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DOTTORATO DI RICERCA IN
SCIENZE BIOMEDICHE E BIOTECNOLOGICHE

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CICLO XXX

Protein engineering and pharmacological approaches to develop novel treatment strategies for coagulation disorders

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Abbreviations

APC: activated protein C

APCC: activated prothrombin complex concentrate

AT: antithrombin

EGF: epidermal growth factor

FcRn: neonatal Fc receptor

FIX: factor IX

FIXa: activated factor IX

FV: factor V

FVa: activated factor V

FVII: factor VII

FVIIa: activated factor VII

FVIII: factor VIII

FVIIIa: activated factor VIII

FX: factor X

FXa: activated factor X

FXI: factor XI

FXIa: activated factor XI

FXIIa: activated factor XII

hFcRn: human neonatal Fc receptor

HRP: horseradish peroxidase

HSA: human serum albumin

mFcRn: mouse FcRn

MSA: mouse serum albumin

NMD: nonsense mediated decay

PC: protein C

PTC: premature termination codon

rFIX: recombinant factor IX

rFX: recombinant factor X

rFVII: recombinant factor VII

rFVIIa: recombinant activated factor VII

TF: tissue factor

TFPI: tissue factor pathway inhibitor

vWF: von Willebrand factor

Chapter 1

General Introduction

1.1. Haemostasis

Haemostasis is a physiological process whereby blood is maintained fluid under normal conditions but is allowed to clot in case of trauma. Various cellular and molecular components are involved in the tight regulation of this mechanism [1]. In the resting state endothelial cells prevent platelet adherence and thus the activation of blood coagulation. Moreover, clot formation in normal blood vessels is also prevented by the synthesis of prostacyclin and heparin-like substances, and by the presence of protein complexes (thrombin-thrombomodulin) leading to generation of anticoagulant proteins (activated protein C, APC) [2].

After vessel injury, damaged endothelial cells expose negatively charged phospholipids and release procoagulant proteins [3]. Platelets adhere to subendothelial tissues *via* von Willebrand Factor (vWF), that acts as a bridge by binding to exposed collagen and to Glycoprotein Ib (GPIb), a specific receptor on platelet surface [2]. The phospholipid composition of the platelet membrane changes, resulting in the exposure of negatively charged phosphatidylserine on the outer leaflet [4][5].

The activation of platelets by thrombin, ADP, thromboxane A2 or epinephrine triggers morphological and biochemical alterations, leading to the secretion of α-granules, containing vWF, fibrinogen, factor V (FV), and δ-granules, containing calcium ions and ADP. Glycoprotein IIb-IIIa (G-IIb-IIIa) expression on platelet membrane surface mediates interaction with fibrinogen, vWF, fibronectin and platelet aggregation, with the formation of a sort of plug that provisionally block blood loss [6].

The exposure of blood to tissue factor (TF) triggers the activation of the coagulation cascade, culminating in thrombin generation. Newly-formed thrombin converts soluble fibrinogen into insoluble fibrin that precipitates at the site of injury, stabilizing the platelet plug. Later, as wound healing occurs, the fibrin clot is degraded by the serine protease plasmin, a process known as fibrinolysis [7].

1.2 Blood coagulation

According to the classical model, described in 1964 by two independent groups [8][9], coagulation is represented as a cascade/waterfall divided into two pathways: an "intrinsic pathway", so called because all the components are present in blood and an "extrinsic pathway", in which the subendothelial cell membrane protein TF is required in addition to circulating components. The initiation of both pathways resulted in activation of factor X (FX) and the eventual generation of a fibrin clot through a common pathway [10]. Although these concepts served for many years as a useful model, more recent clinical and experimental observations [11] explain how the classical cascade hypothesis does not fully reflect the events of hemostasis in vivo. A cell-based model of coagulation explains, in a more physiological way, how coagulation evolves in consequence of an injury, underlying the roles of cellular components [12]. Several cell types play different roles in the coagulation process, due to their procoagulant or anticoagulant properties. Blood platelets and TF-bearing microparticles (MPs) support procoagulant reactions, supplying negatively charged phospholipids essential for the correct assembly of macromolecular complexes. Vascular endothelial cells play a key role in maintaining the anticoagulant properties of the vasculature.

Formation of an impermeable platelet and fibrin clot at the site of the vessel injury is essential, but it is also required that procoagulant substances remain localized on damaged site. This localization is mediated by the different distribution of procoagulant reactions on specific cell surfaces.

According to this model, coagulation proceeds as a sequence of events localized on the site of vessel injury and occurs in distinct overlapping steps: initiation, amplification and propagation.

Initiation phase

The process of blood coagulation is triggered by the exposure of TF to flowing blood. TF is an integral membrane protein constitutively expressed on cells such as fibroblasts and smooth muscle cells, but not resting endothelium; it constitutes an hemostatic envelope normally not in contact with blood [6]. Upon endothelium disruption, TF is exposed to flowing blood, where it can be bound by factor VII (FVII) or activated factor VII (FVIIa), present at trace levels in plasma. The new-formed TF/FVIIa complex activates small amounts of FX and factor IX (FIX). Activated FX

(FXa) associates with its cofactor, activated factor V (FVa), and forms the so called "prothrombinase complex" on the surface of TF-bearing cells [13], leading to the conversion of small amounts of circulating prothrombin (II) into thrombin (IIa) (fig.1.1).

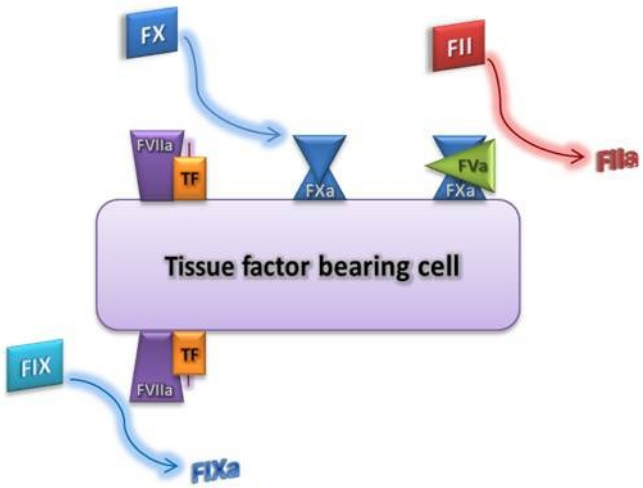

Figure 1.1. *Factor VIIa bound to TF activates both factor X and factor IX. Factor Xa formed by factor VIIa/TF binds to factor Va and converts a small amount of prothrombin to thrombin.*

The active form of FV derives from the plasma pool and from the α-granules of activated platelets [14]. FV can also be activated by thrombin and FXa [15] or by noncoagulant proteases [16].

It is worth noting that this initiation phase only results in a trace amount of thrombin after FXa activation, because FXa and thrombin are rapidly neutralized by antithrombin (AT) and the complex FVIIa-TF-FXa by the tissue factor pathway inhibitor (TFPI).

Amplification phase

The small amount of thrombin generated during the initiation phase exerts several important functions:

- complete activation of platelets, with the induction of higher levels of procoagulant activity [17] and the release of partially activated forms of FV onto their surfaces;
- activation of the pro-cofactors FV and FVIII on the activated platelet surface and the concomitant dissociation of the FVIII/vWF complex, permitting vWF

to mediate additional platelet adhesion and aggregation at the site of injury (fig. 1.2);

- conversion of factor XI to its active form (FXIa), which enhances the amount of platelet surface FIXa, increasing the supply of FXa and thereby acting as a "booster" of thrombin generation on the platelet surface [18].

By the end of the amplification phase, the "stage" is set for large-scale thrombin generation in the propagation phase.

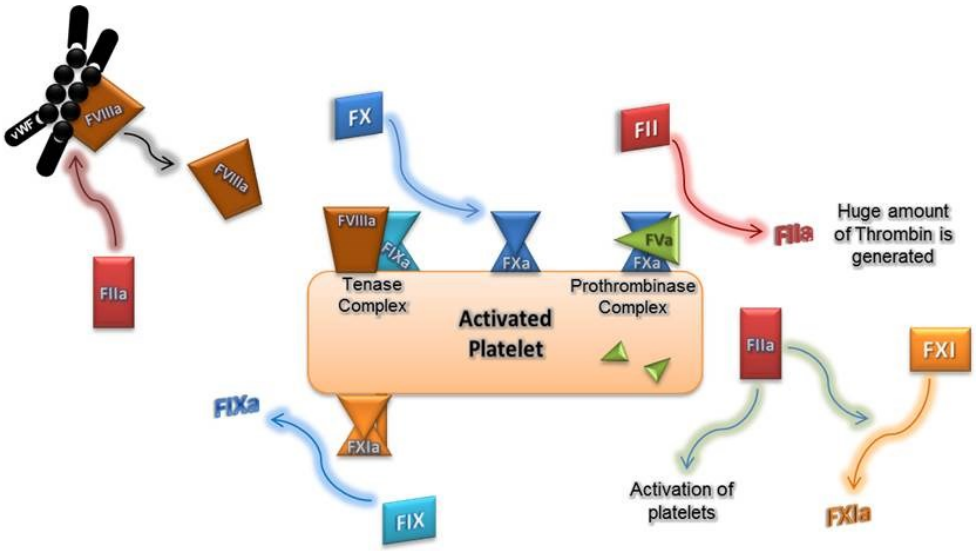

Figure 1.2. *On the surface of an activated platelet, factor IXa formed on the TF-bearing cell can incorporate into a Xase complex. Additional factor IXa is formed by platelet-bound factor XIa. Factor Xa formed on the platelet membrane surface is channelled into prothrombinase complexes, leading to a burst of thrombin generation.*

Propagation phase

FIXa generated during the initiation phase can now bind to its cofactor FVIIIa, FX and calcium on negatively charged surfaces, mostly on platelets membrane, in order to form the "tenase complex", leading to the generation of a large amount of FXa. Because FXa cannot move effectively from the TF-bearing cell to the activated platelet, FXa must be provided directly on the platelet surface by the FIXa/FVIIIa complex, which is considered to be around 50-fold more efficient in this process compared to the FVIIa-TF complex. FXa then participates in the assembly of the prothrombinase complex together with FVa and calcium, which results in an explosive generation of thrombin sufficient to clot fibrinogen [6][12][19]. Hence more than 95% of the total amount of thrombin production takes place after initial clot formation, during propagation phase [20].

Termination phase

Once a fibrin clot is formed, coagulation must be limited to avoid thrombotic occlusion in normal areas of the vasculature [21].

The TF/FVIIa activity is limited by the Kunitz type inhibitor TFPI [22][23], secreted by the endothelium. TFPI binds to FXa forming a quaternary complex with TF/FVIIa that quickly limits coagulation [24].

The serine protease inhibitor (serpin) AT [25] is the major thrombin-inactivating protein [26] and it also neutralizes other enzymes of the coagulation system, thus protecting the circulation from free enzymes and limiting the clotting process to the site of injury. Circulating AT is a relatively inefficient serpin but its activity can be stimulated by heparin or heparin-like molecules such as sulfated glycosaminoglycans [27].

The protein C (PC) anticoagulant system inhibits the procoagulant functions of FVIIIa and FVa, the cofactors involved in the tenase and prothrombinase complexes, respectively [28]. The key component is PC, a vitamin K-dependent zymogen activated by thrombin bound to the membrane protein thrombomodulin (TM) on the surface of endothelial cells. Upon binding to TM, thrombin becomes more effective in activating PC than clotting fibrinogen or activating platelets [29], thus effectively changing from a procoagulant to an anticoagulant molecule.

APC cleaves a few peptide bonds of FVIIIa and FVa, resulting in their inactivation [30]. APC can also cleave the intact form of FV, converting it to an anticoagulant cofactor of APC in the inactivation of FVIIIa in the tenase complex [6].

1.3 Factor VII

Human coagulation factor VII (fig. 1.3) is the zymogen for a plasmatic vitamin K-dependent serin protease. It is synthesized in liver as a single chain polypeptide of approximately 50 kDa and it circulates in plasma at a concentration of 0.5 μg/mL (10 nM).

Biosynthesis and post-translational modifications

FVII is encoded by the *F7* gene, which is located on the long arm of chromosome 13, region q34-qter. The gene spreads about 12.8 kb of genomic DNA and contains nine exons and eight introns.

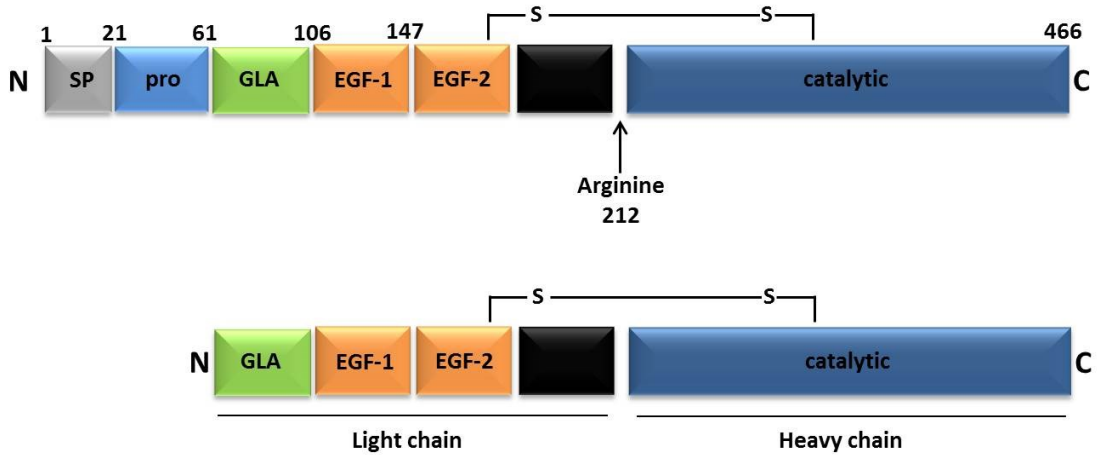

Figure 1.3. *Schematic representation of FVII (top) and activated FVII (bottom) protein domains.*

Like the other coagulation serin proteases, FVII encounters different post-translational modifications, needed to ensure its proper secretion, function and half-life.

The signal peptide (residues 1-20) mediates association of the nascent polypeptide with the cytosolic face of the endoplasmic reticulum (ER). Cleavage of the signal sequence by the signal peptidase then releases the mature amino-terminus into the lumen of the ER and is required for the translocation to the secretory pathway [31].

The vitamin K-dependent coagulation factors have some conserved disulfide bonds. Generally, three disulfide bonds occur within each EGF (Epidermal Growth Factor)-like domain, and several disulfide bonds occur within the serine protease catalytic domain [31]. In addition, a disulfide bond between Cys^{195} and Cys^{322} connects the light and heavy chains of the protein to prevent their dissociation after activation [32].

The precursor of FVII contains a propeptide (residues 21-60) that directs γ-carboxylation of 10 glutamic acid residues within the Gla domain, a process mediated by the vitamin K-dependent γ-glutamyl carboxylase enzyme [33]. In the presence of CO_2, O_2 and vitamin K hydroquinone (KH_2) the enzyme is able to carboxylate a peptide yielding Gla residues, vitamin K epoxide and H_2O. The vitamin K epoxide formed is subsequently reduced by a thiol or the enzyme vitamin K epoxide reductase to regenerate KH_2 [34].

Upon transit through the Golgi apparatus a series of additional modifications occur:

- N-glycosylation of two asparagine residues, one in the linker region (Asn^{205}) and one in the catalytic domain (Asn^{382}), important for an efficient biosynthesis and secretion;
- O-glycosylation of two serine residues in the EGF1-like domain (Ser^{112} and Ser^{120}), whose functional significance is not yet known [35].

Just prior to secretion from the cell propetide cleavage occurs in the trans-Golgi compartment, releasing the zymogen form of the mature protein (406 aa) [36].

Activation, structure and function

FVII mostly circulates as zymogen, with traces (about 1%) of FVIIa [37]. Activation of FVII occurs by the cleavage of a single peptide bond between Arg^{212} and Ile^{213}; this results in the formation of a two-chain FVIIa molecule consisting of a light chain of 152 amino acids (Gla, EGF-1, EGF-2 domains) and a heavy chain of 254 amino acids (catalytic domain) held together by a single disulfide bond between Cys^{195} and Cys^{322}. Conversion of FVII to FVIIa is catalyzed by a number of proteases, including thrombin, FIXa, FXa, FXIa and activated factor XII (FXIIa) [38]. Comparison of these proteins has shown that FXa, in association with phospholipids, has the highest potential to activate FVII [39]. It has also been reported an autocatalytic mechanism of FVII activation following complex formation of FVIIa with TF, which may play a key role in the initiation of extrinsic coagulation in normal hemostasis [40].

Cleavage of the peptide bond between Arg^{212} and Ile^{213} generates the mature N-terminal residue Ile^{213} and enables conformational changes that create the active enzyme. The new N-terminus becomes buried with its non-polar side chain in a hydrophobic environment and its charged α-amino nitrogen forms a salt bridge with the carboxyl group of Asp^{403} side chain. This rearrangement allows the formation of the oxyanion hole and enables the creation of catalytic site active conformation [41].

FVIIa is a trypsin-like enzyme and catalyses the hydrolysis of peptide bonds within a polypeptide chain to produce two new smaller peptides. In 1964, FVIIa proteolytic activity was observed first against FX; a few years later, it was demonstrated that TF interacts with FVII and the formed intermediate was catalytically active [42]. The reaction product of TF and FVII is a potent activator of FX and also of FIX [43]. The kinetics of substrate hydrolysis by FVIIa were later extensively investigated [44]. Binding of FVIIa to TF increases the catalytic efficiency of FVIIa of more than 100 fold. The interaction of FVIIa with TF is Ca^{2+}-dependent; Ca^{2+} saturation of Gla domain is

likely responsible for this increase in affinity, since deletion of Gla in FVIIa results in loss of affinity for TF. Ca^{2+} may stabilize energetically important hydrophobic contacts of Gla with TF [45]. In addition, Ca^{2+} increases FVIIa affinity for FX by conformational changes in FVIIa and FX that are essential for the interaction of these proteins with phospholipids [46].

1.4 Factor IX

Human coagulation factor IX, also called Christmas factor or antihemophilic B factor, is a single chain glycoprotein synthesized in liver and circulating in blood like zymogen at a concentration of 5 μg/ml [47]. The gene for FIX (*F9*) consists of eight exons and seven introns, is approximately 34 kb long, and is located on the long arm of the X chromosome at Xq27.1 [48].

It is synthesized as a precursor protein of 461 amino acids containing a 28-residue signal peptide and an 18-residue leader propeptide. During biosynthesis, FIX undergoes several post-translational modifications. These include γ-carboxylation of the first 12 Glu residues, glycosylation at Asn^{203}, Asn^{205}, Ser^{99}, Ser^{107}, Thr^{215} and Thr^{218}, partial hydroxylation of Asp^{110}, sulphatation of Tyr^{201}, phosphorylation of Ser^{204} and removal of the signal and the propeptide sequences [49]. The resulting mature protein consists of 415 amino acids of 55 kDa molecular weight.

The first 40 amino acids constitutes the Gla domain, containing Gla residues responsible for the high-affinity binding of calcium ions [50], so conferring to FIX its biological activity. Then the protein consists of a short hydrophobic segment (residues 87-92), two EGF-like domains (EGF1: residues 93-130, and EGF2: residues 131-173), an activation peptide (residues 192-226) and a C-terminal serin protease module (residues 227-461) [51] (fig.1.4).

FIX can be activated to FIXa by either the TF/FVIIa/Ca^{2+} complex (extrinsic pathway) or FXIa/Ca^{2+} (intrinsic pathway) [52][43]. This activation involves proteolytic cleavages in FIX at the Arg^{191}-Ala^{192} and Arg^{226}-Val^{227} bonds with the concomitant release of a 35-residue activation peptide [52]. The FIXa thus formed contains a light chain (residues 47-191) and a heavy chain (residues 227-461) held together by a single disulfide bond. The light chain consists of the Gla, EG1 and EGF2 domains, whereas the heavy chain contains the serine protease domain that features the catalytic triad of residues Ser^{411}, His^{267} and Asp^{315}.

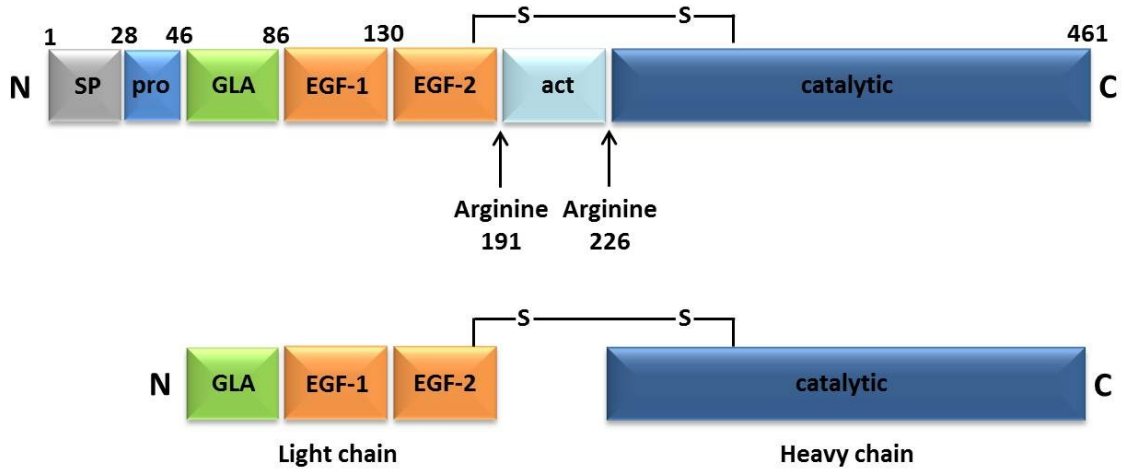

Figure 1.4. *Schematic representation of FIX (top) and activated FIX (bottom) protein domains.*

The FIXa generated during the clotting process then combines with its cofactor activated factor VIII (FVIIIa) on the platelet surface to activate FX in the coagulation cascade. In this assembly, the protease domain and possibly the EGF2 domain of FIXa are thought to provide the primary specificity in binding to FVIIIa.

1.5 Factor X

Human coagulation factor X is a vitamin K-dependent plasma glycoprotein, with a molecular weight of 58.8 kDa. It circulates in blood as an inactive two-chain zymogen protein at a concentration of 8 μg/ml, with a 32-48 hours half-life . The gene encoding FX is located on chromosome 13 at position 13q34, consists of eight exons and seven introns and is approximately 27 kb long [53].

FX is synthesized in the liver as an inactive precursor, in a single chain form. It contains a pre-pro leader sequence of 40 amino acids [54], comprising a signal peptide (SP, residues 4-19) followed by a pro-peptide (residues 23-40). The signal peptide targets the protein for translocation in the endoplasmic reticulum, where it is cleaved off by a signal peptidase [55]. In the trans-Golgi compartment the single chain FX precursor is processed in the two-chain form by two proteolytic cleavages at Arg^{179} and Arg^{182}, with the removal of an internal tripeptide, and this intra-chain cleavage can precede the release of the propeptide [56]. As all vitamin K-dependent proteins, the biosynthesis of FX involves several post-translational modifications, including formation of disulfide bonds, γ-carboxylation of glutamic acid residues, β-

hydroxylation (Asp[103]), and N- and O-linked glycosylation (Asn[221], Asn[231], Thr[199] and Thr[211]) [57][31].

The mature form of the protein consists of a light chain (139 residues) and a heavy chain (306 residues), linked by a disulfide bond between residues Cys[172] and Cys[342] [58]. The light chain contains a Gla and two EGF-like domains (EGF-1 and EGF-2), whereas the heavy chain contains the serine protease domain (fig.1.5).

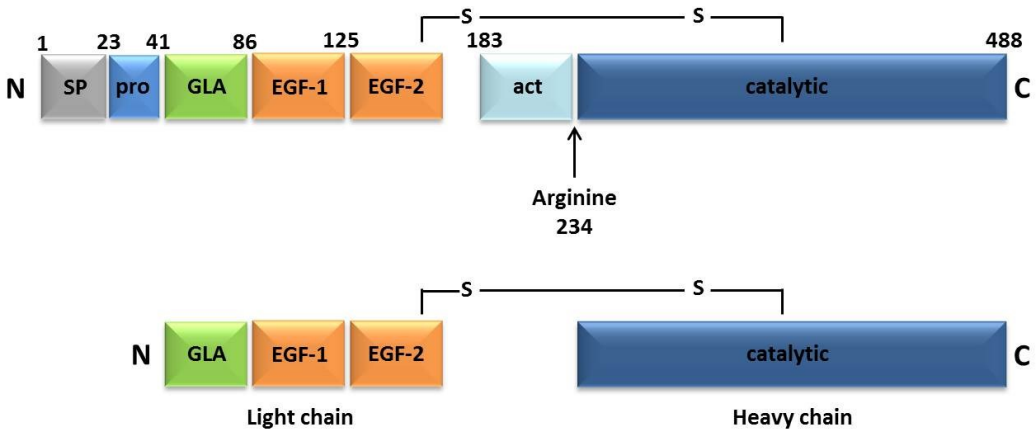

Figure 1.5. *Schematic representation of FX (top) and activated FX (bottom) protein domains.*

FX is activated by a proteolytic cleavage of the peptide bond between Arg[234] and Ile[235] in the N-terminal region of the heavy chain, giving rise to FXa and a 52-aminoacid glycopeptide [59]. Activation of FX to FXa occurs through multiple pathways. In the presence of Ca^{2+} and phospholipids, FX can be activated by both the extrinsic FVIIa/TF complex, in the initiation phase of coagulation, and the intrinsic FIXa/FVIIIa complex, in the subsequent propagation phase [60].

FXa reversibly associates with its cofactor FVa on a negatively charged phospholipidic membrane surface, in the presence of Ca^{2+} ions, to form the prothrombinase complex, which catalyze the conversion of prothrombin into thrombin, leading to the formation of the fibrin clot [2]. While FXa catalyzes prothrombin cleavage, the macromolecular interactions that stabilize prothrombinase lead to a profound enhancement in catalytic efficiency, indicating that prothrombinase, not FXa, is the physiologically relevant enzyme leading to explosive thrombin generation [61]. FXa also cleaves other protein substrates involved in blood coagulation, like FV, FVII, FIX and PC.

Finally, FXa (FXa-α) catalyzes the cleavage of at least two peptide bonds in its own heavy chain. The faster cleavage, between Arg[469] and Gly[470], liberates a 19-residue

glycopeptide from the C-terminus of the heavy chain. This reaction leads to the conversion of FXa from the α to the β-form, that are both functional [62]. A second autoproteolytic cleavage within the Arg^{366}-Arg^{376} autolysis loop releases a peptide containing the active-site serine residue from the C-terminal region of the heavy chain, resulting in the FXa γ-form, a species that has little or no catalytic activity [63].

1.6 Haemophilia

Haemophilia is an inherited bleeding disorder caused by deficiency or dysfunction of FVIII (haemophilia A, HA) or FIX (haemophilia B, HB). Since these proteins play a fundamental role in coagulation, their dysfunction cause decreased and delayed thrombin generation, giving rise to defects in clot formation that lead to haemorrhagic diathesis. These disorders are inherited as X-linked recessive traits, so male individuals are affected and female individuals are asymptomatic or mildly affected carriers. Haemophilia account for about 95% of all congenital coagulopathies, being the global prevalence of HA 1/5000 males and that of HB 1/30000 males [64][65].

On the basis of residual coagulant activity in blood, haemophilia is classified into three main forms:

- mild, when the factor level is comprised between 5-40 IU/dL (5-40% of normal);
- moderate, with a level between 1-5 IU/dL (1-5% of normal);
- severe, that occurs with level below 1 IU/dL (<1% of normal) and accounts for about 50% of cases [66].

More than 2000 unique molecular defects in the gene encoding FVIII (*F8*) have been described (Worldwide Factor VIII Variant Database, http://www.factorviii-db.org/). Point mutations accounts for 67% of molecular defects, small insertions and deletions represent 25% of such defects and 6% of all mutations are large deletions. In patients with severe HA, molecular characterization starts with the detection of inversions of intron 22 (reported in 40-45% of severe patients) and intron 1 (1-6% of severe patients) [67][68].

For FIX gene (*F9*) 1095 unique variants have been described so far (Factor IX Variant Database, http://www.factorix.org). Missense mutations are the most frequent (55%), followed by small deletions (16%), splicing (10%) and nonsense (8%) mutations. Large deletions and promoter mutations are relatively rare.

The clinical pictures of patients with haemophilia A and B are largely similar. The risk of haemorrhage depends both on the severity of clotting factor deficiency and on the age of the patient. The earliest and most serious complication in neonates with severe haemophilia is intracranial haemorrhage, which occurs in 1-4% of cases [69]. Bleeding after surgery is an important complication in patients affected by all severities of the pathology. In severe haemophilia, spontaneous muscle haemorrhage occurs in the lower legs, buttocks and forearms [70]. Another common and frequent manifestation is non-traumatic intra-articular bleeding (haemarthrosis), that increase with age and occurs especially in ankle, elbow and knee [71]. Recurrent haemorrhages at the same joint cause inflammation of the synovial tissue, with the development of haemophilic arthropathy and finally chronic joint deformity, pain, muscle arthropathy and functional impairment [72][73]. In patients with moderate haemophilia, spontaneous bleeds are infrequent; haemorrhages tend to occur after injury, trauma or surgery. Mild haemophilia is not generally associated with spontaneous bleeding, with nearly all incidents caused by trauma or surgery.

Table 1: Clinical and molecular characteristics of haemophilia A and B (adapted from [74])

Characteristics	Haemophilia A	Haemophilia B
Gene location	Xq28	Xq27.1-q27.2
Common clynical symptoms	Haemarthroses	Haemarthroses
Bleeding frequency	12-30 episodes/year(severe)	12-30 episodes/year(severe)
Most frequent gene defects	Intron 22 inversion	Missense mutations
FVIII/FIX half-life (hours)	8-12	18-24
Inhibitor incidence	~30-50%	<5%

Current treatment strategies and limitations

The main treatment for haemophilia is replacement therapy, consisting in the administration of the deficient clotting factor to achieve adequate haemostasis. The injected concentrated factor can be either plasma-derived or recombinant, and may be administered "on-demand" or in the frame of a prophylactic regimen.

