activated protein C (APC) proteolytically inactivates the FVIIIa and FVa, the essential cofactors of the intrinsic tenase and prothrombinase complexes, respectively. Both inactivation reactions are greatly stimulated by ***protein S***,[23,24] another vitamin K-dependent protein which acts as a cofactor of APC. Optimal inactivation of FVIIIa also requires APC-cleaved FV as an additional APC cofactor. The physiological importance of the protein C pathway is underscored by the increased risk of venous thrombosis associated with hereditary deficiencies of protein C and protein S.[25]

Antithrombin[26] is a liver-derived anticoagulant protein belonging to the serpin family. It inhibits several coagulation serine proteases, including FIXa, FXa, FXIa, FXIIa and thrombin. The efficiency of the inhibition is greatly increased by heparin, mimicked *in vivo* by endhotelial glycosaminoglycans. Antithrombin deficiency is a strong risk factor for venous thrombosis.[25]

Coagulation factor V

Coagulation factor V (FV),[27] also known as proaccelerin or labile factor, was discovery by the Norwegian physician Paul Owren in 1947.[28] It is mainly synthesized in the liver and

circulates in plasma as a 330-kDa single-chain glycoprotein, with a concentration of 20-25 nM and a half-life of ~13 hours.[29] Some FV (~20%) is stored in platelet α-granules,[30] where it is bound to the soluble protein multimerin-1.[31] Although megakaryocytes are able to synthesize FV,[32] it has been shown that the platelet FV pool derives from endocytosis of plasma FV by bone-marrow megakaryocytes.[33,34] During its transport to the α-granules, the internalized FV undergoes an extensive intracellular processing that makes platelet FV structurally and functionally different from plasma FV.[35,36]

Structure and functions of FV(a)

Human FV is encoded by the *F5* gene, which is localized on the long arm of chromosome 1 (1q21-25).[37] The gene spans ~80 kb and consists of 25 exons and 24 introns.[38] Its 7-kb mRNA[39] encodes a mature protein of 2196 amino acids, organized in six domains (A1-A2-B-A3-C1-C2). The A and C domains are highly homologous to the corresponding domains of FVIII,[40] whereas the extremely large B domain has a unique sequence. In particular, this domain contains a basic region (residues 963-1008*) and an acidic region (residues 1493-1537) which engage in a high-affinity interaction that maintains FV in its inactive conformation.[41]

The "life cycle" of FV is illustrated in Figure 2. FV is activated by thrombin or FXa *via* limited proteolysis at Arg709, Arg1018 and Arg1545.[42] These cleavages remove the B-domain and release activated FV (FVa), which consists of a ~105 kDa heavy chain (A1-A2) and a ~72 kDa light chain (A3-C1-C2) non-covalently linked *via* a calcium ion. FVa binds to the serine-protease FXa on the surface of negatively charged phospholipids to form the prothrombinase complex, which converts prothrombin to thrombin. As a non-enzymatic

***Note:** All *F5* nucleotides and FV amino acids in this Introduction are numbered according to the classical nomenclature (based on Jenny *et al*. 1987),[39] which is still the most widely used within the FV community.

cofactor of FXa, FVa accelerates prothrombin activation by several orders of magnitude.[43] This property makes it essential for efficient thrombin formation and thus indispensable to life.

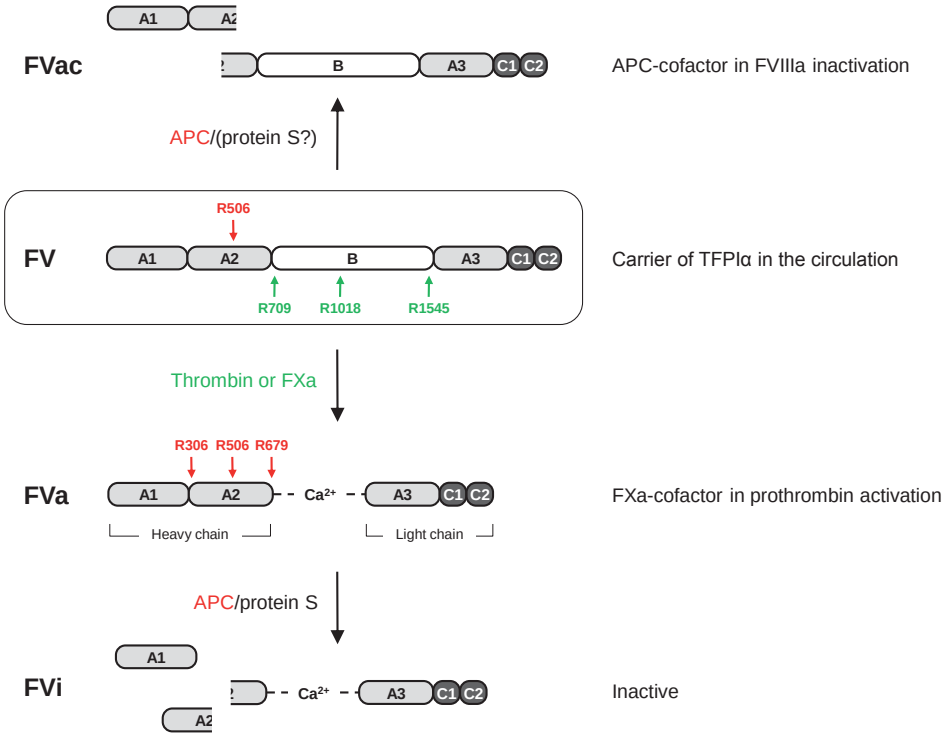

Figure 2. Structure and functions of FV(a).
Native single-chain FV is boxed. FV can be cleaved by APC to yield an anticoagulant cofactor of APC (FVac, top), or by thrombin or FXa to give a procoagulant cofactor of FXa (FVa, bottom). FVa is eventually inactivated (FVi) by APC/protein S. APC-cleavage sites are shown in red, thrombin- and FXa-cleavage sites are shown in green.

FVa is inactivated by the APC-mediated proteolysis of the heavy chain at Arg306, Arg506 and Arg679.[44,45] Cleavage at Arg506 is kinetically preferred and usually occurs first, but cleavage at Arg306 is required for complete FVa inactivation. Cleavage at Arg679 contributes little to FVa inactivation. The APC-cofactor protein S accelerates FVa

inactivation by stimulating cleavage at Arg306.[46] When FVa is incorporated in the prothrombinase complex, it is protected from APC-mediated inactivation by its interactions with FXa and prothrombin.[46,47]

In contrast to plasma FV, platelet FV is stored as a partially activated molecule. Following platelet activation, it is released from the α-granules, exposed on the platelet surface and fully activated by FXa (preferably) or thrombin.[48] Platelet FVa is partially resistant to inactivation by APC.[49]

While FVa is strongly procoagulant, its precursor FV has anticoagulant activity as an additional cofactor of the APC/protein S complex in the inactivation of FVIIIa.[50-52] Expression of this APC-cofactor activity requires FV to be cleaved by APC at Arg506[53] and to be intact at the Arg1545 thrombin-cleavage site.[54]

FV also acts as a carrier of TFPIα in plasma,[20,55] protecting this anticoagulant protein from truncation and/or clearance. In fact, >50% of circulating TFPIα is bound to FV *via* an interaction between the basic C-terminus of TFPIα and the acidic region in the B domain of FV.[56] Recently, FV splicing variants (FV-short) with increased affinity for plasma TFPIα have been described (see below).

Role of FV(a) as regulator of the haemostatic balance

The numerous pro- and anticoagulant functions of FV(a) make it a pivotal regulator of the haemostatic balance. Not surprisingly, mutations in the *F5* gene can result in a thrombotic or haemorrhagic phenotype.[27,57] In particular, the common Arg506Gln mutation (FV Leiden),[58] which abolishes one of the APC-cleavage sites on FV(a), is associated with APC resistance and an increased risk of venous thrombosis.[59,60] In contrast, mutations that impair the synthesis and/or function of FV(a) lead to bleeding manifestations.[61]

FV deficiency

FV deficiency states can originate from genetic or acquired conditions. Inherited FV deficiencies include classical FV deficiency (linked to the *F5* gene),[61] combined deficiency of FV and FVIII (linked to the *LMAN1* and *MCFD2* genes)[62] and the Québec platelet disorder (linked to the *PLAU* gene),[63] whereas acquired FV deficiency is most often due to the development of anti-FV antibodies (FV inhibitors).[64,65] Virtually all FV deficiency states are associated with a bleeding diathesis, whose severity depends on the reduction in FV levels and to whether or not the platelet FV pool is also affected.

This thesis focusses on classical FV deficiency[66-68] (also known as Owren parahemophilia, OMIM +227400), which is a rare bleeding disorder with an incidence of 1 in 1 million and an autosomal recessive pattern of inheritance. Patients present with prolonged PT and APTT, but the diagnosis must be confirmed with specific assays for FV antigen and activity.

Like other coagulation disorders, FV deficiency comes in two types: type 1 is a quantitative defect characterized by proportionally decreased levels of FV antigen and activity, whereas type 2 is a qualitative defect with a normal or sub-normal antigen level, but reduced activity. Type 2 FV deficiency is extremely rare and only two cases have been described so far.[69,70]

Based on (plasma) FV activity levels, FV deficiency is classified as severe (FV:C <1%), moderate (FV:C 1-5%) and mild (FV:C 5-30%), although the correlation between FV levels and clinical phenotype is not clearcut. The most common symptoms are easy bruising, epistaxis, mucosal bleeding and menorrhagia in females.[71,72] Approximately one quarter of patients also experience joint and muscle bleeds, whereas life-threatening intracranial and gastro-intestinal haemorrhages are rare and confined to the most severe cases. Patients with partial FV deficiency (FV:C ~50%) are usually asymptomatic.

Since no FV concentrate or recombinant FV preparation is available, the management of bleeding episodes in FV-deficient patients still relies on fresh frozen plasma and platelet concentrates.[73] While most patients respond well to this therapy, repeated plasma transfusions may lead to several complications, including volume overload, the transmission of infectious agents, immune reactions and transfusion-related acute lung injury (TRALI). In contrast to haemophilia A, where substitutive therapy with FVIII often triggers the development of inhibitory antibodies, FV inhibitor development in FV-deficient patients treated with fresh frozen plasma is a rare occurrence.

Modulators of the bleeding tendency in FV deficiency

Experiments in mice models have indicated that the complete absence of FV is not compatible with life,[74] but minimal FV expression is sufficient to rescue the perinatal lethality of FV knock-out mice.[75] The same applies to humans, where many patients with apparently undetectable FV levels experience only moderate bleeding symptoms. This observation suggests the existence of compensatory mechanisms which ameliorate the clinical phenotype of FV-deficient patients.[68] Previous studies from our laboratory have identified platelet FV[76] and plasma TFPIα levels[55] as candidate modulators of the bleeding tendency in severe FV deficiency. In particular, we have shown that patients with undetectable plasma FV often have traces of residual platelet FV which can support enough thrombin generation to prevent fatal hemorrhages.[76] Furthermore, we have observed that, due to the interaction between FV and TFPIα in plasma, FV-deficient patients have low TFPIα levels (~20-30% of normal), which considerably reduces the FV requirement for minimal hemostasis.[55] Therefore, low TFPIα levels are beneficial to FV-deficient patients and variations in TFPIα levels could modulate the bleeding tendency in patients with equally low

FV levels. A third, as yet uncharacterized, compensatory mechanism acting on the intrinsic coagulation pathway has also been reported in an 83-year-old FV-deficient patient (FV:C <2%) with no spontaneous bleeding.[77]

Genetics of FV deficiency

FV deficiency is caused by loss-of-function mutations in the *F5* gene.[61,78] Currently, 153 *F5* mutations are listed in the Human Genome Mutation Database (accessed in March 2015), most of which are associated with FV deficiency. The vast majority are missense/nonsense point mutations (66%) or small insertions/deletions (21%), whereas splicing mutations represent only 10% of the total and gross gene rearrangements are strikingly under-represented (only 2 large deletions[79,80] and 1 duplication[81]) (Figure 3).

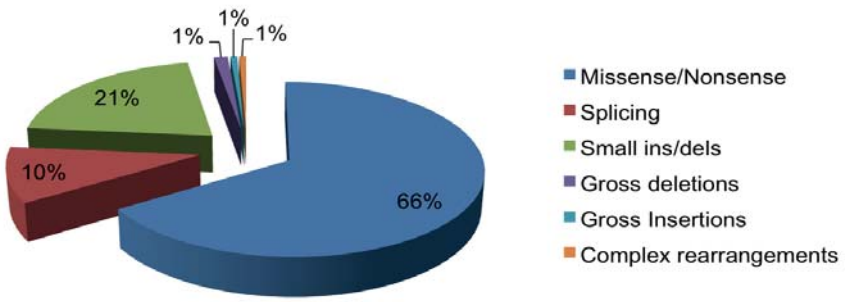

Figure 3. *F5* mutational spectrum.
Relative abundance of the different types of mutation in the *F5* gene as reported in the Human Genome Mutation Database (accessed in March 2015).

Remarkably, premature termination codons introduced by nonsense and frameshift mutations tend to cluster in exon 13, which encodes the large B domain. Splicing mutations, whose contribution to the mutational spectrum is probably underestimated, tend to be associated with severe clinical phenotypes, with a high incidence of life-threatening bleeding manifestations (*e.g.* intracranial haemorrhages).[82-86]

In general, each family with FV deficiency has its own "private" *F5* mutation. Only few mutations, such as Tyr1702Cys[87] and Arg712Stop,[88] have been found in several apparently unrelated patients. Therefore, *F5* mutation screening needs to be carried out in each individual patient. Although all FV-deficient patients are treated in the same way irrespective of the underlying mutation(s), a molecular diagnosis may be desirable for the purposes of genetic counseling, prenatal diagnosis and (in perspective) personalized molecular therapy.

Other bleeding disorders linked to the *F5* gene

The East Texas bleeding disorder[89] is an autosomal dominant coagulopathy characterized by prolonged PT and APTT but normal values of all coagulation factors. Linkage analysis in the affected family pointed at *F5* as the most likely candidate gene. Sequencing of this gene identified a mutation in exon 13 (2440A>G), which co-segregated with the bleeding phenotype. Further studies showed that this mutation up-regulates an alternatively spliced FV isoform called FV-short[90] (Figure 4). This isoform lacks 702 amino acids of the B domain (residues 756-1458), including the basic region, leaving the acidic region fully available to interact with TFPIα.[91] As a consequence, FV-short binds TFPIα with markedly higher affinity than full-length FV. FV-short is present in all individuals (accounting for ~5% of all plasma FV with a large inter-individual variation), but its up-regulation by the 2440A>G mutation results in 10-fold increased levels of plasma TFPIα and a bleeding tendency.

More recently, a different mutation (*F5* 2678C>G, FV Amsterdam) was identified in a Dutch family with a similar bleeding disorder and normal levels of all coagulation factors.[92] Also this second mutation enhances an alternative splicing event leading to a FV isoform with an

in-frame deletion of 623 amino acids within the B domain (residues 834-1458) and increased affinity for TFPIα. This "Amsterdam FV-short isoform" (Figure 4) does not seem to occur in normal individuals.

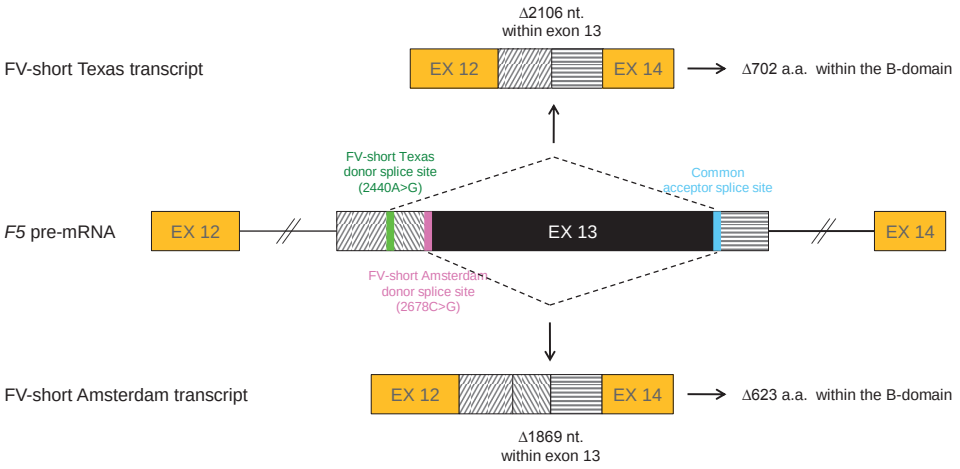

Figure 4. FV-short splicing variants.
Schematic representation of the portion of the *F5* pre-mRNA that undergoes alternative splicing. The donor splice sites activated by the East Texas and Amsterdam mutations are shown in green and pink, respectively. The common acceptor splice site is shown in light blue.

Outline of this thesis

This thesis focuses on the molecular genetics of FV deficiency and on emerging therapeutic approaches. Conventional mutation screening strategies, based on the amplification and sequencing of each exon and its splicing junctions, fail to identify one or both mutations in 10-20% of patients with severe FV deficiency. These patients may harbour gross gene rearrangements (which are invisible to direct sequencing of individual exons) or splicing mutations (which may be easily overlooked if "disguised" as synonymous/missense exonic mutations or if located deep in introns). Identifying the mutation(s) responsible for FV deficiency in each individual patient may be important not only for a better understanding of

the molecular mechanisms of the disease, but also for developing personalized molecular therapies. Particularly splicing mutations, which are often associated with a severe bleeding phenotype, are amenable to RNA therapy with mutation-specific antisense molecules.

Chapter 2 of this thesis reviews the general mechanism of pre-mRNA splicing and the available options to modulate this process for therapeutic purposes. **Chapter 3** describes the development and validation of an in-house multiplex ligation-dependent probe amplification (MLPA) assay for the detection of gross *F5* gene rearrangements and its application to 14 genetically unexplained FV-deficient patients. **Chapter 4** is a preliminary report of a novel splicing mutation affecting the canonical donor splice site of *F5* intron 3 in a FV-deficient patient with an extremely severe clinical presentation. **Chapter 5** describes the identification and characterization of a *F5* splicing mutation initially mistaken for a neutral (synonymous) polymorphism in a patient with severe FV deficiency. In addition, it illustrates the use of a morpholino antisense oligonucleotide for the correction of this splicing defect in an *in vitro* minigene model. **Chapter 6** extends this concept by presenting the correction of a *F5* deep-intronic mutation using two types of antisense molecules (a morpholino oligonucleotide and an engineered small nuclear RNA) and two experimental models (a *F5* minigene and the patient's own megakaryocytes differentiated *ex vivo* from circulating hematopoietic progenitors). **Chapter 7** illustrates the impact of plasma full-length TFPIα levels on the thrombin generation and bleeding tendency of two related FV-deficient patients homozygous for the same *F5* missense mutation, pointing out TFPIα as a possible therapeutic target in severe FV deficiency. Finally, **Chapter 8** integrates the information of all preceding chapters and discusses it in the context of current literature.

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2

Pre-mRNA splicing as a target for personalized molecular therapy

3

Identification of a novel large deletion in a patient with severe factor V deficiency using an in-house *F5* MLPA assay

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Abstract

Factor V (FV) deficiency is a rare autosomal recessive bleeding disorder caused by mutations in the *F5* gene. FV-deficient patients in whom no mutation or only one mutation is found may harbour large gene rearrangements, which are not detected by conventional mutation screening strategies. The aim of this study was to develop and validate a multiplex ligation-dependent probe amplification (MLPA) assay for the detection of large deletions and duplications in the *F5* gene. Twenty-two MLPA probes targeting 19 of the 25 exons and the upstream and downstream regions of the *F5* gene were designed and tested in 10 normal controls, a patient with a known heterozygous deletion of *F5* exons 1-7 (positive control) and 14 genetically unexplained FV-deficient patients. MLPA results were confirmed by digital PCR on a QuantStudio™ 3D Digital PCR System. The *F5*-specific probes yielded a reproducible peak profile in normal controls, correctly detected the known deletion in the positive control and suggested the presence of a novel deletion of exons 9-10 in a patient with undetectable FV levels and only one identified mutation. Follow-up by chip-based digital PCR, long-range PCR and direct sequencing confirmed that this patient carried a heterozygous *F5* deletion of 1823 bp extending from intron 8 to intron 10. Bioinformatics sequence analysis pinpointed repetitive elements that might have originated the deletion. In conclusion, we have developed and validated an MLPA assay for the detection of gross *F5* gene rearrangements. This assay may represent a valuable tool for the molecular diagnosis of FV deficiency.

Note: The *F5* gene mutations described in this chapter are annotated according to the HGVS recommendations. The corresponding annotation according to the classical nomenclature (Jenny *et al*. 1987) is as follows:

HGVS nomenclature	Classical nomenclature
g.42208_44031del	Deletion extends from nt. IVS8+1311 to nt. IVS10+378
p.Ile433_Gln537del	Deletion spans amino acids 405-509
p.Phe551Ser	Phe523Ser

Introduction

Coagulation factor V (FV) deficiency is a rare autosomal recessive bleeding disorder associated with mutations in the *F5* gene.[1-3] The human *F5* gene spans ~80 kb on the long arm of chromosome 1 (1q23) and comprises 25 exons. To date, ~150 point mutations and small insertions/deletions have been described in this gene. In contrast, only two large deletions have been reported: an interstitial deletion of chromosome 1q involving the (whole?) *F5* gene in a 15-year-old girl (FV:C 9%) who carried a missense mutation (p.Ser262Trp) on the other *F5* allele,[4] and a heterozygous 205-kb deletion spanning *F5* exons 1-7 (plus the whole *SELP*, *SELL* and *SELE* genes) in a South-African baby (FV:C 1%) who carried a splicing mutation (*F5* 1975+5G>A) on the other allele.[5] The remarkable under-representation of large deletions in the *F5* mutational spectrum is probably due to the fact that gross gene rearrangements are invisible to conventional mutation screening strategies, which are based on the amplification and sequencing of individual exons.

Multiplex ligation-dependent probe amplification (MLPA)[6-8] is the technique of choice for the detection of copy-number variations in genes of interest. In this PCR-based gene dosage assay, the number of copies of specific target sequences in a genome is determined by comparison with reference sequences assumed to be present in two copies in the same genome. For each target and reference sequence, two oligonucleotides (*i.e.* a 'probe') are designed that anneal side by side to the template DNA. After incubating the probes with the genomic DNA sample, annealed oligonucleotides are ligated to generate products of different lengths, each corresponding to a target or reference sequence. Ligation products are then amplified using a pair of universal primers (one of which is fluorescently labelled) and PCR products are separated by capillary electrophoresis, yielding a characteristic peak profile. The height of each target peak relative to the heights of the reference peaks in the

same profile provides information on the number of copies of that particular target sequence in the DNA sample.

Since no commercial MLPA probes for the *F5* gene are available, we developed and validated an in-house MLPA assay with self-designed *F5*-specific probes, and applied it to the identification of gross gene rearrangements in genetically unexplained FV-deficient patients.

