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Blood coagulation factor X: molecular biology, inherited disease, and engineered therapeutics

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Abstract

Blood coagulation factor X/Xa sits at a pivotal point in the coagulation cascade and has a role in each of the three major pathways (intrinsic, extrinsic and the common pathway). Due to this central position, it is an attractive therapeutic target to either enhance or dampen thrombin generation. In this brief review, I will summarize key developments in the molecular understanding of this critical clotting factor and discuss the molecular basis of FX deficiency, highlight difficulties in expressing recombinant factor X, and detail two factor X variants evaluated clinically.

Keywords Factor X · Factor Xa · Recombinant proteins · Coagulation · Protein therapeutic

Abbreviations

TF	Tissue factor
FX	Factor X
FXa	Factor Xa
FVIIa	Factor VIIa
FIXa	Factor IXa
FVIIIa	Factor VIIIa
FVa	Factor Va
rFX	Recombinant factor X
Gla	Gamma-carboxyglutamic acid
EGF	Epidermal growth factor
RVV-X	Factor X activating enzyme from *Vipiera russulli*
CHO	Chinese hamster ovary
HEK	Human embryonic kidney
DOAC	Direct oral anticoagulants
aPCC	Activated prothrombin complex concentrates
TFPI	Tissue factor pathway inhibitor
wt	Wild-type
aPTT	Activated partial thromboplastin time

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Highlights

- The molecular and structural understanding of factor X has been established over the past century.
- Over 100 factor X mutations have been identified at the molecular level which led to factor X deficiency.
- Two recombinant factor Xa variants have been evaluated clinically for the treatment of bleeding and reversal of factor Xa inhibitors.
- New biochemical insights over the past two decades point to multiple approaches in which factor X or factor Xa can be modified for therapeutic use.

Introduction

The blood coagulation response is initiated following damage to the vasculature. This disruption exposes several molecules to the flowing blood, including tissue factor (TF), which triggers the hemostatic response to form a blood clot. Damaged vascular surfaces recruit activated blood cells such as platelets to the site of injury and provide an important anionic membrane surface that localizes coagulation factors [1]. These proteins are involved in a series of proteolytic activation reactions that ultimately results in the conversion of prothrombin to thrombin.

Blood coagulation factor X (FX) occupies a central position in the coagulation system and is a key driver of thrombin generation. Factor X is converted to activated

FX (FXa) by either the extrinsic (TF-FVIIa) or intrinsic (FVIIIa-FIXa) pathway [2]. In the common pathway, FXa reversibly associates with its cofactor FVa on an anionic membrane surface in the presence of calcium ions to form prothrombinase, the physiologic activator of prothrombin [1]. Due to its direct impact on thrombin generation, the regulation of prothrombinase or its individual components (FXa and FVa), has a major impact on blood clot formation [3].

Our understanding of the function of FX in the coagulation system developed over 100 years of research (Fig. 1). In 1904, Paul Morawitz postulated that there was a critical activator of blood coagulation that acted on prothrombin, which he termed thrombokinase [4]. Fifty years later, J Haskell Milstone isolated, purified, and characterized thrombokinase and made several important observations including that thrombokinase existed in an inactive form and needed to be activated to act on prothrombin [5, 6]. Through the 1960s–1970s, with refinements in purification procedures, FX was isolated in large quantities from human and bovine plasma [7]. These studies, spearheaded by many groups, led to a highly productive phase in which the biochemical nature of FX was unraveled, its enzymology was studied, its amino acid sequence determined, and its activation by the intrinsic and extrinsic pathways was evaluated [8, 9]. In the 1980s, the molecular genetics phase of FX started with the cloning of the cDNA and gene [10]. These advances helped uncover the molecular basis of naturally occurring mutations causing FX deficiency [11–13]. It also enabled researchers to express FX recombinantly and perform mutagenesis studies to better understand structure/function relationships at the molecular level. This information laid the groundwork to make recombinant (r)FX/FXa variants that are therapeutically useful.