On-demand treatment is infusion of the factor at the time of bleeding. The appropriate dose, frequency and number of infusions depend on the type and severity of the bleed [75]. Prophylaxis consists in the intravenous injection of the factor to prevent bleeding and joint destruction, with the objective of preserving normal musculoskeletal function. Primary prophylaxis is long term and requires treatment 2-3 times per week, starting at very young age (≤2 years) before joint disease develops,

whereas secondary prophylaxis begins after the onset of joint disease [76][77]. Many approaches to prophylaxis exist, and the optimum regimen remains to be defined.

The main limitation with existing standard products is their short half-lives (8-12 h for FVIII and 18-24h for FIX), and thus frequent administrations are necessary. One strategy to overcome this issue is the bioengineering of recombinant factors in order to extend their half-lives (see Chapter 5).

The most serious complication of replacement therapy is the development of inhibitors, i.e. antibodies against the infused factor. An inhibitor is a polyclonal high-affinity IgG antibody that specifically neutralizes the procoagulant activity of the relevant clotting factor, rendering management of bleeds difficult. The development of such inhibitors results from a multicausal immune response involving both patient-related (genetics, family history) and treatment-related (intensity of exposure, product type) factors. In severe haemophilia A, FVIII inhibitors form in 30% of patients, while in severe haemophilia B the incidence is around 3-5% [78]. Inhibitors are characterized in two ways, by the titre and by the anamnestic response. The titre is defined as the inhibitory capacity of patient's plasma to neutralize clotting factors in normal plasma, and is quantified in Bethesda Units (BU). This characterization is important because patients with a low titre (0.5-5 BU) and with low responding inhibitors can be treated with increased doses of the missing factor. Patients with a high titre (>5 BU) or high responding inhibitors can be treated effectively only with bypassing agents, unless the inhibitor is eradicated [79]. Immune tolerance induction (ITI) can be used to eradicate inhibitors and involves frequent injections of concentrates over many months [80]. For patients with a high titre, the only effective therapies are bypassing agents, that circumvents the need for FVIII or FIX by generating thrombin through other mechanisms. The two principal bypassing agents are activated prothrombin complex concentrate (APCC) and recombinant activated factor VII (rFVIIa). Both agents have shown efficacy and safety in clinical trials [81][82], although some patients seem to have a better response to one agent than the other [83].

A different strategy for haemophilia treatment is gene therapy, that has the potential to lessen disease severity through continuous production of FVIII or FIX after one administration of a gene vector. Nathwani et al. [84] have reported an important milestone in a gene therapy trial for haemophilia B. Gene therapy in haemophilia A showed instead a substantial limitation due to the large size of *F8* cDNA, that exceeds

the normal packaging capacity of adeno-associated viral vectors [85]. Recently, very promising results from two clinical trials on HA and HB were published [86][87].
Additionally, other technologies attempt to improve haemostasis by reducing the effect of natural anticoagulants [88][89].
Finally, another approach is the use of a bispecific antibody (ACE910) that promotes thrombin generation by binding both activated FIX (FIXa) and FX, thus mimicking the cofactor activity of FVIIIa [90].

1.7 Rare bleeding disorders: FVII and FX deficiency

FVII deficiency

Inherited FVII deficiency is the most common of rare bleeding disorders, its incidence being 1 in 500,000 [91]. It is inherited in an autosomal recessive manner and affects males and females equally.
Studies on *F7* knock-out mice indicate that the absence of FVII is incompatible with life [92], so it is thought that FVII deficiency could not be associated with a complete lack of functional FVII [93][94].
This disease is phenotypically variable and has a wide range of clinical manifestations: mild (muco-cutaneous) bleeds are the most frequent, while 10% to 15% exhibit more severe and potentially life-threatening haemorrhages (central nervous system, gastrointestinal or haemarthrosis) [95][96]. About one-third of patients with FVII deficiency tend to remain asymptomatic during their life [97]. The most frequently reported bleeding symptoms among mild FVII deficiency are epixtasis, gum bleeding, easy bruising and menorrhagia (69% of females) [97]. Severe bleedings usually occur soon after birth or in infants. In newborns (<1 month) presenting bleeding manifestations were, as ranked for frequency, central nervous system, gastro-intestinal, cephalohaematoma and umbilical bleeding.
Several therapeutic options are currently available, including fresh-frozen plasma and prothrombin complex concentrates, but FVII concentration in both are low and therefore they are not optimal treatments. Plasma-derived FVII concentrates may be an alternative option but large volume infusions and the short half-life (4-6h) of FVII make this administration option less appealing. Recombinant FVIIa is to be considered nowadays the optimal protein replacement therapy.

Prophylaxis is debated in FVII deficiency but has been used in those with severe bleeding [98]. However, the short FVII half-life contributes to difficulty establishing standardized prophylactic protocols.

FX deficiency

Hereditary FX deficiency is a rare blood coagulation disorder, with a prevalence of 1 to 1,000,000 for the severe form in the general population [99]. It is inherited as an autosomal recessive trait and is characterized by considerable clinical heterogeneity, which poorly correlates with FX levels and usually presents with variable bleeding tendency [100]. While mucocutaneous symptoms tend to occur frequently in patients, they can also suffer from severe haemarthroses, severe postoperative haemorrhage, central nervous system and gastrointestinal bleeding [101].

FX deficiency can present as a mild, moderate or severe deficiency state [99][102]. Mild FX deficiency (FX functional activity 6-10 IU/dL) is characterized by easy bruising and/or menorrhagia. In moderate FX deficiency (1-5 IU/dL) bleeding is associated with trauma or surgery, while severe FX deficiency (<1 IU/dL) may present in neonates and tends to exhibit the most severe phenotype.

For treatment of bleeding episodes, guidelines have typically recommended antifibrinolyitcs and FX replacement using plasma or prothrombin complex concentrate [103]. However, experts now consider the optimal therapy for rare coagulation disorders the use of single-factor concentrates for specific replacement of the deficient factor. In this view, a high-purity human plasma-derived FX has recently been developed and approved in the United States and Europe [104][105].

1.8 Aim of the present work

Haemophilia A and B as well as factor VII or X deficiencies are well-characterized haemorragic genetic disorders, whose etiology, even at the molecular level, has been extensively described in the last decades. Treatment options for patients affected by these diseases are currently available and in recent years lot of efforts have been put in developing better therapeutic strategies, in terms of efficacy, safety and patients' compliance. However, present treatment methods still have important limitations and research in the field is boosted towards the implementation of current therapeutic options or the discovery of alternative and innovative procedures.

In this thesis we provide consistent examples of novel treatment strategies for bleeding disorders, via protein engineering or pharmacological/molecular approaches. The different systems here proposed intend to (i) provide solid selection criteria for an individually-targeted therapy of patients carrying specific mutations (personalized medicine) and to (ii) ameliorate pharmacokinetic features of therapeutic proteins in order to reduce the burden of treatment and to increase patients' compliance.

In the first part of the thesis, we studied the well-described molecular event of ribosome readthrough (chapter 2) in the context of nonsense mutations causing haemophilia B or factor VII deficiency. In particular, we aimed at dissecting the complex mechanisms underlying the responsiveness of nonsense mutations to the pharmacological induction of ribosome readthrough. For this purpose, we took advantage of an *in vitro* platform for the expression of FIX nonsense variants, in order to provide evidence for the selection of HB patients treatable with readthrough-inducing drugs (chapter 3). As a novel investigation, we aimed at studying ribosome readthrough in order to elucidate the molecular mechanisms underlying phenotypes' severity in FVII deficiency, by two paradigmatic examples (chapter 4).

In the second part of this work, we exploited protein engineering approaches to extend coagulation factors half-life, aimed at overcoming important limitations of conventional patients' treatment and prophylaxis. Among the several known strategies, we choose the fusion with human serum albumin (HSA) (chapter 5). In the frame of bypassing therapy for haemophilias, we aimed at providing next-generation rFVIIa-HSA fusion proteins by taking advantage of albumin engineered variants with improved pharmacokinetic features (chapter 6). Furthermore, we also proposed a fusion protein between FX and HSA, in order to fulfill the unmet need of a recombinant product for FX deficiency therapy (chapter 7).

Chapter 2

Ribosome readthrough

2.1 Nonsense mutations

Nonsense mutations, defined as single nucleotide base changes within a gene that result in an in-frame premature termination codon (PTC), account for ~11 % of all described gene lesions that are associated to inherited human diseases [106] (www.hgmd.org).

Biostatistical analysis of human genes show that TGA is the most frequent stop codon (49.5% of genes), followed by TAA and TAG (28.1% and 22.4%, respectively) [107]. When disease-causing stop codons are compared with authentic ones, it results that the proportion of TAG is higher (40.4%) while TGA and TAA proportions are lower (38.5% and 21.1% respectively) [106]. This can be explained by the frequency of the most common transition (C->T) that is responsible for 46% of nonsense mutations. In fact, this is also confirmed by the triplets that are most frequently mutated to nonsense codons, CGA (Arg, 21%) and CAG/CAA (Gln, 19% and 6%, respectively).

When a mutation introduces a PTC at the genomic level, the resulting messenger RNA (mRNA) can undergo degradation by the nonsense-mediated decay (NMD) process [108]. NMD is a quality control pathway that allows the elimination of aberrant and abnormal transcripts inside the cell. This process could be triggered during the first round of translation, the so called “pioneer round” [109]. During the pioneer round, in normal condition, the ribosome removes all the exon junction complexes (EJC), that are placed about 20-24 nucleotides upstream of every exon-exon junction during the splicing process. If a PTC is located at least 50-55 nucleotides upstream of the exon-exon junction, the ribosome stalls and the EJC remains stably associated with the mRNA. An RNA-dependent helicase, Upf1, is recruited to the mRNA by translation release factors (eRFs) and bridges the ribosome and the downstream EJC to form an active NMD complex that triggers rapid exosome-mediated decay of the mRNA (fig. 2.1) [110].

Transcripts that bear PTCs in the last exon or less than 50-55 nucleotides upstream of an exon-exon junction are predicted to escape NMD. This is probably due to the steric hindrance of the ribosome, that displaces the EJC before recognizing the PTC.

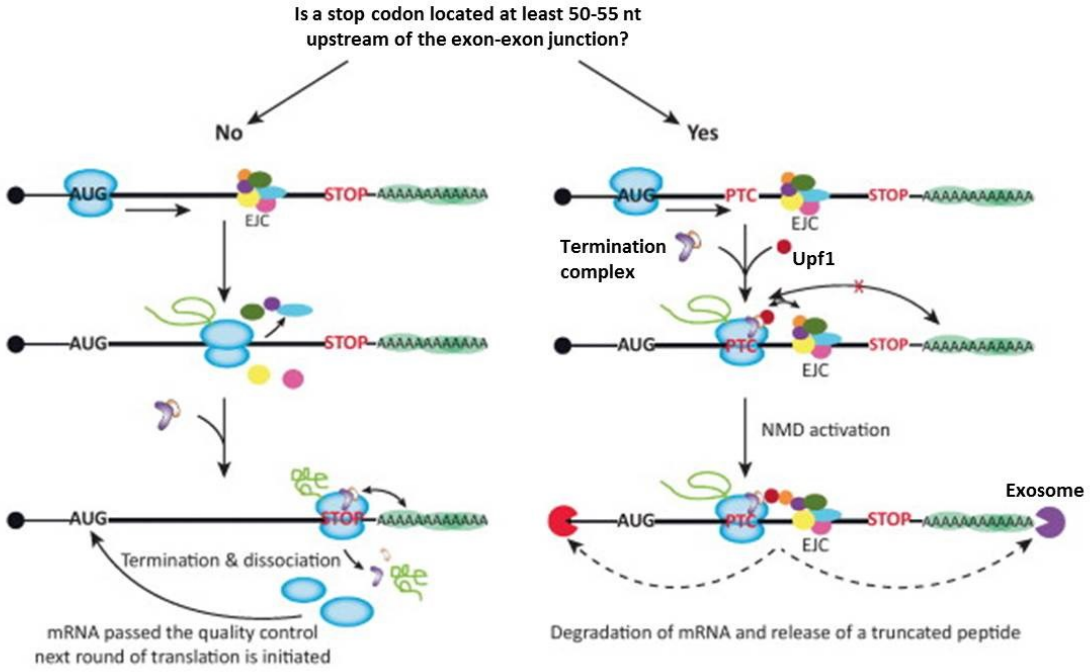

Figure 2.1. *Schematic representation of the NMD pathway versus normal termination* [111].

2.2 Molecular mechanism of ribosome readthrough

When the ribosome arrives at a stop codon, there is no corresponding tRNA. Instead, a release factor enters the assembly site and synthesis is terminated, releasing the completed polypeptide. However, occasionally stop codon recognition by eRF1 (eukaryotic release factor 1) can be superseded by selected aminoacyl-tRNAs, resulting in stop codon suppression (fig. 2.2).

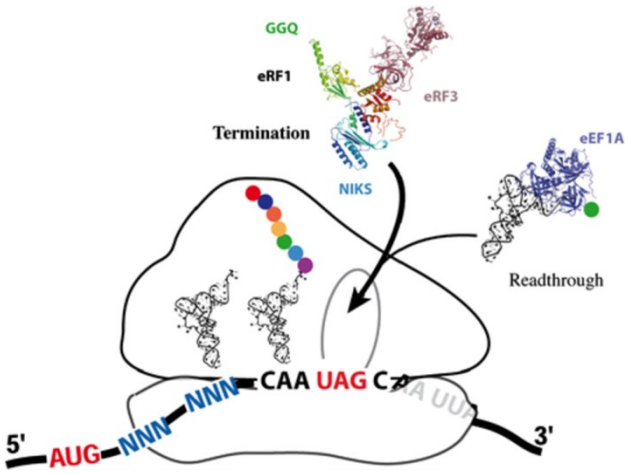

Figure 2.2. *Schematic diagram of the competition between termination and readthrough* [112]. *In eukaryotes, two eukaryotic release factors, eRF1 and eRF3, mediate translation termination. The eRF1 protein recognizes stop codons through its N-terminal domain and triggers peptidyl-tRNA hydrolysis by activating the peptidyl transferase centre of the ribosome through the highly conserved NIKS and GGQ motifs* [113].

This low-frequency event (10^{-4}) is called "ribosome readthrough", and corresponds to the incorporation of a near-cognate tRNA, or natural suppressor, at the stop codon, allowing translation to continue in the same frame until the ribosome reaches the next stop.

The efficiency of readthrough, which inversely correlates with translation termination efficiency, can vary depending on many factors. Firstly, the basal level of termination suppression at natural stop codons is lower than the one occurring on PTCs [114]. This is due to the role played by the polyA-binding protein (PABP) in translation termination. Because it is bound to the polyA tail, PABP is normally close to the termination complex at normal stop codons, allowing it to interact with eRF3 and stimulate polypeptide chain release [115]. In contrast, termination at a PTC usually does not occur in proximity to the polyA tail, which limits the interaction between eRF3 and PABP and leads to prolonged ribosomal pausing at PTCs (fig. 2.3).

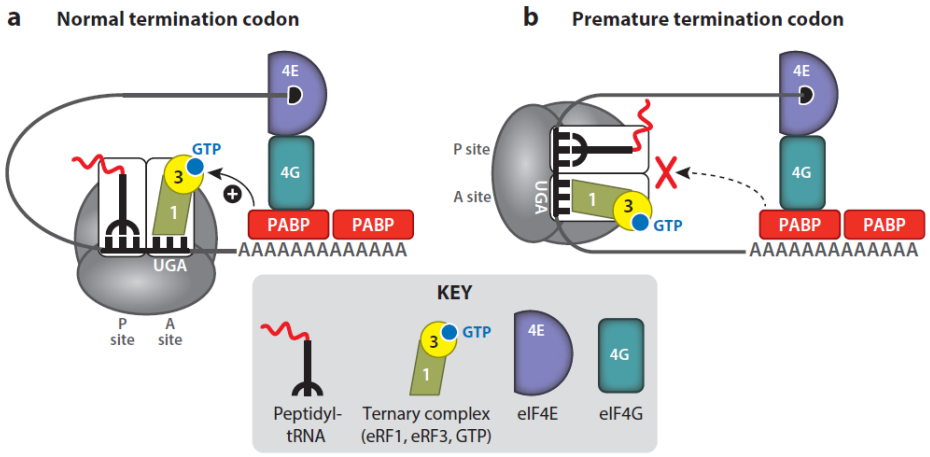

Figure 2.3. *Schematic representation of normal (a) and premature (b) termination codons* [116].

Moreover, the nucleotide context strongly influences readthrough efficiency. As a matter of fact, the different stop codons are suppressed with variable efficiencies (in humans, UGA≥UAG>UAA, where UGA is the most prone to readthrough) and the level of suppression depends, in part, on the identity of the nucleotide immediately downstream from the stop codon (table 2) [117].

Table 2: Levels of basal readthrough depending on the different stop codons and possible sequence contexts. Values are reported as percentage of full-length polypeptides on total protein translated in vitro [117].

Codon	Readthrough	Codon	Readthrough	Codon	Readthrough
UGA-C	3.8	UAG-C	0.9	UAA-C	0.2
UGA-A	1.0	UAG-A	1.1	UAA-A	0.5
UGA-U	0.7	UAG-U	0.8	UAA-U	0.4
UGA-G	0.6	UAG-G	1.6	UAA-G	0.2

In addition, readthrough efficiency can be influenced by drugs impairing ribosome fidelity, such as aminoglycosides. They are a class of antibiotics that interfere with protein synthesis. They all have a common 2-deoxystreptamine ring structure, which binds to the ribosome decoding center [118]; this binding induces a conformational change similar to the transition caused by a tRNA binding, thereby reducing the fidelity of normal translational processes and allowing stop codon readthrough.

The type of stop codon determines also the type of amino acid that will be incorporated in the nascent polypeptide chain. In fact, only a subset of predictable suppressor tRNA (near-cognate tRNAs, differing in only one position of the codon-anticodon pairing) are actually incorporated at the various stop codons. Tyrosine, glutamine and lysine can be inserted, with different frequencies, on UAG and UAA codons, while tryptophan is the one most frequently incorporated over UGA codons, followed by cysteine and arginine [119].

2.3 Nonsense suppression therapy

Nonsense mutations can lead to the premature termination of translation and the synthesis of a truncated protein, with potentially deleterious effects. Induction of ribosome readthrough represents a valuable strategy in order to suppress the PTC and thus restore the synthesis of a full-length polypeptide (fig. 2.4).

In 1996 PTC suppression was first described as a potential therapy for diseases caused by nonsense mutations [120]. The potential of this approach was then demonstrated *in vivo* for the first time in a mouse model of Duchenne Muscular Distrophy (DMD) [121]. Since then, approximately 100 studies have investigated the effectiveness of nonsense suppression as a possible treatment for nearly 40 different diseases [122]. Among the disease models successfully challenged with the drug-induced readthrough approach, important examples are Duchenne/Becker muscular dystrophies [123], cystic fibrosis [124] and spinal muscular atrophy [125].

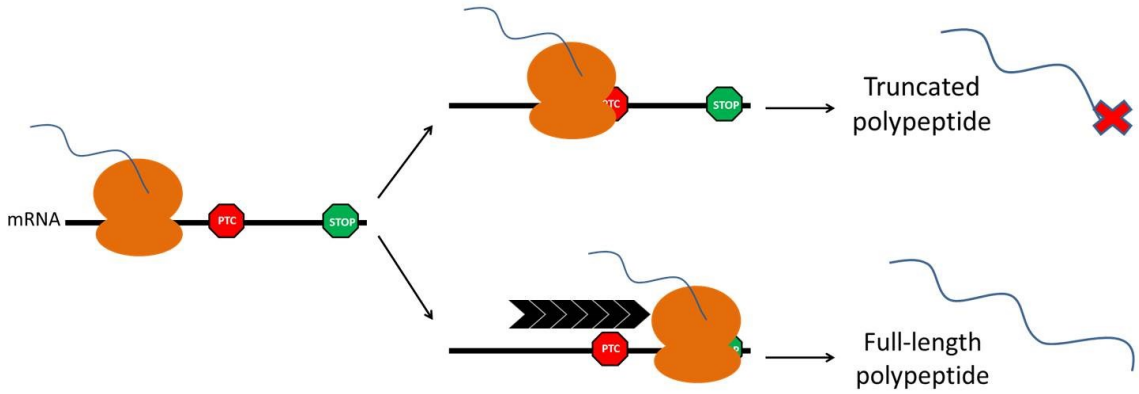

Figure 2.4. *Representation of ribosome readthrough over a premature stop codon, with the concomitant synthesis of a full-length polypeptide.*

All these studies indicate that aminoglycosides such as gentamicin can restore a significant amount of protein function, but only in a fraction of patients with nonsense mutations. In addition, one of the major drawbacks of aminoglycosides is their well-known toxicity due to their accumulation in the proximal tubular cells of the renal cortex (nephrotoxicity) and the cochlear hair cells (ototoxicity).

This highlights the importance of identifying alternative small molecules; in fact, several emerging compounds have been developed in the last years [111]. Among them, PTC124 (Ataluren) [126] and RTC compounds [127] are the most promising. In particular, Ataluren has been shown to be active in multiple cell and animal models and has entered clinical trials for DMD [128][129] and cystic fibrosis [130].

Chapter 3

Specific factor IX mRNA and protein features favor drug-induced readthrough over recurrent nonsense mutations.

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Based on: Blood, 2017, Apr 20; 129(16): 2303-2307.

3.1 Background and rationale

Together with other innovative approaches, the development of compounds promoting ribosome readthrough over nonsense mutations, and thus synthesis of full-length proteins, is receiving increasing attention for therapeutic purposes. This approach could be of particular interest for treatment of coagulation factor disorders, where even a tiny increase in functional protein levels would significantly mitigate the bleeding phenotype [131]. However, the evaluation of readthrough-inducing drug efficacy in different disorders showed a wide outcome variability [132][133], with poor response being observed in several patients [134][135].

The rescue of functional protein levels results from a complex interplay between messenger RNA and protein components: (1) the well-recognized nucleotide context of the premature stop codon (PTC), influenced by the triplet (TGA≥TAG>TAA) and the downstream nucleotide, and (2) the impact of amino acid changes inserted in the full-length protein during PTC mis-recognition [119]. The latter, a key determinant of mutation responsiveness, has never been addressed in haemophilias.

In order to provide a systematic investigation by a rational selection of nonsense mutations, in this study we evaluated quantitative and qualitative aspects of drug-induced readthrough for nonsense mutations causing haemophilia B (table 3).

Table 3. Features of HB-causing nonsense mutations according to the number of patients and type of nonsense triplet. The induced-readthrough score, ranging from 0 (unfavorable sequence) to 10 (highly favorable) was adapted from *in vitro* studies on G418-induced readthrough by Manuvakhova et al. [117].

HB Patients (n)	Nonsense Mutation	Exon	Nucleotide change	CpG	PTC Sequence	Readthrough score
73	p.R75X	2	c.223C>T	Yes	TGA-G	4
70	p.R294X	8	c.880C>T	Yes	TGA-A	4.1
65	p.R379X	8	c.1135C>T	Yes	TGA-G	4
63	p.R298X	8	c.892C>T	Yes	TGA-A	4.1
22	p.R162X	5	c.484C>T	Yes	TGA-C	6.3
11	p.W240X	6	c.720G>A	No	TGA-C	6.3
5	p.R384X	8	c.1150C>T	Yes	TGA-T	2.7
7	p.W240X	6	c.719G>A	No	TAG-C	2.9
3	p.Q370X	8	c.1108C>T	No	TAG-T	4.8
4	p.Y330X	8	c.990C>A	No	TAA-G	0.8
1	p.L103X	4	c.308T>A	No	TAA-A	0.56

3.2 Materials and methods

Mutation selection

The investigated nonsense mutations were chosen to include all those recurrent at CpG sites with the best-predicted susceptibility to readthrough (TGA codon) and four additional PTCs selected as controls. Altogether, these mutations have been reported in 324/469 (70%) HB patients with nonsense mutations (www.factorix.org) (table 3).

Expression vectors

Expression vectors for recombinant factor IX (rFIX) nonsense and missense variants were created by site-directed mutagenesis of FIX cDNA cloned in pCMV5, by using the QuickChange® XL Site-Directed Mutagenesis Kit (Agilent, Santa Clara, CA, USA). Sequences of the forward mutagenic primers are reported in the table below. Reverse oligonucleotides were complementary to the forward ones. All expression vectors have been validated by sequencing.

Variant	Primer sequence
FIX-21X	5′CACCATCTGCCTTTTA**TGA**TATCTACTCAGTGC3′
FIX-21W	5′CACCATCTGCCTTTTA**TGG**TATCTACTCAGTGC3′
FIX-21C	5′CACCATCTGCCTTTTA**TGT**TATCTACTCAGTGC3′
FIX-21R	5′CACCATCTGCCTTTTA**CGA**TATCTACTCAGTGC3′
FIX-28X	5′CTCAGTGCTGAA**TGA**ACAGGTTTGTTTCC3′
FIX-45X	5′CAAAATTCTGAATCGGCCA**TAG**AGGTATAATTCAGG3′
FIX-75X	5′GTAGTTTTGAAGAAGCA**TGA**GAAGTTTTTGAAAAC3′
FIX-103X	5′GTCCAATCCATGT**TAA**AATGGCGGCAGTTG3′
FIX-103Y	5′GTCCAATCCATGT**TAC**AATGGCGGCAGTTG3′
FIX-103Q	5′GTCCAATCCATGT**CAA**AATGGCGGCAGTTG3′
FIX-162X	5′GTACTGAGGGATAT**TGA**CTTGCAGAAAACC3′
FIX-240X	5′CAGGTCAATTCCCT**TGA**CAGGTTGTTTTGAATG3′
FIX-240X	5′CAGGTCAATTCCCT**TAG**CAGGTTGTTTTGAATG3′
FIX-240Y	5′CAGGTCAATTCCCT**TAC**CAGGTTGTTTTGAATG3′
FIX-294X	5′CATACAGAGCAAAAG**TGA**AACGTGATTCGAATTATTCC3′
FIX-298X	5′CAAAAGCGAAATGTGATT**TGA**ATTATTCC3′

FIX-330X	5′GTGCTAAACAGC**TA<u>A</u>**GTTACACCTATTTGC3′
FIX-330Q	5′GTGCTAAACAGC**<u>CAG</u>**GTTACACCTATTTGC3′
FIX-370X	5′GATCAGCTTTAGTTCTT**<u>T</u>AG**TACCTTAGAGTTCCAC3′
FIX-379X	5′GAGTTCCACTTGTTGAC**<u>T</u>GA**GCCACATGTC3′
FIX-384X	5′CGAGCCACATGTCTT**<u>T</u>GA**TCTACAAAGTTCACC3′
FIX-384W	5′CGAGCCACATGTCTT**<u>T</u>G<u>G</u>**TCTACAAAGTTCACC3′

Modified oligonucleotides (underlined) and triplets (bold) are indicated.

Transfection

The human embryonic kidney HEK293 (parental) cell lines were maintained under 37°C, 5% CO_2 using Dulbecco's modified Eagle's Medium supplemented with 10% FBS, 2mM GlutaMAX (Invitrogen, Carlsbad, CA, USA), 100U/ml penicillin and 100 µg/ml streptomycin. Cells in OptiMEM medium (Gibco, Gaithersburg, MD, USA) added with 5 µg/ml vitamin K were transfected with 2 or 4 µg of plasmid DNA in 12 or 6-well plates, in the presence of a 1:1 ratio of Lipofectamine® 2000 Reagent (Invitrogen), according to the manufacturer's protocol. Transfected cells were incubated with an optimized geneticin (G418, Sigma-Aldrich, St Louis, MO, USA) concentration (100 µg/ml) [132]. Media and cell lysates were collected 48 hours later.

Evaluation of rFIX activity

Activity of rFIX variants was evaluated by standard activated partial thromboplastin time (aPTT)-based coagulation assays. FIX-depleted plasma (HemosIL, Instrumentation Laboratory, Lexington, MA, USA) was supplemented with rFIX-containing conditioned medium. Coagulation times were measured upon addition of a contact activator (SynthASil, Hemosil) and $CaCl_2$ on a ACLTOP700 instrument (Instrumentation Laboratory). Activity was also assessed through a commercially available chromogenic assay (Hyphen Biomed, Neuville-sur-Oise, France), according to manufacturer's protocol. Coagulation times (aPTT-based assay) or optical density values (chromogenic assay) from serial dilutions of rFIX-wild type (WT) were used as reference. The specific activity of rFIX variants was calculated as the ratio between coagulant activity and protein levels expressed as % of the rFIX-wt used in the coagulation assays.

Evaluation of rFIX levels and isoforms

Secreted rFIX levels were evaluated by enzyme-linked immunosorbent assay (ELISA), using a commercially available kit (Affinity Biologicals, Ancaster, ON, Canada), according to manufacturer's protocol. Plasma-derived human FIX (Haematologic Technologies, Essex Junction, VT, USA) was used as reference.

FIX isoforms were evaluated by western blotting. Media or cell lysates were separated by SDS-PAGE in Bis-Tris precast polyacrylamide (4-12%) gels (Invitrogen) with MES-SDS as the running buffer, and then transferred to a 0.45 μm nitrocellulose membrane. Blocking of membranes was carried out by incubation with 5% w/v milk in PBS buffer over-night at 4°C. Protein bands were revealed by a polyclonal goat anti-human FIX (APGAFIX; Affinity Biologicals) and anti-goat horseradish peroxidase(HRP)-conjugated (A50-101P; Bethyl Laboratories, Montgomery, TX, USA) antibodies. Blotting images were acquired by chemiluminescence detection on the ChemiDoc MP System (Bio-Rad Laboratories, Hercules, CA, USA) and were analyzed by Image Laboratory Software version 4.0 (Bio-Rad).

3.3 Results and discussion

In this study we evaluated the responsiveness to readthrough-inducing drugs of the most recurrent HB-causing nonsense mutations by exploiting an *in vitro* expression platform (fig. 3.1).