Patients and Methods

Study subjects

Genomic DNA was obtained from the following subjects: a) 10 unrelated normal controls (5 males and 5 females) used to assess the reproducibility of the *F5* MLPA assay; b) a carrier of the described deletion of *F5* exons 1-7 (***i.e.*** the mother of the patient reported in ref.[5]), who served as a positive control to validate the assay; and c) 14 patients with severe (FV:C ≤5%, n=5) or partial (FV:C 11-48%, n=9) FV deficiency, in whom no mutation or only one mutation was identified by conventional mutation screening strategies. The study was approved by the local Ethical Committees and conducted according to the Helsinki Declaration.

F5 MLPA assay

MLPA probe design. Two sets of 11 *F5*-specific probes with lengths between 88 and 148 nucleotides (nt) were designed according to the guidelines provided by MRC-Holland, Amsterdam, the Netherlands (www.mrc-holland.com) and purchased from Integrated DNA Technologies (Leuven, Belgium), as previously described.[9] The first set (probe mix A)

includes probes for exons 1, 4, 7, 10, 13 (proximal portion), 16, 19, 22 and 24, as well as intron 3 and the 3'-UTR. The second set (probe mix B) includes probes for the promoter region as well as intron 2 and exons 6, 9, 11, 13 (distal portion), 14, 15, 17, 18 and 21. Each *F5*-specific probe mix was combined with a commercial reference probe mix (SALSA MLPA kit P200-A1 Human DNA Reference 1, MRC-Holland), which contains 14 probes (172-250 nt in length) mapping to other genes scattered over different chromosomes, including the X and Y chromosomes. The reference probe mix also contains control probes for DNA amount (Q-fragments, 64-82 nt) and for complete DNA denaturation during the MLPA reaction (D-probe, mapping to 14q32).

MLPA reaction. MLPA ligation and amplification reagents were obtained from MRC-Holland. MLPA reactions were carried out on 100 ng genomic DNA according to the manufacturer's instructions. After adding GeneScan™ 600 LIZ® dye Size Standard and Hi-Di™ formamide (Life Technologies, Bleiswijk, The Netherlands), amplification products were denatured at 80°C for 2 minutes and separated by capillary electrophoresis on an ABI 3730 DNA Analyzer (Life Technologies).

Peak analysis. Peak profiles were analysed using Coffalyser.Net software (MRC-Holland) and dosage quotients were calculated for each target (*i.e. F5*-specific) probe as follows. First, the height of each *F5*-specific peak was divided by the sum of the heights of all reference peaks in the same sample (except the peaks corresponding to the D-probe and to the sex chromosome probes). Then, the ratio obtained for each *F5*-specific probe in the test sample was divided by the corresponding ratio obtained in a control sample run in parallel. A dosage quotient of 0.8-1.15 is considered normal and indicates the presence of two copies of the corresponding target sequence. Dosage quotients of 0.35-0.65 and 1.35-1.55 indicate the

presence of one copy (heterozygous deletion) and three copies (heterozygous duplication), respectively, of the corresponding target sequence.

Digital PCR

Digital PCR (dPCR)[10,11] allows accurate quantification of nucleic acid templates by partitioning the PCR reaction mix over a large number of wells, so that each well contains a single copy or no copies of the target region. Based on the assumption of Poisson distribution of copies, the number of template copies originally present in the sample can be recalculated from the number of wells in which amplification has successfully occurred.

dPCR was performed on a QuantStudio™ 3D Digital PCR System (Life Technologies). Reaction mixtures were prepared by combining 30 ng of genomic DNA with the QuantStudio™ 3D Digital PCR Master Mix and a custom TaqMan assay (Sigma-Aldrich, Milan, Italy) for *F5* exon 10 (target) or *F11* exon 5 (control). Each reaction mixture was loaded onto a QuantStudio™ 3D Digital PCR Chip, which contains 20,000 wells, and cycled under standard conditions for 40 cycles. End-point fluorescence data were collected and analysed using the QuantStudio™ 3D Digital PCR Instrument and the QuantStudio™ 3D AnalysisSuite™ according to the manufacturer's instructions.

Long-range PCR

Long-range PCR was performed using the Expand Long Template PCR System Kit (Roche Applied Science, Almere, The Netherlands) according to the manufacturer's instructions. The exact deletion breakpoints were mapped by direct sequencing of the PCR products.

Bioinformatics analysis

The genomic region surrounding the newly identified deletion was analysed with RepeatMasker (www.repeatmasker.org) to look for repeated sequences that may have mediated the recombination event. Linkage disequilibrium within and around the *F5* locus was visualised using Haploview.[12]

Results

Development and validation of the *F5* MLPA assay

To test the self-designed probes, the *F5* MLPA assay was initially carried out on DNA samples from 10 normal controls. Both probe mixes yielded the expected peak profiles (Figure 1, upper panels) and the assay reproducibility in normal DNA samples was generally good (average coefficient of variation of normalised peak height: 11.9% for probe mix A and 11.4% for probe mix B; average coefficient of variation of normalised peak area: 12.1% for probe mix A and 12.6% for probe mix B). Only the shortest probes of both probe mixes (corresponding to exon 1 and to the promoter region, respectively) occasionally produced double peaks, possibly due to the high GC-content of these probes. Since peak height was slightly more reproducible than peak area, we decided to rely on peak height for the quantitative analysis of peak profiles.

When the *F5* MLPA assay was applied to a positive control DNA sample carrying the previously described deletion of *F5* exons 1-7,[5] all *F5*-specific probes targeting sequences located upstream of intron 7 showed markedly decreased peaks (Figure 1, middle panels). Quantification of peak heights showed dosage quotients between 0.49 and 0.65 for these

probes and normal dosage quotients for all other probes (Figure 1, lower panels), indicating that the assay is indeed sensitive to copy number variations in the *F5* gene.

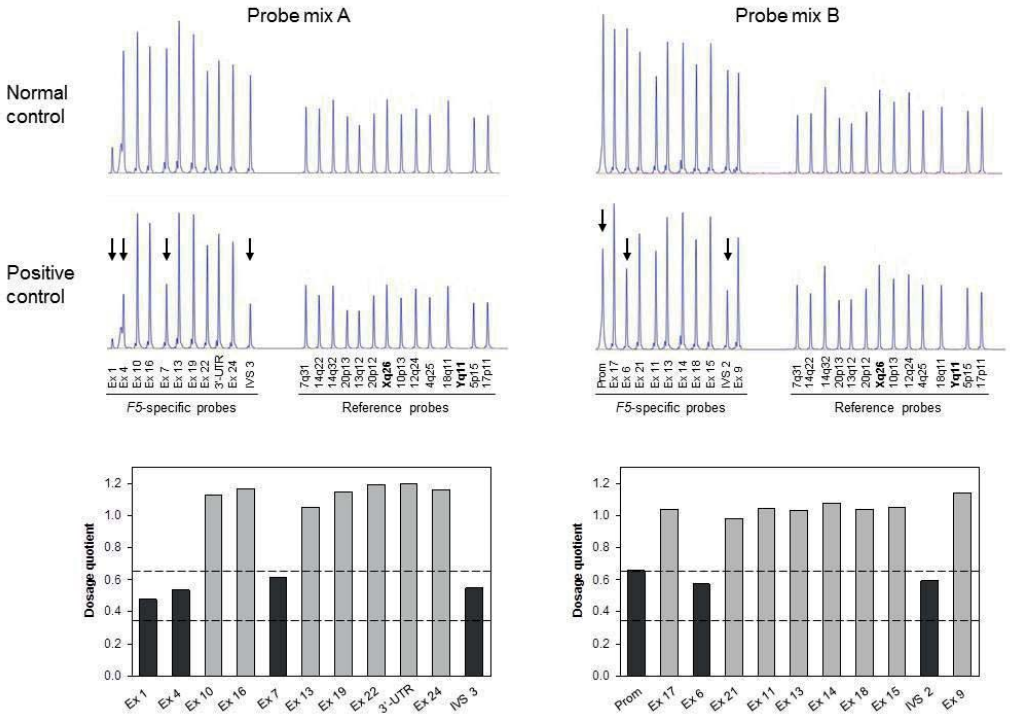

Figure 1. Validation of the in-house *F5* MLPA assay in a positive control sample.
The peak profiles obtained with probe mixes A (left) and B (right) in a normal control (upper panels) and in a positive control carrying the described deletion of *F5* exons 1-7 (middle panels) are shown. Both individuals are female, as indicated by the absence of signal for the Y chromosome probe. The target and reference sequences recognised by each probe are indicated below the corresponding peaks. The peaks that are relatively lower in the positive control are indicated by arrows. Peak profiles were analysed as described under Methods and the dosage quotient of each probe was plotted in a bar diagram (lower panels). Probes with a dosage quotient between 0.35 and 0.65 (promoter region, exons 1, 4, 6 and 7, and introns 2 and 3), suggestive of a heterozygous deletion, are marked in black.

Identification and characterisation of a novel *F5* large deletion

We then analysed DNA samples from 14 FV-deficient patients in whom no mutation or only one mutation had been identified using conventional screening methods. One patient with severe FV deficiency (P10) showed remarkably low peaks for exons 9 and 10 (dosage quotients of 0.55 and 0.60, respectively), suggestive of a heterozygous deletion of these exons (Figure 2).

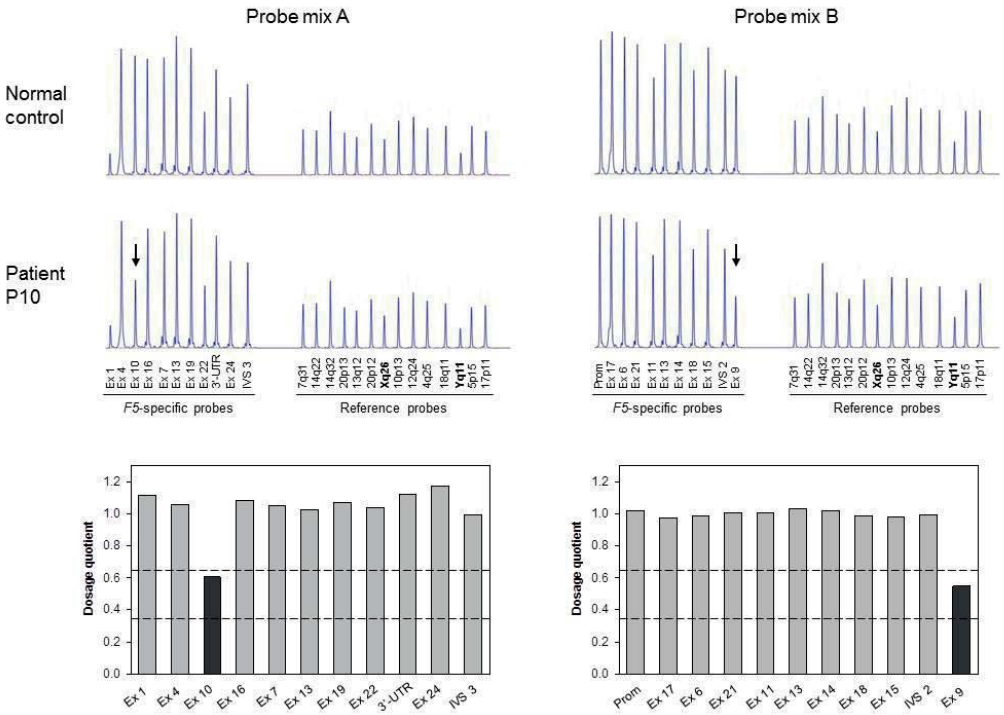

Figure 2. Detection of a novel *F5* large deletion in a FV-deficient patient.
The peak profiles obtained with probe mixes A (left) and B (right) in a normal control (upper panels) and in a patient with severe FV deficiency (P10, middle panels) are shown. Both individuals are male, as indicated by the successful amplification of the Y chromosome probe. The target and reference sequences recognised by each probe are indicated below the corresponding peaks. The peaks that are relatively lower in the patient are indicated by arrows. Peak profiles were analysed as described under Methods and the dosage quotient of each probe was plotted in a bar diagram (lower panels). Probes with a dosage quotient between 0.35 and 0.65 (exons 9 and 10), suggestive of a heterozygous deletion, are marked in black.

The presence of a deletion was verified by dPCR (Figure 3), which allows absolute quantification of copy numbers without reference to a standard curve.

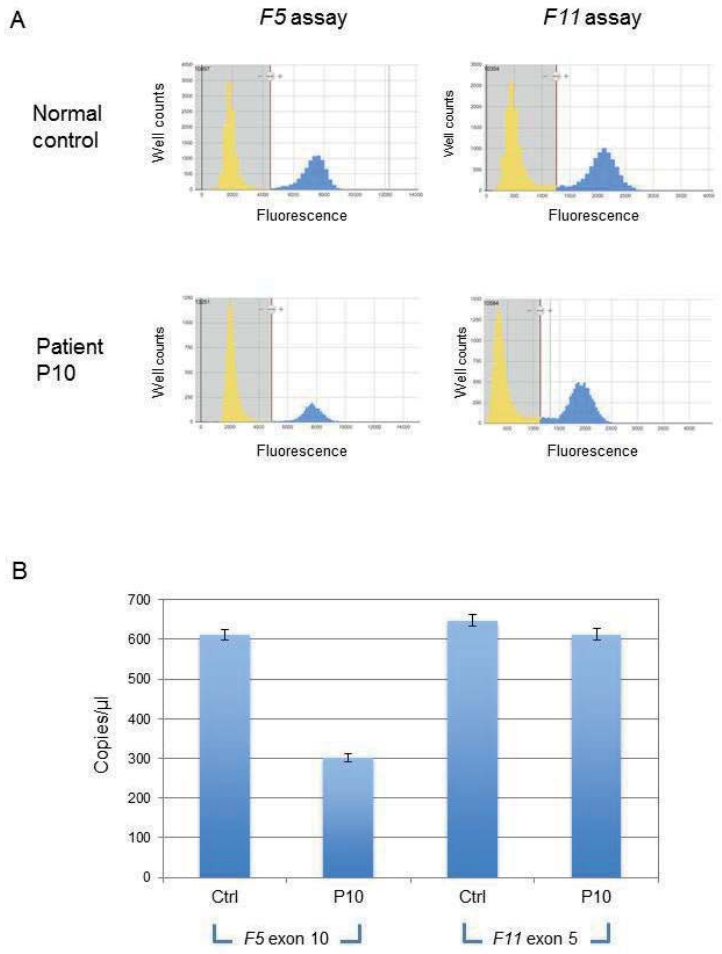

Figure 3. Evaluation of the deletion in the *F5* gene using dPCR.

A) Histogram view of the results obtained with QuantStudio™ 3D AnalysisSuite™ (absolute quantification mode). Yellow peaks correspond to wells in which no amplification was detected; blue peaks indicate PCR-amplified spots.

B) Graphic representation of the results of the assay. The number of copies of the target region is shown for both *F5* and *F11* assays. Bars represent the mean ± the precision of the assay as calculated by the software.

Analysis of the *F11* gene, used as a control, showed that patient P10 and a normal control had the same number of *F11* copies (on average 612 copies/µl and 648 copies/µl, respectively); conversely, analysis of the *F5* gene revealed that the patient had half as many copies of *F5* exon 10 as the normal control (on average 301 copies/µl *vs.* 611 copies/µl) (Figure 3).

The deletion was further confirmed by long-range PCR using primers located in exons 7 and 11 (Figure 4A), which had normal dosage quotients in the MLPA assay (Figure 2) and were therefore assumed to fall outside the deleted region. Amplification of this large genomic fragment yielded only the expected 8713-bp band in the normal control, but also a lower band (corresponding to the deleted allele) in the patient. In fact, the deleted allele was amplified much more efficiently than the normal allele, probably due to its considerable (~2 kb) smaller size. In order to map the exact deletion breakpoints, nested PCR reactions were carried out using forward primers located progressively closer to exon 9, until the deleted allele failed to amplify and only the normal band was observed in the patient (Figure 4A). This indicated that the forward primer used in this reaction (IVS8cF) fell inside the deleted region and that the proximal breakpoint of the deletion was located between primers IVS8bF and IVS8cF. Direct sequencing of the patient's IVS8bF-11R fragment eventually showed that the deletion (g.42208_44031del) encompassed 1823 bp of genomic sequence between introns 8 and 10 (Figure 4A). Bioinformatics analysis revealed that the deleted region was delimited by an AluJb sequence[13] in intron 8 and a MIRc (mammalian-wide interspersed repeat, variant c) sequence[14] in intron 10 (Figure 4A). These short interspersed elements (SINEs), which are extremely common in the human genome, share partial sequence homology and may have originated the deletion *via* misalignment of chromatids and homologous recombination at meiosis.[15]

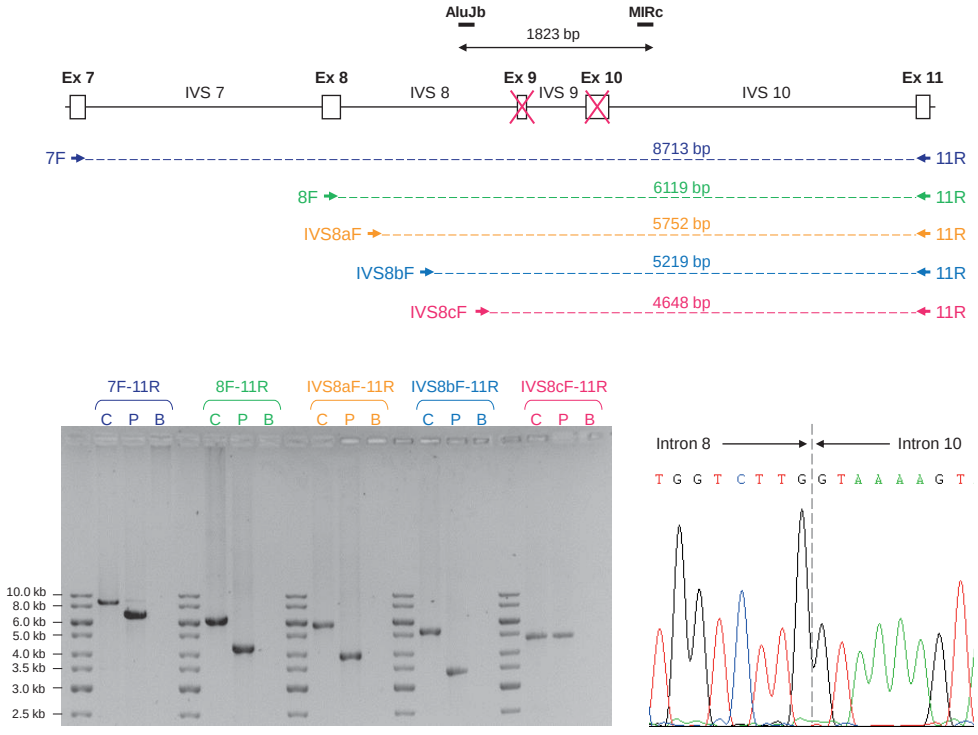

Figure 4A. Characterisation of the newly identified *F5* deletion.

Schematic representation of the relevant portion of the *F5* gene, showing the extension of the deletion, the positions of the repeated sequences (AluJb and MIRc) presumably responsible for the unequal homologous recombination event, and the PCR primers used to map the deletion. The expected length of each amplicon in base pairs (bp) is indicated. Bottom left: PCR products obtained with the different primer pairs in a normal control (C), in FV-deficient patient P10 (P) and in a no-DNA control (blank, B). Bottom right: Sequencing chromatogram showing the junction between introns 8 and 10 in the deleted allele.

Interestingly, the putative recombination event took place close to the region that separates the two main haploblocks of the *F5* gene in the Caucasian population (Figure 4B).

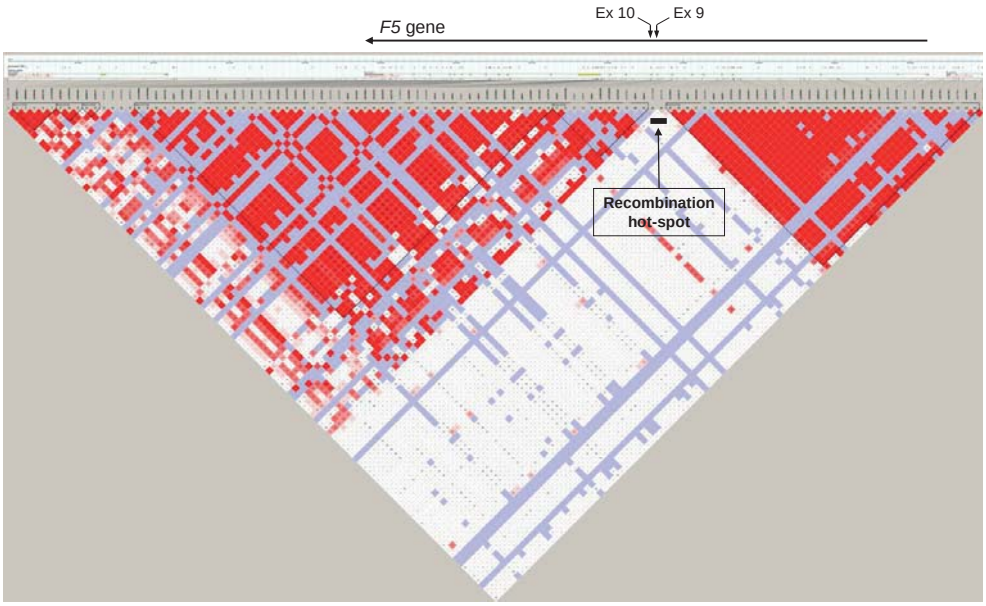

Figure 4B. Characterisation of the newly identified *F5* deletion.

Linkage disequilibrium plot of a portion of chromosome 1 generated using Haploview software and the genotype data of the CEU population (Utah residents of Caucasian descent). The plot shows that the region found to be deleted in patient P10 lies near a recombination hot-spot within the *F5* gene.

Discussion

Approximately 10-20% of all FV-deficient patients remain genetically unresolved, as no mutation or only one mutation is found by amplification and sequencing of the *F5* coding sequence and splicing junctions. These patients might harbour gross gene rearrangements, which are not detected by the conventional mutation screening strategies. Therefore, we have developed and validated an in-house MLPA assay for the detection of large deletions or duplications in the *F5* gene. This assay showed good reproducibility in normal controls, correctly recognised a previously described deletion of *F5* exons 1-7 and successfully identified a novel deletion of exons 9 and 10 in a partially unresolved FV-deficient patient.

This is the third large deletion to be described in the *F5* gene and the second to be characterised in detail. In combination with the missense mutation (p.Phe551Ser, Paraboschi *et al*, manuscript in preparation) which had been previously identified by direct sequencing of the *F5* gene and which is predicted to be damaging (SIFT score 0), this large deletion accounts for the patient's severe FV deficiency.

Since MLPA is considered an explorative technique, the deletion was verified using both dPCR and long-range PCR. Compared to conventional real-time qPCR, dPCR can easily resolve two-fold differences in template copy numbers and proved to be a very efficient and elegant method to confirm the existence of the deletion. Long-range PCR with progressively nested primers was instrumental in mapping the extent of the deletion, which caused the proximal portion of intron 8 to fuse with the distal portion of intron 10. Although this chimeric intron could be correctly spliced out of the primary transcript and the resulting mature mRNA, lacking exons 9 (100 bp) and 10 (215 bp), would maintain the correct reading frame, the encoded mutant protein would lack 105 amino acids in the A2 domain (p.Ile433_Gln537del) and is unlikely to be properly folded and secreted.