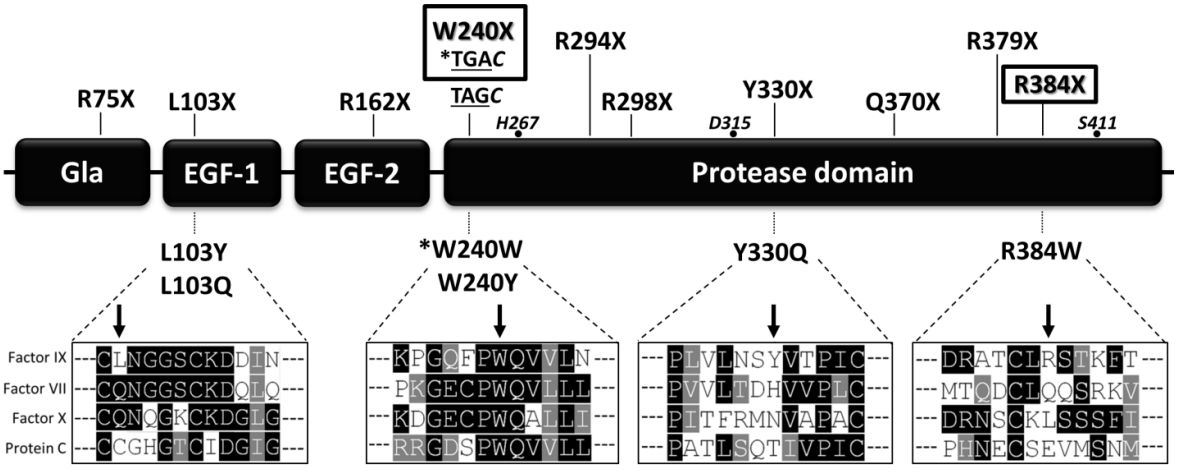

Figure 3.1. *Schematic representation of FIX organization and relative position of nonsense mutations (top) and of predicted missense changes arising from readthrough (bottom). The sequence alignments of the selected amino acid positions among the homologous FIX (NP_000124.1), FVII (NP_000122.1), FX (NP_000495.1), and protein C (NP_000303.1) are indicated below the corresponding missense variants. The asterisk indicates that readthrough over the W240X(TGAC) is expected to reinsert the authentic residue (tryptophan). The catalytic triad residues (black circles) are also numbered.*

We therefore expressed the rFIX nonsense variants (fig. 3.1) in HEK293 cells and challenged them with a well-established readthrough-inducing agent, geneticin (G418), to assess the key outcome, which is the increase in FIX activity levels over the undetectable basal levels. As shown in figure 3.2, G418 elicited a poor rescue, if any, for the large majority of mutations, considering as threshold for its potential therapeutic meaning levels >2% of rFIX-WT. Remarkably, the rFIX-R384X (TGAT; chromogenic 7.1 ± 0.6%; pro-coagulant 7.5 ± 0.7%) and the rFIX-W240X (TGAC; 5.6 ± 0.7% and 5.2 ± 0.6%) displayed a robust rescue. By comparison, the rFIX-W240X(TAGC), which is the topologically overlapping nonsense variant at the W240X PTC differing in the nonsense triplet, did not appreciably respond to G418.

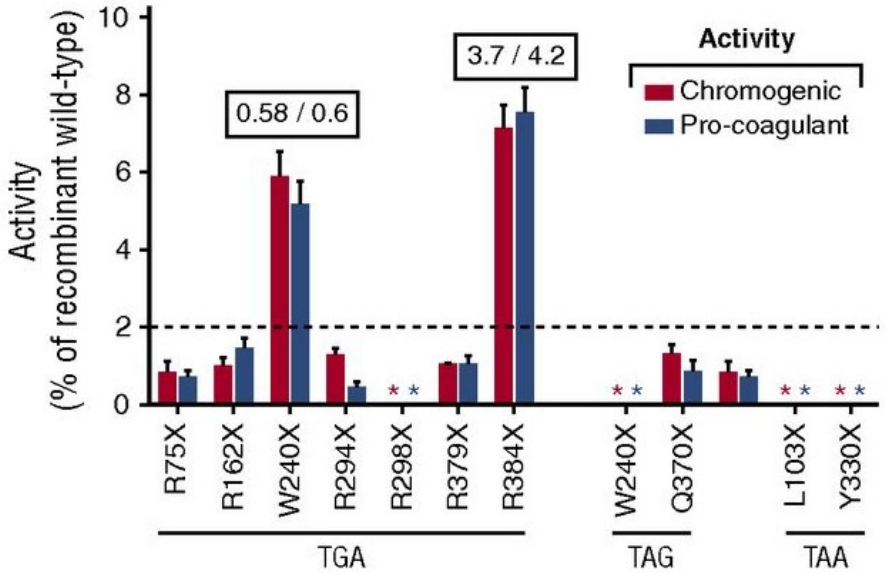

Figure 3.2. *FIX activity levels in medium from cells expressing the rFIX nonsense variants upon treatment with G418, evaluated by chromogenic (red bars) and aPTT-based (blue bars) assays. Numbers above the bars report the activity/antigen ratio of the most responsive variants. Nonsense variants are indicated by amino acid numbering and grouped according to the 3 nonsense triplets (theoretical readthrough susceptibility, TGA≥TAG>TAA). The dotted line represents the selected threshold of 2%. The activity in medium from untreated cells was undetectable for all variants and the undetectable activity after treatment is indicated by asterisks. Results are reported as mean ± standard deviation from at least 3 independent experiments*

To dissect the molecular bases of these observations, we investigated the intracellular and secreted FIX species by western blotting, and expressed the most probable missense variants expected by readthrough [119]. The interpretation of results illustrates different scenarios.

Expression of the p.R384X mutation showed both full-length and truncated forms at the intracellular level (fig. 3.3). However, only full-length FIX was secreted in medium, which accounted for 1.9 ± 0.3% of rFIX-WT (fig. 3.3). This value was much lower than

the measured activity (7.5 ± 0.7%), thus pointing out hyperactive features, with an estimated fourfold increased activity/antigen ratio.

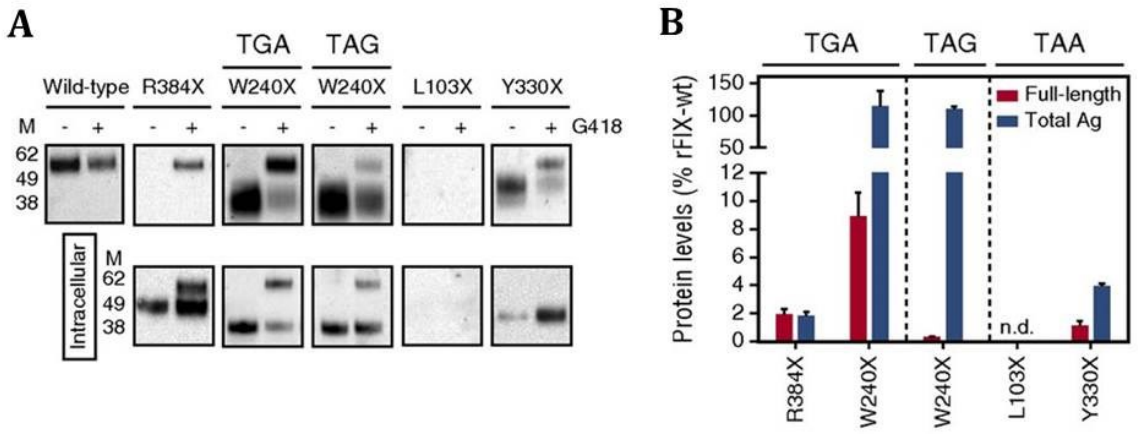

Figure 3.3. A) *Western blotting analysis on secreted (top) and intracellular (bottom) rFIX proteins transiently expressed from HEK293 cells untreated (–) or treated (+) with G418. The W240X(TGAC) and R384X stop codons, producing the highest rescue, are compared with PTCs displaying barely detectable (W240X and Y330X) or undetectable (L103X) readthrough. For the W240X PTCs, both nonsense triplets are indicated. The images are representative of at least 3 independent experiments.* ***B)*** *Secreted full-length (red bars) and total (blue bars) rFIX levels after G418 treatment. Full-length rFIX levels were calculated by densitometric analysis of western blots shown on the left and total antigen by ELISA. Results are reported as mean ± standard deviation from at least 3 independent experiments. M, molecular weight marker; Ag, antigen; n.d., not detectable.*

Consistently, the most probable missense variant (rFIX-R384W) arising from readthrough (fig. 3.4), albeit with reduced secretion (8.0 ± 3.3%), revealed an increased specific activity (3.7 ± 0.5-fold). Similar to the thrombophilic R384L substitution (FIX Padua) [136], the R384W substitution confers gain-of-function features, thus magnifying the drug effects that are higher than those expected from the readthrough score. It is worth noting that the p.R384X nonsense mutation, an example of recurrent C>T change at a CpG site, has been detected in only 5 HB patients (2 moderate), a number much lower than that of the other TGA PTCs (n = 22 to 73; table 3).

For the rFIX-W240X (TGAC), the amount of full-length rFIX protein evaluated by western blotting was much higher (8.8 ± 1.8% of rFIX-WT) than for the rFIX-W240X (TAGC) variant (0.25 ± 0.1%), despite the indistinguishable secreted levels measured by ELISA (fig. 3.3). This is attributable to counterbalancing amounts of truncated molecules. Intriguingly, the sequence context of the favorable W240X (TGAC) PTC predicts, as the most probable event during readthrough, the reinsertion of

tryptophan (82% of events), which would lead to the WT FIX molecule. Although with much lower frequency, cysteine (14%) and arginine (4%) could also be introduced, but the p.W240C [137] and p.W240R [138] missense changes, found in severe HB patients, are associated with barely detectable FIX levels. Consistently, the rFIX-W240Y missense variant, predicted to arise from readthrough over the unfavorable W240X (TAGC) PTC, was secreted with very poor efficiency (2.5 ± 1% of rFIX-WT; fig. 3.4). These coherent findings support that the highly conserved tryptophan (fig. 3.1) is the sole residue compatible with the functional improvement observed in response to G418 for W240X (TGAC). For this variant, the apparently reduced activity/antigen ratio (0.6 ± 0.1) is very likely produced by the secretion of truncated molecules, which are also abundant at the intracellular level (fig. 3.3).

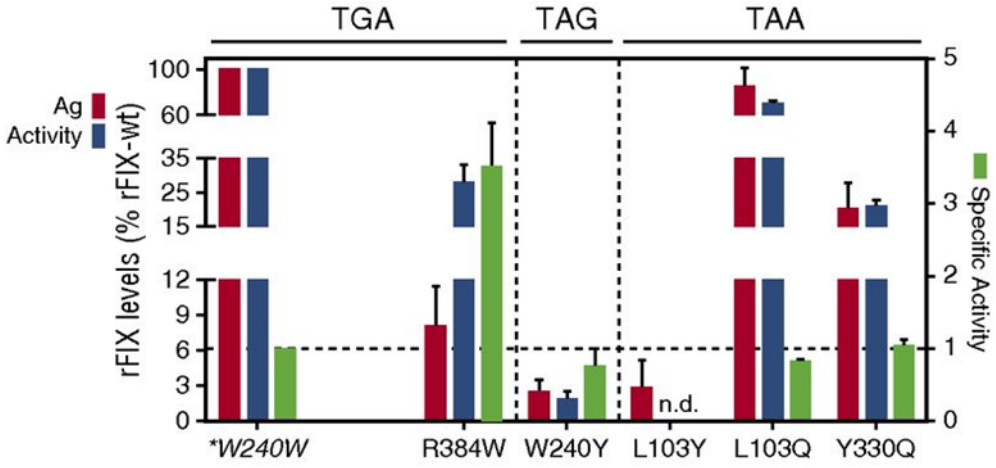

Figure 3.4. *Antigen (red bars), pro-coagulant activity (blue bars) levels, and activity/antigen ratio (specific activity, light green bars) of the most probable rFIX missense variants arising from misrecognition of TGA (R384W), TAG (W240Y), and TAA (L103Y/L103Q and Y330Q) stop codons. Readthrough over the W240X (TGAC) PTC is predicted to reintroduce the authentic amino acid (tryptophan, *W240W). The dashed-line indicates the specific activity of WT rFIX. Results are reported as mean ± standard deviation from at least 3 independent experiments. Ag, antigen; n.d., not detectable.*

Altogether, these data corroborate the remarkable nucleotide sequence-related differences in susceptibility to drug-induced readthrough, and anticipates unexpected differences in the response of patients with apparently identical nonsense mutations.

The balance between mechanisms accounting for poor response of the W240X (TAGC) also pertain to the other investigated unfavorable nonsense mutations, as in the paradigmatic examples of p.L103X (TAAA) and p.Y330X (TAAG). Expression of the predicted missense variants indicates that these moderately conserved positions

are functionally tolerant to amino acid changes (fig. 3.1, fig. 3.4), but their biosynthesis is prevented by inefficient, predicted (table 3), and observed (fig. 3.2, 3.3), ribosome readthrough.

Prompted by the results obtained in this part of the study, we decided to take into account other rationally-selected nonsense mutations. In fact, nonsense mutations occurring in sequences encoding for protein regions that are poorly conserved would represent candidates for therapeutic readthrough. In particular, suppression of nonsense mutations affecting regions that are intracellularly removed would make negligible the impact of missense changes on secretion and function of the mature protein. Therefore we selected as paradigmatic examples three nonsense mutations affecting the FIX signal and pro-peptide: the p.G21X (TGA-T) in the variable hydrophobic core, the p.C28X (TGA-A) at the intracellular cleavage site and the p.K45X (TAG-A), affecting the pro-peptide cleavage site (fig. 3.5).

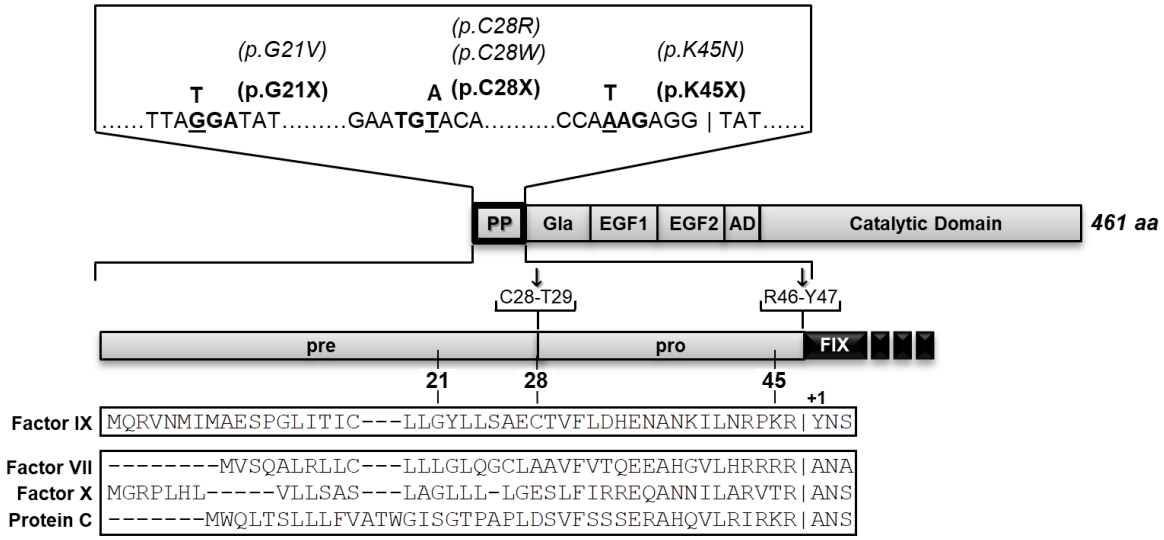

Figure 3.5. *Schematic representation of FIX with the selected nonsense mutations and features of the pre-pro-peptide region. Upper panel. The selected nonsense mutations (bold) as well as the nucleotide changes (underlined) and contexts, and the HB-related missense variants (italic) (www.factorix.org) are indicated above the pre-pro-peptide (PP) scheme. Middle panel. Schematic representation of FIX domain organization. Lower panel. Representation of the pre-pro-peptide (grey) and mature (black) FIX, with the pre- (C28-T29) and pro-peptide (R46-Y47) cleavage sites indicated on top. Positions 21, 28 and 45 are indicated above the pre-pro-peptide amino acid sequence of human FIX (NP_000124.1), together with that of FVII (NP_062562.1), FX (NP_000495.1) and protein C (NP_000303.1). The +1 residue of mature FIX (legacy numbering), following the pro-peptide cleavage site (| symbol), is shown.*

Expression of the rFIX-28X and rFIX-45X nonsense variants in HEK293 cells did not result in appreciable secreted FIX levels. Conversely, the full-length FIX form was

clearly detectable by Western Blotting in medium from rFIX-21X expressing cells, with estimated levels of 0.4±0.3% of rFIX-wt (fig. 3.6).

Treatment with G418 resulted in a remarkable increase of secreted FIX protein levels for the rFIX-21X (4.1±0.5% of rFIX-wt), but had only a negligible impact on the rFIX-28X (0.05±0.01%) and rFIX-45X (0.06±0.03%) variants (fig. 3.6). Interestingly, while the rFIX-21X and rFIX-28X displayed the full-length rFIX proteins, in medium from the rFIX-45X-expressing cells we detected a FIX form compatible with retention of the propeptide sequence, in accordance with previous studies [139].

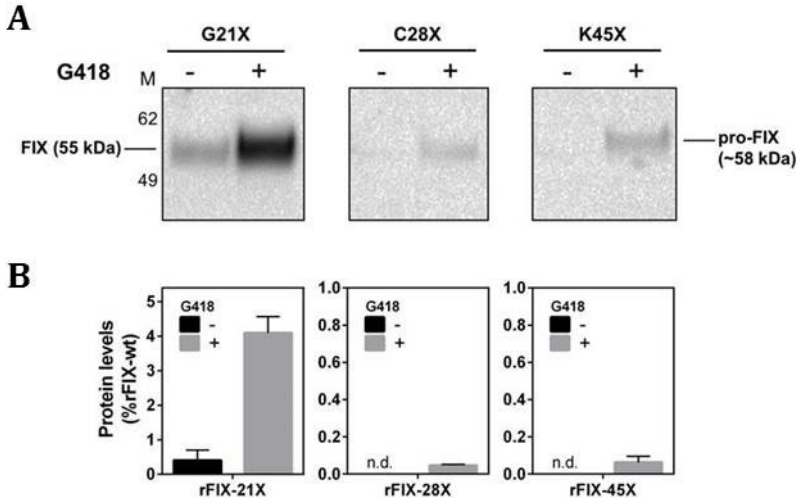

Figure 3.6. *Evaluation of drug-induced readthrough over FIX nonsense mutations.* ***A)*** *Western blotting analysis of secreted rFIX proteins transiently expressed from HEK293 cells untreated (-) or treated (+) with 100 µg/ml geneticin (G418). M, molecular weight marker. Pro-FIX, protein form retaining the pro-peptide.* ***B)*** *Secreted rFIX levels (% of rFIX-wt) measured by ELISA before (-) and after (+) G418 treatment.*

The very low protein levels achieved for the rFIX-28X and rFIX-45X variants, with the latter resulting in partially-processed secreted rFIX, prevented their functional evaluation. Strikingly, measurement of coagulation times in medium from rFIX-21X-expressing cells showed a FIX coagulant activity corresponding to 0.6±0.1% and 4.0±0.3% of rFIX-wt before and after geneticin treatment, respectively (fig. 3.7). Overall data, and particularly the parallel increase of full-length FIX protein and functional levels, indicated the secretion of FIX molecules with normal specific activity (activity/antigen ratio, 0.96±0.11) (fig. 3.7).

To provide experimental evidence for the properties of FIX molecules induced by translational readthrough, we investigated the impact on FIX secretion and activity of the probable missense changes (G21W/C/R) predicted to arise from readthrough of

the PTC at position 21. Expression studies showed that the secreted levels of the missense variants were unaffected (rFIX-21W, 89.0±22.2% of rFIX-wt) or slightly reduced (rFIX-21C, 63.2±14.3%; FIX-21R, 39.3±8.3%). Interestingly, aPTT-based assays revealed virtually normal specific activities (rFIX-21W, 0.91±0.06; rFIX-21C, 0.84±0.08; rFIX-21R, 0.98±0.04) (fig. 3.7) comparable to that of full-length rFIX-21X.

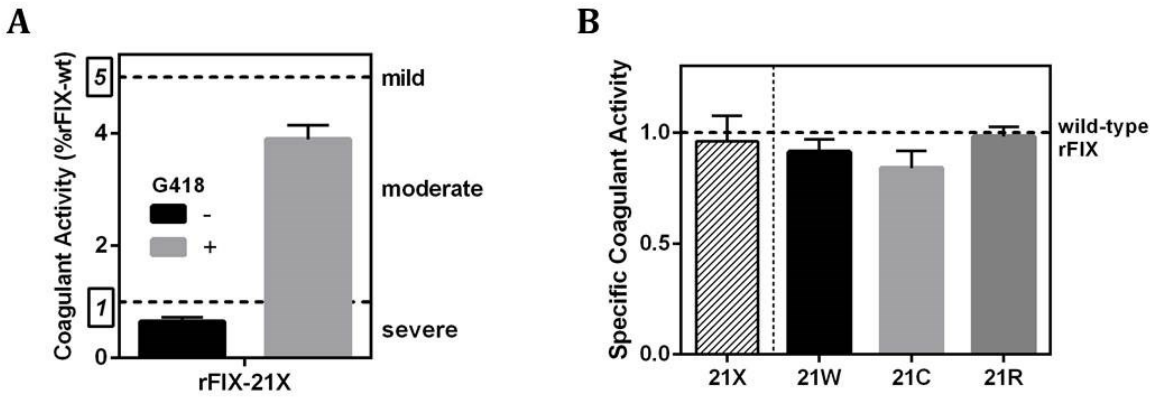

Figure 3.7. A) *FIX coagulant activity levels (% of rFIX-wt) in medium from cells expressing the rFIX-21X nonsense, either untreated (black bar) or treated (grey bar) with G418. Boxed numbers indicate activity levels corresponding to 1% and 5% rFIX activity and thus translating into severe or mild phenotypes.* ***B)*** *FIX specific coagulant activity, expressed as activity/antigen ratio, of nonsense and missense variants at position 21. The dashed line indicates the specific activity of rFIX-wt, used as reference. Results are expressed as mean ± standard deviation calculated from three independent experiments.*

Therefore, we demonstrated that only the p.G21X mutation in the pre-peptide core was responsive. This finding is consistent with the high variability across coagulation factor serine proteases (fig. 3.5) of this region that we experimentally demonstrated to tolerate the various amino acid changes (G21W/C/R) predicted by readthrough, and resulting in appreciable secreted functional levels. Consistent with the required hydrophobic feature of the pre-peptide core, the apolar tryptophan and the polar cysteine had negligible or weak effects, whereas the positively charged arginine displayed the strongest impact (fig. 3.8). This was also corroborated by the in vivo observation that the introduction of valine (p.G21V) is associated with mild HB. On the other hand, the p.I17Q, disrupting the hydrophobic core of the pre-peptide, required for secretion of FIX, has been associated with very low protein levels and severe HB [140]. Further, nonsense mutation suppression in this region would permit a substantial protein expression because, if all PTC types are considered, the readthrough-mediated introduction of charged amino acids is by far the less likely event [119].

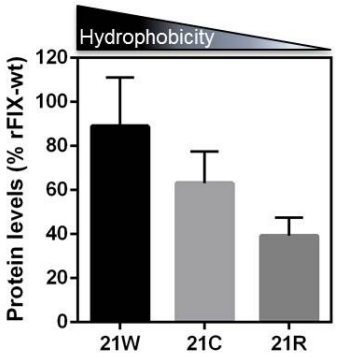

Figure 3.8. *Secreted protein levels (% of rFIX-wt) of rFIX missense variants (W/C/R) predicted to arise from readthrough at position 21. The gradient of hydrophobicity is shown on top. Results are expressed as mean ± standard deviation calculated from three independent experiments.*

The favorable context of this region, combined with that of the nucleotide sequence prone to readthrough, would also explain the detectable FIX expression observed at basal level, which indicates the occurrence of spontaneous readthrough. Furthermore, once secreted and have lost the affected pre-peptide, the FIX form arising from readthrough over the p.G21X nonsense or from missense variants displayed normal features, as indicated by their specific activity. Taken together these observations support that nonsense mutations in the pre-peptide hydrophobic core, or amino acid substitutions compatible with its features [141], would be associated with a less pronounced degree of clinical severity.

The poor responsiveness to geneticin of the p.C28X and p.K45X mutations appears to be related to constraints, albeit partially known, dictated by intracellular cleavage sites. The p.C28X occurs at the pre-peptide cleavage site, and the introduction of tryptophan, predicted to be the most frequent readthrough-mediated event, severely impairs FIX levels in HB patients (p.C28W), similarly to other HB-related changes (p.C28R/G/Y). Albeit with very reduced probability, the re-introduction of cysteine is also predicted, which might explain the very low levels of full-length FIX observed upon induction.

The p.K45X occurs at the key conserved pro-peptide residues (fig. 3.5) pivotal for the correct processing of coagulation factors [139]. Readthrough at this PTC is predicted to incorporate amino acids other than the authentic one, and missense changes at this site (p.FIX-K43W/Q/N/L, p.FIX-K45N) cause severe HB [142][143][141].

These data specifically identified the p.G21X nonsense mutation as candidate for treatment with readthrough-inducing agents, with expected rescued FIX levels that, if translated into patients, will be well above the therapeutic threshold (fig. 3.7). When translated to other coagulation factors, these findings indicate nonsense mutations in the pre-peptide core as potentially responsive to therapeutic readthrough. More generally, and also in other disease-proteins, this rational approach could be extended to nonsense mutations in protein regions removed during processing and moderately conserved (activation peptides, B-domain, etc.) [144] to select responsive mutations, and thus patients, to be challenged with readthrough-inducing drugs, also in the view of the new emerging compounds.

Chapter 4

Differential functional readthrough over homozygous nonsense mutations contributes to the bleeding phenotype in coagulation factor VII deficiency

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4.1 Background and rationale

By causing the premature termination of protein synthesis, nonsense mutations frequently predict a null phenotype, which is almost completely incompatible with life in the setting of factor VII deficiency [145]. Ribosome readthrough, based on misrecognition of the nonsense triplet, may induce the biosynthesis of trace amounts of full-length FVII [132]. The TGA, TAG and TAA stop codons, and their sequence context, predict a variable degree of readthrough efficiency in terms of the amount of full-length protein produced [117].

At the protein level, extreme differences are expected for the most probable substituted residues [119], ranging from the wild-type amino acid reinsertion to missense changes impairing secretion and function. The consequences would be of great importance for disorders such as FVII deficiency, in which even tiny increases in functional protein levels could ameliorate the haemorrhagic phenotype of patients.

Among all the homozygous *F7* nonsense mutations, the p.Cys132X and p.Arg462X, associated with moderate or asymptomatic conditions, respectively, represent noticeable exceptions (table 4).

Table 4. Homozygous *F7* nonsense mutations: haemorrhagic phenotypes and expected readthrough output

Mutation	Exon (NMD)	Protein Domain	Sequence Context	Predicted suppression efficiency *	Predicted amino acid insertion	Bleeding symptoms	FVIIc (%) **	Ref
p.Ser112X	4 (+)	EGF1	TGA A	1.0%	$Trp^{0.82}$ - $Cys^{0.14}$ - $Arg^{0.04}$	Ic	<1	[146]
p.Cys132X	4 (-)	EGF1	TGA C	3.8%		Um, Ep	<1	[147]
p.Gln287X	8 (-)	Catalytic	TAG G	0.8%	$Tyr^{0.92}$- $Gln^{0.05}$ - $Lys^{0.03}$	Gi, Um, Hr, Gn	<1	[148]
p.Lys376X	8 (-)	Catalytic	TAG G	0.8%		Ic	<1	[132]
p.Gln442X	8 (-)	Catalytic	TAG T	1.6%		Ic, Gi, Um, Hr	<1	[148]
p.Arg462X	8 (-)	Catalytic	TGA G	0.6%	$Trp^{0.82}$ - $Cys^{0.14}$ - $Arg^{0.04}$	None	3-5	[149]

Ep, epistaxis; FVIIc, factor VII coagulant activity; Gi, gastrointestinal; Gn, gingival; Hr, hemoarthrosis; Ic, intracranial; NMD, nonsense-mediated decay; Um, umblical. *Efficiency of spontaneous readthrough-mediated suppression of stop codons based on the type of nonsense triplet and the downstream nucleotide [117]. **FVIIc was measured by the use of human thromboplastin.

Whereas it has been previously demonstrated that the p.Arg462X mutation produces small amounts of a truncated gain-of-function FVII variant [149], the molecular basis

underlying the moderate symptoms associated with the homozygous p.Cys132X mutation are unknown. Comparison among expected suppression scores, although obtained for other gene contexts and in different expression systems, suggests that the Cys132X stop codon displays the most favorable prediction of readthrough (table 4). Nonsense changes can be further classified by the potential readthrough-induced re-insertion of the wild-type residue. This analysis highlighted that the p.Ser112X, differently from the other mutations, would not permit the readthrough-mediated re-insertion of the natural serine and, bearing a stop codon located 93 bases from the downstream splicing junction, is expected to induce "in vivo" nonsense-mediated FVII mRNA decay [150]. These observations, and the extremely different bleeding symptoms, prompted us to thoroughly investigate quantitative and qualitative aspects of the p.Cys132X and p.Ser112X spontaneous and drug-induced readthrough.

4.2 Materials and methods

Expression vectors

Expression vectors were created by mutagenesis of the human FVII cDNA, cloned into the pCMV5 plasmid, by using the QuickChange® XL Site-Directed Mutagenesis Kit (Agilent). Sequences of the forward mutagenic primers are reported in the table below. Reverse oligonucleotides were complementary to the forward ones. All expression vectors have been validated by sequencing.

Variant	**Primer sequence**
FVII-Ser112X	5'CCAGTGTGCC**T<u>G</u>A**AGTCCATGCC3'
FVII-Cys132X	5' CTATATCTGCTTC**TG<u>A</u>**CTCCCTGCCTTCGAG3'
FVII-Ser112W	5' CCAGTGTGCC**T<u>GG</u>**AGTCCATGCC3'
FVII-Ser112C	5' GGGACCAGTGTGCC**T<u>GT</u>**AGTCCATGCCAGAATG3'
FVII-Ser112R	5' GATGGGGACCAGTGTGCC**<u>CG</u>A**AGTCCATGCCAGAATG3'
FVII-Cys132W	5' CTATATCTGCTTC**TG<u>G</u>**CTCCCTGCCTTCGAG3'
FVII-Cys132R	5' CTATATCTGCTTC**<u>C</u>GC**CTCCCTGCCTTC3'

Modified oligonucleotides (underlined) and triplets (bold) are indicated.