Bioinformatics analysis suggested that the deletion might have originated *via* unequal crossing-over between two different but partially homologous SINEs located in introns 8 and 10 of the *F5* gene. Remarkably, a similar mechanism (homologous recombination between two AluY repeats) was also proposed to underlie the previously described deletion of *F5* exons 1-7.[5] In fact, the role of transposable elements in mediating unequal crossing-overs and generating large deletions or duplications is well documented.[15] For example, the relatively high frequency of large deletions observed in the antithrombin gene (*SERPINC1*) has been attributed to the large number of Alu repeats present in this gene.[16]

Gross gene rearrangements are believed to play a major role in the aetiology of human diseases, but tend to be under-explored because of the lack of straightforward techniques for their detection. The development of commercial MLPA assays for several coagulation-related genes has led to the identification of large deletions/duplications in patients with various coagulation disorders, including haemophilia A[17-19] and B,[20] von Willebrand disease[21,22] and the deficiencies of antithrombin,[16,23-25] protein C[25] and protein S.[25-30] Although gross rearrangements of the *F5* gene may also be more prevalent than previously suspected, only one of the 14 genetically unexplained FV-deficient patients investigated in our study turned out to carry a large deletion. Possible causes of FV deficiency in the other patients, which had already been screened for mutations in the *F5* coding region and splicing junctions, may be apparently synonymous mutations affecting splicing,[31] deep-intronic splicing mutations,[9] mutations in regulatory portions of the gene (promoter, 3'-untranslated region) and perhaps extragenic mutations.

Conclusion

We have designed and validated an in-house MLPA assay for the detection of gross *F5* gene rearrangements. This assay may represent a valuable tool for the molecular diagnosis of FV deficiency.

Acknowledgements

The authors wish to thank Ms. Widad Rifi from MRC-Holland for helpful suggestions. This study was supported by a VIDI grant (nr. 917-76-312, to E. Castoldi) from the Dutch Organisation for Scientific Research (NWO).

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4

Novel *F5* splicing mutation in a factor V-deficient patient with multiple intracranial haemorrhages

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Preliminary report

Abstract

Coagulation factor V (FV) deficiency is a rare autosomal recessive bleeding disorder caused by mutations in the *F5* gene. Here, we report the identification of a novel homozygous splicing mutation (*F5* c.373+2T>C) in a FV-deficient patient with a particularly severe bleeding phenotype. Since this variant affects the conserved +2 position of the donor splice site of intron 3, it is likely to impair *F5* pre-mRNA splicing by causing exon 3 skipping and/or the activation of a nearby cryptic splice site. However, analysis of the patient's *F5* cDNA could not be performed (yet). Correct splicing might be restored using a specifically modified U1 small nuclear RNA.

Note: The *F5* gene mutation described in this chapter is annotated according to the HGVS recommendations. The corresponding annotation according to the classical nomenclature (Jenny *et al*. 1987) is as follows:

HGVS nomenclature	**Classical nomenclature**
c.373+2T>C	IVS3+2T>C

Coagulation factor V (FV) deficiency[1,2] is a rare autosomal recessive bleeding disorder caused by deleterious mutations in the *F5* gene, which spans ~80 kb on the long arm of chromosome 1 and contains 25 exons.[3] Symptoms range from mucosal and post-traumatic bleeding, which are present in virtually all patients, to life-threatening intracranial and gastro-intestinal hemorrhages, which are rare and confined to the most severe cases.[4]

FV-deficient patients show a large genetic heterogeneity, each family having its own private mutation. In fact, >100 different *F5* mutations have been described to date, the majority of which are missense or nonsense variants. Splicing mutations represent only ~10% of the *F5* mutational spectrum, but they tend to be associated with severe bleeding manifestations.[5-11]

In this study we investigated the molecular basis of FV deficiency in an 18-month-old Omani girl (FV:C <1%) that came to clinical attention because of multiple intracranial hemorrhages in the first days of life. Despite immediate prophylaxis with fresh frozen plasma twice weekly, she experienced an additional intracranial bleeding while on prophylaxis. Since then, her prophylactic regimen has been upgraded to three fresh frozen plasma infusions per week. However, this could not prevent global developmental delay as a result of the previous bleeding events. Her parents are consanguineous (Figure 1).

After obtaining informed consent, blood was collected from the proband and her family members at Sultan Qaboos University Hospital (Muscat, Sultanate of Oman) in compliance with local ethical regulations. Citrated plasma and genomic DNA were shipped to Maastricht for further analysis. The study was conducted according to the Helsinki Declaration.

Direct sequencing of the proband's *F5* coding region and splicing junctions detected a novel homozygous mutation (*F5* c.373+2T>C), affecting the second nucleotide of intron 3 (Figure 1) and therefore likely to impair pre-mRNA splicing. Both parents (I-1 and I-2) and the proband's eldest sister (II-1) were heterozygous for the same mutation, whereas the

proband's other sister (II-2) did not carry the mutation. The observed *F5* c.373+2T>C genotypes were consistent with the plasma FV antigen and activity levels determined by ELISA (Zymutest Factor V ELISA kit, NODIA, Amsterdam, The Netherlands) and by a sensitive prothrombinase-based assay, respectively (Figure 1). In particular, hardly any FV antigen and no FV activity could be detected in the proband's plasma.

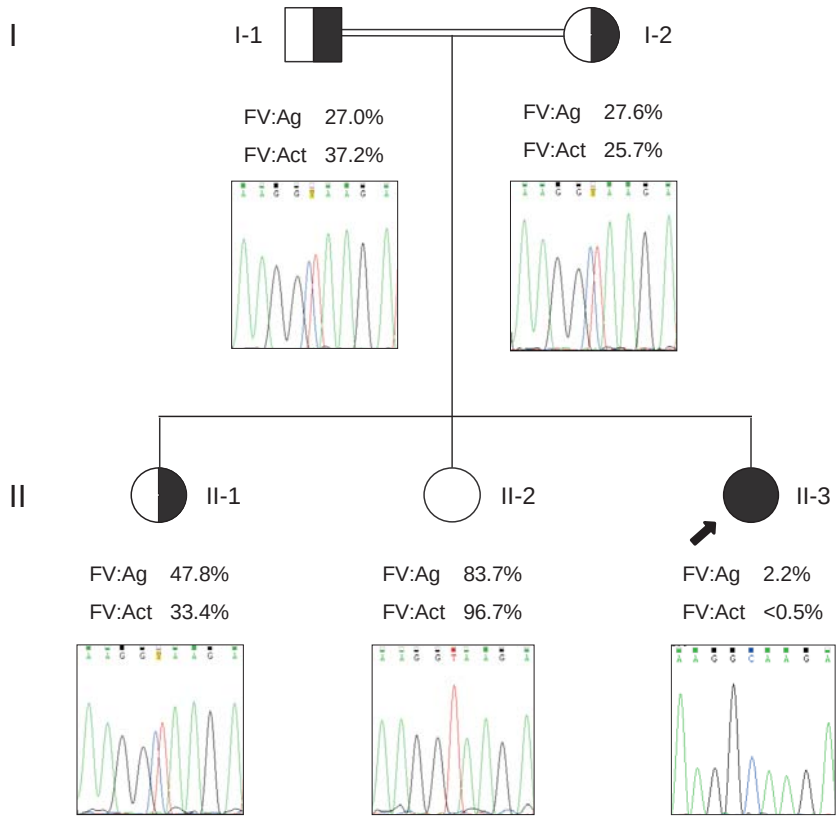

Figure 1. Pedigree of the FV-deficient family, *F5* genetic analysis and FV levels.
The proband is marked by an arrow. Carriership of the *F5* c.373+2T>C mutation, as illustrated by the sequencing chromatograms of the *F5* exon 3/intron 3 junction, is indicated by half-closed (heterozygosity) or closed (homozygosity) symbols. Plasma FV antigen (FV:Ag) and activity (FV:Act) levels are reported below each subject.

The *F5* c.373+2T>C mutation affects positon +2 of the canonical donor splice site of intron 3, reducing its complementarity to the U1 small nuclear RNA (U1snRNA), which normally recognizes the donor splice site during pre-mRNA splicing. *In silico* analysis indicated that the mutation reduces the donor splice site consensus score from 0.99 to undetectable (according to the NNsplice software) or from 0.96 to 0.69 (according to the Human Splicing Finder software) (Figure 2A).

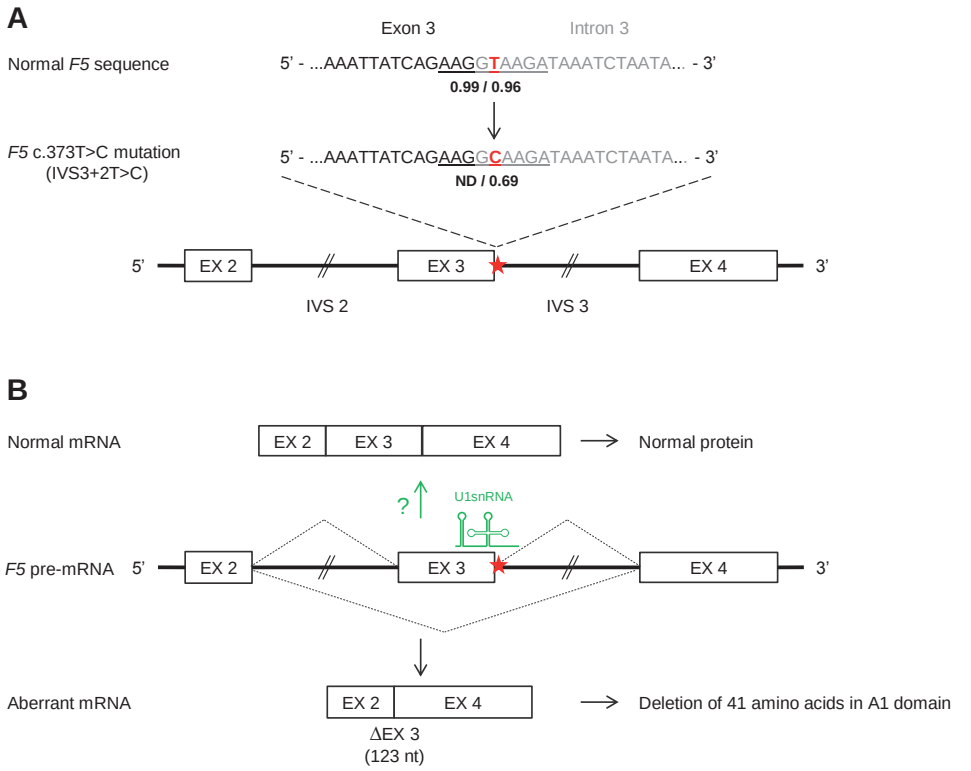

Figure 2. *In silico* analysis of the *F5* c.373+2T>C mutation and possible splicing correction strategy.

A) Schematic representation of the genomic region surrounding the *F5* c.373+2T>C mutation (in red). The donor splice site consensus scores of the normal and mutated sequences, provided by the NNsplice/Human Splicing Finder softwares, are indicated below each sequence. ND, not detectable.

B) Predicted effect of the *F5* c.373+2T>C mutation on pre-mRNA splicing (exon 3 skipping) and possible strategy to restore correct splicing using an engineered U1snRNA (in green) specifically modified to recognise the mutated donor splice site.

Since position +2 is part of the absolutely conserved GT dinucleotide of the donor splice site consensus sequence (MAG/GTRAGT),[12] the mutation is predicted to disrupt *F5* pre-mRNA splicing. Abolition of a canonical donor splice site most often results in skipping of the preceding exon, unless nearby cryptic splice sites can take over the donor splice site function.[13,14] Accordingly, the most likely consequence of the *F5* c.373+2T>C mutation is skipping of exon 3 (123 nt), also in view of the fact that this alternative splicing event occurs in normal individuals as well.[9] The resulting mRNA would maintain the correct reading frame, but it would encode a mutant FV lacking 41 amino acids in the A1 domain (Figure 2B), which is unlikely to be correctly folded and secreted. Alternatively, activation of a pre-existing cryptic splice site located upstream or downstream of the mutated splice site would result in partial deletion of exon 3 or partial retention of intron 3, probably leading to a frameshift and premature stop codon. Unfortunately, however, we have not (yet) been able to obtain a new blood sample from the proband for *F5* cDNA analysis. On the other hand, cloning of a *F5* minigene suitable to study the effects of the c.373+2T>C mutation on pre-mRNA splicing is hampered by the large size of *F5* intron 3 (>11 kb).

The proband's extremely severe clinical phenotype, requiring life-long prophylaxis with fresh frozen plasma three times a week (with consequent circulatory overload), justifies the search for alternative therapeutic options. Since the mutation disrupts a donor splice site, it might be targeted with antisense therapy using an engineered U1snRNA specifically modified to perfectly match the mutated splice site and thereby restore correct splicing (Figure 2B). The feasibility of this approach has been already shown for other splicing mutations responsible for coagulation factor deficiencies.[15-17] However, mutations affecting position +2 of the donor splice site tend to be resistant to U1snRNA-mediated splicing correction.[16,18]

In conclusion, this preliminary study confirms the association between *F5* splicing mutations and severe clinical manifestations of FV deficiency. The exact impact of the *F5* c.373+2T>C mutation on pre-mRNA splicing awaits experimental investigation by *F5* cDNA analysis in the proband and/or in a minigene model. The latter would also offer the possibility to test the efficacy of therapeutic strategies based on engineered U1snRNA.

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5

Characterisation of an apparently synonymous *F5* mutation causing aberrant splicing and factor V deficiency

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Abstract

Coagulation factor V (FV) deficiency is a rare autosomal recessive bleeding disorder. We investigated a patient with severe FV deficiency (FV:C <3%) and moderate bleeding symptoms. Thrombin generation experiments showed residual FV expression in the patient's plasma, which was quantified as 0.7 ± 0.3% by a sensitive prothrombinase-based assay. *F5* gene sequencing identified a novel missense mutation in exon 4 (c.578G>C, p.Cys193Ser), predicting the abolition of a conserved disulphide bridge, and an apparently synonymous variant in exon 8 (c.1281C>G). The observation that half of the patient's *F5* mRNA lacked the last 18 nucleotides of exon 8 prompted us to re-evaluate the c.1281C>G variant for its possible effects on splicing. Bioinformatics sequence analysis predicted that this transversion would activate a cryptic donor splice site and abolish an exonic splicing enhancer. Characterization in a *F5* minigene model confirmed that the c.1281C>G variant was responsible for the patient's splicing defect, which could be partially corrected by a mutation-specific morpholino antisense oligonucleotide. The aberrantly spliced *F5* mRNA, whose stability was similar to that of the normal mRNA, encoded a putative FV mutant lacking amino acids 427-432. Expression in COS-1 cells indicated that the mutant protein is poorly secreted and not functional. In conclusion, the c.1281C>G mutation, which was predicted to be translationally silent and hence neutral, causes FV deficiency by impairing pre-mRNA splicing. This finding underscores the importance of cDNA analysis for the correct assessment of exonic mutations.

Note: The *F5* gene mutations described in this chapter are annotated according to the HGVS recommendations. The corresponding annotation according to the classical nomenclature (Jenny *et al*. 1987) is as follows:

HGVS nomenclature	**Classical nomenclature**
c.578G>C (p.Cys193Ser)	668G>C (Cys165Ser)
c.1281C>G (p.Val427_Lys432del)	1371C>G (Deletion spans amino acids 399-404)
c.1285G>T	1375G>T

Introduction

Factor V (FV)[1,2] is an essential clotting factor present in plasma (~80%) and platelets (~20%). It is produced in the liver and secreted in plasma as a single-chain protein with the same domain structure as factor VIII (A1-A2-B-A3-C1-C2). Limited proteolysis by thrombin or factor Xa converts FV to its active form (FVa), which acts as a non-enzymatic cofactor in prothrombin activation and accelerates this reaction >1000-fold. FVa activity is down-regulated by activated protein C (APC)-mediated proteolysis of the heavy chain. Experiments in mice have shown that the complete absence of FV is not compatible with life,[3] but traces of FV are sufficient to rescue the perinatal lethality of $FV^{-/-}$ mice.[4]

In humans, FV deficiency is a rare autosomal recessive bleeding disorder.[5-7] The most common symptoms are mucosal and post-traumatic bleeding, while haemarthroses and muscle haematomas are less frequently observed and life-threatening haemorrhages are rare.[8] Residual platelet FV is a better predictor of clinical severity than plasma FV[9-11] and plasma tissue factor pathway inhibitor (TFPI) levels may act as an additional phenotype modulator.[12]

FV deficiency is caused by *F5* gene mutations, of which ~150 have been described to date.[13,14] Splicing mutations currently represent only ~10% of the *F5* mutational spectrum, but their proportion is likely to be underestimated, because many splicing mutations fail to be identified. In fact, deep-intronic mutations that activate intronic pseudo-exons[15] are not detected by conventional mutation screening strategies, which focus on the coding region of the gene. Moreover, exonic mutations that are translationally silent or predict a conservative amino acid change are usually dismissed as neutral or benign, whereas they might activate cryptic splice sites or alter splicing regulatory sequences, such as exonic splicing enhancers

(ESEs) and silencers (ESSs).[16-18] Such splicing defects can only be detected by cDNA analysis, which is however not routinely performed.

Here we report an apparently synonymous *F5* variant which, far from being neutral, causes aberrant splicing and virtually abolishes protein expression.

Materials and Methods

Blood collection and work-up

The study was approved by the Human Subjects Committee of the University of North Carolina and was conducted in accordance with the Declaration of Helsinki. After obtaining the patient's informed consent, blood was drawn in 3.2% citrate (9/1, vol/vol) and centrifuged at 2500*g* for 15 minutes to prepare platelet-poor plasma, which was stored at -80°C.

Thrombin generation

Thrombin generation was measured in platelet-poor plasma with the Calibrated Automated Thrombogram (CAT) method,[19] essentially as described.[12]

Measurement of FV levels

Plasma FV antigen level was measured using an in-house ELISA based on commercial polyclonal antibodies (Affinity Biologicals, Ancaster, ON, Canada). Plasma FV activity was measured with a prothrombinase-based assay, as described.[12] The assay was performed in 8-fold on independent plasma dilutions and calibrated with pooled normal plasma. The detection limit of this assay in plasma is ~0.5%.[12]

Genomic DNA analysis

Genomic DNA was isolated from peripheral blood leukocytes by salting-out. All 25 exons and splicing junctions of the *F5* gene were amplified and sequenced as previously described.[11]

***F5* cDNA analysis**

Blood was collected from the patient and two normal controls in PAXgene Blood RNA tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland). Total RNA was isolated using TRIzol Reagent (Invitrogen, Breda, The Netherlands) and reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Bedford, MA, USA), as described before.[11] The *F5* cDNA was amplified in 20 overlapping fragments, each spanning at least one splicing junction, and analysed by agarose gel electrophoresis. *F5* cDNA fragments spanning exons 8-10 were excised from gel, purified with the QIAquick Gel Extraction Kit (Qiagen, Venlo, The Netherlands) and sequenced.

***In silico* sequence analysis**

The impact of the *F5* c.1281C>G mutation on splice site and regulatory sequences was investigated *in silico* using NNSPLICE version 0.9 (http://www.fruitfly.org/seq_tools/splice.html), Human Splicing Finder (http://www.umd.be/HSF/)[20] and ESE Finder 3.0 (http://rulai.cshl.edu/tools/ESE/).[21]

***F5* minigene model**

The effects of the *F5* c.1281C>G (natural) and 1285G>T (control) mutations on pre-mRNA splicing were studied using a minigene model, as previously described.[22] To this end, a wild-

type genomic fragment encompassing *F5* exon 8, intron 8 and exon 9 (g.40512-43071) was cloned within the *FN1* intron of the pTB vector. The c.1281C>G and c.1285G>T mutations were separately introduced in the wild-type construct by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies, Amstelveen, The Netherlands) and the sequences of all three constructs were verified by direct sequencing.

COS-1 and HepG2 cells were cultured as described before.[22] Cells were seeded in 6-well plates and transiently transfected with 3 μg of wild-type or mutant *F5* minigene construct using Lipofectamine[2000] (Invitrogen). After 48 hours, cells were harvested in TRIzol Reagent (Invitrogen) and total RNA was isolated and reverse-transcribed. *F5* minigene mRNA was specifically amplified using primers located in *F5* exon 8 and in the distal *FN1* exon of the pTB vector, and the splicing pattern was analysed by agarose gel electrophoresis.

A 25-mer phosphorodiamidate morpholino oligonucleotide (PMO) complementary to the *F5* pre-mRNA bearing the c.1281C>G mutation and covering the mutation site and the putative ESE element (5'-TGTGTCTCTCACCTGGGCTCTGATA-3', g.40867-40891, mutation site underlined) was obtained from GeneTools (Philomath, OR, USA). The ability of this antisense PMO ("rescuer") to correct the splicing defect induced by the c.1281C>G mutation was tested in COS-1 and HepG2 cells transfected with the *F5* 1281G minigene construct. A similar PMO with an irrelevant sequence ("control") was used as a negative control. PMOs (0-20 μM) were administered to cells using EndoPorter Reagent in aqueous formulation (6 μM), essentially as described.[22] After 48 hours, cells were harvested in TRIzol Reagent and mRNA was isolated, reverse-transcribed and analysed by PCR and agarose gel electrophoresis.

Recombinant FV expression

The c.578G>C mutation and the deletion of codons 427-432 were introduced in the pMT2/FV construct by site-directed mutagenesis. Successful mutagenesis was confirmed by direct sequencing. COS-1 cells were seeded in 6-well plates and transiently transfected with 3 μg of wild-type or mutant pMT2/FV construct using Lipofectamine^{2000} (Invitrogen). Conditioned media were collected 48 hours post-transfection, centrifuged at 2000*g* for 10 minutes and analysed for FV antigen (ELISA) and activity (prothrombinase-based assay). FV antigen and activity levels from quadruplicate transfections were averaged and mock medium was used as a negative control. Control experiments in which known amounts of purified FV were added to mock medium indicated that the prothrombinase-based test can be reliably performed on the background of (conditioned) medium.

Results

Case history

The patient is a 20-year-old male with severe FV deficiency (FV:C <3%). Because he was adopted, little is known about his early medical history. The diagnosis of FV deficiency was made at the age of 15 years, when he presented with prolonged epistaxis. Subsequently, he was treated with fresh frozen plasma for several muscle bleeds induced by mild trauma.

The patient had not received any fresh frozen plasma or platelet concentrates in the three months preceding blood collection for the present study.

Thrombin generation and FV levels

To assess the patient's overall coagulation potential, thrombin generation was measured in the patient's plasma and control plasma triggered with 1-50 pM TF (Figure 1A,B). Thrombin generation in the patient's plasma was detectable at all TF concentrations, suggesting that the patient has residual FV expression. To prove this, thrombin generation was re-measured at 10 pM TF in the absence and presence of increasing concentrations of APC (Figure 1C,D), the specific inhibitor of FVa. APC progressively reduced thrombin generation in control plasma and completely abolished thrombin generation in the patient's plasma, indicating that the thrombin generation observed in the absence of APC was indeed supported by FV.

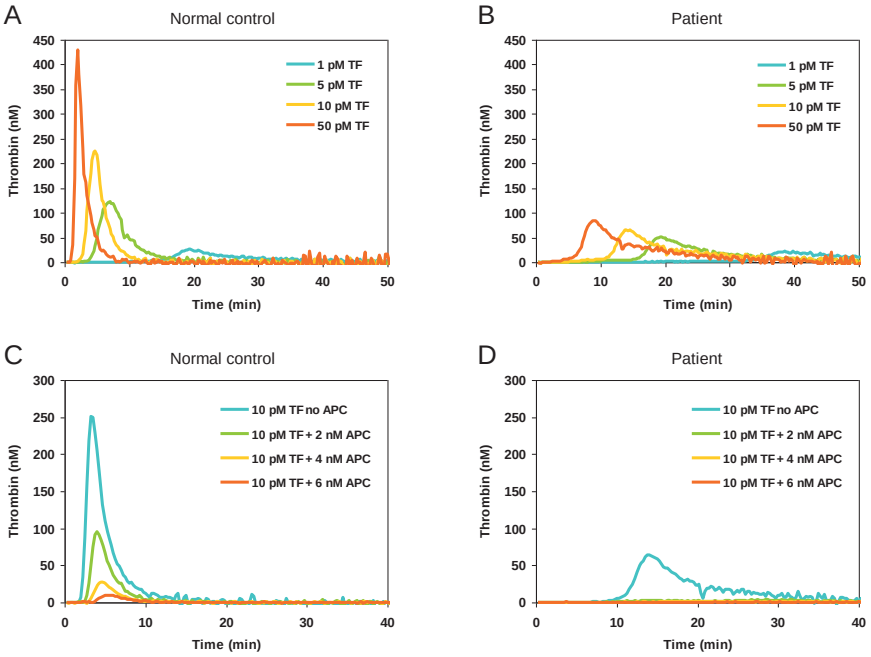

Figure 1. Thrombin generation in patient's plasma.
Platelet-poor plasma from a normal control (A,C) and from the FV-deficient patient (B,D) was activated with the indicated concentrations of TF and thrombin generation was measured by Calibrated Automated Thrombography. A,B: TF-titration (1-50 pM) of thrombin generation in the absence of APC. C,D: APC-titration (0-6 nM) of thrombin generation triggered with 10 pM TF.

The FV antigen level in the patient's plasma was 0.4%. The FV activity level, as determined in 8-fold using a very sensitive prothrombinase-based assay in highly diluted plasma, was found to be significantly different from the blank and quantified as 0.7±0.3% of normal plasma.

Genetic analysis

F5 mutation screening identified two novel heterozygous transversions in the patient's DNA: c.578G>C in exon 4 and c.1281C>G in exon 8. The former predicts the substitution of Cys^{193} by a Ser and consequent destruction of a disulfide bridge (Cys^{167}-Cys^{193}) within the A1 domain of FV. The latter does not predict an amino acid change (Val427Val) and is located far from splicing junctions. Therefore, it was initially regarded as a rare neutral variant.

Since the p.Cys193Ser mutation alone could not explain the patient's barely detectable FV levels, *F5* cDNA analysis was performed to check for possible splicing defects. Amplification of a *F5* cDNA fragment spanning exons 8-10 yielded only the expected 269-bp product in two normal controls, but an additional - smaller - product in the patient (Figure 2), suggesting that half of the patient's *F5* mRNA was spliced incorrectly in this region. Sequencing of the aberrant cDNA fragment indicated that this lacked the last 18 nucleotides of exon 8 (Figure 2). As judged from the intensity of the gel bands, this in-frame deletion did not compromise the stability of the aberrantly spliced mRNA.

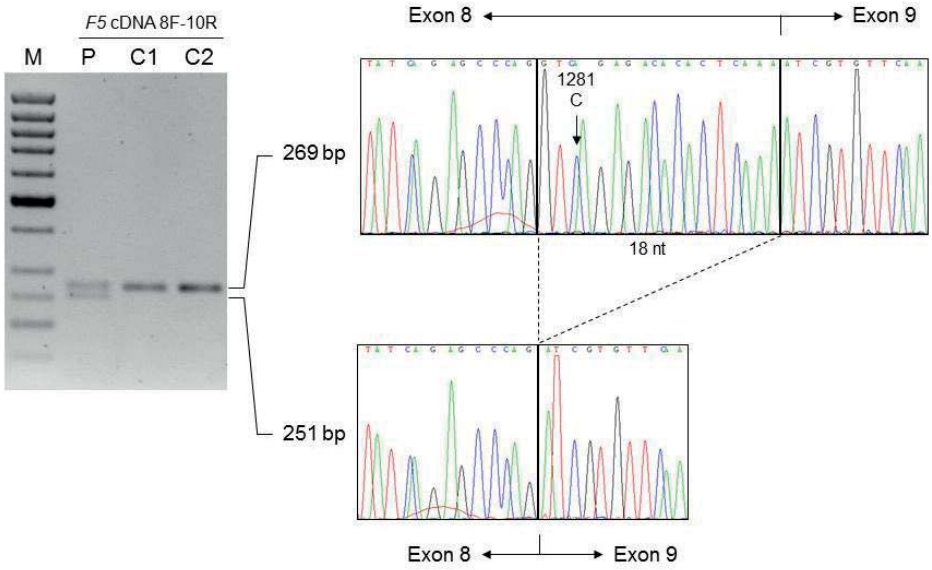

Figure 2. *F5* cDNA analysis.
Total RNA was isolated from the patient's blood cells and reverse-transcribed to cDNA. A *F5* cDNA fragment spanning exons 8-10 was amplified in the patient (P) and in two normal controls (C1, C2) and analysed by agarose gel electrophoresis (left) and direct sequencing (right). M,molecular weight marker. The patient's aberrantly spliced cDNA lacks the last 18 nucleotides (nt) of exon 8. Noteworthy, the 1281 position is normal (C) in the correctly spliced transcript and falls in the deleted region in the aberrantly spliced transcript.

Bioinformatics sequence analysis

F5 exon 8 was analysed *in silico* for the presence of splice site consensus and regulatory sequences, and the c.1281C>G mutation was re-evaluated for its possible impact on pre-mRNA splicing (Figure 3A). This analysis showed that the c.1281C>G mutation activates a cryptic donor splice site in exon 8, increasing its consensus score from 0.81 in the absence of the mutation to 0.97 in the presence of the mutation (NNSPLICE software) or from 92.64 to 96.51 (Human Splicing Finder software). Due to its high consensus score, this splice site may effectively compete with the canonical donor splice site (consensus score of 0.98 and 88.47 according to the NNSPLICE and Human Splicing Finder softwares, respectively). In

addition, the c.1281C>G mutation destroys a putative ESE sequence recognised by the trans-acting splicing factor SRSF2 (consensus motif: GRYYcSYR), which might contribute to define exon 8. As a consequence, in the presence of the mutation, the cryptic splice site is predicted to become the preferred donor splice site for intron 8, resulting in the deletion of 18 nucleotides from the mature mRNA.

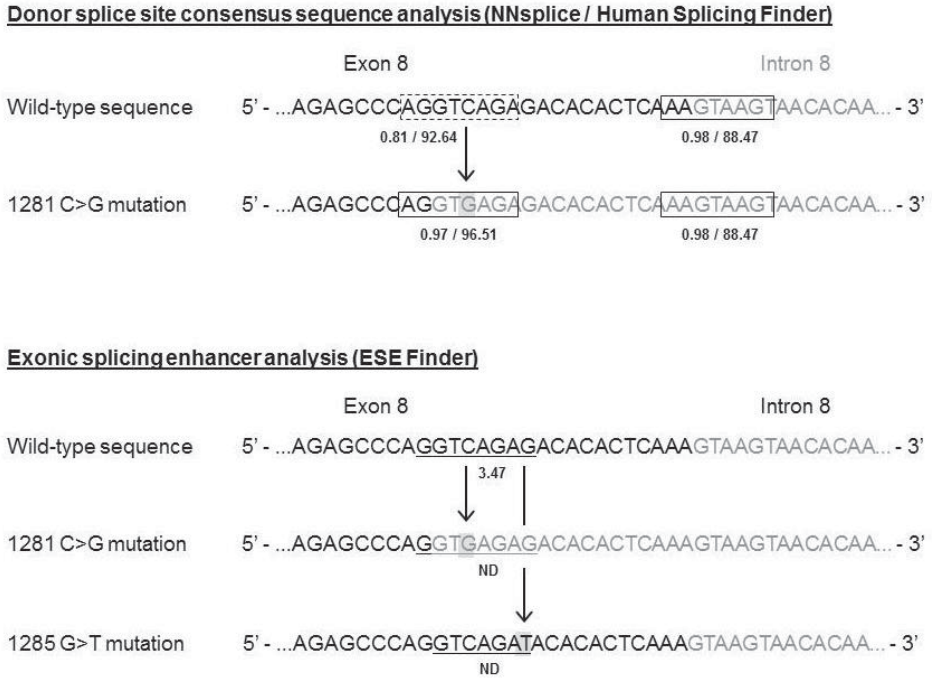

Figure 3A. Bioinformatic analysis of the c.1281C>G and c.1285G>T mutations.
In silico analysis of the *F5* sequence surrounding the c.1281C>G mutation for the presence of splice site consensus sequences (top) and splicing regulatory elements (bottom). Identified donor splice site consensus sequences are boxed and the corresponding consensus scores (provided by the NNSPLICE / Human Splicing Finder softwares) are reported underneath. The cryptic donor splice site present in the wild-type sequence is boxed with a dashed line. The putative exonic splicing enhancer (ESE) element is underlined and its score (provided by the ESE Finder software by comparison with the consensus sequence GRYYcSYR) is reported underneath. ND, not detectable (*i.e.* below the threshold of 2.383). Exonic sequence is shown in black, intronic sequence in grey. Mutated nucleotides are shaded in light grey.

Characterisation of the c.1281C>G mutation in a minigene model

To prove that the c.1281C>G mutation causes the splicing abnormality observed in the patient's *F5* cDNA, we transfected COS-1 and HepG2 cells with *F5* minigene constructs containing exon 8 (without or with the c.1281C>G mutation), intron 8 and exon 9, and analysed the splicing pattern of the transcribed mRNA. While the wild-type minigene produced only the correctly spliced mRNA, the 1281G minigene produced predominantly aberrantly spliced mRNA, lacking the last 18 nucleotides of *F5* exon 8 (Figure 3B), which indicates that the c.1281C>G mutation is indeed responsible for the splicing defect observed in the patient's *F5* mRNA. Remarkably, very little residual correct splicing was observed in the presence of the c.1281C>G mutation, despite the fact that the canonical donor splice site is still intact. This suggested that disruption of the ESE sequence might also contribute to the shift in splicing pattern induced by the c.1281C>G mutation. To verify whether the ESE element is required for the recognition of the canonical donor splice site, we devised a control mutation (c.1285G>T) which destroys the ESE element (Figure 3A) without creating an alternative donor splice site or altering the consensus score of the canonical donor splice site. When this mutation was introduced in the wild-type *F5* minigene and expressed in COS-1 or HepG2 cells, only correctly spliced mRNA was observed (Figure 3B), indicating that the ESE element is unlikely to contribute to exon 8 definition.

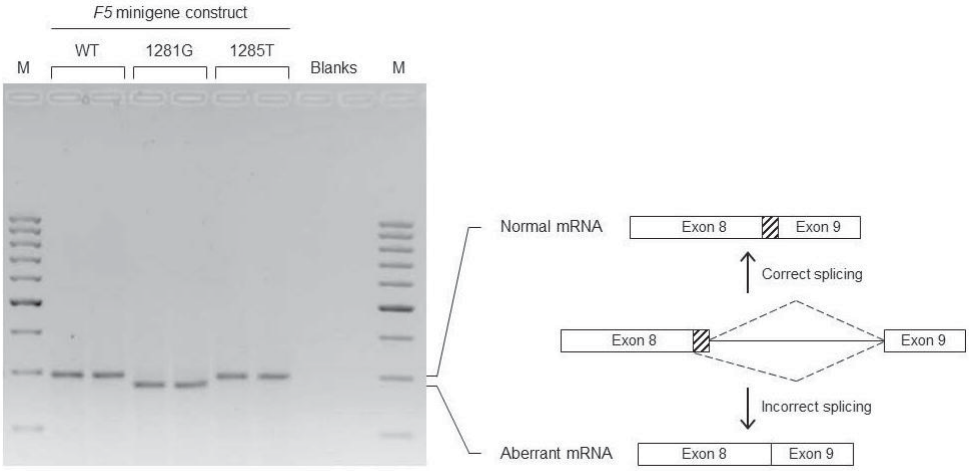

Figure 3B. Splicing assays of the c.1281C>G and c.1285G>T mutations.
HepG2 cells were transfected with the *F5* wild-type (WT), 1281G or 1285T minigene constructs, and output mRNA was analysed 48 hours post-transfection. Results of duplicate transfections are shown. Similar results were obtained in COS-1 cells (not shown). Blanks, reverse-transcription blank and PCR blank. M, molecular weight marker. In the scheme, the sequence of 18 nucleotides that is deleted in the aberrantly spliced mRNA is hatched.

Splicing defects can be corrected by antisense-based RNA therapy,[23,24] which makes use of specific antisense oligonucleotides to hide incorrect splicing signals on the pre-mRNA. To test whether the *F5* c.1281C>G mutation is amenable to antisense-based therapy, cells transfected with the *F5* 1281G minigene construct were treated with a mutation-specific (rescuer) PMO (Figure 4). Increasing concentrations of this PMO caused progressive normalisation of the splicing pattern, reaching a correction level of ~50% at 20 μM rescuer PMO (Figure 4). In contrast, no correction of the splicing defect was observed with the same concentration of control PMO (Figure 4).

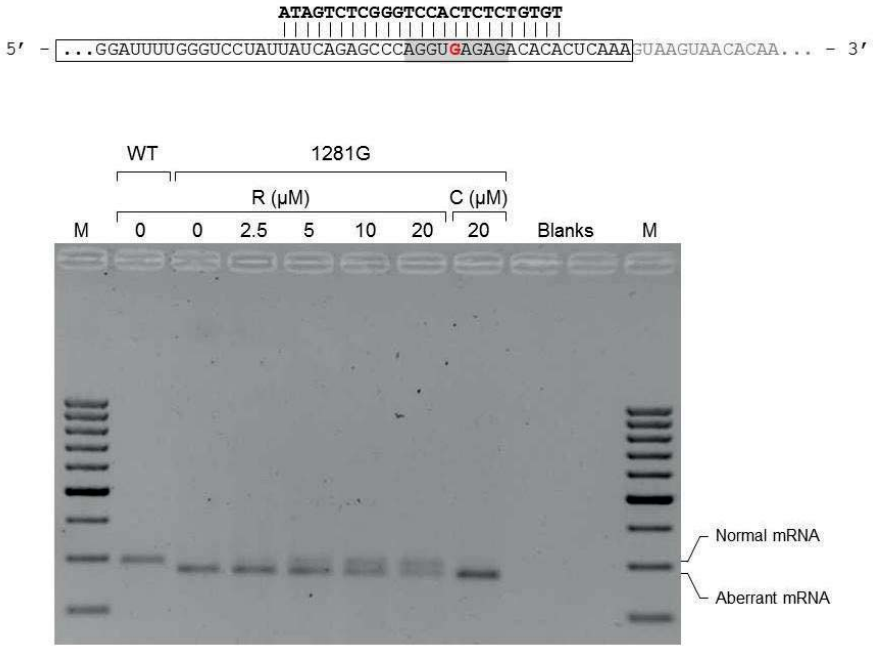

Figure 4. Correction of the splicing pattern by phosphorodiamidate morpholino oligonucleotides (PMO).

Top: Schematic representation of the mutant *F5* pre-mRNA, including the distal portion of exon 8 (boxed black sequence) with the c.1281C>G mutation (red) and the proximal portion of intron 8 (grey sequence). The nucleotides that are part of the cryptic splice site and/or ESE consensus sequences are highlighted in grey. The rescuer PMO (boldface sequence) is shown hybridised to the pre-mRNA.

Bottom: HepG2 cells were transfected with the *F5* wild-type (WT) or 1281G minigene constructs and treated with the indicated concentrations of rescuer (R) or control (C) PMO, and output mRNA was analysed 48 hours post-transfection. Similar results were obtained in COS-1 cells (not shown). Blanks, reverse-transcription blank and PCR blank. M, molecular weight marker.

Expression and characterisation of FV mutants

The patient's aberrantly spliced *F5* mRNA lacks the last 18 nucleotides of exon 8, corresponding to the codons for amino acids 427-432 in the A2 domain of FV. Since this deletion does not affect the reading frame, it does not target the mRNA for degradation by

the nonsense-mediated decay pathway,[25] allowing for the possibility that the aberrant mRNA is translated into a protein, which however may not be stable and/or secretable. To check whether the mutant protein is expressed at all, a pMT2/FV construct lacking codons 427-432 was created and expressed in COS-1 cells. Analysis of the conditioned media revealed some FV antigen (11.6±1.6% of wild-type FV, $p<0.001$ *vs.* mock medium) but no FV activity (0.18±0.02% of wild-type FV, p=n.s. *vs.* mock medium), indicating that the aberrantly spliced *F5* mRNA does not encode a functional protein.

Finally, the FV p.Cys193Ser mutant was also expressed as a recombinant protein. The FV antigen level in the conditioned media was 2.6±0.3% ($p<0.001$ *vs.* mock medium), whereas FV activity was barely detectable (0.32±0.09% of wild-type FV, p=0.017 *vs.* mock medium).

Discussion

Patients with plasma FV levels in the 0-5% range are expected to be homozygous or compound heterozygous for *F5* mutations, but 10-20% of cases remain genetically unexplained.[26] Failure to identify one or both mutations by direct sequencing of the coding region may signify the presence of gross gene rearrangements[26,27] or deep-intronic splicing mutations,[11] which are not detected by conventional mutation screening strategies. Alternatively, it may be due to misclassification of exonic variants, which are typically assessed only on the basis of their predicted effects on translation, whereas they may just as well impair pre-mRNA splicing, as recently shown for missense mutations in the *FGB* and *F11* genes[28,29] as well as for a synonymous mutation in the *LMAN1* gene.[30] In fact, evidence is accumulating that synonymous variants significantly contribute to human disease by affecting not only pre-mRNA splicing, but also mRNA stability and translation efficiency.[31]

In this study, we present a patient with hardly detectable FV levels and apparently only one detrimental *F5* mutation (c.578G>C, predicting the p.Cys193Ser substitution in the A1 domain). Cys^{193} is evolutionarily conserved from zebrafish to human, as well as in the A1 domains of factor VIII and ceruloplasmin, and forms an intra-domain disulfide bridge with equally conserved Cys^{167}.[32] Abolition of this disulfide bridge by the p.Cys193Ser mutation is likely to impair folding of the A1 domain and to compromise the stability and/or secretion of the mutant FV, as confirmed by the expression experiments.

The second *F5* variant identified in the patient (c.1281C>G in exon 8) was initially considered a rare neutral polymorphism, because it does not predict an amino acid change and it is located far from splicing junctions. However, a closer examination led us to establish that the *F5* c.1281C>G mutation actually affects pre-mRNA splicing, based on the following evidence: a) the observation that half of the patient's *F5* mRNA lacks the last 18 nucleotides of exon 8, while this deletion is not present in the patient's genomic DNA; b) the *in silico* prediction that the c.1281C>G mutation activates a cryptic donor splice site (and abolishes a putative ESE element) in exon 8; c) the finding that cells transfected with the *F5* 1281G minigene construct produce almost exclusively aberrantly spliced mRNA; and d) the ability of a mutation-specific antisense PMO to restore correct splicing of the mutant pre-mRNA. In contrast to the deep-intronic mutation that we targeted in a previous study,[22] the c.1281C>G mutation is located in an exon and the corresponding antisense PMO covers 25 nucleotides of exonic sequence and extends up to 6 nucleotides from the canonical donor splice site. This proximity may explain the incomplete correction of the splicing pattern even at relatively high PMO concentrations (20 μM), as observed for similar exonic mutations in the *ATM* gene.[33] Nonetheless, antisense-based RNA therapy might represent an alternative therapeutic option for this patient. The fact that disruption of the ESE sequence (as in the *F5*

1285T minigene) or its masking by the rescuer PMO is compatible with correct splicing suggests that this element is not required for recognition of the canonical donor splice site. Alternatively, the spliceosome and the splicing factor SRSF2 may compete for binding to the overlapping cryptic splice site/ESE sequences and the c.1281C>G mutation might tip the balance in favour of the spliceosome.

Since deletion of the last 18 nucleotides of exon 8 did not alter its reading frame or stability, the aberrantly spliced *F5* mRNA could be theoretically translated into a viable protein lacking amino acid residues 427-432 in the A2 domain. However, expression of the aberrantly spliced mRNA in COS-1 cells resulted in poor protein secretion and undetectable FV activity in conditioned media, suggesting that the deleted protein is grossly misfolded and hence largely retained/degraded within the cell. In addition, these findings confirm that the c.1281C>G mutation, which causes the splicing aberration, is the second mutation responsible for the patient's FV deficiency.

Conclusion

The FV-deficient patient investigated here is doubly heterozygous for the *F5* c.578G>C (p.Cys193Ser) and c.1281C>G (splicing) mutations, which are both causative and together explain the barely detectable FV level in the patient's plasma. Residual FV expression in the patient (<1%) is most probably attributable to a small percentage of normal splicing of the 1281G allele. Given the extremely low FV requirement for minimal haemostasis,[4,10,34] residual FV expression, in combination with the low plasma level of full-length TFPI (35.5% of pooled normal plasma, data not shown),[12] explains the comparatively moderate bleeding tendency of this patient.

Overall, our findings illustrate how an exonic variant, predicted to be translationally silent and hence neutral, can actually be detrimental by disrupting the splicing code and impairing pre-mRNA processing. This underscores the importance of cDNA analysis for the correct assessment of exonic mutations and may have implications for the annotation of exonic variants identified by next-generation sequencing.

Acknowledgements

The authors wish to thank the patient and his adoptive family for their willingness to participate in this study. In addition they thank Dr. M. Baralle from the International Center for Genetic Engineering and Biotechnology (Trieste, Italy) for providing the pTB vector and Prof. F. Bernardi from Ferrara University (Italy) for providing the pMT2/FV construct. Prof. T.M. Hackeng is gratefully acknowledged for critically reading the manuscript. This study was supported by a VIDI grant (nr. 917-76-312 to EC) from the Dutch Organisation for Scientific Research (NWO).

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6

Antisense-based RNA therapy of factor V deficiency: *in vitro* and *ex vivo* rescue of a *F5* deep-intronic splicing mutation

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Blood 2013; 122: 3825-3831

Abstract

Antisense molecules are emerging as a powerful tool to correct splicing defects. Recently, we identified a homozygous deep-intronic mutation (*F5* c.1296+268A>G) activating a cryptic donor splice site in a patient with severe coagulation factor V (FV) deficiency and multiple life-threatening bleeding episodes. Here we assessed the ability of two mutation-specific antisense molecules (a morpholino oligonucleotide (MO) and an engineered U7snRNA) to correct this splicing defect. COS-1 and HepG2 cells transfected with a *F5* minigene construct containing the patient's mutation expressed aberrant mRNA in excess of normal mRNA. Treatment with mutation-specific antisense MO (1-5 μM) or a construct expressing antisense U7snRNA (0.25-2 μg) dose-dependently increased the relative amount of correctly spliced mRNA up to ~30-fold and ~100-fold, respectively, whereas control MO and U7snRNA with irrelevant sequences were ineffective. Patient-derived megakaryocytes obtained by differentiation of circulating progenitor cells did not express FV, but became positive for FV at immunofluorescence staining after administration of antisense MO or U7snRNA. However, treatment adversely affected cell viability, mainly because of the transfection reagents used to deliver the antisense molecules. Our data provide *in vitro* and *ex vivo* proof-of-principle for the efficacy of RNA therapy in severe FV deficiency, but additional cytotoxicity studies are warranted.