Expression studies on recombinant variants

HEK293 cells were cultured as described in paragraph 3.2. Cells were seeded in 12-well plates and transfected with 2 μg of plasmid DNA in the presence of a 1:1 ratio of

Lipofectamine® 2000 Reagent (Invitrogen), according to the manufacturer's protocol. Transfected cells were maintained for 48 hours in OptiMEM medium (Gibco) supplemented with 5 μg/ml vitamin K and 100 μg/ml G418 (Sigma-Aldrich) [132]. All transfections were performed at least in triplicate.

Protein levels of recombinant FVII (rFVII) variants in medium were evaluated by ELISA with immobilized (sheep, PA1-43043; ThermoFisher Scientific, Rockford, IL, USA) and secondary (sheep, CL20030HP; Cedarlane, Burlington, Ontario, Canada) polyclonal anti-human FVII antibodies. Known concentrations of plasma-derived human FVII (Haematologic Technologies) were used as a reference, and, in our hands, the lowest detection limit was 0.4 ng/mL FVII.

Functional and procoagulant activity assays in plasma

The generation of FXa in FVII-deficient plasma (George King, Overland Park, KS, USA) was performed as previously described [151]. Briefly, FVII-deficient plasma was supplemented with rFVII-containing medium; coagulation was triggered by the addition (1/100 of the reaction volume) of Innovin (Dade® Innovin®, Siemens Healthcare, Marburg, Germany) and the reaction was monitored by fluorogenic substrates for FXa (150 μM, SpectroFluor FXa; Sekisui Diagnostics, Lexington, MA, USA) prepared in reaction buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 5 mM CaCl2, and 0.1 % PEG-8000). The generation of FXa was measured as fluorescence emission (Relative Fluorescence Units, RFU; 360 nm excitation, 465 nm emission) over time on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific, Helsinki, Finland).

Prothrombin time (PT)-based coagulation assays were performed with FVII-depleted plasma (HemosIL) supplemented with rFVII-containing conditioned medium. Coagulation times were measured upon addition of RecombiPlasTin 2G (HemosIL) and $CaCl_2$ on a ACLTOP700 instrument (Instrumentation Laboratory).

Lag and coagulation times obtained with serial dilutions of rFVII-wt were used to construct the reference curves, which were optimized for the determination of low activity levels. All assays were conducted at least in duplicate.

4.3 Results and discussion

The two selected nonsense mutations, affecting the same FVII region/domain (EGF1), share the TGA nonsense triplet but differ in the downstream nucleotide (table 4), which has been identified as an efficiency hallmark for both spontaneous and drug-induced readthrough. These sequence features were mimicked by introducing the two premature stop codons in the *F7* cDNA, cloned into the pCMV5 expression plasmid (fig. 4.1).

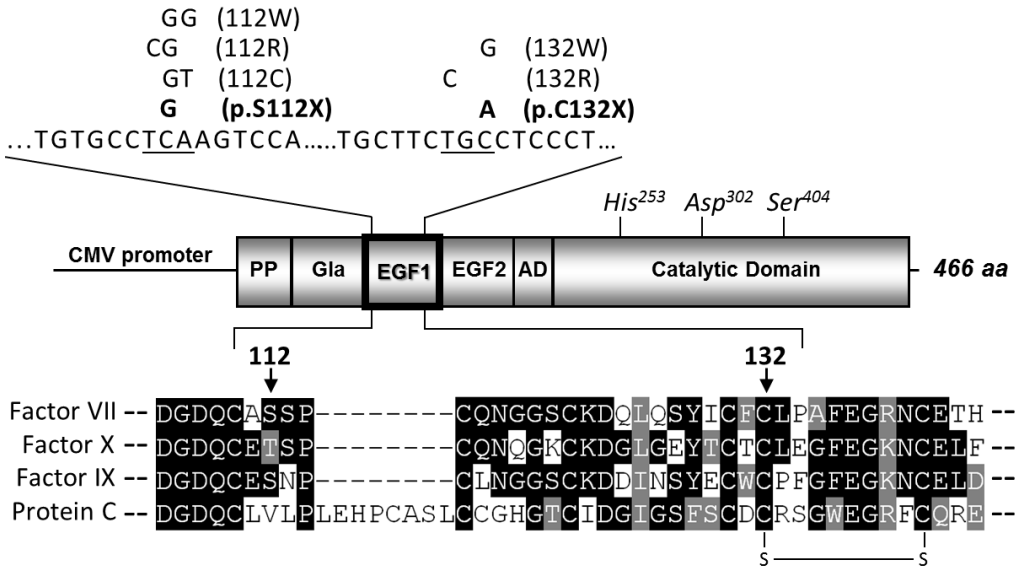

Figure 4.1. *FVII organization and recombinant variants. The natural nonsense (bold) and designed missense variants are indicated above the partial nucleotide sequence of F7 exon 4, as well as the triplet involved in mutations (underlined). The alignment of the EGF1 region among the homologous human FVII (NP_000122.1), FX (NP_000495.1), FIX (NP_000124.1) and protein C (NP_000303.1) is reported with the 112 and 132 positions marked by black arrows. The intrachain Cys132–Cys141 disulfide bridge is also indicated.*

Transient expression studies in HEK293 cells revealed basal levels of secreted protein for rFVII-132X (0.9%± 0.8% of rFVII-wt), whereas those of rFVII-112X were barely detectable above the detection limit of the FVII ELISA assay (fig. 4.2). To magnify the occurrence of readthrough-mediated biosynthesis of FVII, we took advantage of the G418 aminoglycoside. Treatment of transfected cells with G418 resulted in secreted FVII levels corresponding to 3.0%±0.8% (rFVII-112X) and 13%±1.6% (rFVII-132X) of those of rFVII-wt.

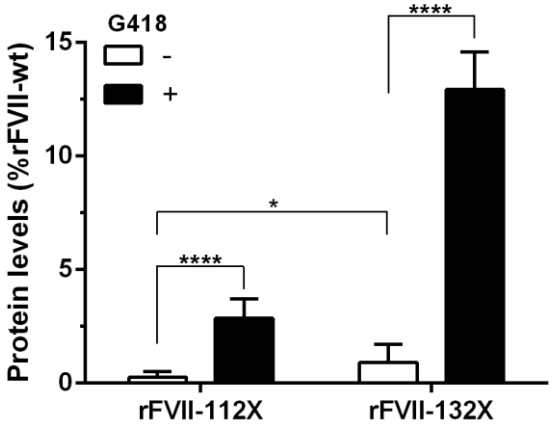

Figure 4.2. *Secreted levels of rFVII nonsense variants transiently expressed in HEK293 cells in the absence (white bars) or in the presence (black bars) of 100 µg/ml G418. Protein levels (mean ± standard deviation) of nonsense variants are indicated as % of rFVII-wt obtained in the same conditions. *, p=0.0358; ****, p<0.0001.*

The functional rescue of rFVII proteins arising from readthrough was investigated with fluorogenic FXa generation assays in FVII-deficient plasma (fig. 4.3). Importantly, these sensitive assays enabled us to analyze the low activity levels associated with the basal expression of the rFVII-132X variant (0.8%±0.1% of rFVII-wt), and also that (0.4%±0.2%) of the rFVII-112X protein, which were barely detectable by ELISA. Treatment with G418 resulted in remarkable shortening of lag times and a parallel increase in FXa generation activity (rFVII-112X, 2.5%±0.6%; rFVII-132X, 12.3%±1.7%) (fig. 4.4).

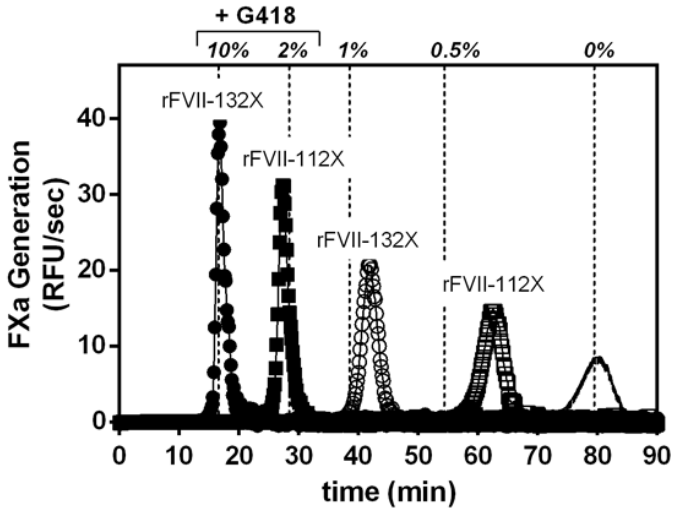

Figure 4.3. *FXa generation assay in FVII-deficient plasma supplemented with rFVII-containing medium. Basal (white symbols) and G418-induced (black symbols) FVII activity are shown. Nonsense variants are indicated on top of curves. The rFVII-wt activity levels are reported. RFU, relative fluorescence units.*

In PT-based assays, the rFVII variants showed basal procoagulant activity below (rFVII-112X, 0.5%±0.1%) or approximately (rFVII-132X, 1.1%±0.2%) 1% of that of rFVII-wt, and a noticeable increase in procoagulant activity after treatment (rFVII-112X, 2.0%±0.4%; rFVII-132X, 11.8%±1.3%) (fig. 4.4). It is worth noting that both p.Ser112X and p.Cys132X stop codons predict the synthesis of truncated FVII proteins, devoid of the catalytic triad and thus of any function.

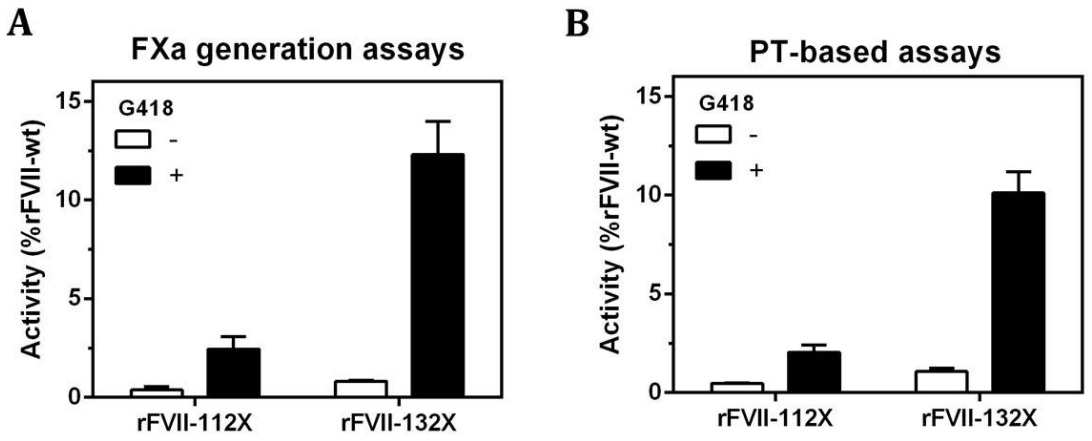

***Figure 4.4**. **A)** Activity levels (mean ± standard deviation, % of rFVII-wt) of nonsense variants in FXa generation assays. Values were obtained by comparison with lag times from serial dilutions of rFVII-wt. **B)** Activity levels (mean ± standard deviation, % of rFVII-wt) of nonsense variants in PT-based generation assays. Values were obtained by comparison with coagulation times from serial dilutions of rFVII-wt.*

Overall, these results suggest the occurrence of both spontaneous and drug-induced readthrough-mediated production of functional full-length FVII. The comparatively high degree of drug-induced readthrough for p.Cys132X also suggests alternative therapeutic strategies in patients, aimed at preventing trauma-related bleeding during surgical interventions.

The misrecognition of stop codons drives the insertion into the full-length protein of different amino acids as a function of the nonsense triplet. Previous studies in *Saccharomyces cerevisiae* have indicated that TGA is suppressed by the incorporation of Trp > Cys > Arg, in order of frequency [119]. With the limitation of translating this information in mammalian cells, the insertion of these residues would have differential implications for the mutations under study, because it would drive the reinsertion of the wild-type residue only for Cys132X. Thus, the missense variants arising from readthrough would be essential to produce residual function for

Ser112X. To investigate secretion and function of the candidate missense variants, we expressed the rFVII-112Trp/Cys/Arg and rFVII-132Trp/Arg variants (fig. 4.1).
A differential impact, related to position and residue, was detected (fig. 4.5). Missense changes at position 112 produced a moderate decrease in secreted protein levels (Trp, 43%±5.7%; Cys, 17%±3.5%; Arg, 20.7%±1.2%) and a parallel decrease in FXa generation (Trp, 44.6%±3%; Cys, 22.3%±1.5%; Arg, 24.7%±3.4%) and PT-based (Trp, 46%±2.8%; Cys, 24.5%±2.1%; Arg, 23.8%±2.5%) assays. Conversely, changes at position 132 did not result in detectable FVII secretion or function.

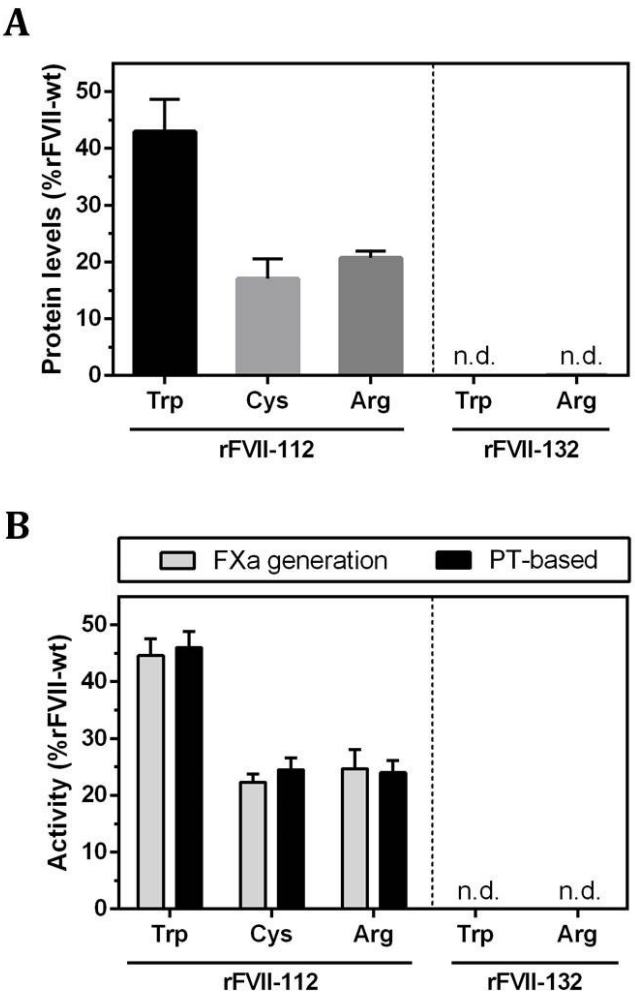

Figure 4.5. *Secretion and functional levels of rFVII variants with candidate missense changes at the nonsense positions.* ***A)*** *Secreted levels of transiently expressed rFVII missense variants.* ***B)*** *Activity levels in FXa generation (grey bars) and PT-based (black bars) assays. Results (mean ± standard deviation) are expressed as % of rFVII-wt. n.d., not detectable.*

Sequence alignment analysis and inspection of the crystal structure of the active site-inhibited FVIIa in complex with TF (2PUQ) [152] provided information that was useful for interpreting the observed differences. Whereas Cys132, as expected from

its key structural role for the intra-chain disulfide bridge with Cys141 within EGF1 [36], is fully conserved among the homologous coagulation members, Ser112 is surface-exposed and only partially conserved (fig. 4.1). Noticeably, the potential presence of very low levels of a FVII protein exposing tryptophan as the most probable residue, instead of the glycosylated serine, would imply important structural differences, which could increase the risk of inhibitor development. Indeed, high-titer inhibitors have been reported after prophylaxis in homozygous patients [153].

The thorough investigation of functional readthrough provides key information to interpret the phenotypes associated with homozygous nonsense changes in the FVII deficient patients, in whom FVII activity levels are hardly distinguishable by laboratory coagulation assays. Interestingly, the integration of data on nonsense and missense variants provides two mutation-specific scenarios.

i) The p.Cys132X displays the most readthrough-favorable sequence context, both for the degree of suppression and for the re-insertion of the wild-type residue, which is the unique condition tolerated at this protein position. The moderate phenotype in the patient, which is unexpected for the homozygous nonsense change, may be explained by the small amount of the wild-type protein, which, based on the spontaneous readthrough of recombinant expression, is approximately 1%.

ii) In the context of the p.Ser112X mutation, readthrough is predicted to occur with lower efficiency and to be unable to restore the wild-type protein, but this protein position significantly tolerates the most probable amino acid substitutions. In turn, the FVII activity observed in the recombinant expression system (around 0.3%) could be ascribed to the additive contribution of the functional missense variants. The overall suppression efficiency of the TGAA context (1%) and the relative frequency of predicted amino acid substitutions at position 112 (Trp, 0.82; Cys, 0.14; Arg, 0.04) were combined with the experimentally observed functional levels of each recombinant missense variant (Trp, 46±2.8%; Cys, 24.5±2.1%; Arg, 23.8±2.5%). The resulting activity values (Trp, 0.37%; Cys, 0.03%; Arg, 0.01%) collectively lead to an estimated residual function of 0.4%, which is similar to that experimentally observed (0.3%) for the Ser112X nonsense variant. Even if these predicted values are only approximate, they help in interpreting the readthrough-mediated protein output.

Moreover, the occurrence of nonsense-mediated decay would further decrease FVII levels by one order of magnitude. The inferred figure is extremely low and in line

with the experimental observation that as little as 0.05% FVII may suffice to trigger a minimal amount of coagulation [154][155]. Altogether, trace levels of three mutated but functional FVII molecules would be compatible with the very severe condition associated with the p.Ser112X, characterized by intracranial bleeding and requiring prophylaxis [146][153].

This integrated and complex picture is based on data from thorough expression of a panel of recombinant molecules but is hardly verifiable in patients' plasma and hepatocytes. Notwithstanding, our experimental findings, and comparison with features of all reported homozygous FVII nonsense mutations, support the notion that the extent of functional readthrough contributes to the bleeding phenotype in patients, and may prevent null conditions even for the most readthrough-unfavorable mutations.

Chapter 5

Half-life extension technologies for therapeutic proteins

5.1 Half-life extension technologies

Coagulation factor products, as well as many other therapeutic proteins, suffer from the limitation of short half-life when infused, with the need of frequent multiple injections to retain a therapeutic effect. One of the main causes underlying the short-term effect of these molecules could be their size, below the kidney clearance threshold (<60 kDa) [156]. Several modifications, either chemical or introduced by molecular manipulation, have been attempted to extend half-life of different types of molecules. The great benefit brought by an extended serum persistence is a high serum concentration of the infused drug, lower dosing frequency, and the relevant advantage of decreasing administered doses without compromising pharmacological efficacy.

Association, conjugation or fusion to protein partners such as albumin currently represents a well-established half-life extension strategy. Albumin and immunoglobulin G (IgG) represent the two most abundant plasmatic proteins and share a really long half-life, which in humans amount to 19-21 days [157][158]. This is mainly due to the binding of a cellular receptor named neonatal Fc receptor (FcRn), which is responsible for a pH-dependent recycling mechanism that protect the ligands from lysosomal degradation (see 5.2). Moreover, albumin fusion can confer to the fusion protein an overall molecular weight above the kidney clearance threshold, limiting renal excretion [159].

Another half-life extension strategy is PEGylation. Covalent binding of polyethylene glycol (PEG) acts by (i) increasing the protein solubility and molecular weight, thus decreasing renal clearance and (ii) by masking the surface and protecting the protein from degradation [160]. This technology has been successfully exploited for different therapeutic proteins [161], but being an exogenous molecule, PEG could induce an immunological response and macrophage-mediated clearance. PEGylated liposomes can be alternatively used; they are artificial phospholipid vesicles with PEG side chains enabling the liposome to act as a carrier. This approach has the advantage of not requiring amino acid changes or covalent modifications, permitting to retain protein structural integrity [162].

Inspired by the need for other polymers with more beneficial properties, biodegradable alternatives to PEG, such as PASylation, have emerged. This strategy is based on biological polypeptides made of proline, alanine and/or serine, which adopt a random coil structure in aqueous buffers [163]. PASylation has been successfully

applied to various biologics, including cytokines, growth factors and enzymes, and validated in diverse animal models [164].

One more strategy involves the fusion of the C-terminus peptide of the hormone human chorionic gonadotropin (hCG) to one or both ends of the target protein. As hCG is found naturally, there is a low likelihood that fusion proteins will be toxic or immunogenic [165].

Finally, other minor strategies are the use of hydroxyethyl starch (HES), fusion with XTEN (an unstructured hydrophilic polypeptide) or hyperglycosylation via the introduction of specific amino acid changes.

5.2 Albumin-based half-life extension

HSA represents the most abundant plasma protein (~40 mg/mL), is very stable and, together with IgGs, has a long half-life, partly due to its molecular weight (66.7 kDa) above renal threshold [159]. These molecules share a common degradation and half-life prolongation mechanism that is mediated by the FcRn, which regulates HSA and IgG half-lives by a rescue mechanism (fig. 5.1) [166][167].

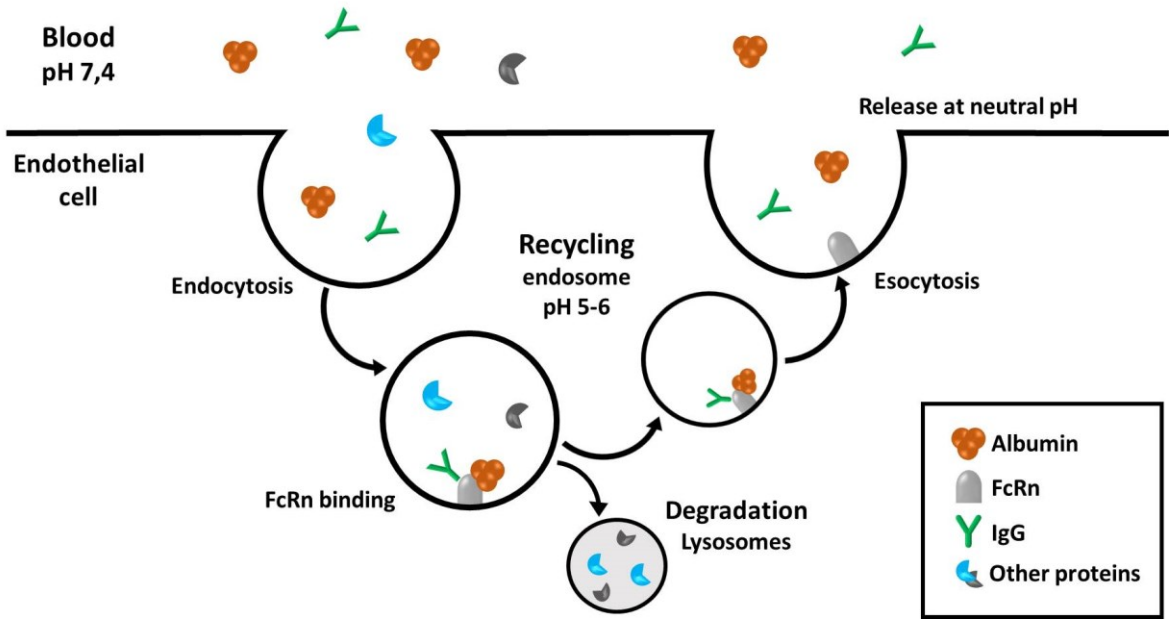

Figure 5.1. *Schematic illustration of albumin and IgG rescue mechanism mediated by the pH-dependent FcRn binding.*

FcRn is a major histocompatibility class-I related molecule composed of a transmembrane heavy chain with three extracellular domains (α1, α2 and α3) noncovalently-associated with the soluble subunit β2-microglobulin [168][169]. FcRn

resides primarily in the endosomal compartments of cells of the vascular endothelia [170] and binds IgG and HSA simultaneously in a pH-dependent manner to opposite sites on the α2-domain of the heavy chain [171]. Inside cells, FcRn function is to rescue endocytosed IgG and HSA from lysosomal degradation by a high-affinity binding into acidic endosomes, where the pH is below 6.5 [172]. Subsequently, the ligand-receptor complex is transported back to the plasma membrane via recycling endosomes, followed by release of IgG and HSA as the pH increases above 7, since affinity of FcRn for HSA decreases about 200-fold from acidic to neutral pH [166].

HSA is structured in three domains named domain I (DI, residues 1-195), II (DII, 196-380) and III (DIII, 381-585), each composed of two subdomains (a and b) with different binding capacities [173][174]. In particular, DIII has been shown to be necessary and sufficient for the pH-dependent binding to FcRn, particularly through histidine residues residing within this domain. Indeed, histidine is the sole amino acid that normally change its protonation state in the relevant pH interval (6.0-7.4). Albumin DIII contains three conserved (His-464, His-510 and His-535) and one non-conserved (His-440) histidine residues, whose mutation has been shown to almost completely abolish binding to FcRn with the exception of the non-conserved His-440 [171][175][176].

The dissection of the molecular bases underlying albumin-FcRn interaction laid the foundation for a rational engineering of albumin aimed at further improving its half-life in the perspective of therapeutic fusion proteins. This is particularly relevant for the impact of HSA fusion on the half-life of the fusion partner, because once injected the fusion molecules compete with the abundant endogenous HSA for FcRn binding, which might limit their recycling and thus half-life [177]. Thus, the identification of HSA variants with an improved FcRn binding capacity will confer a competitive advantage. Interestingly, the observation that mouse albumin (MSA) binds both human (hFcRn) and mouse FcRn (mFcRn) more tightly than HSA, a finding partly due to proline at position 573 that is highly conserved in DIII in several species with the exception of humans, led to the HSA K573P variant that showed a 12-fold increased affinity to FcRn and an extended half-life (1.5 fold) in mouse models [178][179].

Since HSA binds very weakly to mFcRn and as such has a short half-life in mice, a major challenge exits with regards to pre-clinical evaluation of human albumin-based fusions. Indeed, mFcRn binds very weakly to HSA, with a binding affinity 10-fold weaker than that of MSA [180][178][179], thus resulting in a preferential rescue of

the endogenous albumin rather than injected HSA. On the other hand, hFcRn binds MSA 10-fold more strongly than HSA [180]. This complex network of interspecies interactions must be taken into account, since it may compromise the half-life evaluation of HSA variants *in vivo*.

5.3 Previous applications of half-life extension technologies to haemostatic agents

One of the major limitations of the current treatment strategies for haemophilias is the short half-life of the infused factors, which is approximately 10-12 hours (h) for FVIII, 18-34 h for FIX and 2.5 h for FVIIa. The most frequently adopted treatment regimen is then the intravenous infusion of FVIII or FIX two-to three-times per week, thus representing a significant burden to patients and healthcare providers. The prolongation of coagulation factor half-life would enable less frequent infusions, reducing the burden of treatment and improving patients' compliance.

Several half-life extended products are currently in development, employing technologies such as Fc or albumin fusion, PEGylation, carboxyl-terminal peptide (CTP) fusion, HESylation, XTEN and hyperglycosylation (table 5).

Table 5. Half-life extended products in development (adapted from [160]).

Technology	Replacement therapy	Product	Half-life extension (vs native)
Fc fusion	FVIII	rFVIIIFc (ELOCTATE™)	19 h (1.5-fold)
	FIX	rFIXFc (ALPROLIX™)	82.1 h (2.4-fold)
	FVIIa	rFVIIaFc	N/A
Albumin fusion	FIX	rIX-FP	102h (4.3-fold)
	FVIIa	rVIIa-FP	8.5 h (3- to 4- fold)
	vWF	rVWF-FP	N/A
PEGylation-direct	FVIII	N8-GP (NN 7088)	19 h (1.5-fold)
	FVIII	BAY 94-9027	18.7 h (1.3-fold)
	FVIII	BAX 855	N/A (1.5-fold)
	FIX	N9-GP (nanocog beta pegol)	110 h
	FVIIa	N7-GP	15 h
PEGylation-indirect (liposomes)	FVIII	PEGLip-FVIII	10.2 h
	FVIIa	PEGLip-FVIIa	N/A
CTP	FIX	FIX-CTP	N/A
	FVIIa	FVIIa-CTP	N/A
HES	FVIII	HES-rFVIII	N/A
	FVIIa	HES-rFVIIa	N/A
XTEN	FVIII	rFVIII-XTEN	N/A
	FVIIa	rFVIIa-XTEN	N/A
Hyperglycosylation	FIX	FIX-T172N-K228N-I251T-A262T	N/A

Note: only half-life data from human studies are included. N/A, not available

It is particularly worth noting that the albumin fusion strategy, that we explored in this work, has been already successfully applied to rFVIIa and rFIX. Recombinant albumin has been fused to rFVIIa via a flexible glycine-serine linker and the resulting fusion protein (rVIIa-FP, CSL Behring) has shown a prolonged (5.8 fold) half-life in rodents compared with rFVIIa [181][182]. Further investigation of pharmacokinetic parameters in haemophilia A mice, rabbits, rats and monkeys confirmed the prolonged half-life, enhanced recovery and reduced clearance, with haemostatic efficacy maintained in rVIIa-FP-treated animals 12h after administration [183]. Moreover, results from the phase I double-blind study were recently reported, showing rVIIa-FP was well tolerated, with enhanced half-life (8.5 h) and reduced clearance (3-4-fold) [184].

Since fusion with albumin resulted in a reduction in biological activity of FIX, a FIX-albumin fusion protein (rIX-FP, CSL Behring) with a proteolytically cleavable linker was developed. Once activated, since the linker is cleaved and FIXa released, this molecule behaves in the same way as endogenous FIXa. After preclinical studies [185][186][187] and clinical trials [188][189][190], rIX-FP has now entered the market with the commercial name Idelvion® (CSL Behring).

Chapter 6

Next-generation FVIIa-albumin fusion proteins for the treatment of haemophilias

6.1 Background and rationale

Replacement therapy, with either plasma-derived or recombinant proteins, represents the gold-standard approach for coagulation factor deficiencies. The advent of rFVIIa has represent an enormous advancement in the treatment of haemophilia, in particular for its ability to act as a by-passing agent in patients with inhibitors [191][192], a severe complication for a clinically-relevant percentage of HA (~30%) and HB (3-5%) patients [193][78]. However, rFVIIa displays the shortest half-life (2.5-3 hours) among clotting proteins [194]. This major limitation drastically reduces its biological properties [195][78] whose improvement still represents a relevant goal to achieve extended half-life and pharmacokinetic profiles.

To overcome such limitations, several approaches have been developed to improve plasma half-life, including fusion with albumin [182]. This well-known strategy relies on the acquired capacity of the fused molecule to undergo the recycling pathway mediated by FcRn, which is responsible for the extraordinary 3-week long half-life of albumin. However, the infused albumin-based therapeutics may suffer from the high abundance of endogenous albumin that may affect binding to FcRn.

In this view, engineered albumin variants with improved properties provide ideal tools to develop unique molecules to be exploited for therapeutic purposes. In particular, tailored mutagenesis of albumin residues would translate in enhanced but still pH-dependent FcRn binding, that may confer a competitive advantage, and thus improved half-life, to the fused molecules *in-vivo*. This has already been proven by the discovery of the HSA K573P variant, that showed a 12-fold increased affinity to FcRn and an extended half-life (1.5 fold) in mouse models [178][179].

Here, we produced and characterized innovative engineered fusion proteins obtained by joining rFVIIa with unique HSA variants (here called $HSA^{KP/R1}$ and $HSA^{KP/R2/R3}$ due to pending patent application) with improved features, in order to provide evidence for their activity as by-passing agents in hemophilia A and B plasma and for their enhanced kinetics in binding hFcRn.

6.2 Materials and methods

Creation of expression vectors

The human FVII cDNA was PCR amplified from the pCMV4-FVII^{wt} construct with the forward primer $^{5'}$AAAAAGCTTATGGTCTCCCAGGCCCTCAG$^{3'}$ (HindIII restriction site underlined) and the reverse primer $^{5'}$AAACTCGAG**CC**GGGAAATGGGGCTCGCAGG$^{3'}$, that suppresses FVII stop codon and introduces XhoI restriction site (underlined) and two cytosines (bold) to maintain the frame with the following linker. The PCR amplicon was then cloned into the pCDNA3 vector through the HindIII/XhoI restriction sites. In order to obtain the secretion of a furin-cleaved activated double-chain FVII (FVIIa^{wt}), a 2RKR (RKRRKR) motif was inserted between Arg^{212}-Ile^{213} [196] by site-directed mutagenesis with the QuickChange II XL kit (Agilent), using the primers $^{5'}$AAACCCCA AGGCCGAAGGAAGAGGAGGAAGAGGATTGTGGGGGGCAAG$^{3'}$ and $^{5'}$CTTGCCCCCCACAAT CCTCTTCCTCCTCTTCCTTCGGCCTTGGGGTTT$^{3'}$. Subsequently, a 32-residue glycine/serine linker [182] was generating by annealing oligonucleotides $^{5'}$GTGCTCG AGCGGGGGATCTGGCGGGTCTGGAGGCTCTGGAGGGTCGGGAGGCTCT$^{3'}$ (XhoI site underlined) and $^{5'}$CACTCTAGATTATCAGGATCCCGACCCTCCAGACCCGCCAGATCCCCC AGAGCCTCCAGAGCCTCCCGACCCTC$^{3'}$ (XbaI site underlined) and subsequent amplification under standard PCR conditions. The resulting linker fragment was digested with restriction enzymes XhoI and XbaI and then cloned into the pCDNA3-FVIIa^{wt} vector digested with the same enzymes. The HSA cDNA sequence corresponding to mature HSA (aa 25-609) was amplified with primers $^{5'}$AA AGGATCCGATGCACACAAGAGTGAGGTTG$^{3'}$ and $^{5'}$AAAGGATCCCTATAAGCCTAAGGCAG CTTGACTTG$^{3'}$ that introduce BamHI sites (underlined). The amplicon was then cloned into the pGEM®-T Easy vector (Promega, Madison, WI, USA), where it was mutagenized with the primers $^{5'}$ CTTTGCCGAGGAGGGTCCAAAACTTGTTGCTGC$^{3'}$ and $^{5'}$ GCAGCAACAAGTTTTGGACCCTCCTCGGCAAAG$^{3'}$ to introduce the K573P substitution (HSA^{KP}). This was used as scaffold to introduce the R1 and R2/R3 substitutions, in order to generate the HSA$^{KP/R1}$ and HSA$^{KP/R2/R3}$ coding sequences. These changes were provided by our collaborator Prof. Andersen, from Oslo University. Finally, HSA coding sequences (wt, KP/R1 or KP/R2/R3) were subcloned downstream the FVIIa-linker sequence into the pCDNA3 vector digested with BamHI restriction endonuclease, to generate the final constructs (pCDNA3-FVIIa-linker-HSA$^{wt\ -\ KP/R1\ -\ KP/R2/R3}$).

Stable transfection

HEK293 cells in OptiMEM medium (Gibco) were transfected with 5 μg of plasmid DNA in 60 mm plates, in the presence of a 1:1 ratio of Lipofectamine® 2000 Reagent (Invitrogen), according to the manufacturer's protocol. After 24 hours, transfected cells were seeded in 100 mm plates at different dilutions (from 1:2000 to 1:24000) and cultured in selective media supplemented with G418. After 14-21 days, single resistant colonies were moved to 24-well plates and expanded. The best clones in terms of protein expression were selected upon PT based-assay in human FVII-deficient plasma. Selected clones were expanded into Nunc™ EasyFill™ Cell Factories (6320 cm^2; Thermo Fisher Scientific), and a total of 20 L of conditioned medium was collected over 10 days, filtered, added with 10 mM benzamidine and stored at -20 °C.

Protein purification

The fusion proteins were purified by ion-exchange chromatography following a three-step protocol. Firstly, conditioned medium was diluted to bring the final NaCl concentration to 50 mM and then loaded on a Q Sepharose Fast Flow column (GE Healthcare, Uppsala, Sweden), previously equilibrated with 20 mM Tris, 50 mM NaCl, 10 mM benzamidine, pH 7.4. Following washing with the same buffer, proteins were eluted with 20 mM Tris, 1 M NaCl and 10 mM benzamidine, pH 7.4 and then dyalised against 20 mM Hepes, 100 mM NaCl, pH 7.4. All these steps were carried out at 4 °C.
Concentrated medium was loaded on an HQ 20 μm Column, 10 x 100 mm, 7.9 mL (Thermo Fisher), previously equilibrated with 20 mM Hepes, 100 mM NaCl, pH 7.4. After washing with the same buffer, proteins were eluted with 20 mM Hepes and a NaCl gradient from 100 mM to 1 M; fractions with the eluted protein were pooled and dyalised against 20 mM Hepes, 150 mM NaCl, pH 7.4. Finally, proteins were loaded on an HQ column equilibrated as before and, after washing, they were eluted with 20 mM Hepes, 100 mM NaCl and a $CaCl_2$ gradient from 0 to 60 mM. Finally, fractions were pooled, dyalised into 20 mM Hepes, 150 mM NaCl, pH 7.4 and concentrated by ultrafiltration with Amicon Ultra Filter tubes (Millipore). Protein concentration was determined by absorbance at 280 nm (molecular weight: 116'000 Da; $E^{0.1\%}_{280nm}$= 1.94).
Protein purity was assessed using NuPAGE 4-12% Bis-Tris gels (Invitrogen) followed by staining with Coomassie Brilliant Blue R-250.

Western Blotting

About 2 ng of each fusion protein (1 ng of rFVIIa) were loaded on Bis-Tris precast polyacrylamide (4-12%) gels (Invitrogen) and separated by SDS-PAGE with MES-SDS as the running buffer, and then transferred to a 0.45 μm nitrocellulose membrane. Blocking of membranes was carried out by incubation with 5% w/v milk in PBS buffer over-night at 4°C. Protein bands were revealed by a polyclonal sheep anti-human FVII (CL20030AP, Cedarlane) or a polyclonal goat anti-human albumin (A80-129A, Bethyl) antibodies and rabbit anti-sheep IgG HRP-conjugated (P0163, DAKO, Agilent) or donkey anti-goat IgG HRP-conjugated (A50-101P, Bethyl) secondary antibodies. Blotting images were acquired by chemiluminescence detection on the ChemiDoc MP System (Bio-Rad) and were analyzed by Image Laboratory Software version 4.0 (Bio-Rad).

Thrombin generation in human plasma

HA patients' plasma was supplemented with rFVIII (1-0.1 ug/ml concentration in plasma, kind gift of Prof. Camire, Children's Hospital of Philadelphia) or rFVIIa (3-1.5 ug/ml)/rFVIIa-HSA (7-3.5 ug/ml) and diluted in 20 mM Hepes, 150 mM NaCl, 0.1% PEG-8000, pH 7.4. Coagulation was triggered by the addition (1/160 of the reaction volume) of PPP-Reagent LOW (Thrombinoscope, Stago, Asnieres sur Seine, France) diluted in 20 mM Hepes, 150 mM NaCl, 5 mM $CaCl_2$, 0.1 % PEG-8000, pH 7.4, added with MP reagent (Thrombinoscope, 1 uM final concentration) as source of phospholipids, and the fluorogenic substrate for thrombin (400 μM, Thrombin Substrate III, EMD Biosciences Inc., La Jolla, CA, USA). The generation of thrombin at 37°C was measured as fluorescence emission (Relative Fluorescence Units, RFU; 360 nm excitation, 465 nm emission) over time on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific).

***In vivo* studies**

HB-balb/c mice were injected retro-orbitally with 0.5 mg/kg of rFVIIa or 1 mg/kg of rFVIIa-HSA($^{wt\text{-}KP/R2/R3}$) to achieve a circulating concentration of 100 nM protein; blood collections were obtained retro-orbitally (from the opposite eye) using non-heparinized natelson tubes into 3.8% sodium citrate (1/10 final volume) at the indicated time-points (0-2-15 minutes, 1-3-12-24 hours). Whole collected blood was centrifuged immediately at 4 °C for 10 minutes at 10,000 RCF and the supernatant

plasma was snap-frozen onto dry ice.

In order to assess the by-passing activity of rFVIIa/rFVIIa-HSA in mouse plasma, thrombin generation assays were performed. Briefly, 25 µl of plasma were mixed with 15 µl of dilution buffer (20 mM Hepes, 150 mM NaCl, 0.1% PEG-8000, pH 7.4) and 10 ul of a Innovin/phosphatidylcoline-phosphatidylserine (PCPS) mixture (Innovin final dilution was 1:30'000; PC:PS ratio was 3:1, with a 4 µM final concentration). Reaction was triggered by the addition of 50 ul of TECHNOTHROMBIN® TGA substrate (Technoclone, Vienna, Austria) and thrombin generation at 33 °C was measured as fluorescence emission (360 nm excitation, 460 nm emission) over time in a Spectramax M2^{e} (Molecular Devices, Sunnyvale, CA, USA) instrument. Raw fluorescence values were compared with a thrombin calibration curve generated using a thrombin calibrator (TECHNOTHROMBIN® TGA Calibrator, Technoclone) to convert the signal to nM of thrombin.

ELISA

96-well ELISA plates (Costar®, Corning, New York, USA) were coated with 100 µl/well of a human IgG1 mutant variant (M252Y/S254T/T256E/H433K/N434F, with specificity for 4-hydroxy-3-iodo-5-nitrophenylacetic acid) (10 µg/ml) in PBS pH 7.4 and incubated over night at 4°C. The wells were blocked with 200 µl PBS, 4% skimmed milk (PBS/M), and incubated for 1h at RT. Human or mouse FcRn-his (10 µg/ml) in PBS/M, 0.005% Tween 20 (PBS/T/M) pH 5.5 was added and incubated for 1 h at RT. Dilution series of the rFVIIa-HSA fusions (5-0.002 µg/ml) were prepared by 1:2 titrations in PBS/T/M pH 5.5, added to wells in duplicates and incubated for 1 h at RT. Bound fusions were detected with alkaline phosphatase-conjugated polyclonal anti-HSA antibody from goat (1:3000) in PBS/T/M (Bethyl Laboratories, Inc), and incubated for 1h at RT. ELISAs were developed by adding p-nitropenylphospate substrate (Sigma-Aldrich) diluted to 10 µg/ml in diethanolamine buffer. Finally, the absorbance was measured at 405 nm using the Sunrise spectrophotometer (TECAN). A volume of 100 µl was added per well in each layer of the ELISA, and the wells were washed three times with 200 µl PBS/T pH 5.5 after each incubation step.

Surface Plasmon Resonance (SPR)

SPR analysis was performed using a Biacore 3000 instrument (GE Healthcare). Following the manufacturer`s protocol, CM5 sensor chips were coupled with rFVIIa-

HSA fusions (~500 RU) using amine coupling chemistry, by injecting 6 µg/ml in 10 mM sodium acetate, pH 4.5, and unreacted moieties were subsequently blocked with 1 M ethanolamine. Phosphate buffer (67 mM phosphate buffer, 0.15 M NaCl, 0.005% Tween 20) at pH 5.5 was used as running buffer and for preparation of serial dilutions of soluble human or mouse FcRn-his (0.03-1 µM), which were injected at 40 µl/min at 25°C. HBS-P buffer (0.01 M Hepes, 0.15 M NaCl, 0.005% surfactant P20) at pH 7.4 was used for regeneration of the flow cells. All binding curves were zero adjusted and the reference cell value was subtracted, before binding kinetics were estimated using the Langmuir 1:1 ligand binding model provided by the BIAevaluation 4.1 software.

6.3 Results and discussion

Recombinant FVIIa has represented an important advancement in the treatment of haemophilias, especially for those patients who develop inhibitory antibodies against FVIII or FIX in response to classical replacement therapy. However, this approach is limited by the short half-life of rFVIIa (~2.5 hours), that makes multiple injections necessary. For this reason a fusion protein with albumin has been developed [182] and it is currently in clinical trial. To further improve the pharmacokinetic features of this molecule, here we produced and characterized next-generation FVIIa-albumin fusion proteins by taking advantage of albumin variants ($HSA^{KP/R1}$ and $HSA^{KP/R2/R3}$) with enhanced binding to FcRn. These variants are potentially able to last longer in circulation than the wild-type protein and so to confer an even more prolonged half-life to the fusion partner.

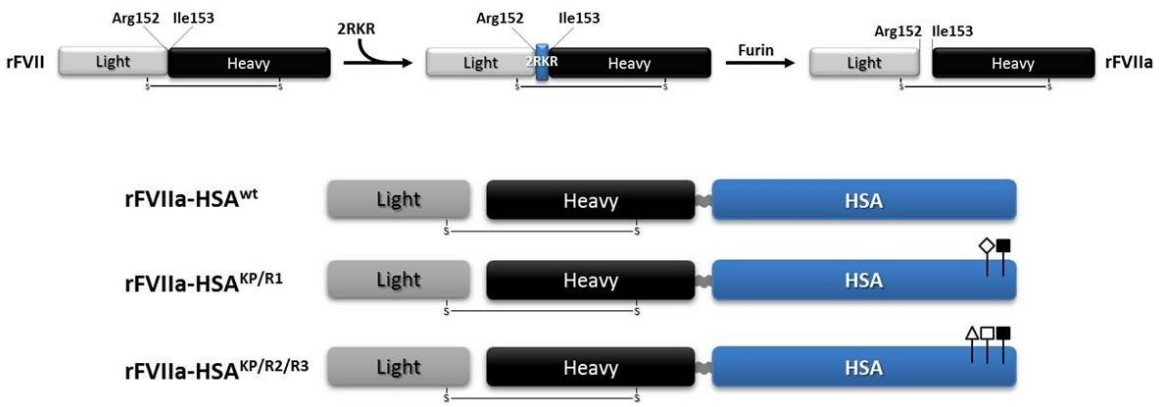

Figure 6.1. *On top, schematic representation of furin-mediated intracellular processing of rFVII engineered with the RKRRKR (2RKR) motif. On the bottom, schematic representation of rFVIIa fused via a flexible glycine-serine linker to wt or engineered HSA.*

In our setup we choose to fuse rFVIIa and albumin(s) via a 32-residue glycine-serine linker [182] and to insert a 2RKR (RKRRKR) motif in the FVII activation site [196] (fig. 6.1). The 2RKR sequence is recognized and cleaved by intracellular furins, allowing to obtain the secretion of a fully-activated molecule, as shown by transient protein expression and by the shorter lag time (57±0,3 vs 491±34 seconds) in FXa generation assays on cell medium (fig. 6.2).

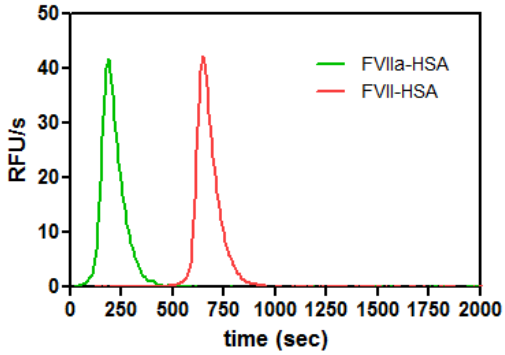

Figure 6.2. *Representative FXa generation assay in FVII-deficient plasma supplemented with rFVII-containing medium. Transient transfection and FXa generation assays were performed as described in 4.2. The assay was normalized for rFVII(a) antigen.*

In order to further characterize the fusion proteins, they were stably expressed in HEK293 cells and subsequently purified by ion-exchange chromatography. Western blotting analysis on purified proteins with anti-human FVII or anti-HSA antibodies showed the presence of both fusion partners, with an overall molecular weight as expected (~120 kDa) (fig. 6.3).

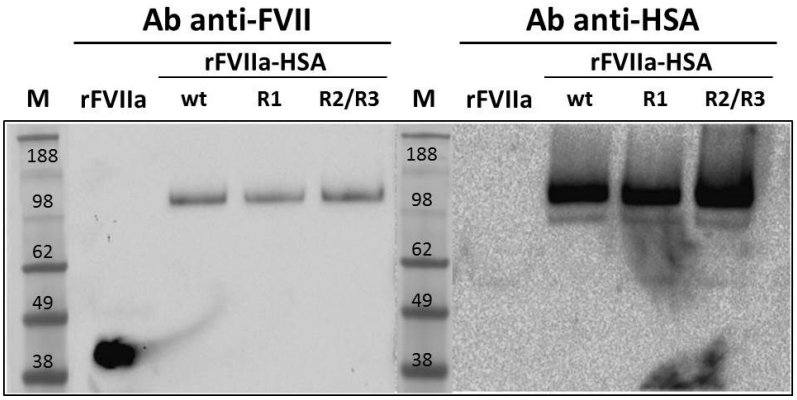

Figure 6.3. *Western Blotting analysis of purified proteins. Commercially available rFVIIa (NovoSeven, NOVO Nordisk, Bagsværd, Denmark) was used as control. M: molecular weight marker.*

To test the ability of our molecules to bypass the activity of FVIII and sustain a consistent thrombin generation *in vitro*, we performed assays on congenital haemophilic A plasma, both in the presence or absence of inhibitory antibodies. As shown in figure 6.4, either rFVIIa-HSA^{wt}, rFVIIa-HSA$^{KP/R1}$ or rFVIIa-HSA$^{KP/R2/R3}$ were able to restore an efficient thrombin generation, with an activity comparable with that of the commercially-available rFVIIa (NovoSeven, NOVO Nordisk).

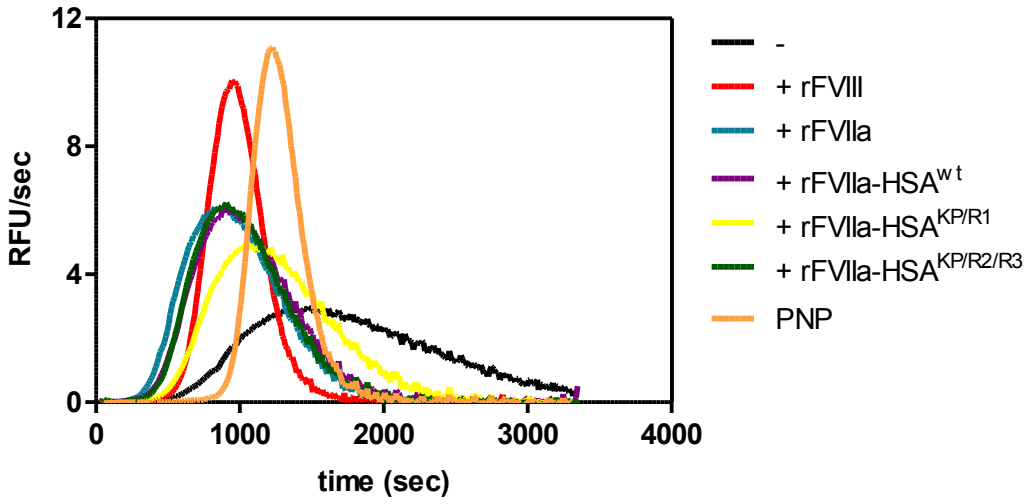

Figure 6.4. *Representative result of a thrombin generation assay in HA plasma without inhibitors. PNP: Pooled Normal Plasma*

Analysis of thrombin generation parameters showed, as expected, a shortening of lag times in comparison to pooled normal plasma (Biophen Normal Control Plasma, Hyphen Biomed) or to haemophilic plasma added with rFVIII, and the generation of the same amount of thrombin (area under curve, AUC), although with a different rate (table 6).

Table 6. Parameters of thrombin generation assays in HA plasma without inhibitors.

	Lag Time (sec)	Rate (RFU/sec)	AUC
HA plasma	300 ± 33	2,91 ± 0,1	4411 ± 243
+ rFVIII	315 ± 17	10,63 ± 0,81	4660 ± 85
+ rFVIIa	184 ± 26	5,33 ± 0,47	4582 ± 388
+ rFVIIa-HSA^{wt}	186 ± 17	5,46 ± 0,58	4590 ± 468
+ rFVIIa-HSA$^{KP/R1}$	218 ± 2	5,3 ± 0,5	4533 ± 383
+ rFVIIa-HSA$^{KP/R2/R3}$	220 ± 54	5,65 ± 0,83	4480 ± 392
PNP	389 ± 70	11,95 ± 1,2	4449 ± 107

It is worth noting that in HA patients' plasma with inhibitors, the addition of rFVIII was not able to restore thrombin generation even at supra-physiological

concentrations, while rFVIIa-HSA variants showed an efficient by-passing activity, comparable to that of rFVIIa (figure 6.5 and table 7), even at increasing titres of FVIII inhibitors.

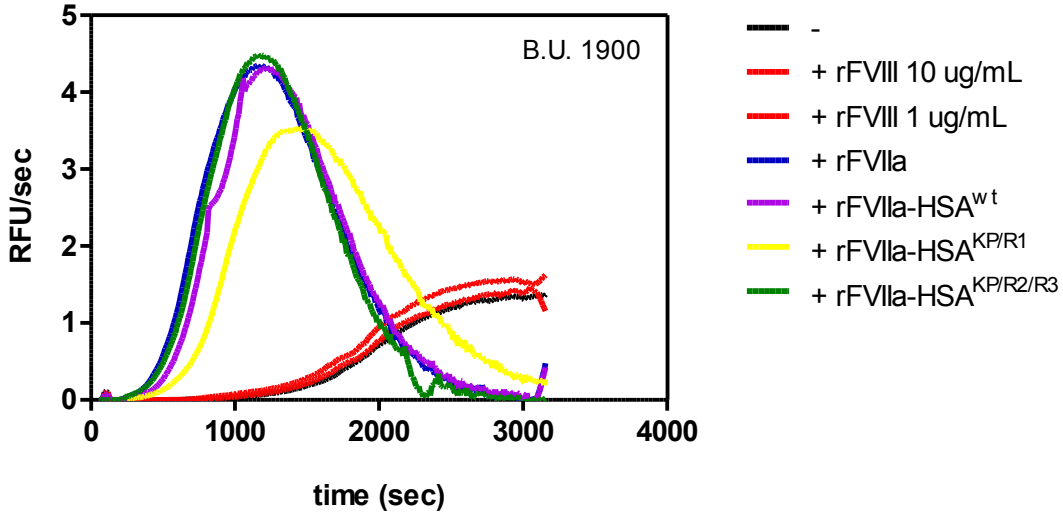

Figure 6.5. *Representative result of a thrombin generation assay in HA plasma with inhibitors. B.U. Bethesda Units.*

Table 7. Parameters of thrombin generation assays performed in plasma from three HA patients with different inhibitors' titre. LT: Lag Time (seconds). AUC: Area Under Curve (RFU*seconds).

	B.U. 7.8			
	+rFVIIa	**+ rFVIIa-HSA^{wt}**	**+ rFVIIa-HSA$^{KP/R1}$**	**+ rFVIIa-HSA$^{KP/R2/R3}$**
LT	263 ± 47	234 ± 68	336 ± 56	262 ± 30
Rate	3,51 ± 0,12	4,07 ± 0,16	3,32 ± 0,24	3,23 ± 0,18
AUC	4476 ± 21	4437 ± 4	4334 ± 1	4325 ± 3
	B.U. 370			
	+rFVIIa	**+ rFVIIa-HSA^{wt}**	**+ rFVIIa-HSA$^{KP/R1}$**	**+ rFVIIa-HSA$^{KP/R2/R3}$**
LT	299 ± 65	262 ± 94	335 ± 58	240 ± 66
Rate	3,92 ± 0,27	4,57 ± 0,19	3,82 ± 0,24	4,17 ± 0,36
AUC	4452 ± 28	4415 ± 37	4268 ± 134	4313 ± 78
	B.U. 1900			
	+rFVIIa	**+ rFVIIa-HSA^{wt}**	**+ rFVIIa-HSA$^{KP/R1}$**	**+ rFVIIa-HSA$^{KP/R2/R3}$**
LT	225 ± 37	216 ± 22	250 ± 1	215 ± 33
Rate	4,03 ± 0,46	4,12 ± 0,29	3,31 ± 0,33	4,11 ± 0,53
AUC	4657 ± 146	4563 ± 95	4419 ± 194	4512 ± 57

These data demonstrate that fusion of albumin, either wt or engineered, did not alter the ability of rFVIIa to restore *in vitro* thrombin generation in plasma of HA patients with inhibitors, the ones who would actually benefit from by-passing therapy with this molecule(s).

In order to obtain a preliminary *in vivo* evaluation of the by-passing activity of the

fusion proteins, we then injected them in HB mice and subsequently evaluated thrombin generation in plasma taken at different time points (experiments were done in collaboration with Prof. Camire's lab in Philadelphia). Both rFVIIa-HSA^{wt} and rFVIIa-HSA$^{KP/R2/R3}$ were able to restore the deficient thrombin generation, as shown by analysis of peak (fig. 6.6, top) and Endogenous Thrombin Potential (ETP, fig. 6.6, bottom) parameters, that were comparable with those obtained with rFVIIa. Interestingly, while peak and ETP values return basically to the baseline (uninjected mice) after 6 hours for rFVIIa, they were still high at 12 and 24 hours for the fusion proteins, indicating their persistence in circulation. This is due to the increased size of chimeras in respect to rFVIIa alone, since in this mouse model (that possesses mFcRn) it is not possible to take into account HSA hFcRn-mediated recycling, given the large abundance of mouse albumin that binds the receptor with a higher affinity.

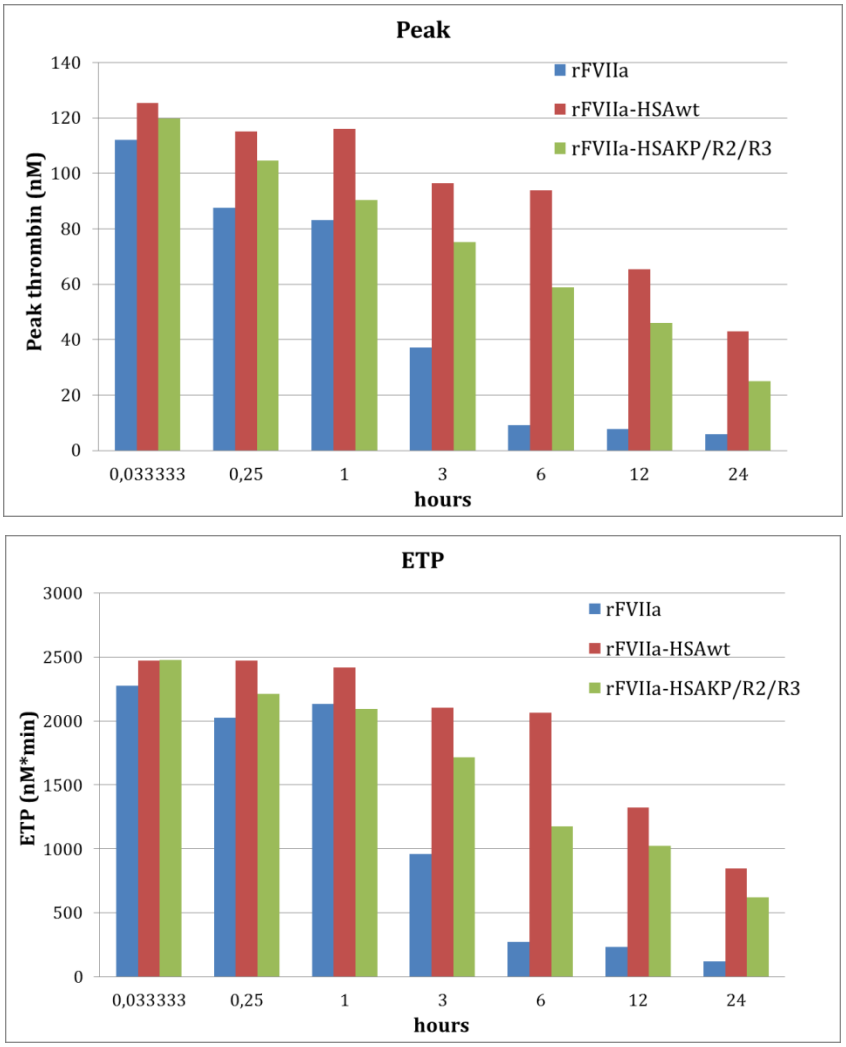

Figure 6.6. *Peak (top) and ETP (bottom) parameters derived from thrombin generation assays on plasma from HB mice injected with rFVIIa (NovoSeven, NOVO Nordisk), rFVIIa-HSA^{wt} or rFVIIa-HSA$^{KP/R2/R3}$. All values are corrected for those measured from time 0 samples and from PBS-injected mice.*

To provide evidence that rFVIIa-HSA$^{KP/R1}$ and rFVIIa-HSA$^{KP/R2/R3}$ bind to FcRn with improved affinity than rFVIIa-HSA^{wt}, in collaboration with Prof. Andersen in Oslo we performed SPR and ELISA-based assays. As shown in figure 6.7 (A-B-C) and 6.8 (A), both fusion proteins comprising the engineered albumin variants have a stronger binding to hFcRn than rFVIIa-HSA^{wt} at pH 5.5; strikingly, kinetic calculations revealed that rFVIIa-HSA$^{KP/R2/R3}$ had >400 fold-improved binding affinity (table 8).