Note: The *F5* gene mutation described in this chapter is annotated according to the HGVS recommendations. The corresponding annotation according to the classical nomenclature (Jenny *et al*. 1987) is as follows:

HGVS nomenclature	**Classical nomenclature**
c.1296+268A>G	IVS8+268A>G

Introduction

Coagulation factor V (FV) is a large glycoprotein produced in the liver and released in the bloodstream as an inactive precursor. Once activated, it acts as a non-enzymatic cofactor of factor Xa (FXa) in the conversion of prothrombin (PT) to thrombin, accelerating this reaction by several orders of magnitude.[1] This essential role in thrombin formation makes FV indispensable to life, as demonstrated by the lethal phenotype of FV knock-out mice.[2] Approximately 80% of FV circulates in plasma, the remaining 20% being stored in platelet α-granules.[3] Although megakaryocytes can synthesise FV,[4] platelet FV originates from secondary endocytosis of plasma FV.[5-7]

FV deficiency[8-10] is a rare bleeding disorder inherited as an autosomal recessive trait and associated with mutations in the *F5* gene. Homozygous and doubly heterozygous individuals present with a strikingly variable bleeding tendency,[11] which is mainly determined by their residual platelet FV.[12-14] Since no FV concentrate or recombinant FV preparation is available, replacement therapy still relies on the administration of fresh-frozen plasma or platelet concentrates, with all well-known associated risks (volume overload, transmission of infectious agents, immune reactions and transfusion-related acute lung injury).

At least 10% of all *F5* mutations are splicing defects,[15] *i.e.* they disrupt the process by which introns are removed from the primary transcript and exons are joined together to form the mature mRNA. The resulting mature transcript often contains a premature stop codon and is rapidly degraded. Accordingly, *F5* splicing mutations are usually associated with severe bleeding symptoms.[14,16-21] Several studies on various genetic disorders have shown that splicing defects are amenable to "RNA therapy" with antisense molecules specifically designed to anneal to the mutant pre-mRNA (without triggering its degradation) and to direct its maturation into the correct transcript (reviewed in refs.[22-25]). In particular, mutations that

disrupt an existing donor splice site can be corrected with U1 small nuclear RNA (snRNA) specifically modified to recognise the mutated splice site,[26] whereas mutations that create a new splice site can be targeted with short antisense oligonucleotides or snRNAs that mask the aberrant splice site and prevent its interaction with the spliceosome. A growing body of *in vitro* and *ex vivo* evidence supports the efficacy and safety of antisense-based RNA therapy in several genetic diseases,[23,24,27] but only few studies have been conducted in bleeding disorders.[28-30]

The aim of the present study was to design and validate a personalised antisense-based RNA therapy for a previously described patient with severe FV deficiency (undetectable FV in both plasma and platelets),[14] who recently suffered from a second spontaneous intracranial haemorrhage. The patient is homozygous for a deep-intronic splicing mutation (*F5* c.1296+268A>G) activating a cryptic donor splice site in intron 8 and causing the inclusion of a pseudo-exon with an in-frame stop codon in the mature transcript.[14] The efficacy and cytotoxicity of two different antisense molecules targeting this mutation were tested in an *in vitro* model and *ex vivo* on patient-derived megakaryocytes.

Materials and Methods

Antisense molecules

Morpholino oligonucleotide (MO). MOs are uncharged analogues of nucleic acids with a phosphorodiamidate backbone. Two 25-mer MOs were purchased from GeneTools (Philomath, OR, USA), one (rescuer) with a sequence complementary to the mutant *F5* pre-mRNA (5'-GGTCACATGGTATCTACTTACCTGT-3', mutation site underlined) and the other (control) with an irrelevant sequence (5'-CCTCTTACCTCAGTTACAATTTATA-3').

Engineered U7snRNA. U7snRNAs are a class of snRNAs involved in the 3'-end processing of histone pre-mRNAs.[31] The U7-SmOPT construct[32] (kindly provided by Dr. D. Schümperli, Bern, Switzerland) consists of a murine U7snRNA gene (including its natural promoter and 3' sequences) cloned in the pSP64 vector. This construct expresses a 62-nt U7snRNA in which the U7-specific Sm-binding site has been replaced by the Sm-binding site (SmOPT) found in spliceosomal snRNAs. Using a mutagenic primer, we replaced the anti-histone pre-mRNA sequence with a sequence complementary to the aberrant splice site in the mutant *F5* pre-mRNA (5'-TCTACTTACCTGTGGCTTTA-3', mutation site underlined). The ability of this engineered anti-*F5* U7snRNA (rescuer) to correct splicing of the mutant *F5* pre-mRNA was compared to that of the original anti-histone U7snRNA (control).

In vitro model: COS-1 or HepG2 cells transfected with *F5* minigene constructs

Cloning of the F5 minigene. A 2572-bp genomic fragment spanning *F5* intron 7 through intron 9 and containing the c.1296+268A>G mutation was amplified from the patient's genomic DNA using the PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies, Amstelveen, The Netherlands) and cloned in the pTB expression vector[33] between the unique Sac II and Nde I restriction sites (Figure 1). This mutant minigene construct was used as a template to obtain the corresponding wild-type construct by site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Agilent Technologies). The sequences of both *F5* minigenes were checked by direct sequencing.

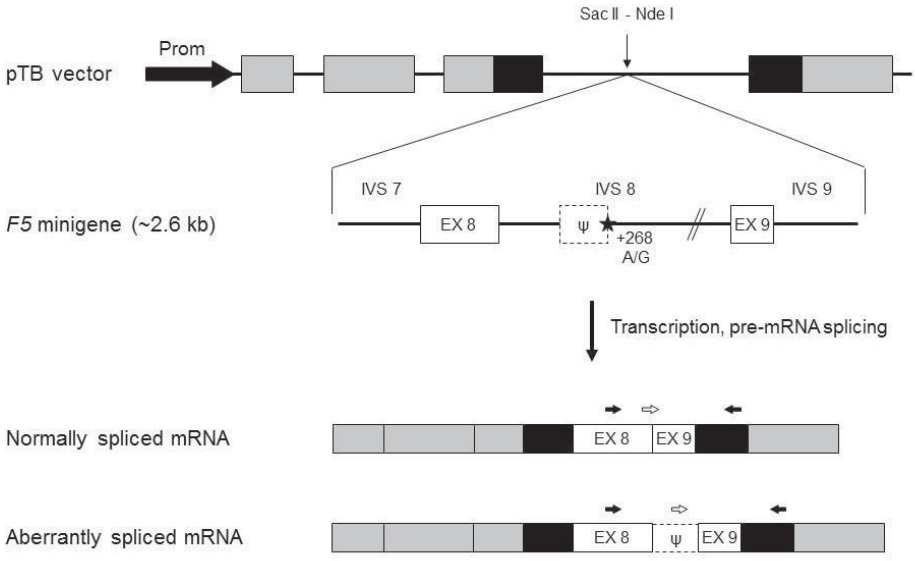

Figure 1. *F5* minigene construct.
Top: Schematic representation of the *F5* minigene construct. The pTB vector contains the promoter (black arrow) and exons 1-3 of the human α-globin gene (grey boxes) as well as exons 24-25 of the fibronectin-1 gene (black boxes). High transcription levels are ensured by an SV40 enhancer (not shown). The *F5* minigene, encompassing the 3' end of intron 7, exon 8, intron 8 (with or without the +268A>G mutation), exon 9 and the 5' end of intron 9 of the *F5* gene, was inserted between the vector's unique Sac II and Nde I restriction sites. The position of the c.1296+268A>G mutation is marked by a star. The dotted box indicates the intronic pseudo-exon (ψ). Bottom: Mature transcripts produced by the *F5* minigene construct. The black arrows represent the primers used for qualitative analysis of the *F5* mRNA; the white arrows represent the specific forward primers used to distinguish between the two differently spliced transcripts using real-time qPCR.

Transfection of COS-1 and HepG2 cells. COS-1 and HepG2 cells were cultured in a humidified incubator at 37 °C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM, Lonza, Verviers, Belgium) supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin. For each experiment, 80% confluent cells were seeded in a 6-well plate. After 24 hours, cells were switched to serum-

free OptiMEM (GIBCO, Invitrogen, Bleiswijk, The Netherlands) and transiently transfected with the wild-type or mutant *F5* minigene construct using Lipofectamine^{2000} (Invitrogen).

Treatment with antisense molecules. Cells were treated with a single dose of antisense molecule (MO or U7-SmOPT construct) administered at the time of transfection with the *F5* minigene construct. MOs (1-10 μM) were delivered using EndoPorter Reagent (GeneTools, 6 μM) in aqueous formulation. The U7-SmOPT constructs (0.25-2 μg, corresponding to a 0.6-4.8× molar excess over the *F5* minigene construct) were delivered by co-transfection with the *F5* minigene using Lipofectamine^{2000} (Invitrogen). After 48 hours cells were harvested for mRNA analysis.

Qualitative mRNA analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) and quantified spectrophotometrically. Total RNA (2 μg) was reverse-transcribed for 2 hours at 37 °C with MultiScribe^{TM} reverse-transcriptase and random primers (Applied Biosystems, Bleiswijk, The Netherlands). The mRNA (cDNA) transcribed from the *F5* minigene construct was amplified using a forward primer located in *F5* exon 8 and a reverse primer located in the distal fibronectin exon of the pTB vector (Figure 1, black arrows). This primer pair amplifies exclusively the *F5* minigene mRNA (and not any endogenous *F5* mRNA) and yields products of different sizes according to the absence (283 bp) or presence (394 bp) of the *F5* pseudo-exon in the mature transcript. PCR products were analysed by agarose gel electrophoresis and direct sequencing.

Quantification of the normal and aberrant F5 transcripts. The normal and aberrant *F5* transcripts were quantified by real-time qPCR on a LightCycler 480 Real-Time PCR instrument (Roche Applied Science, Almere, The Netherlands). Two different forward primers, located at the exon 8-9 junction (5'-TCAGAGACACACTCAAAATCG-3') and in the pseudo-exon (5'-AGTAATACAAAGGATCTGAGAC-3'), respectively (Figure 1, white

arrows), and a common reverse primer in the distal fibronectin exon of the pTB vector, were used in separate reactions to specifically amplify the normal (reference) and aberrant (target) *F5* transcripts derived from the *F5* minigene construct. Amplification reactions were carried out in a volume of 10 µL, including 100 ng total cDNA, 5 µL LightCycler 480 SYBR Green I Master mix (Roche) and 0.4 µM of each primer. An initial denaturation step (10 min at 95 °C) was followed by 40 cycles of amplification (denaturation: 20 sec at 95 °C, annealing: 20 sec at 57 °C, extension: 30 sec at 72 °C). The specificity of amplification products was checked by melting curve analysis (42-95 °C). Fluorescence curves were analysed with the LightCycler 480 Software version 1.5 and relative quantification was performed with the $2^{-\Delta\Delta Ct}$ method. All samples were assayed in duplicate.

Cytotoxicity assays

Potential cytotoxic effects of the antisense molecules and/or delivery agents were evaluated with the xCELLigence System (Roche). Details are reported in the Supplemental Data file.

***Ex vivo* model: patient's *ex-vivo* differentiated megakaryocytes**

Isolation and differentiation of circulating progenitor cells. The study was approved by the Ethical Committee of Padua Academic Hospital and conducted according to the Declaration of Helsinki. After obtaining informed consent, venous blood was drawn from the FV-deficient patient and from a normal control in 0.129 M trisodium citrate. Megakaryocytes were differentiated from circulating haematopoietic progenitor cells as previously described.[34] Briefly, mononuclear cells were isolated by centrifugation on Histopaque-1077 (Sigma-Aldrich, Milan, Italy) density gradient, resuspended in serum-free Iscove's modified Dulbecco's medium (Celbio, Milan, Italy) and seeded in 24-well plates containing a glass

coverslip at the bottom of each well. Cells were cultured at 37 °C and 5% CO_2 in medium supplemented with 2 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen) and 1% insulin-transferrin-selenium (Invitrogen), as well as 50 ng/mL thrombopoietin (TPO, Peprotech, London, UK) and 10 ng/mL interleukin-3 (IL-3, Peprotech) to induce differentiation towards the megakaryocytic lineage.

Treatment with antisense molecules. At day 9 of culture, part of the cells were treated with rescuer MO (2.5 µM or 5.0 µM) or rescuer U7snRNA construct (200 ng or 400 ng), as described above. After 24 hours, glass coverslips were retrieved and processed for FV immunostaining. Untreated and treated cells from other wells were collected in TRIzol reagent (Invitrogen, 200 µL/well) for *F5* mRNA analysis.

F5 mRNA analysis. Total RNA isolation, reverse transcription and quantification of *F5* transcripts by real-time qPCR were carried out as described above, except for the use of a different reverse primer (located in *F5* exon 10).

FV immunostaining. Cells adhering to the glass coverslips were fixed with 2% paraformaldehyde and permeabilised with 0.5% Triton X-100. FV expression was visualised with a mouse monoclonal antibody directed against the human FV heavy chain (#5146, Hematologic Technologies, Essex Junction, VT, USA) and a FITC-labeled goat anti-mouse IgG secondary antibody (Chemicon International, Milan, Italy). Cell nuclei were stained with 1.5 µg/mL Hoechst 33258 (Sigma-Aldrich). Slides were examined with a Leica DMI6000CS fluorescence microscope (Leica Microsystems CMS, Wetzlar, Germany) using a 63x/1.40 oil immersion objective. Images were acquired by means of a DFC365FX camera and analysed with Leica LAS-AF 3.1.0 software.

Results

Splicing correction strategy

The *F5* c.1296+268A>G mutation introduces a strong donor splice site deep in intron 8, causing the inclusion of an intronic pseudo-exon with an in-frame stop codon in the mature *F5* mRNA. In order to correct this aberrant splicing event, we designed antisense ("rescuer") MO and engineered U7snRNA molecules targeting the mutation site, with the aim to induce pseudo-exon skipping. MO and U7snRNA molecules with irrelevant sequences were used as controls.

In vitro model

To develop an *in vitro* model to test the antisense molecules, we transiently transfected COS-1 cells with a *F5* minigene construct containing the c.1296+268A>G mutation (Figure 1) and analysed minigene mRNA by qualitative and real-time qPCR.

The *F5* minigene produced aberrantly spliced mRNA (containing the pseudo-exon) and correctly spliced mRNA (not containing the pseudo-exon) in a typical ratio of 5-10:1 (Figure 2A, first two lanes). Treatment of transfected cells with 1-10 μM rescuer MO caused a dose-dependent decrease in the aberrant transcript and a parallel increase in the normal transcript, indicating progressive correction of *F5* pre-mRNA splicing (Figure 2A). The ratio between aberrant and normal mRNA decreased from 9.50 in untreated cells to 0.09 in cells treated with 10 μM rescuer MO, corresponding to a relative enrichment in the normal mRNA of 103 times. To verify whether the effect of rescuer MO on *F5* pre-mRNA splicing was specific, we performed additional transfection experiments to compare rescuer and control MO (Figure 2B). Treatment of transfected cells with 5 μM rescuer MO caused most of the *F5*

minigene pre-mRNA to be spliced correctly, whereas the same concentration of control MO was ineffective. The ratio between aberrant and normal mRNA decreased from 4.42 in untreated cells to 0.16 in cells treated with rescuer MO (correction factor 28-fold), whereas it hardly changed in cells treated with control MO (ratio 6.08, correction factor 0.7-fold). Finally, when COS-1 cells were transfected with the wild-type *F5* minigene construct, not carrying the c.1296+268A>G mutation, only the correctly spliced mRNA was expressed and the administration of either rescuer or control MO did not affect its expression or splicing pattern (Figure 2B).

Similar experiments were carried out with the U7-SmOPT constructs. When COS-1 cells were co-transfected with the mutant *F5* minigene construct and increasing amounts of rescuer U7snRNA construct (0.25-2 μg, corresponding to a 0.6-4.8× molar excess over the *F5* minigene construct), a dose-dependent decrease in the aberrant transcript and a parallel increase in the normal transcript were observed (Figure 2C). The ratio between the aberrant and normal *F5* mRNA decreased from 12.07 in untreated cells to 0.14 in cells treated with 2 μg rescuer U7snRNA construct, indicating progressive correction of pre-mRNA splicing up to a correction factor of 84-fold. The ability of rescuer U7snRNA to specifically restore normal splicing of the *F5* minigene transcript was confirmed in independent transfections (Figure 2D), where the rescuer U7snRNA construct (2 μg) decreased the ratio between aberrant and normal *F5* mRNA from 2.89 in untreated cells to 0.03 (correction factor 106-fold), whereas the control U7snRNA construct showed no corrective potential (3.44, correction factor 0.8-fold). No effect of rescuer or control U7snRNAs was observed in COS-1 cells transfected with the wild-type *F5* minigene construct.

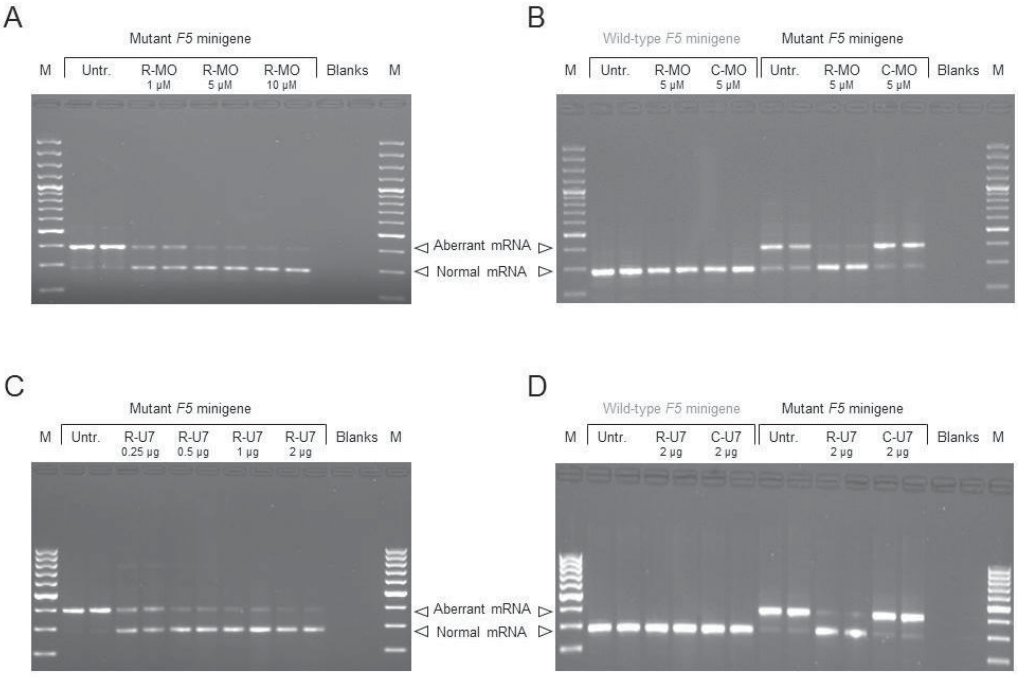

Figure 2. Splicing correction in COS-1 cells.
Panels A-B: COS-1 cells were transfected with the mutant or wild-type *F5* minigene construct and either left untreated (Untr.) or treated with the indicated concentrations of rescuer MO (R-MO) or control MO (C-MO). Panels C-D: COS-1 cells were transfected with the mutant or wild-type *F5* minigene construct and either left untreated (Untr.) or co-transfected with the indicated amounts of the U7-SmOPT construct expressing anti-*F5* (rescuer) U7snRNA (R-U7) or control U7snRNA (C-U7). 2 µg U7-SmOPT construct corresponds to a molar excess of 4.8× over the *F5* minigene construct. After 48 hours RNA was isolated, reverse-transcribed and analysed by PCR and gel electrophoresis. Representative gels are shown. M, 100 bp marker.

Since the liver is the physiological site of FV production *in vivo*, the ability of rescuer MO and U7snRNA to correct splicing of the mutant *F5* pre-mRNA was also tested in HepG2 cells. These cells were transfected with the mutant *F5* minigene construct and either left untreated or treated with 5 µM rescuer MO or 2 µg of the rescuer U7snRNA construct (Figure 3A). The ratio between aberrant and normal mRNA was 6.44±1.64 (mean ± standard

deviation of 4 replicate transfections) in untreated cells and decreased to 0.20±0.03 in cells treated with rescuer MO (correction factor 32-fold, p=0.005) and to 0.034±0.003 in cells treated with rescuer U7snRNA (correction factor 189-fold, p=0.004) (Figure 3B), similar to what observed in COS-1 cells. In a control experiment, control MO and U7snRNA with irrelevant sequences did not affect *F5* pre-mRNA splicing (data not shown).

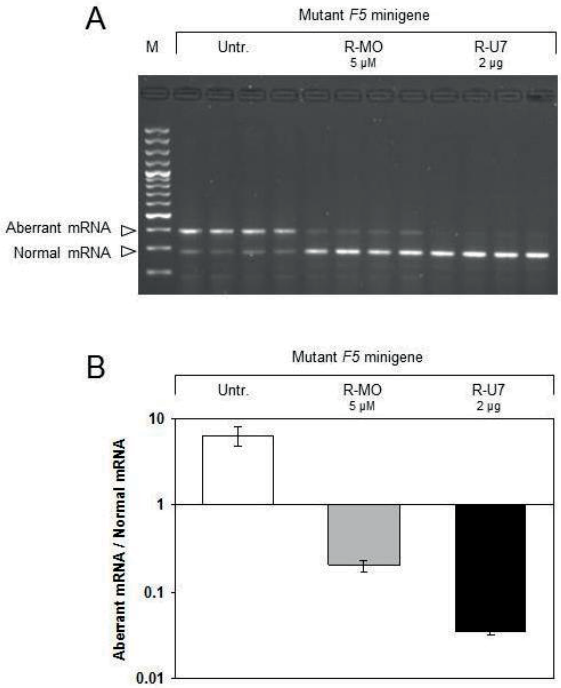

Figure 3. Splicing correction in HepG2 cells.

HepG2 cells were transfected with the mutant *F5* minigene construct and either left untreated (Untr.) or treated with 5 µM rescuer MO (R-MO) or co-transfected with 2 µg of the U7-SmOPT construct expressing anti-*F5* (rescuer) U7snRNA (R-U7). 2 µg U7-SmOPT construct corresponds to a molar excess of 4.8× over the *F5* minigene construct. After 48 hours RNA was isolated, reverse-transcribed and analysed by PCR and gel electrophoresis (A) and real-time qPCR (B). The gel shows the effects of 5 µM R-MO and 2 µg of rescuer U7snRNA construct on *F5* pre-mRNA splicing in HepG2 cells transfected with the mutant *F5* minigene (quadruplicate transfections). M, 100 bp marker. Results of the quantification experiments are expressed as the ratio between the aberrant and normal *F5* mRNA, plotted on a logarithmic scale. The quantification data represent the mean ± standard deviation of the four replicates.

Treatment with rescuer or control antisense molecules affected the viability and/or proliferation of COS-1 cells and (to a much lesser extent) HepG2 cells (Supplemental Data file).

***Ex vivo* model**

Haematopoietic progenitor cells from the FV-deficient patient and a normal control were cultured in serum-free medium in the presence of TPO and IL-3 to induce differentiation towards the megakaryocytic lineage. While control cells rapidly developed into FV-expressing megakaryocytes (Figure 4F), the patient's cells did not show any sign of FV expression even after 9 days of culture (Figure 4A). However, treatment with rescuer MO (2.5-5.0 μM) or rescuer U7snRNA construct (200 ng and 400 ng) effectively restored FV expression in the patient's cells (~80% positivity, Figure 4B-E). Although this technique does not lend itself well to quantitative evaluation, green fluorescence was more intense (suggesting higher FV expression) in patient's megakaryocytes treated with U7snRNA than in those treated with MO, whereas no clear-cut correlation with the intensity of treatment was noticed. Moreover, also in this model, administration of antisense molecules was associated with significant cytotoxicity, as demonstrated by the marked reduction in the number of viable cells following treatment.

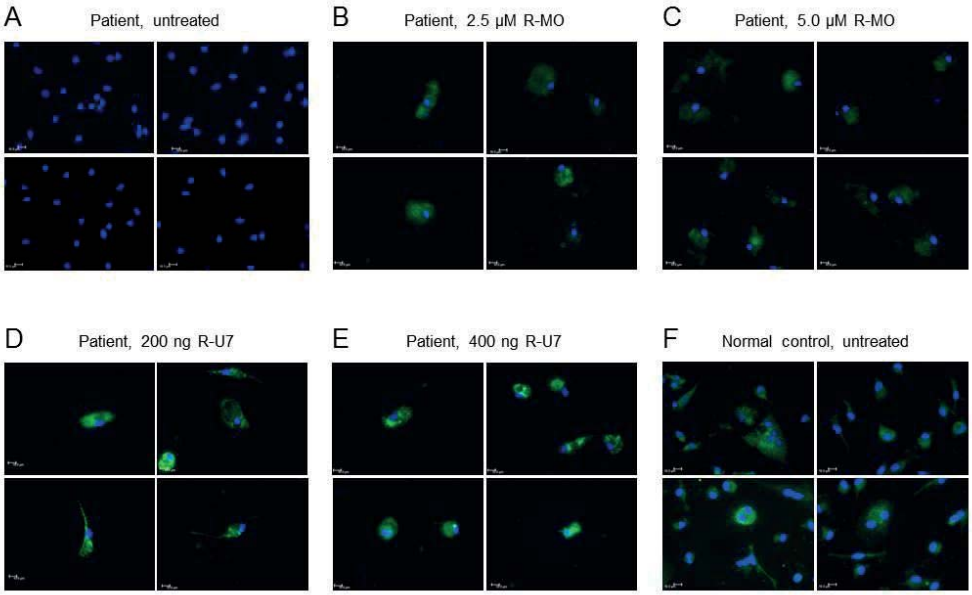

Figure 4. *Ex vivo* splicing correction in patient's megakaryocytes.
Haematopoietic progenitor cells were isolated from peripheral blood and differentiated towards the megakaryocytic lineage as described under Methods. At day 9 of culture, the FV-deficient patient's cells were either left untreated (A) or treated with rescuer MO (R-MO) or with the U7-SmOPT construct expressing rescuer U7snRNA (R-U7) at the indicated concentrations (B-E). Untreated cells from a normal control are shown for comparison (F). After immunofluorescence staining with Hoechst 33258 (cell nuclei, in blue) and with a FITC-labelled anti-mouse antibody recognising the primary anti-FV antibody (FV, in green), cells were examined under a Leica DMI6000CS fluorescence microscope using a 63x/1.40 oil immersion objective at room temperature. Images were acquired using a DFC365FX camera and analysed with Leica LAS-AF 3.1.0 software. Each panel represents the overlay of two images of the same preparation stained with Hoechst 33258 and FITC-labelled antibody, respectively. Four representative microscope fields are shown for each condition.

For a more quantitative assessment of the efficacy of antisense molecules in patient's megakaryocytes, total RNA was isolated from untreated and treated megakaryocytes and *F5* mRNA was analysed by real-time qPCR (Figure 5). The ratio between aberrant and normal *F5* transcripts in untreated megakaryocytes (158.3) was similar to that observed in the patient's platelets (194.8, unpublished observation) and indicated that <1% of *F5* mRNA is

spliced correctly in this patient. Treatment with rescuer MO and rescuer U7snRNA construct corrected the splicing pattern by 2-3 orders of magnitude in a roughly dose-dependent manner. At the concentrations used, rescuer U7snRNA was somewhat more effective than rescuer MO in restoring normal splicing, in line with the protein expression data (and with the *in vitro* splicing correction experiments).

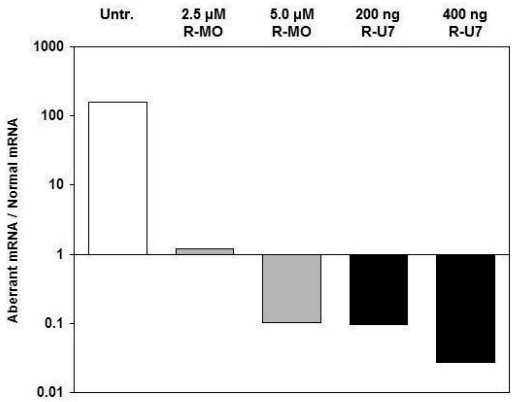

Figure 5. *Ex vivo* splicing correction in patient's megakaryocytes.
Haematopoietic progenitor cells were isolated from peripheral blood and differentiated towards the megakaryocytic lineage as described under Methods. At day 9 of culture, the FV-deficient patient's cells were either left untreated or treated with rescuer MO (R-MO) or with the U7-SmOPT construct expressing rescuer U7snRNA (R-U7) at the indicated concentrations. Following isolation and reverse-transcription of total RNA, the *F5* mRNA splicing pattern in untreated and treated patient's megakaryocytes was analysed by real-time qPCR. Results are expressed as the ratio between the aberrant and normal *F5* mRNA, plotted on a logarithmic scale.

Discussion

Antisense-based RNA therapy is rapidly emerging as a promising tool to correct splicing defects.[22-24,27] Unlike classical gene therapy, where a functional copy of the defective gene (cDNA) under control of a strong promoter is introduced in the patient's cells, RNA therapy relies on short mutation-specific antisense oligonucleotides or engineered snRNAs that

anneal to the mutant primary transcript and re-direct its maturation into the correct mRNA. By acting at the RNA rather than DNA level, this form of molecular therapy does not affect the transcriptional regulation of the targeted gene (or any other gene).

Despite the encouraging results obtained in several genetic diseases, the potential utility of RNA therapy in coagulation disorders is only just beginning to be explored.[25] Among all coagulation factor deficiencies, FV deficiency represents a particularly suitable target for RNA therapy, because: a) no FV concentrate or recombinant FV preparation is available for substitutive therapy; b) the FV requirement for adequate haemostasis is extremely low, making a few % FV sufficient to prevent life-threatening bleeding;[19,35-37] and c) the large size of the *F5* cDNA makes conventional gene therapy particularly challenging. Here we present the first application of RNA therapy to severe FV deficiency and we provide *in vitro* and *ex vivo* evidence that mutation-specific antisense molecules can effectively rescue a *F5* splicing mutation.

The mutation that we have targeted (*F5* c.1296+268A>G) activates a cryptic donor splice site deep in intron 8, causing the retention of an intronic pseudo-exon in the mature mRNA.[14] Although this is presently the only known *F5* splicing defect that can be corrected through MO- or U7snRNA-mediated pseudo-exon skipping, deep-intronic splicing mutations may be more prevalent than previously suspected.[38] In fact, deep-intronic splicing mutations potentially amenable to this corrective approach have been identified in the *F8*,[39-42] *FGB*[30] and *FGG*[43] genes.

COS-1 and HepG2 cells transfected with a *F5* minigene construct containing the c.1296+268A>G mutation provided a suitable *in vitro* model to test the antisense molecules, even if the relative proportion of correctly spliced mRNA was much higher in this model (10-20%) than in the patient's platelets (~0.5%, as estimated by real-time qPCR). This is not

surprising considering that: a) the splicing pattern/efficiency is known to vary in different cell types; b) the minigene transcript is different from the full-length *F5* transcript, which may affect the susceptibility of the aberrantly spliced mRNA to nonsense-mediated decay; and c) the transcript ratio in the minigene model also varied according to cell status and transfection conditions (data not shown). However, treatment of transfected cells with mutation-specific antisense molecules restored normal splicing in a specific and dose-dependent manner. In particular, the highest concentrations of antisense MO and U7snRNA increased the relative proportion of the correctly spliced mRNA by 1-2 orders of magnitude. Similar correction efficiencies, if achieved *in vivo*, would be more than sufficient to bring the patient's FV level in the safe range.

The *F5* c.1296+268A>G mutation was originally identified in the homozygous state in a patient with undetectable FV and a life-threatening bleeding diathesis.[14] To prove that our antisense MO and U7snRNA could rescue the patient's FV deficiency, we showed that they could effectively correct the *F5* mRNA splicing pattern and restore FV expression in the patient's *ex vivo* differentiated megakaryocytes. Although megakaryocytes are not the physiological site of FV production *in vivo*,[5-7] they have been shown to synthesise FV in culture,[4] offering us the opportunity to test the antisense molecules on the patient's own cells without a liver biopsy. Unfortunately, the antisense molecules could not be tested directly on the patient's hepatocytes, but their efficacy in correcting the splicing defect in HepG2 cells transfected with the mutant *F5* minigene construct suggests that they would be able to restore FV expression in the patient's hepatocytes as well, especially considering the fact that the liver is a preferential site of accumulation of systemically injected MOs.[44,45]

Treatment with rescuer (and control) MO and U7snRNA molecules adversely affected the viability and/or proliferation of COS-1 and (to a much lesser extent) HepG2 cells in *in vitro*

cytotoxicity assays. However, toxic effects were largely attributable to the transfection reagents used to deliver the antisense molecules rather than to the antisense molecules themselves. Since the delivery of antisense molecules to target cells *in vivo* does not rely on transfection reagents, but rather on free uptake by body cells (MOs)[46] or on targeted delivery using engineered viral vectors (U7snRNAs),[47] major adverse effects in treated patients appear unlikely. In fact, antisense molecules have shown excellent safety profiles in several *in vivo* studies of other genetic diseases, both in animal models and in human volunteers, being well tolerated even after systemic administration.[44,47] Nevertheless, since each antisense molecule has a unique sequence, off-target effects can never be excluded.

In summary, our data show that specific antisense molecules targeting the c.1296+268A>G mutation can correct splicing of the mutant *F5* pre-mRNA *in vitro* and restore FV synthesis in the patient's megakaryocytes *ex vivo*. These findings provide proof-of-principle for the efficacy of RNA therapy for this particular *F5* gene mutation and support RNA therapy as a possible alternative to substitutive therapy with blood derivatives in severe FV deficiency.

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Supplemental material

Cytotoxicity assays

Methods

Potential cytotoxicity of the antisense molecules and/or delivery agents was evaluated with the xCELLigence System (Roche), which uses impedance measurements for real-time monitoring of cell behaviour.[1] Subconfluent COS-1 and HepG2 cells were seeded in an E-plate 96 and the impedance of the cell layer (cell index) was recorded every hour for 5 days. The plate was kept at 37 °C and 5% CO_2 throughout the experiment. Based on control experiments, cells were seeded at a density of 5000 cells/well (COS-1) or 7500 cells/well (HepG2) and allowed to adhere and grow for 24 hours before treatment with 5 μM MO (and 6 μM EndoPorter Reagent) or 2 μg U7-SmOPT construct (and 0.5 μL Lipofectamine) or the delivery agents alone. During the first 2 days after treatment, impedance was recorded every 15 minutes to closely monitor cell viability and proliferation. All measurements were done in triplicate. Wells containing medium without cells were used as a blank. Data were analysed with the RTCA Software 1.2 (Roche).

Cell viability 24 hours post-treatment with antisense molecules and/or delivery agents was also assessed with the MTT assay (Sigma-Aldrich), which was carried out in 8-plo according to the manufacturer's instructions.

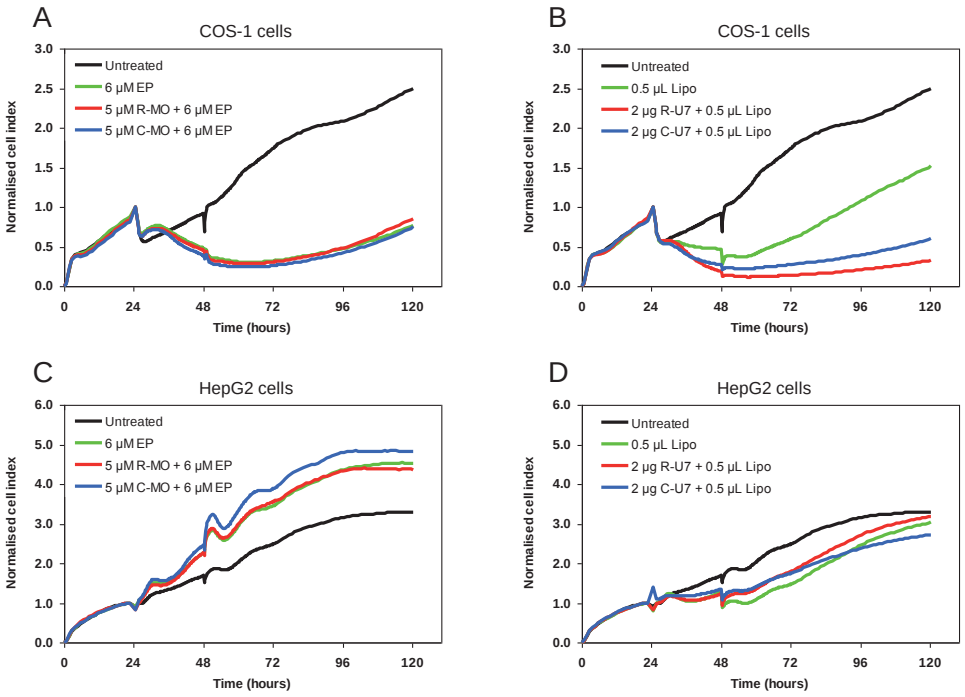
Results

To test whether the antisense molecules or the reagents used to deliver them to the cells cause any cytotoxicity, we monitored the proliferation of COS-1 and HepG2 cells in the absence and presence of treatment using xCELLigence technology (Supplemental Figure).

Untreated COS-1 cells grew steadily and reached a cell index of ~2.5 after five days. Treatment with rescuer or control MO caused an initial decrease in the cell index, indicative of cell stress, but after two days cells started to recover and resumed proliferation (Supplemental Figure, panel A). Interestingly, administration of EndoPorter Reagent alone had exactly the same effect (Supplemental Figure, panel A), suggesting that the toxicity of MO treatment is attributable to the delivery agent rather than to the antisense molecules themselves. Treatment of COS-1 cells with rescuer or control U7snRNA constructs was also associated with transient toxicity, but this effect was only partially mediated by the transfection agent (Lipofectamine) used to deliver the constructs to the cells (Supplemental Figure, panel B).

Untreated HepG2 cells grew steadily reaching a plateau at a cell index of ~3.3 (Supplemental Figure, panel C). Treatment with rescuer or control MO seemed to enhance rather than decrease their proliferation (Supplemental Figure, panel C), whereas treatment with rescuer or control U7snRNA constructs had only a minor adverse effect on the cell index (Supplemental Figure, panel D), suggesting that HepG2 cells are less sensitive than COS-1 cells to cytotoxicity induced by the antisense molecules and/or their respective delivery agents.

Essentially the same results were obtained when cell viability was assessed using the MTT assay (data not shown).



Supplemental figure. Cytotoxicity assays.

COS-1 (A, B) and HepG2 (C, D) cells were seeded in the wells of an E-plate 96 and allowed to adhere and grow at 37 °C for 24 hours before treatment with the indicated antisense molecules and/or delivery agents (EP, EndoPorter Reagent; R-MO, rescuer MO; C-MO, control MO; Lipo, Lipofectamine; R-U7, U7-SmOPT construct expressing rescuer U7snRNA; C-U7, U7-SmOPT construct expressing control U7snRNA). The cell index, reflecting cell adhesion, growth and proliferation, was monitored continuously for a total of 5 days with xCELLigence technology. Each condition was tested in triplicate and average cell index tracings are shown. The "spikes" in the signal at 24 and 48 hours are due to the removal of the plate from the incubator to administer the treatment (at 24 hours) and to refresh the medium (at 48 hours).

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7

Plasma tissue factor pathway inhibitor modulates bleeding tendency in severe factor V deficiency

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In preparation

8

General Discussion

Coagulation factor V (FV) deficiency[1-4] is a rare autosomal recessive bleeding disorder caused by *F5* gene mutations that impair FV protein synthesis and/or function. Affected patients experience a heterogeneous bleeding diathesis which is only weakly correlated with plasma FV levels. In patients with undetectable plasma FV, symptoms range from mild bleeding episodes (easy bruising, epistaxis, gum bleeding and menorrhagia) to life-threatening intracranial or gastrointestinal hemorrhages.[5] Although a FV concentrate is under development,[6] administration of fresh frozen plasma (and/or platelet concentrates) is presently the only option for the prevention and treatment of bleeding episodes in these patients.[7] This form of therapy is however not optimal, especially in case of long-term treatment or life-long prophylaxis, because regular plasma transfusions are associated with several risks and complications (volume overload, transmission of infectious agents, allergic reactions, transfusion-related acute lung injury).[8]

In clinical laboratories the diagnosis of FV deficiency relies entirely on clotting tests and on the measurement of FV levels in plasma. *F5* mutation screening is rarely performed, because it requires a specialized molecular genetic facility, it is expensive and labor-intensive, and it does not influence treatment decisions. However, identification of the responsible mutation(s) is the only way to definitely prove the genetic nature of FV deficiency in a patient and to firmly establish the carrier status in his/her family members. In turn, this information may be used for genetic counseling of affected individuals (especially in countries where consanguineous marriages are common) and for prenatal diagnosis of the most severe forms.[9] Moreover, the molecular diagnosis opens the possibility for the development of personalized therapeutic strategies based on molecular approaches, such as the correction of splicing defects using antisense molecules[10-13] or the read-through of nonsense mutations using aminoglycosydes.[14,15]

Conventional mutation screening techniques, based on the amplification and sequencing of each individual exon of the *F5* gene, identify both mutations in a large proportion of FV-deficient patients, but still leave 10-20% of cases genetically unsolved. Mutations may be overlooked either because they reside in regions of the gene that are not routinely screened (promoter region, introns, 3'-UTR) or because they are not detectable by amplification and sequencing of individual exons (large deletions and duplications involving multiple exons). In other cases mutations are identified, but their deleterious effects on gene expression are not recognized. This is typically the case for exonic splicing mutations, which are often mistaken for rare synonymous or benign-missense polymorphisms. Unraveling these mutations "in disguise"[16] is not only necessary for a correct molecular diagnosis of the disease, but can also point out new mechanisms of regulation of gene expression.

Specific contributions of this thesis

The work described in this thesis has addressed the molecular genetics of FV deficiency as a starting point for the development of personalized approaches of molecular therapy, leading to the following original contributions:

1) Development and validation of a *F5* MLPA assay for the efficient and cost-effective detection of large deletions or duplications of the *F5* gene (Chapter 3);

2) Broadening of the *F5* mutational spectrum by six novel mutations, including a large deletion and two splicing mutations "in disguise" (Chapters 3-7);

3) Proof-of-principle of antisense-based correction of two different *F5* splicing defects (Chapters 5 and 6);

4) Evidence for the role of plasma full-length TFPI as a phenotype modulator and a possible therapeutic target in FV deficiency (Chapter 7).

Development and validation of a *F5* MLPA assay

Structural variation, including copy number variations (*i.e.* deletions, duplications and insertions) as well as translocations and inversions of larger (>1 kb) genomic fragments, has been recognized as a common feature of the human genome and a major cause of genetic disease.[17,18] However, the role of copy number variations in the pathogenesis of FV deficiency has remained largely unexplored, mainly due to the lack of suitable tools to identify these genetic defects in the *F5* gene. In fact, of the two *F5* large deletions identified so far, the first has been discovered more or less by chance as part of a major cytogenetic defect (interstitial deletion of the long arm of chromosome 1) responsible for the patient's mental retardation,[19] while the other was initially suspected on the basis of the patient's apparent homozygosity (actual hemizygosity) for all SNPs located in the 5' portion of the gene and subsequently mapped through a laborious long-range PCR approach.[20]

To overcome these difficulties, we have now developed an in-house multiplex ligation-dependent probe amplification (MLPA) assay for the efficient detection of large deletions and duplications of *F5* gene exons (**Chapter 3**). This assay makes use of specific probes which, once hybridized to their target (*F5*) and reference (other genes) sequences on the patient's genomic DNA and ligated, can be amplified with a single primer pair, allowing the relative quantification of multiple *F5* gene regions within a single PCR reaction.[21,22] Due to the large size of the *F5* gene (80 kb, 25 exons) and to the numerous (sequence, length and melting temperature) requirements that MLPA probes must fulfill,[23] probe design was technically quite challenging. First of all, we could not accommodate all *F5*-specific probes in a single MLPA reaction, but we had to divide the probes in two sets, requiring two separate MLPA reactions to cover the whole gene. Secondly, we were unable to design suitable probes for exons 5, 8, 12, 20, 23 and 25, and had to place the probes for exons 2 and

3 in the adjacent intronic regions (intron 2 and 3). In order to achieve an adequate coverage of the whole *F5* locus, we included two different probes for the large exon 13, as well as probes for sequences located upstream and downstream of the *F5* gene. This resulted in a total of 22 *F5*-specific probes evenly distributed throughout the gene. When tried out on control DNA samples, all probes yielded specific and reproducible peaks of the expected size, except for the shortest probe of each set, which produced very low peaks and occasional double peaks, possibly due to aspecific binding to partially homologous target sequences elsewhere in the genome. These probes definitely require further optimisation. However, the ability of our in-house *F5* MLPA assay to unequivocally detect and define a previously described large deletion[20] makes it a reliable method to screen for the presence of *F5* large deletions or duplications in patients' samples. Due to its relative simplicity of execution, this assay can be performed in any molecular genetics laboratory, providing an efficient and cost-effective tool for the rapid detection of *F5* copy number variations in FV-deficient patients.