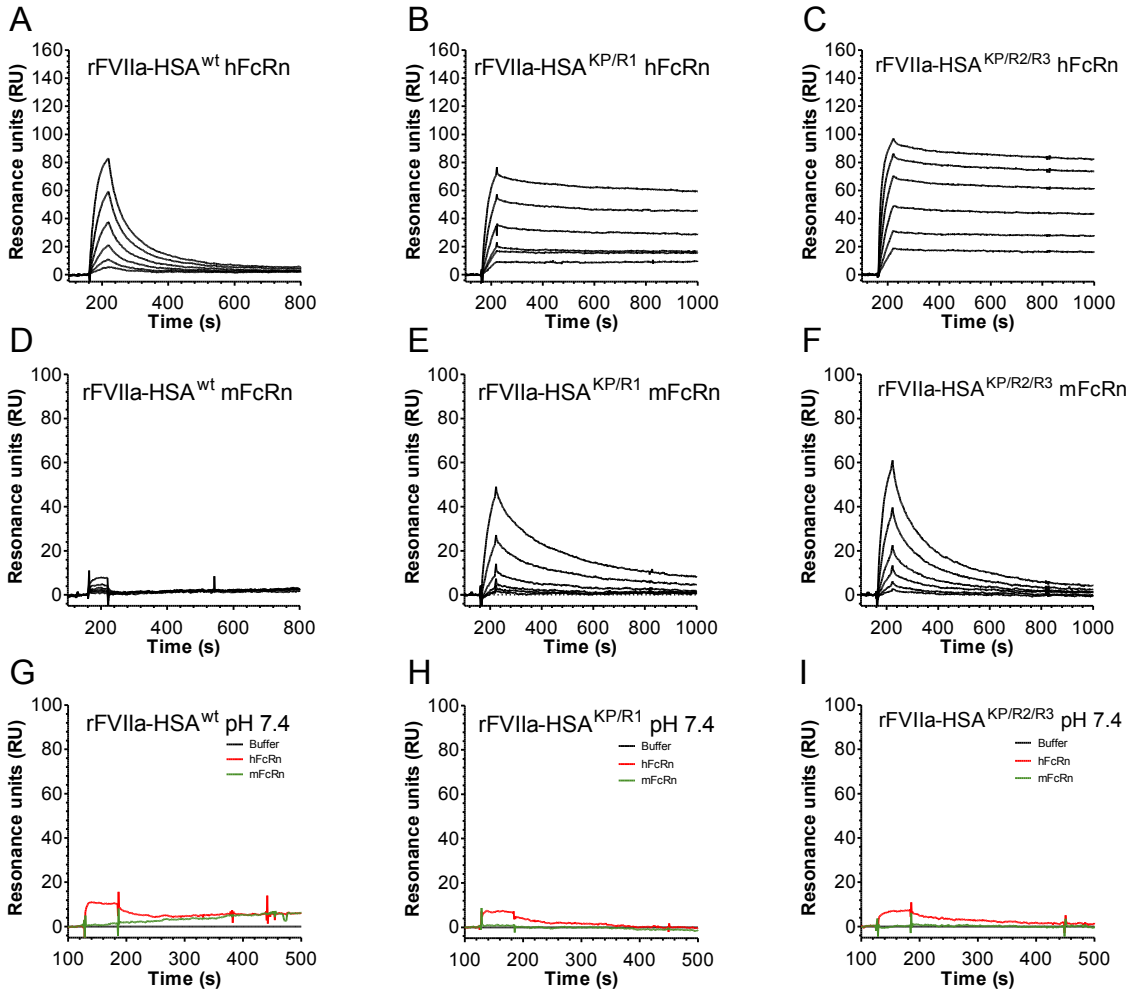

Figure 6.6. *Representative sensorgrams showing binding of titrated amounts of soluble human or mouse FcRn (0.03-1 μM) to immobilized rFVIIa-HSA fusions.*

It must be underlined that both fusion variants do not bind to hFcRn at pH 7.4 (fig. 6.6, G-H-I), meaning that they would be recycled back in circulation once intracellularly rescued. Improved albumins were also able to bind mFcRn, although with much less affinity than the human one (fig. 6.6, D-E-F, fig. 6.7-B and table 8).

Table 8. Kinetic rate constants for binding of hFcRn/mFcRn to rFVIIa-HSA fusions. Constants were obtained using a Langmuir binding model supplied by the BIAevaluation 4.1 software.

	K_a (10^3/Ms)	K_d (10^{-4}/s)	K_D (nM)
hFcRn			
rFVIIa-HSA^{wt}	45.7	114	311
rFVIIa-HSA$^{KP/R1}$	41.2	1.4	3.45
rFVIIa-HSA$^{KP/R2/R3}$	124	0.87	0.71
mFcRn			
rFVIIa-HSA$^{KP/R1}$	0.92	28.8	3130
rFVIIa-HSA$^{KP/R2/R3}$	20.3	58.2	287

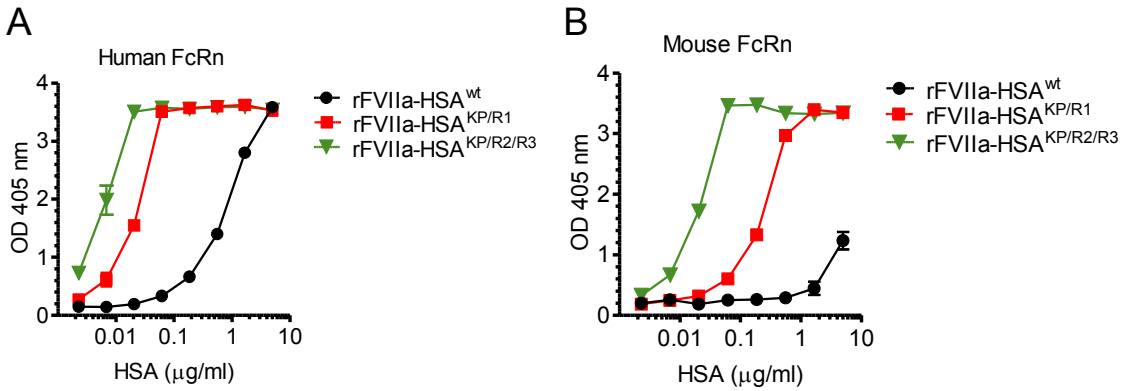

Figure 6.8. *ELISA showing binding of hFcRn-his (A) or mFcRn-his to titrated amounts of rFVIIa-HSA fusions (5-0.002 µg/ml). The ELISA was performed at pH 5.5.*

These albumin variants remarkably improved binding features would potentially translate into a superior pharmacokinetics for the fusion proteins. However, the different interspecies interactions between MSA/HSA and FcRn (see paragraph 5.2) may compromise the half-life evaluation of HSA variants *in vivo.* To overcome these limitations, a humanized mouse strain transgenic for hFcRn and knockout for mFcRn and albumin has recently been developed [197]. Notably, administration of HSA to this mouse model resulted in a serum half-life (24 days) comparable to that found in humans. This was due to the absence of endogenous MSA, acting as a competitor, and the presence of a functional hFcRn, thus making this novel mouse model a more appropriate rodent surrogate for the evaluation of pharmacokinetics of HSA-based drugs.

Injection of rFVIIa-HSA variants in these mice will permit a consistent evaluation of their half-life, proving for the first time that fusion of unique HSA engineered variants can remarkably increase coagulation factors pharmacokinetic features. Based on the promising *in vitro* and *in vivo* data here presented, we expect in the future to provide

patients with next-generation rFVIIa-HSA variants characterized by superior half-life as novel therapeutic tools for haemophilia with inhibitors.

Chapter 7

The carboxyl-terminal region of coagulation factor X as natural linker for albumin fusion

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7.1 Background and rationale

Congenital FX deficiency is one of the most severe form of rare bleeding disorders but, at variance from haemophilia or FVII deficiency, has lacked specific treatment for decades, with APCC and fresh frozen plasma (FFP) being the most frequent interventions [198]. However, APCC and FFP present several limitations [102][199] that led to the recent advent of plasma-derived FX (pdFX), shown to be effective for on-demand treatment, prophylaxis and pre-surgery intervention and displaying an half-life of approximately 30 hours in patients [105][104].

While the *in vitro* expression of FX is rather straightforward, as witnessed by the numerous FX variants so far characterized [6,7], the recombinant technologies have not been exploited to treat FX deficiency but mainly to engineer FX as by-passing agent for haemophilia [200]. Moreover, these technologies would offer the opportunity to translate the fusion strategy to HSA, successfully exploited to remarkably increase half-life of other recombinant coagulation factors. However, the creation of chimeras with HSA requires the identification of the proper fusion strategy to preserve protein properties in terms of secretion and function.

The extensive studies in the coagulation field and particularly with FVII and FIX highlighted the importance of a linker, either the glycine/serine (GS) flexible amino acid stretch for FVII [182] or the cleavable for FIX [185], to guarantee protein secretion and function. The requirement of the linker for FVII and FIX might stem from the fact that the carboxyl-terminal region of these proteins is essential for secretion [201][202]. Differently, it has been previously demonstrated that the deletion of up to 21 residues in the FX carboxyl-terminal region did not affect secretion as well as amidolytic and pro-coagulant activity [203]. This prompted us to explore the FX carboxyl-terminus (residues 470-488) as a natural linker for fusion purposes. So, we decided to express and characterize recombinant FX differentially fused to HSA.

7.2 Materials and methods

Expression vectors

FX and FX-HSA- The human FX cDNA was PCR amplified from the pCMV4-ss-pro-II-FX construct [204] with the forward primer $^{5'}$AAAAAGCTTATGGCGCACGTCCGAGG$^{3'}$ (HindIII restriction site underlined) and the reverse primers $^{5'}$AAACTCGAGTCACTTT AATGGAGAGGACGTTATG$^{3'}$ (to create pFX) or the $^{5'}$AAAAAGCTTCTTTAATGGAGAGGA

CGTTATGACC3' (to create pFX-HSA), both incorporating the restriction sites (XhoI and HindIII, respectively; underlined) and with the latter suppressing the FX stop codon. The first amplicon was then cloned into the pCDNA3 vector through the HindIII/XhoI restriction sites to generate the pFX vector.

The HSA cDNA sequence corresponding to mature HSA (aa 25-609) was amplified with primers 5'AAAAAGCTTGATGCACACAAGAGTGAGGTTGC3' and 5'AA ACTCGAGCTATAAGCCTAAGGCAGCTTGAC3' that introduce the HindIII and XhoI sites (underlined), respectively. The amplicon was then cloned into the pCDNA3 vector together with the FX devoid of the stop codon through the HindIII/XhoI sites to generate the pFX-HSA vector. The central HindIII site, inserting two additional codons between FX and HSA, was removed by mutagenesis (Agilent Technologies).

FX-GS-HSA- The glycine/serine(GS)-encoding linker was inserted into the pCDNA3 vector as described [182]. The FX cDNA was amplified with primers 5'AAAAAGCTTAT GGCGCACGTCCGAGG3' and 5'AAACTCGAGCCCTTTAATGGAGAGGACGTTATGACC3' and cloned upstream of the GS-encoding sequence by HindIII/XhoI restriction sites (underlined). The HSA was cloned through BamHI restriction sites (underlined) after PCR amplification with primers 5'AAAGGATCCGATGCACACAAGAGTGAGGTTGC3' and 5'AAAGGATCCCTATAAGCCTAAGGCAGCTTGAC3'.

FX-CL-HSA- The cleavable linker(CL)-encoding sequence, resembling that recognized by FVIIa, was created by annealing of primers 5'GTGCTCGAGCG GGGGATCTGGCGGGTCTCCTGAACGAGATAATGCTCTTACTCGT3' and 5'CACTCTAGATT ATCAGGATCCCGACCCTCCAGACCCGCCAGAAACTGAACGAGTAAGGCATTAT3' followed by cloning into the pCDNA3 backbone through XhoI and XbaI restriction sites. The subsequent cloning procedures were as described for the pFX-GS-HSA construct.

All plasmids have been validated by sequencing.

Creation of stable clones and evaluation of secreted protein levels

HEK293 cells were stably transfected as described in paragraph 6.2. FX levels in media were evaluated by ELISA (Cedarlane) and known concentrations of plasma-derived human FX (Haematologic Technologies) were used as reference.

Detection of fusion proteins in media was performed by ELISA-based assays, with a capture polyclonal anti-HSA antibody (Bethyl Laboratories, Montgomery, TX, USA) followed by detection with a polyclonal HRP-conjugated anti-human FX antibody (Cedarlane), and Western blotting analysis with polyclonal sheep anti-human FX (Cedarlane) and rabbit anti-sheep HRP-conjugated (DAKO, Agilent) antibodies.

Thrombin generation and coagulation assays

Thrombin generation assays were performed as previously described for FVII [151]. Briefly, FX-deficient plasma was supplemented with rFX(HSA)-containing medium; coagulation was triggered by the addition (1/100 of the reaction volume) of Innovin (Dade® Innovin®, Siemens Healthcare) and the reaction was monitored by fluorogenic substrates for thrombin (250 μM, Thrombin Substrate III, EMD Biosciences Inc) prepared in reaction buffer (20 mM Hepes pH 7.4, 150 mM NaCl, 5 mM CaCl2, and 0.1 % PEG-8000). The generation of thrombin was measured as fluorescence emission (Relative Fluorescence Units, RFU; 360 nm excitation, 465 nm emission) over time on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific).

Prothrombin time (PT)-based coagulation assays were performed with FX-depleted plasma (HemosIL) supplemented with rFX(HSA)-containing conditioned medium. Coagulation times were measured upon addition of a RecombiPlasTin 2G (HemosIL) and $CaCl_2$ on a ACLTOP700 instrument (Instrumentation Laboratory).

Time-course activation was performed with rFVIIa (NOVO Nordisk) and Innovin and quenching of sample aliquots in 50 mM EDTA at the selected time points. Activity was assayed toward the activated FX (FXa) fluorogenic substrate (250 μM, SpectroFluor FXa; Sekisui Diagnostics) by measuring fluorescence emission over time on a microplate fluorometer (Fluoroskan Ascent FL, Thermo Fisher Scientific).

Half-life evaluation

Eight-week-old wild-type C57/BL6 mice (n=6 per group) were tail vein injected with FX (5 μg) or FX-HSA variants (10 μg) concentrated by centrifugal filter devices from conditioned media and diluted in PBS, as previously described [205]. Blood samples were collected from the tail vein at the selected time points and protein levels evaluated by ELISA, which does not cross react with murine FX.

FX levels at 5 minutes post-injection were used to evaluate protein recovery. FX values at the following time points (4, 8, 16, 20, 24 and 36h) were considered to estimate FX half-life. All procedures were approved and conducted under the guidelines established by the Italian Ministry of Health.

Data analysis

The statistic software GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA) was used to analyze data. The lag time parameter in thrombin generation assays was extrapolated from the first derivative of relative fluorescence units (RFU) as a function of time (minutes). PT-based assays were analyzed by a two-phase decay non-linear fit of coagulation times. The amidolytic activity was determined by a linear regression analysis of the initial rate of fluorescence emission. Statistical differences in protein levels were evaluated by the t-test.

7.3 Results and discussion

In this study we explored recombinant DNA technologies to provide for the first time a recombinant FX molecule as an ideal tool for replacement therapy in FX-deficient patients. Since the limited half-life of injected proteins represents one of the major limitations, with a great impact on patient compliance and costs, we explored the fusion strategy with albumin, one of the best established approaches to confer an extended half-life to therapeutic proteins (see chapter 5).

The simplest method is the direct tandem fusion, but for many therapeutic proteins the absence of a linker resulted in impaired expression or function of the chimera. Based on the analysis of recurrent features of natural linkers within multi-domain proteins, several types of artificial linkers have been designed, with either flexible, rigid or cleavable properties [206]. These categories, mainly consisted of repetitive units of amino acid stretches, confer optimal protein-to-protein connection by i) allowing interaction and/or spatial separation between domains conferred by small or hydrophilic amino acids (i.e. glycine/serine) (flexible linkers), ii) maintaining distance between domains through helical structures or proline-rich sequences (rigid linkers), and iii) allowing disjoining of fused domains by protease-sensitive sequences (cleavable linkers) [206].

Since we demonstrated that the FX carboxyl-terminal region is dispensable for secretion as well as amidolytic and pro-coagulant activity [203], we explored this region as a “natural” linker sequence for fusion purposes. As a matter of fact this region in FX is i) composed of amino acids that are predicted to have high propensity to be found in naturally-occurring protein linkers, ii) predicted to be unstructured, with a length (19 residues) in line with large natural linkers, and iii) displays partly flexible and rigid properties [207]. These virtually unique features led us to express

and characterize a fusion protein between FX and HSA by direct tandem fusion (FX-HSA). As comparison we investigated the FX-HSA chimera separated i) through a rationally-designed cleavable linker resembling the FX activation site recognized by FVIIa (FX-CL-HSA), chosen to drive disjoining of HSA after activation, or ii) by a flexible glycine/serine [182] linker (FX-GS-HSA)(fig. 7.1).

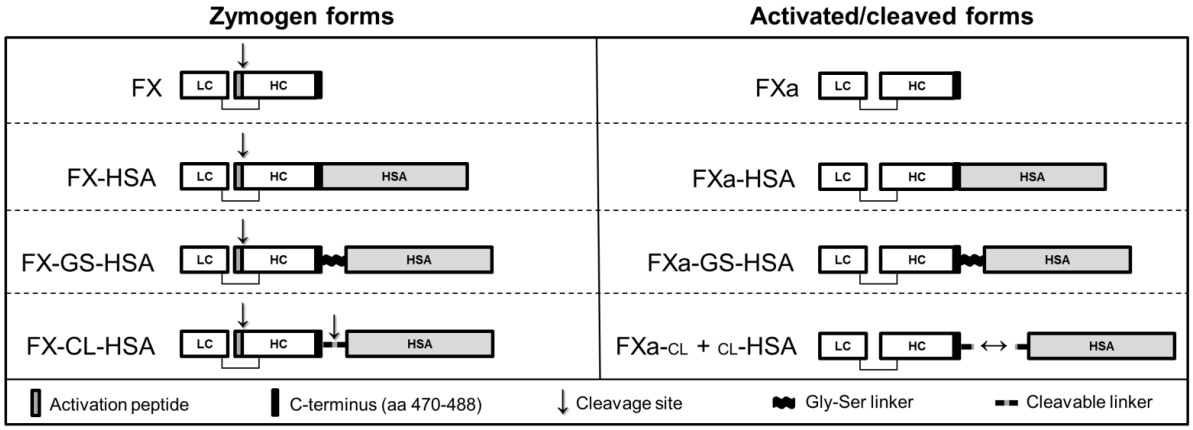

Figure 7.1. *Schematic representation of FX variants as zymogen (left) or activated (right) forms. Fusion proteins were designed as direct tandem fusion by exploiting the FX carboxyl-terminal region as natural linker (FX-HSA), or by intervening a flexible 32-residue glycine/serine linker (FX-GS-HSA)* [182] *or a rationally-designed 30-residue cleavable linker, flanked by glycine/serine residues, resembling the natural FVIIa cleavage site within FX activation peptide (FX-CL-HSA). LC, light chain; HC, heavy chain; HSA, human serum albumin, mature protein (aa 25-609).*

We characterized the three fusion variants from stably-expressing HEK293 cells. Through Western blotting analysis and ELISA-based assays we clearly detected the fusion variants with the expected molecular weights (~130 kDa) and possessing both fusion partners (fig. 7.2).

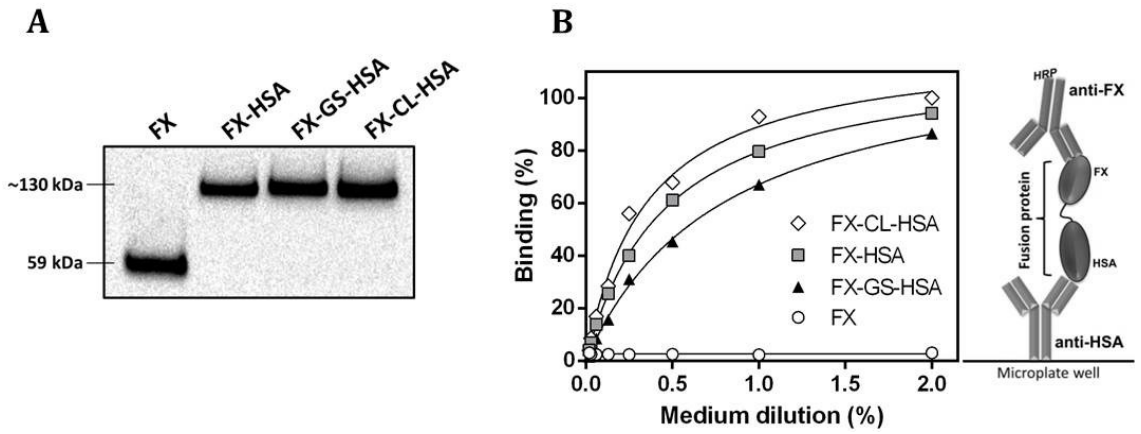

Figure 7.2. ***A)*** *Western blotting analysis of FX and of fusion variants with anti-FX polyclonal antibodies.* ***B)*** *ELISA-based assays with coated anti-HSA and detecting anti-FX antibodies (scheme). Serial dilutions of medium containing fusion proteins (indicated as percentage) were evaluated in comparison with unfused FX exploited as negative control.*

At variance, FX alone was not detected by the combined ELISA. The expression of stable clones produced similar amounts of wild-type FX and fusion variants, as indicated by both Western blotting and ELISA.

The pro-coagulant activity of the proteins was evaluated in FX-deficient plasma by thrombin generation and prothrombin time (PT)-based assays (fig. 7.3).

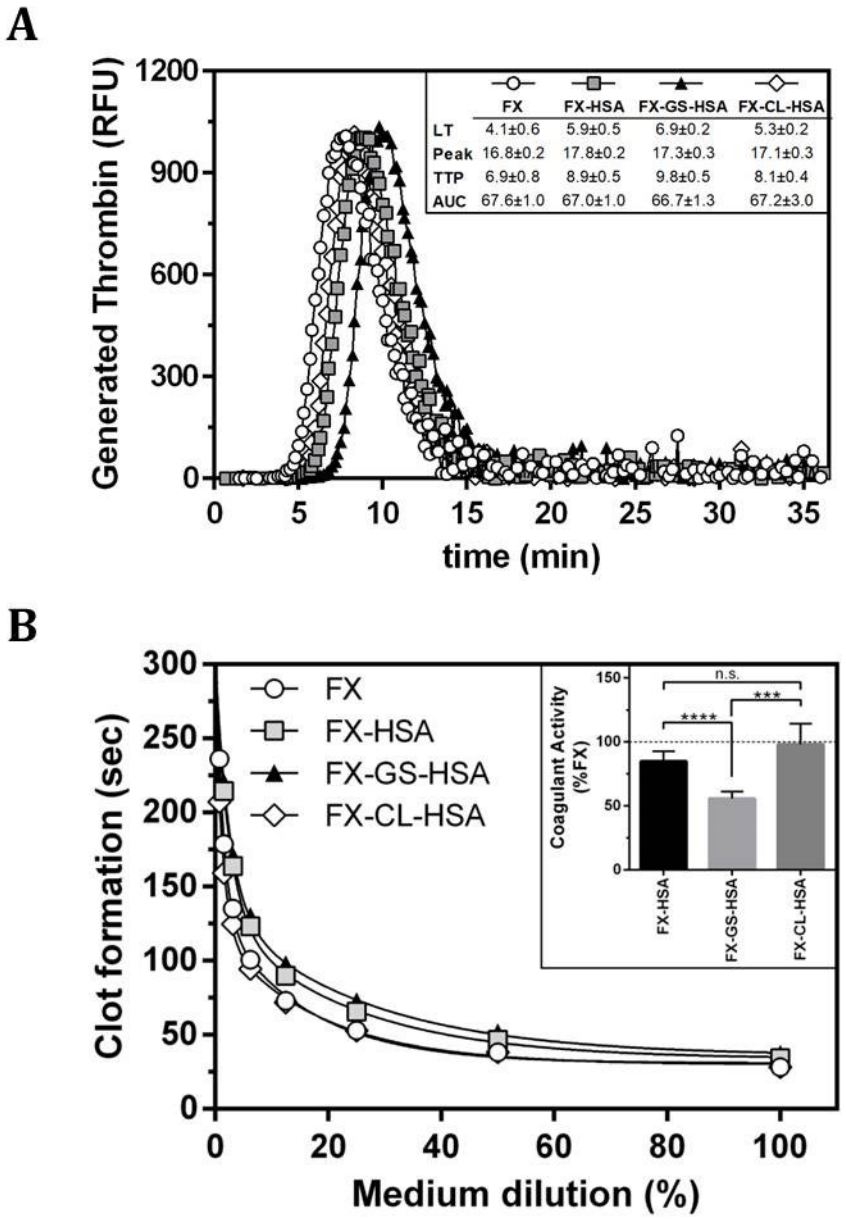

Figure 7.3. A) *Thrombin generation activity of fusion variants after extrinsic activation in commercially-available congenital FX-deficient plasma in comparison with FX alone. Inset. Thrombin generation parameters. LT, lag time (min); TTP, Time to peak (min); Peak (RFU); AUC, area under curve (RFU*min).* ***B)*** *Prothrombin time (PT)-based assays with serial dilutions of fusion proteins and FX alone as control. Inset. Coagulant activity (% of FX alone) of fusion proteins. Results are expressed as mean ± standard deviation. RFU, Relative Fluorescence Units. ****, p<0.0001 ; ***, p=0.0009; n.s., not significant (p=0.1155).*

In both systems we observed that all fusion variants were characterized by appreciable activity, with the FX-HSA and FX-CL-HSA displaying functional features comparable to that of FX (FX-HSA, 84.7±7.8%; FX-CL-HSA, 98.0±16.4%) (fig. 7.3, inset). Conversely, the FX-GS-HSA variant showed a significantly lower efficiency in both assays, with a coagulant activity of 55.7±5.5% of FX.

To dissect the functional impact of the differentially-fused albumin on FX, we investigated the activation profile of the three fusion variants in the presence of the physiological activator FVIIa by Western blotting with anti-FX antibodies. Although in the presence of uncleaved FX, a common observation with recombinant FX in conditioned medium [208], all fusion proteins, upon incubation with rFVIIa, showed the band corresponding to the activated form (~117 kDa) (fig. 7.4) resulting from the zymogens (~130 kDa) that have lost the FX activation peptide (52 residues; ~13 kDa).

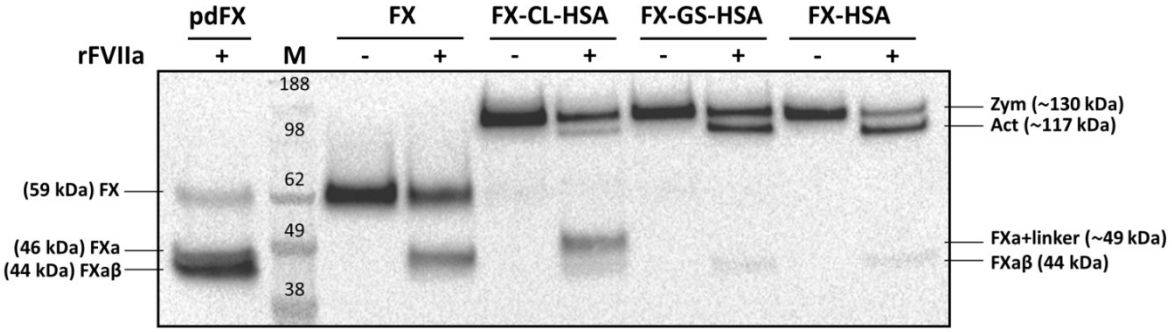

Figure 7.4. *Western blotting analysis of FX and fusion variants in the absence (-) or in the presence (+) of rFVIIa/Innovin. The FXa+linker form is referred to the FXa form disjoined from the FX-CL-HSA fusion protein and retaining part of the linker upstream of the cleavage site (see fig. 7.1). Relative molecular weights for each form are shown. Plasma-derived FX (pdFX) was used as external control. Zym, zymogen forms of fusion variants; Act, rFVIIa-activated fusion proteins; M, molecular weight marker.*

Moreover, the ratio between bands corresponding to the zymogen and the activated forms point towards a defective activation of FX-GS-HSA as compared to the FX-HSA and FX-CL-HSA variants, a finding coherent with its reduced coagulant activity. Noticeably, the ratio between the activated and zymogen forms observed in FX-HSA is higher to that observed for FX, pointing out an efficient activation. Interestingly, and in accordance with the rationally-designed fusion strategy, only the FX-CL-HSA showed a band of approximately 49 kDa attributable to the FXaα form that, upon rFVIIa cleavage at the linker site, has lost HSA. The slightly higher molecular weight of this fragment, as compared to that of FXaα alone, is due to the persistence of the

linker portion upstream of the rFVIIa cleavage site (fig. 7.1). Finally, all fusion variants showed traces of the FXaβ form (~44 kDa), arising from auto-proteolytic cleavage at the R469-G470 site that releases the downstream HSA. These data demonstrate that the FX-HSA direct fusion variant without linker is characterized by coagulant activity comparable to that of FX alone. Moreover, the activation profiles of the fusion proteins were confirmed upon incubation with a non-physiological activator, Russell Viper Venom (RVV) (fig. 7.5).