Application of our in-house *F5* MLPA assay to 14 genetically unresolved patients with partial or severe FV deficiency identified a novel large deletion involving *F5* exons 9-10, which was subsequently confirmed by digital PCR (dPCR) and fine-mapped by long-range PCR and direct sequencing (**Chapter 3**). Despite this encouraging result, we had expected to find more *F5* copy number variations in this selected population of FV-deficient patients, where conventional *F5* mutation screening methods had already failed to identify one or both mutations. However, the genetic nature of the FV deficiency had not been confirmed in all patients by family studies, and the alternative diagnosis of combined FV and FVIII deficiency[24] had not always been excluded in the patients with partial deficiency. Moreover, we may have missed single-exon deletions/duplications of the few exons not covered by our

probes or of the regions (promoter, exon 1) whose probes do not yield optimal peaks in our MLPA assay. Alternatively, it is possible that large deletions/duplications are a truly rare cause of FV deficiency. In fact, a sequence analysis conducted using the RepeatMasker software (www.repeatmasker.org) showed that repeated elements that often mediate gross gene rearrangements[25] are less represented in the *F5* gene than in the homologous *F8* gene (unpublished observation). This is the case for Alu sequences (making up 4.1% of the *F5* sequence *vs.* 7.9% of the *F8* sequence), LINEs (23.8% *vs.* 44.2%) and particularly LTR elements (1.9% *vs.* 14.7%).

The performance of MLPA assays is strongly dependent on pre-analytical variables, such as the DNA extraction method, the purity and quality of the DNA preparation and the composition of the buffer in which it is stored. Moreover, polymorphisms or mutations in the probe target sequences (especially if located close to the probe ligation site) can interfere with probe ligation, thereby giving rise to a false positive result, which may be interpreted as a single-exon deletion.[26,27] Therefore, MLPA findings should always be confirmed with an independent method, typically long-range PCR and/or qPCR. In our study, we have used dPCR, a relatively new microfluidic technique which allows the sensitive detection and accurate quantification of even tiny amounts of template DNA.[28,29] The extreme sensitivity of dPCR is achieved by partitioning a qPCR mix in a large number of wells (*e.g.* 20,000), each of which ends up containing only one or no template molecule, and by counting the number of wells in which amplification takes place. This trick makes dPCR able to resolve small differences in the number of copies of template DNA, such as those involved in heterozygous deletions or duplications of the target sequence, which are challenging to detect by conventional qPCR. Although dPCR finds most of its applications in oncology[30,31] and microbiology,[32,33] its extreme sensitivity has also been successfully exploited in the

coagulation field for the non-invasive prenatal diagnosis of haemophilia A and B in maternal plasma DNA.[34]

Broadening of the *F5* mutational spectrum

To date, 153 *F5* gene mutations have been reported (Human Genome Mutation Database, accessed in March 2015), mostly missense and nonsense mutations. This thesis describes six novel *F5* loss-of-function mutations, including a large deletion, two missense mutations and three splicing mutations (Table 1).

Table 1. Novel *F5* mutations described in this thesis

Mutation type	Mutation*	Molecular mechanism	Chapter
Large deletion	Deletion extends from IVS8+1311 to IVS10+378	1.8-kb deletion including exons 9-10	3
Missense	668G>C (Cys165Ser)	Loss of a highly conserved disulfide bridge in the A1 domain	5
	1918T>G (Trp582Gly)	Replacement of a highly conserved residue in the A2 domain	7
Splicing	IVS3+2T>C	Classical splicing mutation disrupting the donor splice site of intron 3	4
	1371C>G	Apparently synonymous exonic mutation impairing pre-mRNA splicing by activating a cryptic donor splice site in exon 8	5
	IVS8+268A>G	Deep-intronic mutation activating an intronic pseudo-exon with an in-frame stop codon	6

*Annotated according to the classical nomenclature of Jenny *et al*.[35]

Large deletion

The detection of *F5* large deletions and their contribution to the *F5* mutational spectrum has been discussed in the previous paragraph.

Missense mutations

The causal role of a missense mutation (amino acid substitution) is often difficult to prove without expressing the variant as a recombinant protein.[36] However, causality may be inferred from the evolutionary conservation of the affected amino acid residue, an *in silico* analysis with prediction algorithms (*e.g.* SIFT or PolyPhen), structural data (if the three-dimensional structure of the protein is available), co-segregation of the genetic variant with the phenotype and absence of the variant in the general population.

Both *F5* missense mutations described in this thesis affect highly conserved amino acid residues. The first (Cys165Ser, **Chapter 5**) abolishes a conserved disulfide bridge in the A1 domain of FV and its causal role has been verified experimentally by *in vitro* expression of the recombinant mutant. The second (Trp582Gly, **Chapter 7**) replaces a bulky Trp located in the core of the A2 domain with a Gly and was predicted to be damaging by both SIFT and PolyPhen. In addition, this mutation showed complete co-segregation with FV levels in two (distantly related) families and was also found in a third (apparently unrelated) family with FV deficiency, all from the Sultanate of Oman. Screening of the Omani general population for this mutation might be warranted.

Splicing mutations

The complexity of the splicing code offers many chances for mutations to disrupt normal pre-mRNA splicing. Mutations can destroy functional donor and acceptor splice sites,

activate cryptic splice sites in exons or introns and weaken/strengthen auxiliary splicing signals (exonic and intronic splicing enhancers and silencers), thereby leading to aberrant splicing events such as exon skipping or intron retention in the mature mRNA.[37] Accordingly, it has been estimated that splicing mutations may represent up to 50% of all pathogenic DNA variants.[38]

The under-representation of splicing mutations in the *F5* mutational spectrum is likely due to the fact that current mutation screening strategies detect only splicing mutations affecting the canonical donor and acceptor splice sites, whereas splicing mutations acting *via* other mechanisms remain largely undetected or unrecognized.[39] This thesis reports an example of a classical splicing mutation, abolishing the donor splice site of *F5* intron 3 (**Chapter 4**), and two unconventional splicing mutations (**Chapters 5** and **6**), which were missed by the initial *F5* mutation screening.

The splicing defect associated with the *F5* IVS3+2T>C mutation (**Chapter 4**) could unfortunately not be characterized in detail due to the lack of a patient's blood sample suitable for RNA isolation and to the technical challenge of cloning a *F5* minigene containing intron 3 (>11 kb). However, the most likely consequence of this mutation is exon 3 skipping, as this aberrant splicing event also occurs physiologically,[40] presumably because of the low consensus score of the acceptor splice site of intron 3 (0.75 according to the NNsplice software).

The *F5* 1371C>G mutation (**Chapter 5**), located in exon 8, was detected by sequencing of the coding region, but its effect on splicing was not immediately recognized. As it is often the case for exonic variants, the mutation was only assessed on the basis of its impact on translation. Since it did not predict an amino acid change, it was interpreted as a (rare) neutral polymorphism. However, an *in silico* sequence analysis indicated that this variant

may activate a cryptic donor splice site potentially competing with the canonical donor splice. This was subsequently confirmed by analysis of the patient's cDNA and by characterization of the isolated variant using a minigene model. Similar examples of apparently synonymous mutations affecting pre-mRNA splicing have been recently described in the *LMAN1*, *VWF* and *F8* genes.[41-43] In fact, evidence is accumulating that synonymous mutations can be pathogenic *via* several mechanisms, not only by impairing correct pre-mRNA splicing,[44] but also by affecting mRNA stability, translation rate and co-translational folding of the nascent protein.[45,46] In other words, synonymous mutations are not always neutral.[47] This should be kept in mind when annotating and prioritizing exonic variants of unknown significance identified by next generation sequencing.

The *F5* IVS8+268A>G mutation (**Chapter 6**) is a typical deep-intronic mutation which activates a cryptic donor splice site in intron 8, resulting in the inclusion of an intronic pseudo-exon with an in-frame stop codon in the mature *F5* mRNA. This mutation was only identified after finding a splicing aberration in the patient's *F5* cDNA.[48] Its causative role was supported by splicing prediction software and experimentally proven by expression in a minigene model. Deep-intronic mutations activating intronic pseudo-exons are more common than previously suspected, and several examples have been described in various genes,[49] including *F8*,[50-53] *FGB*[54] and *FGG*.[55] However, deep-intronic mutations are still largely overlooked, because introns are usually not sequenced and cDNA analysis is rarely performed in patients.

Overall, our findings underscore the importance of analyzing the patient's cDNA in order to detect splicing defects. Unfortunately, cDNA analysis is not routinely performed, as extraction of RNA from blood cells requires an especially collected blood sample. Moreover, RNA processing and analysis are labor-intensive and generally more demanding than DNA

analysis. In the absence of cDNA analysis, evaluation of variants of unknown significance using splicing prediction software might help identify potential effects on splicing, at least those mediated by the creation or destruction of splice sites, whereas the ability of these algorithms to predict functional auxiliary splicing signals is still quite limited.[43] Therefore, *in silico* predictions should be (ideally) always supported by experimental verification in minigene models. On the other hand, targeted resequencing of introns in genetically unsolved patients could lead to the identification of deep-intronic mutations potentially affecting splicing, as recently shown for hemophilia A patients in which no mutation was found by sequencing of the *F8* coding sequence.[50,51]

Two of the FV-deficient patients described in this thesis (**Chapters 4** and **6**), who were homozygous for splicing mutations, had a particularly severe bleeding phenotype, characterized by recurrent intracranial haemorrhages. A review of the literature shows that this is often the case for patients who are homozygous for *F5* splicing mutations (Table 2). This is remarkable because intracranial hemorrhages are otherwise a rather rare (6%) manifestation of the disease, even in the subgroup of patients with undetectable FV, where the incidence is 12.5%.[5]

The reason for the association between *F5* splicing mutations and severe clinical phenotype is unclear, but may relate to the fact that splicing defects often result in grossly abnormal mRNA containing a premature stop codon, which triggers mRNA degradation by the nonsense-mediated decay (NMD) pathway.[40,48,56] Even if the correct reading frame is preserved and the aberrantly spliced mRNA is not degraded, the encoded protein does not fold properly and usually fails to be secreted.[57-59]

Table 2. Characteristics of FV-deficient patients with homozygous *F5* splicing mutations

Patient characteristics	FV:C	Bleeding symptoms	Mutation*	Reference
Omani female toddler Consanguineous parents	<1%	Multiple intracranial hemorrhages	IVS3+2T>C (homozygous)	This thesis (Chapter 4)
Iranian female Consanguineous parents	5%	Epistaxis	IVS8+6T>C (homozygous)	Dall'Osso *et al.*[40]
Italian male, 31 years old	<1%	Umbilical bleeding at birth Recurrent epistaxis Joint & muscle hematomas Hemothorax 2x intracranial hemorrhage	IVS8+268A>G (homozygous)	Castoldi *et al.*[48] This thesis (Chapter 6)
Chinese male, 37 years old	1.6%	Gum bleeding Hemorrhage after appendectomy Intracranial bleeding at 37 years	IVS8-2A>G (homozygous)	Fu *et al.*[60]
Female baby Parents distantly related	<1%	Umbilical bleeding at birth Gum bleeding Multiple intracranial hemorrhages	IVS10-1G>T (homozygous)	Schrijver *et al.*[61]
Slovenian male	<1%	Gastro-intestinal bleeding Gum, joint and muscle bleeding Intracranial bleeding at 11 years	IVS18-12T>A (homozygous)	Lunghi *et al.*[56]
Iranian male, 19 years old Consanguineous parents	<1%	Mouth and nose bleeding Repeated hemarthrosis Hematuria Bleeding after dental extractions	IVS19+3A>T (homozygous)	Asselta *et al.* [57]

*Annotated according to the classical nomenclature of Jenny *et al.*[35]

Antisense-based RNA therapy of FV deficiency

Recent advances in molecular biology techniques make it possible to correct splicing defects by means of specific antisense molecules that anneal to a target pre-mRNA and thereby influence splicing decisions.[12,62,63] These antisense molecules include specifically modified U1 small nuclear RNA (U1snRNA), which can be used to recognize donor splice sites weakened by mutation, as well as synthetic antisense oligonucleotides and engineered U7snRNA, which can be employed to hide incorrect splicing signals introduced by mutation.

These approaches can also be fruitfully combined to maximize splicing correction.[64] Efforts to develop, test and implement antisense-based RNA therapeutic strategies are presently ongoing for several genetic diseases,[12,13,65-67] including coagulation disorders.[62]

Severe FV deficiency would be a particularly suitable target for this form of molecular therapy, because current treatment (based on the administration of fresh frozen plasma) is not optimal, while gene therapy is hampered by the large size of the *F5* cDNA. Moreover, a minimal increase in the FV levels would be already sufficient to turn the severe clinical phenotype often associated with *F5* splicing mutations into a milder bleeding tendency.[68] Finally, the liver (*i.e.* the physiological site of FV synthesis in humans) represents a privileged target for antisense-based therapy, because systemically injected antisense oligonucleotides tend to accumulate in this organ,[69] while engineered snRNAs can be specifically delivered to the liver using appropriate viral vectors.[70,71]

In this thesis we present *in vitro* evidence for the efficacy of antisense molecules in correcting the effects of two different *F5* splicing mutations associated with FV deficiency. In **Chapter 5** we targeted the *F5* 1371C>G mutation, which activates a cryptic donor splice site in exon 8 and causes the in-frame deletion of the last 18 nucleotides of exon 8 from the mature mRNA. Using a minigene model in liver (HepG2) cells, we showed that a morpholino antisense oligonucleotide directed against this mutation specifically and dose-dependently decreases the amount of the aberrantly spliced transcript in favour of the normal transcript. However, we could not achieve complete normalization of the splicing pattern even at relatively high morpholino concentrations (up to 20 μM). This might be due to interference of the antisense oligonucleotide with the assembly of the spliceosome on the nearby canonical donor splice site, either by direct steric hindrance or indirectly by masking a putative exonic splicing enhancer element which may help define exon 8.

In **Chapter 6** we designed and tested a morpholino antisense oligonucleotide as well as an engineered U7snRNA directed against the *F5* IVS8+268A>G mutation, which activates a cryptic donor splice site deep in intron 8, causing the retention of an intronic pseudo-exon with an in-frame stop codon in the mature mRNA. Also in this case, both antisense molecules were able to specifically and dose-dependently restore normal splicing, improving the ratio between the normal and aberrant transcript by 1-2 orders of magnitude at the highest concentrations used. Remarkably, this was not only observed in a minigene model of the patient's mutation, but also in the patient's own megakaryocytes differentiated *ex vivo* from circulating hematopoietic progenitors. In fact, treatment with the mutation-specific morpholino antisense oligonucleotide or U7snRNA also restored FV protein synthesis in the patient's megakaryocytes, as demonstrated by FV immunofluorescence staining. On the other hand, treatment with antisense molecules also produced some cytotoxic effects, which however were largely attributable to the transfection agents used for delivery of the antisense molecules rather than to the antisense molecules themselves.

Since in humans both plasma and platelet FV derive from the liver, and since splicing is a tissue-specific process, the ideal model to screen antisense molecules for their ability to correct a *F5* splicing defect would be the patient's hepatocytes. However, a biopsy of the patient's liver is hardly ever available. Hence the relevance of our megakaryocyte model, which makes it possible to test antisense molecules on the patient's own cells obtained by a minimally invasive technique (venipuncture) and to check not only for *F5* splicing correction, but also for restoration of FV protein synthesis. In the near future this model might be replaced by patient's hepatocytes differentiated *ex vivo* by induced pluripotent stem cell (iPSC) technology.[72,73]

Overall, our findings provide *in vitro* and *ex vivo* proof-of-principle for the efficacy of antisense-based therapy in at least some forms of FV deficiency. However, before this molecular therapy can be applied to FV-deficient patients, several issues will have to be addressed, including the choice of antisense molecule (synthetic oligonucleotides *vs.* U7snRNA), its efficient delivery to hepatocytes, the duration of the antisense effect and possible side-effects. First of all, a suitable animal model will have to be developed to study the bio-distribution and pharmacokinetics, as well as the *in vivo* efficacy and safety, of the antisense molecules and their delivery agents. While transgenic mice expressing the human *F7* gene in the liver have been successfully used to test a U1snRNA-based splicing correction strategy,[71] a mouse model would be less suitable for FV deficiency, as FV biology is quite different in mice and humans. In particular, platelet FV (the major determinant of bleeding tendency in FV-deficient patients) is of megakaryocyte rather than liver origin in mice and not strictly required for protection from spontaneous or mild bleeding.[74,75] Once the efficacy and safety of antisense-based therapy have been established in animal models, translation to humans will have to overcome additional difficulties related to the genetic heterogeneity of FV-deficient patients (requiring a different and personalized antisense molecule, with its own pharmacological and toxicological profile, for each individual case) and to the general lack of interest of the pharmaceutical industry for rare ("orphan") diseases.

Role of plasma TFPI in severe FV deficiency

Previous studies by our group[76,77] indicated that the plasma levels of TFPIα and FV are highly correlated and that FV-deficient patients have particularly low antigen levels of free TFPIα (~35%) and full-length TFPIα (~24%). This was explained with the observation that a

large fraction of plasma full-length TFPIα is actually bound to FV, which is likely to protect full-length TFPIα from truncation and/or clearance in the circulation.[76,78] Recent developments have confirmed and extended these findings by showing that FV has a low-abundance splicing variant, known as FV-short, which binds full-length TFPIα with high affinity and stabilizes it in the circulation.[79]

In our original study we also proposed that the low full-length TFPIα level would be beneficial to FV-deficient patients, as it reduces the FV requirement for minimal thrombin generation to <1%.[76] Since patients with undetectable plasma FV often have residual platelet FV, the low full-length TFPIα levels allow these traces of platelet FV to support minimal thrombin generation and to rescue the patients from life-threatening bleeding.[80] Based on these findings and on the large inter-individual variation of plasma full-length TFPIα levels,[81] we predicted that plasma full-length TFPIα would be an important modulator of the bleeding phenotype in severe FV deficiency.[3,76] Unfortunately, however, this hypothesis can hardly be tested in epidemiological studies, due to the rare occurrence of the disease and to the genetic heterogeneity of FV-deficient patients.

In **Chapter 7** of this thesis we provide evidence for the role of plasma full-length TFPIα as a modulator of the clinical severity of FV deficiency. In fact, by comparing two related FV-deficient patients with equally undetectable plasma FV levels due to the same homozygous *F5* mutation, but strikingly different bleeding tendencies, we observed that the full-length TFPIα level was twice as high in the severe bleeder (proband A, 66%) than in the mild bleeder (proband B, 34%). This difference was reflected in their thrombin generation capacities and could be largely (though not entirely) abolished by the addition of inhibitory anti-TFPI antibodies, suggesting that the elevated full-length TFPIα level was indeed responsible for the severe bleeding symptoms of proband A. A parallel difference in plasma

full-length TFPI levels was observed between the probands' fathers, suggesting a genetic origin. However, no mutation was identified by direct sequencing of the *TFPI* gene. Therefore, we are now considering the possibility of conducting a whole exome sequencing to identify the possible cause of the elevated full-length TFPI level in proband A.

Full-length TFPIα, which represents only ~10% of all plasma TFPI, is the most biologically relevant form of plasma TFPI, because it expresses the highest anticoagulant activity. In contrast, the far more abundant truncated forms of TFPIα, lacking the Kunitz-3 domain and/or the C-terminus are considerably less potent anticoagulants. Although a genome-wide linkage study has provided evidence that a locus on chromosome 2 located close to the *TFPI* gene may influence the level of total TFPI in plasma,[82] the major genetic determinants of plasma full-length TFPIα are unlikely to reside within the *TFPI* gene. In fact, the TFPIα secreted by endothelial cells undergoes several processes in the circulation, which influence its survival in the full-length form. First of all, TFPIα is susceptible to cleavage by numerous plasma proteases, including leukocyte-derived proteases (neutrophil elastase, cathepsin G),[83] thrombin,[84] FXa,[85] FXIa,[86] plasmin,[87] APC,[88] factor VII activating protease (FSAP),[89] mast cell chymase[90] and matrix metalloproteinases.[91] Moreover, full-length TFPI has been reported to bind to FV[76,78] (particularly FV-short),[79] protein S[92] and high-density lipoproteins (HDLs),[93] which may protect it from truncation and/or clearance, whereas truncated TFPIα binds preferentially to low-density lipoproteins (LDLs).[93] In fact, there is growing evidence that TFPI physiology is tightly inter-twined with lipid metabolism.[94-96] Furthermore, since platelets contain 8-10% of all full-length TFPIα present in blood,[93,97] platelet activation status might also contribute to the plasma full-length TFPIα pool. Based on these considerations, the genetic determinants of plasma full-length TFPI level are likely to be scattered over several genes belonging to different pathways, including coagulation,

fibrinolysis, inflammation, platelet activation and lipid metabolism. Accordingly, a recent meta-analysis has reported evidence for the association between plasma full-length TFPIα levels and genetic variation in the *TFPI*, *PROS1*, *F5*, *APOE* and *GLA* genes,[96] while genome-wide studies are ongoing in our and other laboratories to identify additional genes involved in the regulation of plasma full-length TFPIα levels. The results of these studies will help us prioritize the variants identified by whole exome sequencing in the two probands investigated in Chapter 7.

Our finding that plasma full-length TFPIα modulates the bleeding tendency of FV-deficient patients also points at plasma TFPI as a possible therapeutic target in severe FV deficiency. In particular, TFPI inhibitors may significantly ameliorate the bleeding phenotype of FV-deficient patients. Since TFPI inhibition has also shown potential as an alternative therapeutic strategy for haemophilic patients, several TFPI inhibitors (including non-anticoagulant sulphated polysaccharides, antibodies, small peptides and aptamers) are already available and are currently in various stages of development as potential pharmacological agents.[98,99] Once approved for haemophilia treatment, these molecules might find off-label application in FV deficiency as well. Moreover, a detailed knowledge of the determinants of plasma full-length TFPIα levels might suggest novel approaches to down-regulate this anticoagulant protein.

Conclusions and future perspectives

In summary, the work described in this thesis provides new insights into the molecular genetics of FV deficiency and paves the way for the development of (personalized) treatment strategies.

Based on our experience, we propose the following workflow for the identification of the genetic defect(s) underlying FV deficiency in patients with a confirmed diagnosis of Owren parahemophilia (Figure 1).

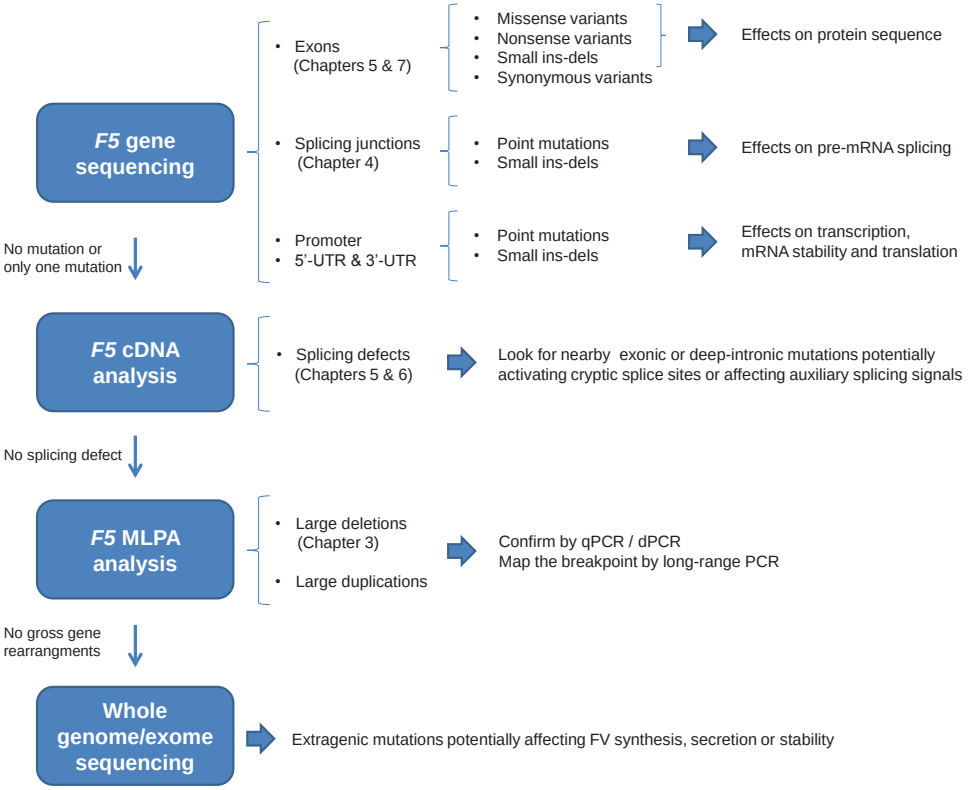

Figure 1. Proposed workflow for the identification of mutations responsible for FV deficiency. First of all, the coding region and all splicing junctions of the F5 gene should be sequenced to identify all exonic variants and exonic/intronic variants affecting the donor/acceptor splice sites. Ideally this screening should also include the proximal promoter, 5'-UTR and 3'-UTR regions. If no mutation or only one mutation is found by this approach, cDNA analysis is recommended to check for splicing defects caused by deep-intronic mutations or exonic/intronic mutations affecting auxiliary splicing signals. If no splicing defect is detected, an MLPA analysis should be performed to look for large deletions/duplications involving multiple F5 gene exons. Finally, if this analysis also fails to reveal any abnormality, whole genome/exome sequencing might be warranted to look for extragenic mutations potentially affecting FV synthesis, secretion and/or stability in the circulation.

As far as treatment is concerned, our work provides *in vitro* and *ex vivo* proof-of-principle that severe FV deficiency caused by (at least) certain splicing mutations is amenable to personalized molecular therapy with antisense oligonucleotides or U7snRNA. The *in vivo* translation of this form of therapy is not yet straightforward, but may take advantage of the experience gained with many other genetic diseases. Alternatively, FV-deficient patients may benefit from therapeutic approaches based on the inhibition of plasma TFPI, which are already under development for the treatment of hemophilia A and B.

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Summary

Nederlandse samenvatting

Summary

Coagulation factor V (FV) is a large liver-derived glycoprotein present in plasma and platelets. After proteolytic activation, it serves as an essential cofactor in the conversion of prothrombin to thrombin, accelerating this reaction by several orders of magnitude. This function makes FV indispensable to life. FV deficiency is a rare autosomal recessive bleeding disorder caused by loss-of-function mutations in the *F5* gene. The associated bleeding tendency is extremely variable and poorly correlated with plasma FV levels. Since FV concentrates or recombinant FV preparations are not available, treatment and prophylaxis of FV-deficient patients still relies on fresh frozen plasma, with potential complications such as volume overload, allergic reactions and transmission of infectious agents.

The work described in this thesis focusses on the molecular genetics of FV deficiency as a starting point for the development of personalized molecular therapies.

Chapter 1 provides a general overview of the hemostatic system and the coagulation cascade, with particular emphasis on the structure, functions and pivotal regulatory role of FV. Moreover, it introduces the genetic bases, clinical manifestations and unresolved issues of FV deficiency. Since the core of this thesis is dedicated to splicing mutations and their correction using antisense-based approaches, **Chapter 2** discusses the process of pre-mRNA splicing, the various mechanisms by which genetic mutations can alter normal splicing and the emerging strategies to correct aberrant splicing using antisense molecules.

Chapter 3 describes the development and validation of a multiplex ligation-dependent probe amplification (MLPA) assay for the efficient detection of large deletions and duplications in the *F5* gene. Application of this assay to 14 genetically unresolved FV-deficient patients led to the identification of a novel ~2-kb deletion spanning intron 8 through intron 10,

demonstrating the potential utility of this in-house assay in the molecular diagnosis of FV deficiency.

Chapters 4-6 share *F5* pre-mRNA splicing as a common theme. **Chapter 4** is a preliminary report of a novel *F5* splicing mutation affecting the canonical donor splice site of intron 3 (IVS3+2T>C*), identified in the homozygous state in a toddler with undetectable plasma FV levels and multiple intracranial hemorrhages. The mutation introduces a mismatch at the invariable +2 position of the donor splice site consensus sequence, most likely leading to exon 3 skipping. However, *F5* mRNA analysis could not be performed yet. **Chapter 5** presents the genetic and functional characterization of a patient with severe FV deficiency and moderate bleeding symptoms. This patient was found to be doubly heterozygous for a missense mutation in exon 4 (Cys165Ser) and an apparently synonymous variant in exon 8 (1371C>G). Analysis of the patient's cDNA, in combination with a detailed *in silico* analysis and splicing assays in a minigene model, indicated that the latter mutation actually disrupts *F5* pre-mRNA splicing, activating a cryptic splice site in exon 8 and causing the in-frame deletion of 18 nucleotides from the mature mRNA. Additional experiments in the minigene model showed that this aberrant splicing event could be corrected in a specific and dose-dependent manner by a morpholino antisense oligonucleotide designed to mask the incorrect splicing signal introduced by the 1371C>G mutation. **Chapter 6** extends the application of antisense technology to a *F5* deep-intronic mutation (IVS8+268A>G) identified in the homozygous state in a patient with severe FV deficiency and life-threatening bleeding manifestations. This mutation causes the activation of a cryptic donor splice site in intron 8, leading to the retention of an intronic pseudo-exon with an in-frame stop codon in the mature mRNA. A mutation-specific morpholino antisense oligonucleotide and an engineered U7 small nuclear RNA (U7snRNA) corrected this splicing defect in a specific and dose-

dependent manner, not only in a minigene model of the *F5* IVS8+268A>G mutation, but also in the patient's own megakaryocytes obtained by *ex vivo* differentiation of circulating hematopoietic progenitors, effectively restoring FV protein expression in these cells. Overall, these data provide proof-of-principle for the efficacy of antisense-based therapeutic approaches in severe FV deficiency.

Chapter 7 reports the case of two distantly related FV-deficient patients who, despite being homozygous for the same *F5* missense mutation (Trp582Gly) and having equally undetectable plasma FV levels, showed very different bleeding tendencies. Phenotyping of the patients' plasma revealed two-fold higher levels of the anticoagulant protein full-length tissue factor pathway inhibitor (TFPI) in the severe bleeder (proband A) than in the moderate bleeder (proband B). Furthermore, thrombin generation experiments in the absence and presence of anti-TFPI antibodies suggested that the difference in plasma TFPI levels may indeed account for the difference in bleeding phenotypes. While the cause of the elevated TFPI level in proband A is still under investigation, these findings support a physiological role for plasma full-length TFPI as a modulator of the bleeding diathesis in severe FV deficiency, pointing at TFPI as an additional therapeutic target in these patients.

Finally, **Chapter 8** puts all findings into perspective, discusses them in the light of current literature and draws some general conclusions. In particular it proposes an optimized workflow for the molecular diagnosis of FV deficiency and it summarizes alternative (and personalized) therapeutic options based on the correction of splicing defects using antisense technology or on the pharmacological inhibition of TFPI.

**F5* nucleotides and FV amino acids in this summary are numbered according to the classical nomenclature, based on Jenny et al. 1987.

Nederlandse samenvatting

Stollingsfactor V (FV) is een groot glycoeiwit dat in de lever wordt aangemaakt en aanwezig is in plasma en bloedplaatjes. De geactiveerde vorm van FV dient als een essentiële cofactor bij de omzetting van protrombine in trombine, waarbij het de trombinevorming orden van grootte versterkt. Hierdoor is FV onmisbaar voor het leven. FV-deficiëntie is een zeldzame autosomaal recessieve aandoening veroorzaakt door mutaties in het *F5* gen. De hieruit volgende bloedingsneiging is variabel en correleert slecht met de FV spiegels in plasma. Omdat FV concentraten of recombinante FV producten niet beschikbaar zijn, zijn FV-deficiënte patiënten nog steeds afhankelijk van vers bevroren plasma voor hun behandeling en profylaxe, met potentiële complicaties zoals circulatoire overbelasting, allergische reacties en overdracht van infecties.

Het werk beschreven in dit proefschrift richt zich op de moleculaire genetica van FV deficiëntie als uitgangspunt voor de ontwikkeling van gepersonaliseerde behandeling door moleculaire therapieën.

Hoofdstuk 1 geeft een algemeen overzicht van het hemostasesystem en de stollingscascade, met bijzondere nadruk op de structuur, functies en centrale regulerende rol van FV. Het introduceert de genetische basis, klinische symptomen en onopgeloste vragen van FV deficiëntie. Aangezien een groot deel van dit onderzoek gewijd is aan splicing afwijkingen en het corrigeren ervan d.m.v. antisense-therapie, beschrijft **hoofdstuk 2** het proces van pre-mRNA splicing, de verschillende mechanismen waarbij mutaties de normale splicing kunnen verstoren, en het gebruik van antisense moleculen om afwijkende splicing te corrigeren.

Hoofdstuk 3 beschrijft de ontwikkeling en validatie van een methode, de zogenaamde multiplex ligatie-afhankelijke probe amplificatie (MLPA) test, voor het efficiënt opsporen van grote deleties en duplicaties in het *F5* gen. Deze assay werd toegepast op 14 genetisch

onopgeloste FV-deficiënte patiënten en leidde tot de ontdekking van een nieuwe grote deletie die exon 9 en 10 omvat. Deze bevinding bewijst de bruikbaarheid van onze in-huis ontwikkelde MLPA test in de moleculaire diagnostiek van FV deficiëntie.

Hoofdstukken 4-6 hebben als gemeenschappelijk onderwerp de pre-mRNA splicing van het *F5* gen. **Hoofdstuk 4** is een voorlopige beschrijving van een nieuwe homozygote *F5* splicing mutatie (IVS3 +2T>C*) in een peuter met een niet-detecteerbare plasma FV spiegel en meerdere intracraniële bloedingen. Deze mutatie introduceert een mismatch op de geconserveerde +2 positie van de donor splice site consensussequentie van intron 3, met exon 3 skipping als meest waarschijnlijk gevolg, alhoewel dit niet bewezen kon worden omdat de *F5* mRNA analyse nog niet kon worden uitgevoerd. **Hoofdstuk 5** behandelt de genetische en functionele karakterisering van een patiënt met een ernstige FV-deficiëntie en een matige bloedingsneiging. Deze patiënt bleek dubbel heterozygoot voor een missense mutatie in exon 4 (Cys165Ser) en een schijnbaar synonieme variant in exon 8 (1371C>G). Analyse van het cDNA van de patiënt, in combinatie met een gedetailleerde *in silico* analyse en splicing assays in een daartoe opgezet minigenmodel, gaf echter aan dat deze mutatie de *F5* pre-mRNA splicing verstoort door het activeren van een cryptische splice site in exon 8, resulterend in een in-frame deletie van 18 nucleotiden van het mRNA. Additionele experimenten in het minigenmodel toonden aan dat deze afwijkende splicing gecorrigeerd kon worden door de toepassing van een specifieke morfolino antisense oligonucleotide die ontworpen was om de onjuiste splicingsequentie bij de 1371C>G mutatie te verbergen. **Hoofdstuk 6** breidt de toepassing van antisense-technologie uit tot een *F5* "diep-intronische" mutatie (IVS8+268A>G) opgespoord in een homozygote patiënt met een ernstige FV deficiëntie en levensbedreigende bloedingen. Deze mutatie veroorzaakt de activering van een cryptische donor splice site in intron 8 die ervoor zorgt dat een

intronische pseudo-exon met een in-frame stopcodon in het mRNA blijft zitten. Een mutatie-specifieke morfolino antisense oligonucleotide en een gemutageniseerde U7 small nuclear RNA (U7snRNA) corrigeerden dit splicing defect op een specifieke en dosis-afhankelijke wijze, niet alleen in een *in vitro* minigenmodel van de *F5* IVS8+268A>G mutatie, maar ook in lichaamseigen megakaryocyten van de patiënt verkregen door *ex vivo* differentiatie van circulerende hematopoietische stamcellen, leidend tot een effectief herstel van de FV eiwit expressie. Deze resultaten onderbouwen de effectiviteit van antisense-gebaseerde therapeutische benaderingen bij ernstige FV deficiënties.

In **hoofdstuk 7** worden twee gerelateerde FV-deficiënte patiënten gerapporteerd, die allebei homozygoot zijn voor de zelfde *F5* missense mutatie (Trp582Gly) met bijbehorende ondetecteerbare plasma FV spiegels, en toch zeer verschillende bloedingsneigingen vertoonen. Fenotypering van het plasma van deze patiënten wees uit dat de ernstige bloeder (proband A) een tweemaal hogere concentratie van het antistollende eiwit full-length tissue factor pathway inhibitor (TFPI) in zijn plasma had dan de matige bloeder (proband B). Bovendien suggereerden trombinegeneratie experimenten in afwezigheid en aanwezigheid van anti-TFPI antilichamen dat het verschil in TFPI plasma concentraties het verschil in bloedingsfenotype kon verklaren. Hoewel de oorzaak van het verhoogde TFPI niveau in proband A nog onduidelijk is, ondersteunen deze bevindingen een fysiologische rol voor full-length TFPI bij het moduleren van bloedingsneigingen bij ernstige FV deficiëntie, waarbij TFPI als mogelijk doel voor behandeling aangewezen kan worden.

In **hoofdstuk 8** worden alle bevindingen in perspectief gezet en besproken in het licht van de huidige literatuur, resulterend in enkele algemene conclusies en aanbevelingen. Als aanbeveling wordt een geoptimaliseerde workflow voor de moleculaire diagnostiek van FV-deficiëntie voorgesteld. Ten slotte geeft het hoofdstuk een overzicht van alternatieve (en

persoonlijke) therapeutische behandelingen op basis van de correctie van splicingdefecten met behulp van antisense-technologie of op basis van farmacologische remming van TFPI.

**F5* nucleotiden en FV aminozuren in deze samenvatting zijn genummerd volgens de klassieke nomenclatuur gebaseerd op Jenny et al. 1987.

Valorisation

Science was originally a curiosity-driven activity with no other purpose than to understand and explain natural phenomena. Only later it was realized that the knowledge gained by exploring nature could be employed to solve practical problems. This dichotomy is still reflected in the current distinction between basic and applied research.

My PhD project was dedicated to the study of the genetics and biochemistry of factor V (FV) deficiency, a rare inherited bleeding disorder for which no optimal treatment is available yet. Most of the work described in this thesis can be regarded as basic research and was done with the primary aim of gaining more insight into the molecular bases of this genetic disease. However, some of my results may find practical application in the near or distant future.

First of all, I have developed and validated a multiplex ligation-dependent probe amplification (MLPA) assay for the detection of large deletions and duplications in the *F5* gene. This assay can be performed in any molecular biology laboratory and may be implemented immediately in the molecular diagnostics of FV deficiency. The company that developed the MLPA technique expressed interest in my assay and invited me for a seminar.

In addition, I have shown in both *in vitro* and *ex vivo* models that some *F5* splicing mutations responsible for (severe) FV deficiency are amenable to antisense-based RNA therapy using antisense oligonucleotides or engineered U7 small nuclear RNA (U7snRNA). Although these studies are promising, the *in vivo* translation of this form of therapy is still hampered by the lack of suitable animal models and by the rare occurrence and genetic heterogeneity of FV deficiency, each mutation requiring a different custom-made oligonucleotide (or U7snRNA) with its own pharmacological/toxicological profile.

Finally, I have provided additional evidence that the level of plasma full-length tissue factor pathway inhibitor (TFPI) is an important determinant of the bleeding tendency in FV-deficient patients. This finding suggests that TFPI might be a suitable target for

pharmacological interventions aimed at preventing life-threatening bleeding in severe FV deficiency. In this respect, FV-deficient patients may benefit from the TFPI inhibitors that are already being developed and tested for the treatment of haemophilia, a much more common bleeding disorder.

Since FV deficiency is a rare disease, with an estimated incidence of 1 in 1 million in the general population, it is unfortunately not an attractive target for the pharmaceutical industry. This is regrettable, as some FV-deficient patients bleed as much and as severely as haemophiliacs, but enjoy a far lower healthcare standard, even in the Western world. Despite remarkable advances in transfusion medicine, the repeated administration of fresh frozen plasma exposes FV-deficient patients to the risk of transfusion reactions, allergic reactions and the transmission of infectious agents, besides taking a toll on the patients' cardiovascular systems on the longer run. On the other hand, the therapeutic development of antisense molecules targeting individual mutations for the personalised treatment of a single patient or only a few patients is hardly feasible, unless the regulations for clinical testing and licensing are specifically modified, as currently being discussed for other rare and/or life-threatening genetic diseases with unmet medical needs (Aartsma-Rus *et al*. Translational and regulatory challenges for exon skipping therapies. *Hum Gene Ther* 2014; 25: 885-892). In this context, pharmacological inhibition of TFPI by means of antibodies, peptides, aptamers or small molecules could represent a valid therapeutic alternative, suitable for all FV-deficient patients irrespective of their mutation(s).

In conclusion, given the complications associated with the current treatment of FV deficiency, it is highly desirable that the long-awaited FV concentrate (currently undergoing pre-clinical testing) and/or other forms of therapy, such as those outlined in this thesis, will become available soon.

Curriculum vitae

List of publications

Curriculum vitae

Francesca Nuzzo was born on 23rd May 1986 in Tricase (Italy). After completing her secondary education at the Scientific High School "Leonardo Da Vinci" in Maglie, she enrolled at Ferrara University, where she obtained a Bachelor's degree in Biology (2008) and a Master's degree in Cellular and Molecular Biology (2010), both *cum laude*. Her Master thesis, entitled: "Minigene systems to study splicing mutations in human coagulation factor IX", was based on a 10-month practical internship at the Department of Biochemistry and Molecular Biology of Ferrara University under the supervision of Dr. M. Pinotti.

In January 2011, she started a PhD project on coagulation factor V deficiency at the Department of Biochemistry of Maastricht University (The Netherlands) under the supervision of Prof. Dr. T. M. Hackeng and Dr. E. Castoldi. In the frame of this project, she also visited the laboratory of Prof. Dr. P. Simioni at Padua University (Italy) to perform experiments on cultured megakaryocytes together with Dr. C. Radu. As a PhD student, she attended several courses on haemostasis and thrombosis organised by the Cardiovascular Research Institute Maastricht (CARIM), the Dutch Society on Thrombosis and Haemostasis (NVTH) and the Dutch Heart Foundation (NHS). Moreover, she presented her work at national and international congresses, receiving an "Award of Excellence" at the Annual Symposium of the Dutch Society on Thrombosis and Haemostasis (Koudekerke, March 2012) and a "Young Investigator Award" at the XXIV Congress of the International Society on Thrombosis and Haemostasis (Amsterdam, July 2013).

List of publications

1. **Nuzzo F**,[#] Radu C,[#] Baralle M, Spiezia L, Hackeng TM, Simioni P, Castoldi E. Antisense-based RNA therapy of factor V deficiency: *in vitro* and *ex vivo* rescue of a *F5* deep-intronic splicing mutation. Blood 2013; 122: 3825-31. [#]Equal contribution.

2. **Nuzzo F**, Paraboschi EM, Straniero L, Pavlova A, Duga S, Castoldi E. Identification of a novel large deletion in a patient with severe FV deficiency using an in house *F5* MLPA assay. Haemophilia 2015; 21: 140-7.

3. **Nuzzo F**, Bulato C, Nielsen BI, Lee K, Wielders SJ, Simioni P, Key NS, Castoldi E. Characterisation of an apparently synonymous *F5* mutation causing aberrant splicing and factor V deficiency. Haemophilia 2015; 21: 241-8.

4. **Nuzzo F**, El-Beshlawi I, Wielders SJ, Castoldi E. Plasma tissue factor pathway inhibitor modulates bleeding tendency in severe factor V deficiency. In preparation.

Congress abstracts

1. **Nuzzo F**, Duckers C, Baralle M, Hackeng TM, Rosing J, Simioni P, Castoldi E. Development of a molecular therapy for severe factor V deficiency caused by a homozygous splicing mutation. Poster presentation at the CARIM Symposium 2011. Maastricht (The Netherlands), 9th November 2011.

2. **Nuzzo F**, Baralle M, Simioni P, Hackeng TM, Castoldi E. *In vitro* rescue of a *F5* deep-intronic splicing mutation using a specific morpholino antisense oligonucleotide. Oral presentation at the Genetica Retraite 2012. Rolduc (The Netherlands), 15-16th March 2012.

3. **Nuzzo F**, Baralle M, Hackeng TM, Rosing J, Simioni P, Castoldi E. Design of an antisense-based molecular therapy for severe factor V deficiency caused by a homozygous splicing mutation. Oral presentation at the Meeting of the Dutch Society on Thrombosis and Haemostasis. Koudekerke (The Netherlands), 25-26th March 2012.

4. **Nuzzo F**, Duga S, Pavlova A, Castoldi E. Development and validation of an MLPA assay for the detection of large deletions or duplications in the *F5* gene. Oral presentation at the Genetica Retraite 2013. Rolduc (The Netherlands), 21st-22nd March 2013.

5. **Nuzzo F**, Bulato C, Simioni P, Key NS, Castoldi E. Characterisation of an apparently synonymous *F5* mutation causing aberrant splicing and factor V deficiency. Oral presentation at the Genetica Retraite 2013. Rolduc (The Netherlands), 21st-22nd March 2013.

6. **Nuzzo F**, Radu C, Baralle M, Spiezia L, Simioni P, Castoldi E. Antisense-based RNA therapy of severe coagulation factor V deficiency: *in vitro* and *ex vivo* rescue of a *F5* deep-intronic splicing mutation. Oral presentation at the XXIV Congress of the International Society on Thrombosis and Haemostasis. Amsterdam (The Netherlands), 1st-4th July 2013.

7. **Nuzzo F**, Bulato C, Nielsen BI, Lee K, Wielders SJ, Simioni P, Key NS, Castoldi E. Characterisation of an apparently synonymous *F5* mutation causing aberrant splicing and factor V deficiency. Oral presentation at the Meeting of the Dutch Society on Thrombosis and Haemostasis. Koudekerke (The Netherlands), 9-10th April 2014.

Awards

Award of Excellence

Annual Symposium of the Dutch Society on Thrombosis and Haemostasis (NVTH)

Koudekerke (The Netherlands), 25-26th March 2012

Young Investigator Award

XXIV Congress of the International Society on Thrombosis and Haemostasis (ISTH)

Amsterdam (The Netherlands), 1st-4th July 2013

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I wish you all good luck, may you reach your goals and move up the career ladder!

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And last but not least, thanks to my **family** and **friends of ever**, who encouraged me to go on because at the end it would be worth it. Well, after these four years, I can tell you that I am not a biochemist neither a writer; but I enjoyed the ride, I loved the lab and, within my limits, I tried to do my best, especially with molecular biology.

…And now, the worst is yet to come: the defense!