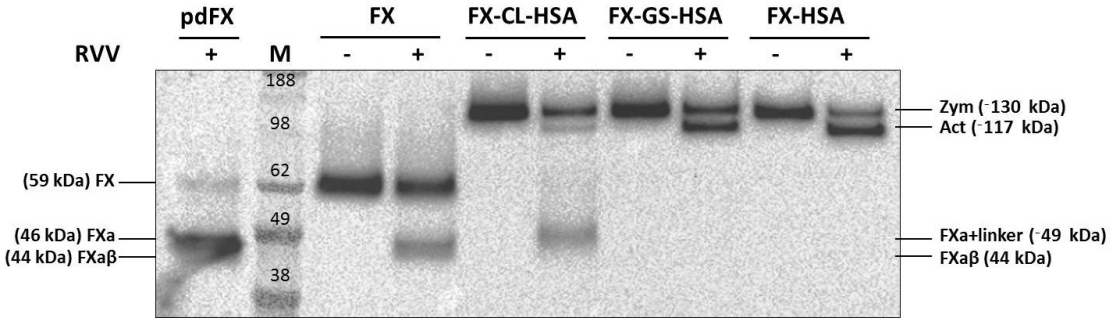

Figure 7.5. *Western blotting analysis of FX and fusion variants in the absence (-) or in the presence (+) of RVV. The FXa+linker form is referred to the FXa form disjoined from the FX-CL-HSA fusion protein and retaining part of the linker upstream of the cleavage site (see fig. 7.1). Relative molecular weights for each form are shown. Plasma-derived FX (pdFX) was used as external control. Zym, zymogen forms of fusion variants; Act, RVV-activated fusion proteins; M, molecular weight marker.*

To corroborate our functional observations we also performed a time-course activation assay, which further confirmed the similar activation profile between FX-HSA and FX alone (fig. 7.6).

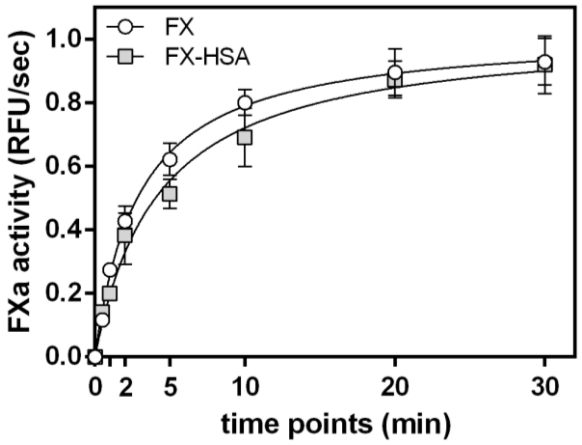

Figure 7.6. *Amidolytic activity after time-course activation of FX and FX-HSA in the presence of rFVIIa and Innovin. The amidolytic activity was evaluated at 0, 30'', 1', 2', 5', 10', 20' and 30' time points. RFU, Relative Fluorescence Units.*

Intrigued by these results we preliminarily investigated the *in-vivo* persistence of FX-HSA. Due to the virtual impossibility of using FX-deficient mice [209][210], which would be also crucial to assess the therapeutic potential of the investigated molecule, we exploited wild-type mice where human FX is easily detectable by a species-specific ELISA. FX and FX-HSA were concentrated from conditioned media and intravenously injected into mice to evaluate the protein levels at different intervals. As shown in figure 7.7, the chimera was detectable by ELISA at 36 hours post-injection whereas the FX alone disappeared at 16 hours. Assuming a therapeutic threshold around 10%, the fusion protein would guarantee a 3/4-fold increase persistence into the circulation (estimated half-life: 220 vs 70 minutes of FX alone).
It is worth noting that the half-life improvement here is due to the albumin steric hindrance, which confers the fusion protein an overall molecular weight (~130 kDa) well above the kidney clearance threshold (60 kDa) [156]. In this model system it is not possible to take into account the FcRn-mediated recycling mechanism, since wild-type HSA binds weakly to mFcRn and it has also to compete with the enormous amount of circulating MSA.

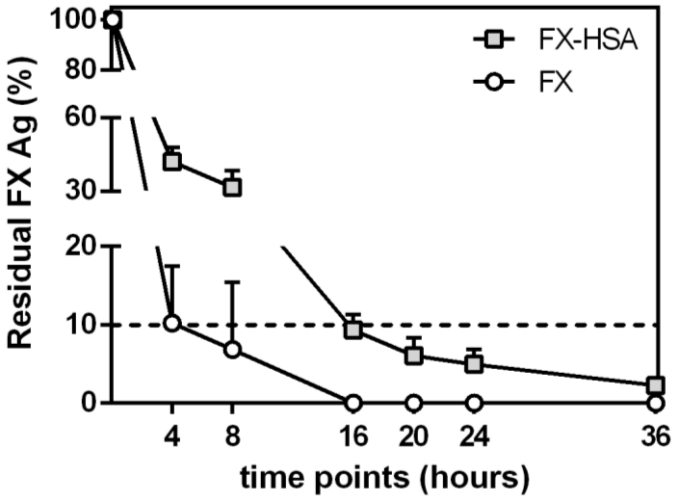

Figure 7.7. *FX-HSA or FX as control were injected (10 or 5 µg/mouse, respectively) in C57/BL6 wild-type mice. Blood samples were collected at different time points and FX antigen measured by a human-specific ELISA. Residual antigen levels are reported as the percentage of FX amount detected at 5 minutes after injection. Data are represented as mean ± standard deviation.*

Altogether these results provide the proof-of-principle that the carboxyl-terminal region of FX represents an ideal natural linker for the direct fusion with partners aimed at conferring improved features, either albumin, or potentially its domains, or

the Fc region of IgGs. In particular, we demonstrated that the conjugation with albumin is compatible with a FX-HSA chimera characterized by efficient activation and robust coagulation properties and with an *in vivo* extended half-life, thus providing with a novel potential therapeutic drug for FX-deficient patients.

Chapter 8

General discussion and conclusions

Congenital bleeding disorders are well-characterized diseases that cause haemorragic symptoms of variable severity. Over the past three decades, research has been pushing towards improvement of the classical treatment strategies such as replacement therapy with the missing factor; however, several limitations still remain, especially in terms of patients' compliance and personalized treatment regimens.

In the present work we addressed these issues by investigating the process of ribosome readthrough over nonsense mutations causing haemophilia B or FVII deficiency, a potentially personalized approach, and by engineering FVIIa and FX through albumin fusion in order to improve their pharmacokinetic features, to overcome limitations of treatment in all patients affected by FVII or FX deficiencies and in HA patients with inhibitors.

Ribosome readthrough has been previously studied as a strategy to improve coagulation disorders [134][132][133][211], but some key determinants of the functional output of this process, such as the impact of the amino acid inserted at the nonsense position, have never been addressed before in the field. Here, by investigating an ample panel of HB-causing nonsense mutations, we showed that specific mRNA and protein constraints limit the number of mutations that could be productively suppressed by readthrough induction. In particular, this correction approach is predicted to be efficient for those mutations that fall in a protein domain removed during biosynthesis (i.e. pre-peptide); moreover, the potential re-insertion of the original residue in place of the premature stop codon strongly favors a productive functional output. Importance of the interplay between nucleotide sequence features and impact of the inserted amino acid was also confirmed by studying two nonsense mutations causing FVII deficiency. In fact, *in vitro* data obtained here permitted us to dissect the molecular mechanisms underlying the different phenotype severity observed in patients.

We are aware of the limitations of the readthrough inducer used in these studies (G418) that, due to its toxicity, cannot be proposed as therapeutics in patients. However, the studies with this potent inducer helps selecting those variants for which readthrough would produce a functionally relevant protein rescue, and thus patients candidate for treatment with the emerging, less-toxic, readtrough-inducing compounds [126][111][212]. In the future, this would possibly lead to a personalized

therapy; in addition, our approach might prevent treatment of patients affected by refractory nonsense mutations.

Bleeding disorders represent an optimal target for this approach, since even tiny increases in functional protein level can be sufficient to considerably ameliorate patients' phenotype. Nevertheless, the principles for selecting responsive mutations/patients proposed here can be also applied to other genetic disorders caused by nonsense mutations, especially those involving defects of enzymes and/or secreted proteins.

Other than the need to develop individualized approaches, the half-life of coagulation factors is a key impactful issue regarding bleeding disorders therapy. One of the systems used to address this problem has been fusing the therapeutic factor to HSA, in order to markedly increase half-life of the chimaeric molecule. In particular, fusion proteins between rFVIIa/FIX and albumin have been developed. We characterized rFVIIa fused with engineered albumin variants having improved pharmacokinetic features; moreover, we proposed to fuse FX with albumin in order to produce an innovative molecule for treatment of FX deficiency.

The rFVIIa-HSA variants showed a by-passing activity in hemophilic plasma comparable to the one of rFVIIa alone, both *in vitro* and *in vivo*. Furthermore, the fusion proteins comprising engineered albumin bind to hFcRn with a strikingly increased affinity compared to wild-type albumin. Experiments in mice transgenic for hFcRn will be of great relevance to prove that rFVIIa-HSA$^{KP/R1\text{-}KP/R2/R3}$ actually have an improved half-life in comparison with rFVIIa-HSA^{wt}. Moreover, it must be verified that rFVIIa would retain its procoagulant and by-passing activity after several hours in circulation and trough the FcRn-mediated albumin-recycling pathway. So, these molecules would have the potential to last remarkably longer in circulation; if translated into hemophilic patients, this would eventually mean needing less frequent injections, which represents a great achievement in terms of prevention of disabling joint bleeding and patients' quality of life.

Finally, we exploited the unique features of FX carboxyl-terminal region to produce a novel fusion protein with albumin, in order to address the need of a recombinant product for FX deficiency therapy. We were then able to show for the first time that fusion with albumin is compatible with a FX-HSA chimaera characterized by robust procoagulant activity; we have also preliminarily demonstrated that albumin actually confers to FX prolonged persistence in circulation in a mouse model. A more detailed

characterization of pharmacokinetic features and *in vivo* activity of this molecule will allow in the future to propose it as a novel therapeutic option for FX-deficient patients. Nevertheless, the FX-HSA^{wt} molecule here proposed could be the platform for albumin engineering aimed at further improving pharmacokinetic properties of the therapeutic protein(s).

References

[1] B. Furie and B. C. Furie, "Molecular and Cellular Biology of Blood Coagulation," *N. Engl. J. Med.*, vol. 326, no. 12, pp. 800–806, 1992.

[2] B. Furie and B. C. Furie, "The molecular basis of blood coagulation," *Cell*, vol. 53, no. 4, pp. 505–518, May 1988.

[3] J. Pearson, "Endothelial Cell Function and Thrombosis," *Baillieres Best Pr. Res Clin Haematol*, vol. 12, no. 3, pp. 329–341, 1999.

[4] E. M. Bevers, P. Comfurius, J. L. M. L. Van Rijn, and H. C. Hemker, "Generation of Prothrombin-Converting Activity and the Exposure of Phosphatidylserine at the Outer Surface of Platelets," *Eur. J. Biochem.*, vol. 122, no. 2, pp. 429–436, Feb. 1982.

[5] E. M. Bevers, P. Comfurius, and R. F. A. Zwaal, "Changes in membrane phospholipid distribution during platelet activation," *Biochim. Biophys. Acta - Biomembr.*, vol. 736, no. 1, pp. 57–66, Dec. 1983.

[6] B. Dahlback, "Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases," *J. Intern. Med.*, vol. 257, no. 3, pp. 209–223, Mar. 2005.

[7] D. Collen, "The Plasminogen (Fibrinolytic) System," *Thromb. Haemost.*, vol. 82, no. 2, pp. 259–270, 1999.

[8] E. W. Davie and O. D. Ratnoff, "Waterfall Sequence for Intrinsic Blood Clotting," *Science (80-.).*, vol. 145, no. 3638, p. 1310 LP – 1312, Sep. 1964.

[9] R. G. Macfarlane, "An Enzyme Cascade in the Blood Clotting Mechanism, and its Function as a Biochemical Amplifier," *Nature*, vol. 202, no. 4931, pp. 498–499, May 1964.

[10] L. Luchtman-Jones and G. J. Broze, "The Current Status of Coagulation," *Ann. Med.*, vol. 27, no. 1, pp. 47–52, Jan. 1995.

[11] C. Kleinschnitz *et al.*, "Targeting coagulation factor XII provides protection from pathological thrombosis in cerebral ischemia without interfering with hemostasis," *J. Exp. Med.*, vol. 203, no. 3, p. 513 LP – 518, Mar. 2006.

[12] M. Hoffman and D. M. Monroe III, "A Cell-based Model of Hemostasis," *Thromb. Haemost.*, vol. 85, no. 6, pp. 958–965, 2001.

[13] D. Monroe III, M. Hoffmann, and H. Roberts, "Transmission of a procoagulant signal from tissue factor-bearing cell to platelets.," *Blood Coagul. fibrinolysis*, vol. 7, no. 4, pp. 459–64, 1996.

[14] J. J. Briedé, J. W. M. Heemskerk, C. v. Veer, H. Coenraad Hemker, and T. Lindhout, "Contribution of Platelet-derived Factor Va to Thrombin Generation on Immobilized Collagen-and Fibrinogen-adherent Platelets," *Thromb. Haemost.*, vol. 85, no. 3, pp. 509–513, 2001.

[15] D. D. Monkovic and P. B. Tracy, "Activation of human factor V by factor Xa and thrombin," *Biochemistry*, vol. 29, no. 5, pp. 1118–1128, Feb. 1990.

[16] D. H. Allen and P. B. Tracy, "Human Coagulation Factor V Is Activated to the Functional Cofactor by Elastase and Cathepsin G Expressed at the Monocyte Surface," *J. Biol. Chem.* , vol. 270 , no. 3 , pp. 1408–1415, Jan. 1995.

[17] Alberio and Dale, "Platelet-collagen interactions: membrane receptors and intracellular signalling pathways," *Eur. J. Clin. Invest.*, vol. 29, no. 12, pp. 1066–1076, Dec. 1999.

[18] F. A. Baglia and P. N. Walsh, "Prothrombin Is a Cofactor for the Binding of Factor XI to the Platelet Surface and for Platelet-Mediated Factor-XI Activation by Thrombin," *Biochemistry*, vol. 46, no. 44, pp. 12886–12887, Nov. 2007.

[19] J. Jesty, E. Beltrami, and G. Willems, "Mathematical analysis of a proteolytic positive-feedback loop: Dependence of lag time and enzyme yields on the initial conditions and kinetic parameters," *Biochemistry*, vol. 32, no. 24, pp. 6266–6274, Jun. 1993.

[20] K. G. Mann, K. Brummel, and S. Butenas, "What is all that thrombin for?," *J. Thromb. Haemost.*, vol. 1, no. 7, pp. 1504–1514, Jul. 2003.

[21] M. Hoffman, "Remodeling the Blood Coagulation Cascade," vol. 16, no. 113, pp. 17–20, 2003.

[22] S. I. Rapaport, "Inhibition of factor VIIa/tissue factor-induced blood coagulation: with particular emphasis upon a factor Xa-dependent inhibitory mechanism," *Blood*, vol. 73, no. 2, p. 359 LP – 365, Feb. 1989.

[23] G. J. Broze, "Tissue factor pathway inhibitor," *Thromb. Haemost.*, vol. 74, no. 1, pp. 90–3, 1995.

[24] V. et al Kumar, *Robbins and Cotran: Pathological basis of disease (7th ed)*. Philadelphia, 2005.

[25] C. van't Veer and K. G. Mann, "Regulation of Tissue Factor Initiated Thrombin Generation by the Stoichiometric Inhibitors Tissue Factor Pathway Inhibitor, Antithrombin-III, and Heparin Cofactor-II," *J. Biol. Chem.* , vol. 272 , no. 7 , pp. 4367–4377, Feb. 1997.

[26] C. H. Beresford and M. C. Owen, "Antithrombin III," *Int. J. Biochem.*, vol. 22, no. 2, pp. 121–128, 1990.

[27] J. I. Weitz, "Heparan sulfate: Antithrombotic or not?," *J. Clin. Invest.*, vol. 111, no. 7, p. 952, 2003.

[28] C. T. Esmon, "The protein C pathway," *CHEST J.*, vol. 124, no. 3_suppl, p. 26S–32S, 2003.

[29] J. Ye, N. L. Esmon, C. T. Esmon, and A. E. Johnson, "The active site of thrombin is altered upon binding to thrombomodulin. Two distinct structural changes are detected by fluorescence, but only one correlates with protein C activation.," *J. Biol. Chem.*, vol. 266, no. 34, pp. 23016–23021, 1991.

[30] B. Dahlbäck, "Progress in the understanding of the protein C anticoagulant pathway," *Int. J. Hematol.*, vol. 79, no. 2, pp. 109–116, 2004.

[31] R. J. Kaufman, "Post-translational modifications required for coagulation factor secretion and function," *Thromb. Haemost.*, vol. 79, no. 6, pp. 1068–1079, 1998.

[32] J. M. Gajsiewicz and J. H. Morrissey, "Structure–Function Relationship of the Interaction between Tissue Factor and Factor VIIa," in *Seminars in thrombosis and hemostasis*, 2015, vol. 41, no. 07, pp. 682–690.

[33] E. Persson, G. Bolt, T. D. Steenstrup, and M. Ezban, "Recombinant coagulation factor VIIa–from molecular to clinical aspects of a versatile haemostatic agent," *Thromb. Res.*, vol. 125, no. 6, pp. 483–489, 2010.

[34] J. W. Suttie, "Synthesis of vitamin K-dependent proteins.," *FASEB J.*, vol. 7, no. 5, pp. 445–452, 1993.

[35] G. Bolt, T. D. Steenstrup, and C. Kristensen, "All post-translational modifications except propeptide cleavage are required for optimal secretion of coagulation factor VII," *Thromb. Haemost.*, vol. 98, no. 5, pp. 988–997, 2007.

[36] F. S. Hagen *et al.*, "Characterization of a cDNA coding for human factor VII," *Proc. Natl. Acad. Sci.*, vol. 83, no. 8, pp. 2412–2416, 1986.

[37] P. Wildgoose, Y. Nemerson, L. L. Hansen, F. E. Nielsen, S. Glazer, and U. Hedner, "Measurement of basal levels of factor VIIa in hemophilia A and B patients.," *Blood*, vol. 80, pp. 25–28, 1992.

[38] R. Radcliffe and Y. Nemerson, "Activation and control of factor VII by activated factor X and thrombin. Isolation and characterization of a single chain form of factor VII.," *J. Biol. Chem.*, vol. 250, no. 2, pp. 388–395, 1975.
[39] S. Butenas and K. G. Mann, "Kinetics of human factor VII activation," *Biochemistry*, vol. 35, no. 6, pp. 1904–1910, 1996.
[40] T. Nakagaki, D. C. Foster, K. L. Berkner, and W. Kisiel, "Initiation of the extrinsic pathway of blood coagulation: evidence for the tissue factor dependent autoactivation of human coagulation factor VII," *Biochemistry*, vol. 30, no. 45, pp. 10819–10824, 1991.
[41] C. Eigenbrot, "Structure, function, and activation of coagulation factor VII," *Curr. protein Pept. Sci.*, vol. 3, no. 3, pp. 287–299, 2002.
[42] Y. Nemerson, "The reaction between bovine brain tissue factor and factors VII and X," *Biochemistry*, vol. 5, no. 2, pp. 601–608, 1966.
[43] B. Osterud and S. I. Rapaport, "Activation of factor IX by the reaction product of tissue factor and factor VII: additional pathway for initiating blood coagulation," *Proc. Natl. Acad. Sci.*, vol. 74, no. 12, pp. 5260–5264, 1977.
[44] Y. Komiyama, A. H. Pedersen, and W. Kisiel, "Proteolytic activation of human factors IX and X by recombinant human factor VIIa: effects of calcium, phospholipids, and tissue factor," *Biochemistry*, vol. 29, no. 40, pp. 9418–9425, 1990.
[45] C. R. Kelly, C. D. Dickinson, and W. Ruf, "Ca2+ binding to the first epidermal growth factor module of coagulation factor VIIa is important for cofactor interaction and proteolytic function," *J. Biol. Chem.*, vol. 272, no. 28, pp. 17467–17472, 1997.
[46] V. J. J. Bom and R. M. Bertina, "The contributions of Ca2+, phospholipids and tissue-factor apoprotein to the activation of human blood-coagulation factor X by activated factor VII," *Biochem. J.*, vol. 265, no. 2, pp. 327–336, 1990.
[47] B. Osterud, B. N. Bouma, and J. H. Griffin, "Human blood coagulation factor IX. Purification, properties, and mechanism of activation by activated factor XI.," *J. Biol. Chem.*, vol. 253, no. 17, pp. 5946–5951, 1978.
[48] S. Yoshitake, B. G. Schach, D. C. Foster, E. W. Davie, and K. Kurachi, "Complete nucleotide sequences of the gene for human factor IX (antihemophilic factor B)," *Biochemistry*, vol. 24, no. 14, pp. 3736–3750, 1985.
[49] A. R. Thompson, "Molecular biology of factor IX," *Hemost. Thromb. Basic Princ. Clin. Pract. 4th edn. Philadelphia, PA, USA Lippincott Williams Wilkins*, pp. 123–134, 2001.
[50] S. P. Bajaj, "Cooperative Ca2+ binding to human factor IX. Effects of Ca2+ on the kinetic parameters of the activation of factor IX by factor XIa.," *J. Biol. Chem.*, vol. 257, no. 8, pp. 4127–4132, 1982.
[51] H. Brandstetter, M. Bauer, R. Huber, P. Lollar, and W. Bode, "X-ray structure of clotting factor IXa: active site and module structure related to Xase activity and hemophilia B," *Proc. Natl. Acad. Sci.*, vol. 92, no. 21, pp. 9796–9800, 1995.
[52] R. G. Di Scipio, K. Kurachi, and E. W. Davie, "Activation of human factor IX (Christmas factor).," *J. Clin. Invest.*, vol. 61, no. 6, p. 1528, 1978.
[53] P. J. Scambler and R. Williamson, "The structural gene for human coagulation factor X is located on chromosome 13q34," *Cytogenet. Genome Res.*, vol. 39, no. 3, pp. 231–233, 1985.
[54] M. R. Fung, C. W. Hay, and R. T. MacGillivray, "Characterization of an almost full-length cDNA coding for human blood coagulation factor X," *Proc. Natl. Acad. Sci.*, vol. 82, no. 11, pp. 3591–3595, 1985.

[55] M. Racchi, H. H. Watzke, K. A. High, and M. O. Lively, "Human coagulation factor X deficiency caused by a mutant signal peptide that blocks cleavage by signal peptidase but not targeting and translocation to the endoplasmic reticulum.," *J. Biol. Chem.*, vol. 268, no. 8, pp. 5735–5740, 1993.

[56] C. Stanton and R. Wallin, "Processing and trafficking of clotting factor X in the secretory pathway. Effects of warfarin," *Biochem. J.*, vol. 284, no. 1, pp. 25–31, 1992.

[57] K. Hansson and J. Stenflo, "Post-translational modifications in proteins involved in blood coagulation," *J. Thromb. Haemost.*, vol. 3, no. 12, pp. 2633–2648, 2005.

[58] R. G. Di Scipio, M. A. Hermodson, S. G. Yates, and E. W. Davie, "A comparison of human prothrombin, factor IX (Christmas factor), factor X (Stuart factor), and protein S," *Biochemistry*, vol. 16, no. 4, pp. 698–706, 1977.

[59] R. J. Baugh and S. Krishnaswamy, "Role of the activation peptide domain in human factor X activation by the extrinsic Xase complex," *J. Biol. Chem.*, vol. 271, no. 27, pp. 16126–16134, 1996.

[60] S. Butenas, C. van't Veer, and K. G. Mann, "Evaluation of the initiation phase of blood coagulation using ultrasensitive assays for serine proteases," *J. Biol. Chem.*, vol. 272, no. 34, pp. 21527–21533, 1997.

[61] K. G. Mann, M. E. Nesheim, W. R. Church, P. Haley, and S. Krishnaswamy, "Surface-dependent reactions of the vitamin K-dependent enzyme complexes," *Blood*, vol. 76, no. 1, pp. 1–16, 1990.

[62] E. L. G. Pryzdial and G. E. Kessler, "Kinetics of Blood Coagulation Factor Xaα Autoproteolytic Conversion to Factor Xaβ Effect of inhibition by antithrombin, prothrombinase assembly, and enzyme activity," *J. Biol. Chem.*, vol. 271, no. 28, pp. 16621–16626, 1996.

[63] A. K. Sabharwal, K. Padmanabhan, A. Tulinsky, A. Mathur, J. Gorka, and S. P. Bajaj, "Interaction of calcium with native and decarboxylated human factor X. Effect of proteolysis in the autolysis loop on catalytic efficiency and factor Va binding," *J. Biol. Chem.*, vol. 272, no. 35, pp. 22037–22045, 1997.

[64] P. M. Mannucci and E. G. D. Tuddenham, "The hemophilias—from royal genes to gene therapy," *N. Engl. J. Med.*, vol. 344, no. 23, pp. 1773–1779, 2001.

[65] F. R. Rosendaal and E. Briet, "The increasing prevalence of haemophilia," *Thromb. Haemost.*, vol. 63, no. 1, p. 145, 1990.

[66] G. C. White 2nd, F. Rosendaal, L. M. Aledort, J. M. Lusher, C. Rothschild, and J. Ingerslev, "Factor VIII and Factor IX Subcommittee (2001) Definitions in hemophilia. Recommendation of the scientific subcommittee on factor VIII and factor IX of the scientific and standardization committee of the International Society on Thrombosis and Haemostasis," *Thromb. Haemost*, vol. 85, no. 3, p. 560.

[67] D. Lakich, H. H. Kazazian, S. E. Antonarakis, and J. Gitschier, "Inversions disrupting the factor VIII gene are a common cause of severe haemophilia A," *Nat. Genet.*, vol. 5, no. 3, pp. 236–241, 1993.

[68] R. D. Bagnall, N. Waseem, P. M. Green, and F. Giannelli, "Recurrent inversion breaking intron 1 of the factor VIII gene is a frequent cause of severe hemophilia A," *Blood*, vol. 99, no. 1, pp. 168–174, 2002.

[69] R. Kulkarni and J. Lusher, "Perinatal management of newborns with haemophilia," *Br. J. Haematol.*, vol. 112, no. 2, pp. 264–274, 2001.

[70] M. D. Carcao, "The diagnosis and management of congenital hemophilia," in *Seminars in thrombosis and hemostasis*, 2012, vol. 38, no. 07, pp. 727–734.

[71] D. Stephensen *et al.*, "Changing patterns of bleeding in patients with severe haemophilia A," *Haemophilia*, vol. 15, no. 6, pp. 1210–1214, 2009.

[72] J. V Luck Jr, M. Silva, C. E. Rodriguez-Merchan, N. Ghalambor, C. A. Zahiri, and R. S. Finn, "Hemophilic arthropathy," *J. Am. Acad. Orthop. Surg.*, vol. 12, no. 4, pp. 234–245, 2004.

[73] L. A. Valentino, "Blood-induced joint disease: the pathophysiology of hemophilic arthropathy," *J. Thromb. Haemost.*, vol. 8, no. 9, pp. 1895–1902, 2010.

[74] G. Dolan *et al.*, "Haemophilia B: Where are we now and what does the future hold?," *Blood Rev.*, 2017.

[75] A. Srivastava *et al.*, "Guidelines for the management of hemophilia," *Haemophilia*, vol. 19, no. 1, 2013.

[76] E. Berntorp *et al.*, "Consensus perspectives on prophylactic therapy for haemophilia: summary statement," *Haemophilia*, vol. 9, no. s1, pp. 1–4, 2003.

[77] L. M. Aledort, R. Haschmeyer, and H. Pettersson, "A longitudinal study of orthopaedic outcomes for severe factor-VIII-deficient haemophiliacs," *J. Intern. Med.*, vol. 236, no. 4, pp. 391–399, 1994.

[78] M. Franchini and P. M. Mannucci, "Inhibitors of propagation of coagulation (factors VIII, IX and XI): a review of current therapeutic practice," *Br. J. Clin. Pharmacol.*, vol. 72, no. 4, pp. 553–562, 2011.

[79] N. S. Key, "Inhibitors in congenital coagulation disorders," *Br. J. Haematol.*, vol. 127, no. 4, pp. 379–391, 2004.

[80] C. R. M. Hay and D. M. DiMichele, "The principal results of the International Immune Tolerance Study: a randomized dose comparison," *Blood*, vol. 119, no. 6, pp. 1335–1344, 2012.

[81] L. J. M. Sjamsoedin *et al.*, "The effect of activated prothrombin-complex concentrate (FEIBA) on joint and muscle bleeding in patients with hemophilia A and antibodies to factor VIII: a double-blind clinical trial," *N. Engl. J. Med.*, vol. 305, no. 13, pp. 717–721, 1981.

[82] N. S. Key *et al.*, "Home treatment of mild to moderate bleeding episodes using recombinant factor VIIa (Novoseven) in haemophiliacs with inhibitors," *Thromb. Haemost.*, vol. 80, no. 6, pp. 912–918, 1998.

[83] G. Young, F. E. Shafer, P. Rojas, and S. Seremetis, "Single 270 μg kg− 1-dose rFVIIa vs. standard 90 μg kg− 1-dose rFVIIa and APCC for home treatment of joint bleeds in haemophilia patients with inhibitors: a randomized comparison," *Haemophilia*, vol. 14, no. 2, pp. 287–294, 2008.

[84] A. C. Nathwani *et al.*, "Adenovirus-associated virus vector–mediated gene transfer in hemophilia B," *N. Engl. J. Med.*, vol. 365, no. 25, pp. 2357–2365, 2011.

[85] K. H. High, A. Nathwani, T. Spencer, and D. Lillicrap, "Current status of haemophilia gene therapy," *Haemophilia*, vol. 20, no. s4, pp. 43–49, 2014.

[86] S. Rangarajan *et al.*, "AAV5–Factor VIII Gene Transfer in Severe Hemophilia A," *N. Engl. J. Med.*, p. NEJMoa1708483, 2017.

[87] L. a. George *et al.*, "Hemophilia B Gene Therapy with a High-Specific-Activity Factor IX Variant," *N. Engl. J. Med.*, vol. 377, no. 23, pp. 2215–2227, 2017.

[88] A. Ellington, A. D. Keefe, and S. Pai, "Aptamers as therapeutics," *Nat Rev Drug Discov*, vol. 9, no. 7, pp. 537–550, 2010.

[89] P. Chowdary *et al.*, "Safety and pharmacokinetics of anti-TFPI antibody (concizumab) in healthy volunteers and patients with hemophilia: a randomized first human dose trial," *J. Thromb. Haemost.*, vol. 13, no. 5, pp. 743–754, 2015.

[90] T. Kitazawa *et al.*, "A bispecific antibody to factors IXa and X restores factor VIII hemostatic activity in a hemophilia A model," *Nat. Med.*, vol. 18, no. 10, pp. 1570–1574, 2012.

[91] G. Mariani, C. L. Lo, F. Bernardi, and M. Pinotti, "Molecular and clinical aspects of factor VII deficiency.," *Blood Coagul. fibrinolysis an Int. J. Haemost. Thromb.*, vol. 9, pp. S83–8, 1998.
[92] E. D. Rosen, J. C. Y. Chan, E. Idusogie, and F. Clotman, "Mice lacking factor VII develop normally but suffer fatal perinatal bleeding," *Nature*, vol. 390, no. 6657, p. 290, 1997.
[93] F. Bernardi *et al.*, "Mutation pattern in clinically asymptomatic coagulation factor VII deficiency," *Hum. Mutat.*, vol. 8, no. 2, pp. 108–115, 1996.
[94] J. H. McVey, E. Boswell, A. D. Mumford, G. Kemball-Cook, and E. G. D. Tuddenham, "Factor VII deficiency and the FVII mutation database," *Hum. Mutat.*, vol. 17, no. 1, pp. 3–17, 2001.
[95] G. Mariani and F. Bernardi, "Factor VII deficiency," in *Seminars in thrombosis and hemostasis*, 2009, vol. 35, no. 04, pp. 400–406.
[96] M. N. D. Di Minno, A. Dolce, G. Mariani, and S. S. Group, "Bleeding symptoms at disease presentation and prediction of ensuing bleeding in inherited FVII deficiency," *Thromb. Haemost.*, vol. 109, no. 6, pp. 1051–1059, 2013.
[97] G. Mariani *et al.*, "Clinical phenotypes and factor VII genotype in congenital factor VII deficiency," *Thromb. Haemost.*, vol. 93, no. 3, p. 481, 2005.
[98] M. Napolitano *et al.*, "Prophylaxis in congenital factor VII deficiency: indications, efficacy and safety. Results from the Seven Treatment Evaluation Registry (STER)," *Haematologica*, vol. 98, no. 4, pp. 538–544, 2013.
[99] P. H. B. Bolton Maggs *et al.*, "The rare coagulation disorders–review with guidelines for management from the United Kingdom Haemophilia Centre Doctors' Organisation," *Haemophilia*, vol. 10, no. 5, pp. 593–628, 2004.
[100] D. S. Millar *et al.*, "Molecular analysis of the genotype-phenotype relationship in factor X deficiency," *Hum. Genet.*, vol. 106, no. 2, pp. 249–257, 2000.
[101] J. Uprichard and D. J. Perry, "Factor X deficiency," *Blood Rev.*, vol. 16, no. 2, pp. 97–110, 2002.
[102] D. L. Brown and P. A. Kouides, "Diagnosis and treatment of inherited factor X deficiency," *Haemophilia*, vol. 14, no. 6, pp. 1176–1182, 2008.
[103] D. Keeling, C. Tait, and M. Makris, "Guideline on the selection and use of therapeutic products to treat haemophilia and other hereditary bleeding disorders," *Haemophilia*, vol. 14, no. 4, pp. 671–684, 2008.
[104] S. K. Austin, C. Brindley, K. Kavakli, M. Norton, and A. Shapiro, "Pharmacokinetics of a high-purity plasma-derived factor X concentrate in subjects with moderate or severe hereditary factor X deficiency," *Haemophilia*, vol. 22, no. 3, pp. 426–432, 2016.
[105] S. K. Austin, K. Kavakli, M. Norton, F. Peyvandi, and A. Shapiro, "Efficacy, safety and pharmacokinetics of a new high-purity factor X concentrate in subjects with hereditary factor X deficiency," *Haemophilia*, vol. 22, no. 3, pp. 419–425, 2016.
[106] M. Mort, D. Ivanov, D. N. Cooper, and N. A. Chuzhanova, "A meta-analysis of nonsense mutations causing human genetic disease," *Hum. Mutat.*, vol. 29, no. 8, pp. 1037–1047, 2008.
[107] J. Sun, M. Chen, J. Xu, and J. Luo, "Relationships among stop codon usage bias, its context, isochores, and gene expression level in various eukaryotes," *J. Mol. Evol.*, vol. 61, no. 4, pp. 437–444, 2005.
[108] M. Khajavi, K. Inoue, and J. R. Lupski, "Nonsense-mediated mRNA decay modulates clinical outcome of genetic disease," *Eur. J. Hum. Genet. EJHG*, vol. 14, no. 10, p. 1074, 2006.

[109] Y. Ishigaki, X. Li, G. Serin, and L. E. Maquat, "Evidence for a pioneer round of mRNA translation: mRNAs subject to nonsense-mediated decay in mammalian cells are bound by CBP80 and CBP20," *Cell*, vol. 106, no. 5, pp. 607–617, 2001.
[110] F. Lejeune and L. E. Maquat, "Mechanistic links between nonsense-mediated mRNA decay and pre-mRNA splicing in mammalian cells," *Curr. Opin. Cell Biol.*, vol. 17, no. 3, pp. 309–315, 2005.
[111] L. Bidou, V. Allamand, J. P. Rousset, and O. Namy, "Sense from nonsense: Therapies for premature stop codon diseases," *Trends Mol. Med.*, vol. 18, no. 11, pp. 679–688, 2012.
[112] L. Bidou, J.-P. Rousset, and O. Namy, "Translational errors: from yeast to new therapeutic targets," *FEMS Yeast Res.*, vol. 10, no. 8, pp. 1070–1082, 2010.
[113] L. Frolova, A. Seit-Nebi, and L. Kisselev, "Highly conserved NIKS tetrapeptide is functionally essential in eukaryotic translation termination factor eRF1," *Rna*, vol. 8, no. 2, pp. 129–136, 2002.
[114] K. K. McCaughan, C. M. Brown, M. E. Dalphin, M. J. Berry, and W. P. Tate, "Translational termination efficiency in mammals is influenced by the base following the stop codon," *Proc. Natl. Acad. Sci.*, vol. 92, no. 12, pp. 5431–5435, 1995.
[115] B. Cosson *et al.*, "Poly (A)-binding protein acts in translation termination via eukaryotic release factor 3 interaction and does not influence [PSI+] propagation," *Mol. Cell. Biol.*, vol. 22, no. 10, pp. 3301–3315, 2002.
[116] K. M. Keeling, X. Xue, G. Gunn, and D. M. Bedwell, "Therapeutics Based on Stop Codon Readthrough," *Annu. Rev. Genomics Hum. Genet.*, vol. 15, no. 1, pp. 371–394, 2014.
[117] M. Manuvakhova, K. Keeling, and D. M. Bedwell, "Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system.," *RNA*, vol. 6, no. 7, pp. 1044–1055, 2000.
[118] B. François *et al.*, "Crystal structures of complexes between aminoglycosides and decoding A site oligonucleotides: role of the number of rings and positive charges in the specific binding leading to miscoding," *Nucleic Acids Res.*, vol. 33, no. 17, pp. 5677–5690, 2005.
[119] S. Blanchet, D. Cornu, M. Argentini, and O. Namy, "New insights into the incorporation of natural suppressor tRNAs at stop codons in Saccharomyces cerevisiae.," *Nucleic Acids Res.*, vol. 42, no. 15, pp. 1–12, 2014.
[120] M. Howard, R. A. Frizzell, and D. M. Bedwell, "Aminoglycoside antibiotics restore CFTR function by overcoming premature stop mutations," *Nat. Med.*, vol. 2, no. 4, pp. 467–469, 1996.
[121] E. R. Barton-Davis, L. Cordier, D. I. Shoturma, S. E. Leland, and H. L. Sweeney, "Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of mdx mice," *J. Clin. Invest.*, vol. 104, no. 4, p. 375, 1999.
[122] H. L. R. Lee and J. P. Dougherty, "Pharmaceutical therapies to recode nonsense mutations in inherited diseases," *Pharmacol. Ther.*, vol. 136, no. 2, pp. 227–266, 2012.
[123] R. S. Finkel, "Read-through strategies for suppression of nonsense mutations in Duchenne/Becker muscular dystrophy: aminoglycosides and ataluren (PTC124)," *J. Child Neurol.*, vol. 25, no. 9, pp. 1158–1164, 2010.
[124] S. M. Rowe *et al.*, "Suppression of CFTR premature termination codons and rescue of CFTR protein and function by the synthetic aminoglycoside NB54," *J. Mol. Med.*, vol. 89, no. 11, p. 1149, 2011.

[125] V. B. Mattis, C.-W. T. Chang, and C. L. Lorson, "Analysis of a read-through promoting compound in a severe mouse model of spinal muscular atrophy," *Neurosci. Lett.*, vol. 525, no. 1, pp. 72–75, 2012.
[126] E. M. Welch *et al.*, "PTC124 targets genetic disorders caused by nonsense mutations," *Nature*, vol. 447, no. 7140, pp. 87–91, 2007.
[127] L. Du *et al.*, "Nonaminoglycoside compounds induce readthrough of nonsense mutations," *J. Exp. Med.*, vol. 206, no. 10, pp. 2285–2297, 2009.
[128] K. Bushby *et al.*, "Ataluren treatment of patients with nonsense mutation dystrophinopathy," *Muscle Nerve*, vol. 50, no. 4, pp. 477–487, 2014.
[129] C. M. McDonald *et al.*, "Ataluren in patients with nonsense mutation Duchenne muscular dystrophy (ACT DMD): A multicentre, randomised, double-blind, placebo-controlled, phase 3 trial," *Lancet*, no. Act Dmd, pp. 1489–1498, 2017.
[130] A. A. Aslam, C. Higgins, I. P. Sinha, and K. W. Southern, "Ataluren and similar compounds (specific therapies for premature termination codon class I mutations) for cystic fibrosis," *Cochrane Database Syst. Rev.*, vol. 2017, no. 1, 2017.
[131] E. Pollak and K. A. High, "Genetic disorders of coagulation," *Oxford Textb. Med.*, vol. 3, pp. 757–767, 2003.
[132] M. Pinotti *et al.*, "Intracellular readthrough of nonsense mutations by aminoglycosides in coagulation factor VII," *J. Thromb. Haemost.*, vol. 4, no. 6, pp. 1308–1314, 2006.
[133] C. Yang *et al.*, "A mouse model for nonsense mutation bypass therapy shows a dramatic multiday response to geneticin," *Proc. Natl. Acad. Sci.*, vol. 104, no. 39, pp. 15394–15399, 2007.
[134] P. D. James *et al.*, "Aminoglycoside suppression of nonsense mutations in severe hemophilia," *Blood*, vol. 106, no. 9, pp. 3043–3048, 2005.
[135] M. Pinotti, L. Rizzotto, a. Chuansumrit, G. Mariani, and F. Bernardi, "Gentamicin induces sub-therapeutic levels of coagulation factor VII in patients with nonsense mutations [1]," *J. Thromb. Haemost.*, vol. 4, no. 8, pp. 1828–1830, 2006.
[136] P. Simioni *et al.*, "X-linked thrombophilia with a mutant factor IX (factor IX Padua)," *N. Engl. J. Med.*, vol. 361, no. 17, pp. 1671–1675, 2009.
[137] G. R. Jayandharan, R. V Shaji, S. Baidya, S. C. Nair, M. Chandy, and A. Srivastava, "Molecular characterization of factor IX gene mutations in 53 patients with haemophilia B in India," *Thromb. Haemost.*, vol. 94, no. 4, pp. 883–886, 2005.
[138] A. R. Thompson, J. M. Schoof, A. F. Weinmann, and S.-H. Chen, "Factor IX mutations: rapid, direct screening methods for 20 new families with hemophilia B," *Thromb. Res.*, vol. 65, no. 2, pp. 289–295, 1992.
[139] J. A. Bristol, B. C. Furie, and B. Furie, "Propeptide processing during factor IX biosynthesis. Effect of point mutations adjacent to the propeptide cleavage site.," *J. Biol. Chem.*, vol. 268, no. 10, pp. 7577–7584, 1993.
[140] P. M. Green, V. E. Mitchell, A. McGraw, E. Goldman, and F. Giannelli, "Haemophilia B caused by a missense mutation in the prepeptide sequence of factor IX," *Hum. Mutat.*, vol. 2, no. 2, pp. 103–107, 1993.
[141] J. M. Montejo, M. Magallon, E. Tizzano, and J. Solera, "Identification of twenty-one new mutations in the factor IX gene by SSCP analysis," *Hum. Mutat.*, vol. 13, no. 2, p. 160, 1999.
[142] M. B. Liddell, D. P. Lillicrap, I. R. Peake, and A. L. Bloom, "Defective propeptide processing and abnormal activation underlie the molecular pathology of factor IX Troed-y-Rhiw," *Br. J. Haematol.*, vol. 72, no. 2, pp. 208–215, 1989.

[143] K. Wulff, W. Schröder, M. Wehnert, and F. H. Herrmann, "Twenty-five novel mutations of the factor IX gene in haemophilia B," *Hum. Mutat.*, vol. 6, no. 4, pp. 346–348, 1995.
[144] K. Ogata, S. R. Selvaraj, H. Z. Miao, and S. W. Pipe, "Most factor VIII B domain missense mutations are unlikely to be causative mutations for severe hemophilia A: implications for genotyping," *J. Thromb. Haemost.*, vol. 9, no. 6, pp. 1183–1190, 2011.
[145] E. D. Rosen *et al.*, "Mice lacking factor VII develop normally but suffer fatal perinatal bleeding," *Nature*, vol. 390, pp. 290–294, 1997.
[146] M. Giansily-Blaizot *et al.*, "Two novel cases of cerebral haemorrhages at the neonatal period associated with inherited factor VII deficiency, one of them revealing a new nonsense mutation (Ser52Stop)," *Blood Coagul. fibrinolysis*, vol. 14, no. 2, pp. 217–220, 2003.
[147] O. Chafa, A.-M. Fischer, A. Reghis, and J. Taponi-Bretaudiere, "Homozygous nonsense mutation (Cys72→ stop) in the human F7 gene: a not life-threatening mutation despite the absence of circulating factor VII," *J. Thromb. Haemost.*, vol. 3, no. 1, pp. 175–177, 2005.
[148] G. R. Jayandharan, A. Viswabandya, S. C. Nair, M. Chandy, and A. Srivastava, "Molecular basis of hereditary factor VII deficiency in India: five novel mutations including a double missense mutation (Ala191Glu; Trp364Cys) in 11 unrelated patients," *Haematologica*, vol. 92, no. 7, pp. 1002–1003, 2007.
[149] A. Branchini *et al.*, "Natural and engineered carboxy-terminal variants: decreased secretion and gain-of-function result in asymptomatic coagulation factor VII deficiency," *Haematologica*, vol. 97, no. 5, pp. 705–709, 2012.
[150] E. Nagy and L. E. Maquat, "A rule for termination-codon position within intron-containing genes: when nonsense affects RNA abundance," *Trends Biochem. Sci.*, vol. 23, no. 6, pp. 198–199, 1998.
[151] A. Branchini *et al.*, "Coagulation factor VII variants resistant to inhibitory antibodies," *Thromb. Haemost.*, vol. 112, no. 5, pp. 972–980, 2014.
[152] K. S. Larsen *et al.*, "Engineering the substrate and inhibitor specificities of human coagulation Factor VIIa," *Biochem. J.*, vol. 405, no. 3, pp. 429–438, 2007.
[153] H. Tokgoz, U. Caliskan, G. LAVIGNE-LISSALDE, and M. GIANSILY-BLAIZOT, "Successful prophylactic use of recombinant activated factor VII (rFVIIa) in a patient with congenital FVII deficiency and inhibitors to FVII," *Haemophilia*, vol. 18, no. 1, 2012.
[154] J. H. Lawson, M. Kalafatis, S. Stram, and K. G. Mann, "A model for the tissue factor pathway to thrombin. I. An empirical study.," *J. Biol. Chem.*, vol. 269, no. 37, pp. 23357–23366, 1994.
[155] S. Butenas, C. van't Veer, and K. G. Mann, "'Normal' thrombin generation," *Blood*, vol. 94, no. 7, pp. 2169–2178, 1999.
[156] D. Sleep, J. Cameron, and L. R. Evans, "Albumin as a versatile platform for drug half-life extension," *Biochim. Biophys. Acta - Gen. Subj.*, vol. 1830, no. 12, pp. 5526–5534, 2013.
[157] T. A. Waldmann and W. Strober, "Metabolism of immunoglobulins," in *Progress in Allergy Vol. 13*, vol. 13, Karger Publishers, 1969, pp. 1–110.
[158] T. Peters, "Serum albumin," *Adv. Protein Chem.*, vol. 37, pp. 161–245, 1985.
[159] E. R. Smith, M. M. X. Cai, L. P. McMahon, D. A. Wright, and S. G. Holt, "The value of simultaneous measurements of urinary albumin and total protein in proteinuric patients," *Nephrol. Dial. Transplant.*, vol. 27, no. 4, pp. 1534–1541, 2011.

[160] P. M. Mannucci, "Half-life extension technologies for haemostatic agents," *Thromb. Haemost.*, vol. 113, no. 1, pp. 165–176, 2015.
[161] N. Flori *et al.*, "Pegylated Interferon-α2a and Ribavirin versus Pegylated Interferon-α2b and Ribavirin in Chronic Hepatitis C," *Drugs*, vol. 73, no. 3, pp. 263–277, 2013.
[162] R. Yatuv, M. Robinson, I. Dayan-Tarshish, and M. Baru, "The use of PEGylated liposomes in the development of drug delivery applications for the treatment of hemophilia," *Int. J. Nanomedicine*, vol. 5, p. 581, 2010.
[163] M. Schlapschy *et al.*, "PASylation: a biological alternative to PEGylation for extending the plasma half-life of pharmaceutically active proteins," *Protein Eng. Des. Sel.*, vol. 26, no. 8, pp. 489–501, 2013.
[164] M. Gebauer and A. Skerra, "Prospects of PASylation® for the design of protein and peptide therapeutics with extended half-life and enhanced action," *Bioorganic Med. Chem.*, 2017.
[165] F. Fares, R. Guy, A. Bar-Ilan, Y. Felikman, and E. Fima, "Designing a long-acting human growth hormone (hGH) by fusing the carboxyl-terminal peptide of human chorionic gonadotropin β-subunit to the coding sequence of hGH," *Endocrinology*, vol. 151, no. 9, pp. 4410–4417, 2010.
[166] C. Chaudhury *et al.*, "The major histocompatibility complex–related Fc receptor for IgG (FcRn) binds albumin and prolongs its lifespan," *J. Exp. Med.*, vol. 197, no. 3, pp. 315–322, 2003.
[167] C. Chaudhury, C. L. Brooks, D. C. Carter, J. M. Robinson, and C. L. Anderson, "Albumin Binding to FcRn : Distinct from the FcRn − IgG Interaction," *Biochemistry*, vol. 45, no. 15, pp. 4983–4990, 2006.
[168] C. M. Story, J. E. Mikulska, and N. E. Simister, "A major histocompatibility complex class I-like Fc receptor cloned from human placenta: possible role in transfer of immunoglobulin G from mother to fetus.," *J. Exp. Med.*, vol. 180, no. 6, pp. 2377–2381, 1994.
[169] A. P. West and P. J. Bjorkman, "Crystal structure and immunoglobulin G binding properties of the human major histocompatibility complex-related Fc receptor," *Biochemistry*, vol. 39, no. 32, pp. 9698–9708, 2000.
[170] D. C. Roopenian and S. Akilesh, "FcRn: the neonatal Fc receptor comes of age," *Nat. Rev. Immunol.*, vol. 7, no. 9, pp. 715–725, 2007.
[171] J. T. Andersen, J. D. Qian, and I. Sandlie, "The conserved histidine 166 residue of the human neonatal Fc receptor heavy chain is critical for the pH-dependent binding to albumin," *Eur. J. Immunol.*, vol. 36, no. 11, pp. 3044–3051, 2006.
[172] M. Raghavan, L. N. Gastinel, and P. J. Bjorkman, "The class I major histocompatibility complex related Fc receptor shows pH-dependent stability differences correlating with immunoglobulin binding and release.," *Biochemistry*, vol. 32, no. 33, pp. 8654–8660, 1993.
[173] S. Sugio, a. Kashima, S. Mochizuki, M. Noda, and K. Kobayashi, "Crystal structure of human serum albumin at 2.5 A resolution," *Protein Eng. Des. Sel.*, vol. 12, no. 6, pp. 439–446, 1999.
[174] T. R. C. Guizado, "Analysis of the structure and dynamics of human serum albumin," *J. Mol. Model.*, vol. 20, no. 10, 2014.
[175] J. T. Andersen *et al.*, "Structure-based mutagenesis reveals the albumin-binding site of the neonatal Fc receptor," *Nat. Commun.*, vol. 3, p. 610, 2012.
[176] M. M. Schmidt *et al.*, "Crystal structure of an HSA/FcRn complex reveals recycling by competitive mimicry of HSA ligands at a pH-dependent hydrophobic interface," *Structure*, vol. 21, no. 11, pp. 1966–1978, 2013.

[177] M. Bern, K. M. K. Sand, J. Nilsen, I. Sandlie, and J. T. Andersen, "The role of albumin receptors in regulation of albumin homeostasis: Implications for drug delivery," *J. Control. Release*, vol. 211, pp. 144–162, 2015.
[178] J. T. Andersen, J. Cameron, A. Plumridge, L. Evans, D. Sleep, and I. Sandlie, "Single-chain variable fragment albumin fusions bind the neonatal Fc receptor (FcRn) in a species-dependent manner: Implications for in vivo half-life evaluation of albumin fusion therapeutics," *J. Biol. Chem.*, vol. 288, no. 33, pp. 24277–24285, 2013.
[179] J. T. Andersen *et al.*, "Extending serum half-life of albumin by engineering neonatal Fc receptor (FcRn) binding," *J. Biol. Chem.*, vol. 289, no. 19, pp. 13492–13502, 2014.
[180] J. T. Andersen, M. B. Daba, G. Berntzen, T. E. Michaelsen, and I. Sandlie, "Cross-species binding analyses of mouse and human neonatal Fc receptor show dramatic differences in immunoglobulin G and albumin binding," *J. Biol. Chem.*, vol. 285, no. 7, pp. 4826–4836, 2010.
[181] S. Schulte, "Use of albumin fusion technology to prolong the half-life of recombinant factor VIIa," *Thromb. Res.*, vol. 122, no. SUPPL. 4, pp. S14–S19, 2008.
[182] T. Weimer, W. Wormsbächer, U. Kronthaler, W. Lang, U. Liebing, and S. Schulte, "Prolonged in-vivo half-life of factor VIIa by fusion to albumin," *Thromb. Haemost.*, vol. 99, no. 4, pp. 659–667, 2008.
[183] S. Zollner *et al.*, "Pharmacological characteristics of a novel, recombinant fusion protein linking coagulation factor VIIa with albumin (rVIIa-FP)," *J. Thromb. Haemost.*, vol. 12, no. 2, pp. 220–228, 2014.
[184] G. Golor *et al.*, "Safety and pharmacokinetics of a recombinant fusion protein linking coagulation factor VIIa with albumin in healthy volunteers," *J. Thromb. Haemost.*, vol. 11, no. 11, pp. 1977–1985, 2013.
[185] H. J. Metzner, T. Weimer, U. Kronthaler, W. Lang, and S. Schulte, "Genetic fusion to albumin improves the pharmacokinetic properties of factor IX," *Thromb. Haemost.*, vol. 102, no. 4, pp. 634–644, 2009.
[186] E. Herzog *et al.*, "Biodistribution of the recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP) in rats," *Thromb. Res.*, vol. 133, no. 5, pp. 900–907, 2014.
[187] M. W. Nolte, T. Nichols, S. Zollner, J. Mueller-Cohrs, I. Pragst, and G. Dickneite, "Improved kinetics of rIX-FP, a recombinant fusion protein linking coagulation factor IX with albumin, in cynomolgus monkeys and hemophilia B dogs," *J. Thromb. Haemost.*, vol. 9, p. 220, 2011.
[188] E. Santagostino *et al.*, "Safety and pharmacokinetics of a novel recombinant fusion protein linking coagulation factor IX with albumin (rIX-FP) in hemophilia B patients," *Blood*, vol. 120, no. 12, pp. 2405–2411, 2012.
[189] U. Martinowitz *et al.*, "Results of a phase I/II open-label, safety and efficacy trial of coagulation factor IX (recombinant), albumin fusion protein in haemophilia B patients," *Haemophilia*, vol. 21, no. 6, pp. 784–790, 2015.
[190] E. Santagostino *et al.*, "Long-acting recombinant coagulation factor IX albumin fusion protein (rIX-FP) in hemophilia B: Results of a phase 3 trial," *Blood*, vol. 127, no. 14, pp. 1761–1769, 2016.
[191] E. Persson, G. Bolt, T. D. Steenstrup, and M. Ezban, "Recombinant coagulation factor VIIa - from molecular to clinical aspects of a versatile haemostatic agent," *Thromb. Res.*, vol. 125, no. 6, pp. 483–489, 2010.
[192] C. L. Kempton and S. L. Meeks, "Toward optimal therapy for inhibitors in hemophilia," *Blood*, vol. 124, no. 23, pp. 3365–3372, 2014.

[193] D. Di Michele, "Inhibitor development in haemophilia B: an orphan disease in need of attention," *Br. J. Haematol.*, vol. 138, no. 3, pp. 305–315, 2007.
[194] E. Erhardtsen, "Pharmacokinetics of recombinant activated factor VII (rFVIIa)," in *Seminars in thrombosis and hemostasis*, 2000, vol. 26, no. 04, pp. 385–392.
[195] M. Morfini *et al.*, "Pharmacokinetic properties of recombinant FVIIa in inherited FVII deficiency account for a large volume of distribution at steady state and a prolonged pharmacodynamic effect," *Thromb. Haemost.*, vol. 112, no. 2, pp. 424–425, 2014.
[196] P. Margaritis, "Gene-based continuous expression of FVIIa for the treatment of hemophilia.," *Front. Biosci. (Schol. Ed).*, vol. 4, pp. 287–299, 2012.
[197] D. C. Roopenian, B. E. Low, G. J. Christianson, G. Proetzel, T. J. Sproule, and M. V. Wiles, "Albumin-deficient mouse models for studying metabolism of human albumin and pharmacokinetics of albumin-based drugs," *MAbs*, vol. 7, no. 2, pp. 344–351, 2015.
[198] A. Shapiro, "Plasma-derived human factor X concentrate for on-demand and perioperative treatment in factor X-deficient patients: pharmacology, pharmacokinetics, efficacy, and safety," *Expert Opin. Drug Metab. Toxicol.*, vol. 13, no. 1, pp. 97–104, Jan. 2017.
[199] P. M. Mannucci, S. Duga, and F. Peyvandi, "Recessively inherited coagulation disorders," *Blood*, vol. 104, no. 5, pp. 1243–1252, 2004.
[200] L. Ivanciu *et al.*, "A zymogen-like factor Xa variant corrects the coagulation defect in hemophilia," *Nat. Biotechnol.*, vol. 29, no. 11, pp. 1028–1033, 2011.
[201] A. Branchini *et al.*, "Natural and engineered carboxy-terminal variants: Decreased secretion and gain-of-function result in asymptomatic coagulation factor VII deficiency," *Haematologica*, vol. 97, no. 5, pp. 705–709, 2012.
[202] S. Kurachi, D. P. Pantazatos, and K. Kurachi, "The carboxyl-terminal region of factor IX is essential for its secretion," *Biochemistry*, vol. 36, no. 14, pp. 4337–4344, 1997.
[203] A. Branchini *et al.*, "The carboxyl-terminal region is NOT essential for secreted and functional levels of coagulation factor X," *J. Thromb. Haemost.*, vol. 13, no. 8, pp. 1468–1474, 2015.
[204] R. M. Camire, P. J. Larson, D. W. Stafford, and K. A. High, "Enhanced γ-carboxylation of recombinant factor X using a chimeric construct containing the prothrombin propeptide," *Biochemistry*, vol. 39, no. 46, pp. 14322–14329, 2000.
[205] C. F. Freguia, R. Toso, E. S. Pollak, V. R. Arruda, M. Pinotti, and F. Bernardi, "Characterization of mild coagulation factor VII deficiency: activity and clearance of the Arg315Trp and Arg315Lys variants in the Cys310-Cys329 loop (c170s)," *Haematologica*, vol. 89, no. 12, pp. 1504–1509, 2004.
[206] X. Chen, J. L. Zaro, and W.-C. Shen, "Fusion protein linkers: property, design and functionality," *Adv. Drug Deliv. Rev.*, vol. 65, no. 10, pp. 1357–1369, 2013.
[207] R. A. George and J. Heringa, "An analysis of protein domain linkers: their classification and role in protein folding," *Protein Eng. Des. Sel.*, vol. 15, no. 11, pp. 871–879, 2002.
[208] M. Monti *et al.*, "Characterization of the intracellular signalling capacity of natural FXa mutants with reduced pro-coagulant activity," *Thromb. Res.*, vol. 123, no. 6, pp. 914–918, 2009.
[209] M. Dewerchin *et al.*, "Blood coagulation factor X deficiency causes partial embryonic lethality and fatal neonatal bleeding in mice," *Thromb. Haemost.*, vol. 83, no. 2, pp. 185–190, 2000.

[210] S. J. Tai *et al.*, "A viable mouse model of factor X deficiency provides evidence for maternal transfer of factor X," *J. Thromb. Haemost.*, vol. 6, no. 2, pp. 339–345, 2008.
[211] M. Pinotti *et al.*, "Ribosome readthrough accounts for secreted full-length factor IX in hemophilia B patients with nonsense mutations," *Hum. Mutat.*, vol. 33, no. 9, pp. 1373–1376, 2012.
[212] L. Du *et al.*, "A New Series of Small Molecular Weight Compounds Induce Read Through of All Three Types of Nonsense Mutations in the ATM Gene," *Mol. Ther.*, vol. 21, no. 9, pp. 1653–1660, 2013.