In this brief review, I will summarize key developments in the molecular characterization of FX, discuss the molecular basis of FX deficiency, highlight difficulties in expressing rFX, and detail two rFXa variants evaluated clinically.

Molecular genetics of factor X

Following the development of recombinant DNA techniques in the mid-to-late 1970s, it became possible to characterize the coagulation factor genes, including *F10*. A critical step that preceded this, is the isolation of FX protein in large quantities from bovine and human plasma using methods refined over 20 years [7, 9]. This allowed for the determination of the amino acid sequence from both the heavy and light chains of FX using classical protein chemistry techniques [14–19]. These efforts revealed many important insights about the function of FX and showed FX had homology with other coagulation factors and trypsin-like enzymes. Further, it provided the critical information to design synthetic oligodeoxyribonucleotides as hybridization probes to screen liver cDNA libraries. In 1984 Fung, Campbell, and MacGillivray published the first description of a cDNA clone for FX [20]. A few months later, the human FX cDNA was published by Earl Davie's laboratory [21]. Not surprisingly, the bovine and human FX cDNAs are very similar. The human FX cDNA consists of 1467 bp with 120 bp coding for the 40 amino acid pre-pro-leader sequence, 1344 bp coding for the mature protein (448 amino acids), which is immediately followed by a stop codon [21]. The poly-adenylation signal (ATTAAA) is located within the cDNA, 7 bp upstream from the stop codon. Important findings from these studies include the recognition that FX is synthesized with a 40 amino acid pre-pro leader sequence consisting of a signal sequence and propeptide and the light and heavy chains are connected by Arg-Lys-Arg (Fig. 2). This tripeptide and the pre-pro-leader are removed by intracellular proteases and

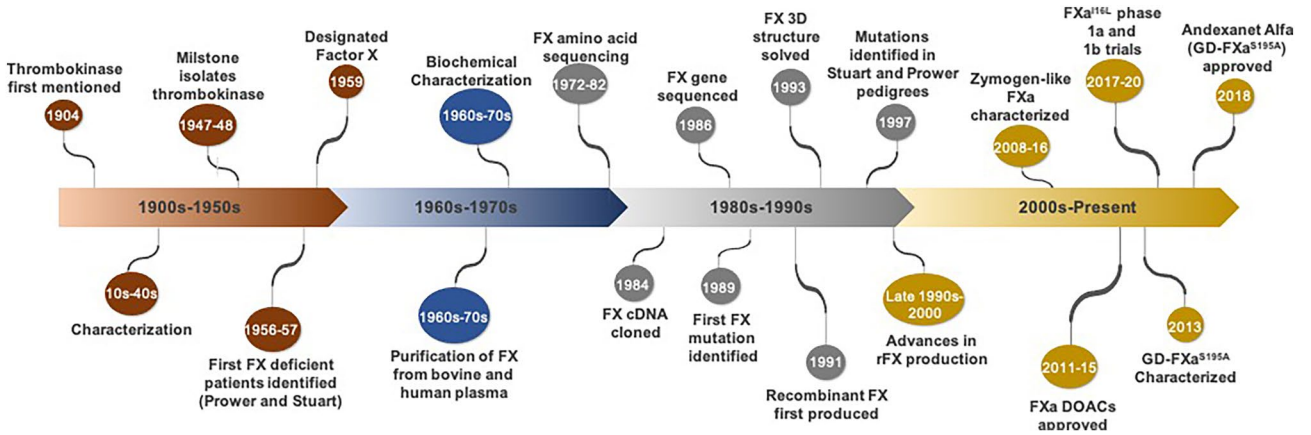

Fig. 1 Timeline of FX research and therapeutic development. Major developments in the understanding of FX are highlighted. Chronological blocks of significance include discovery/early characterization (red); protein biochemistry/understanding FX/FXa function (blue); molecular biology/genetics/structural biology (grey); mutagenesis/FXa inhibitors/FXa therapeutics (yellow)

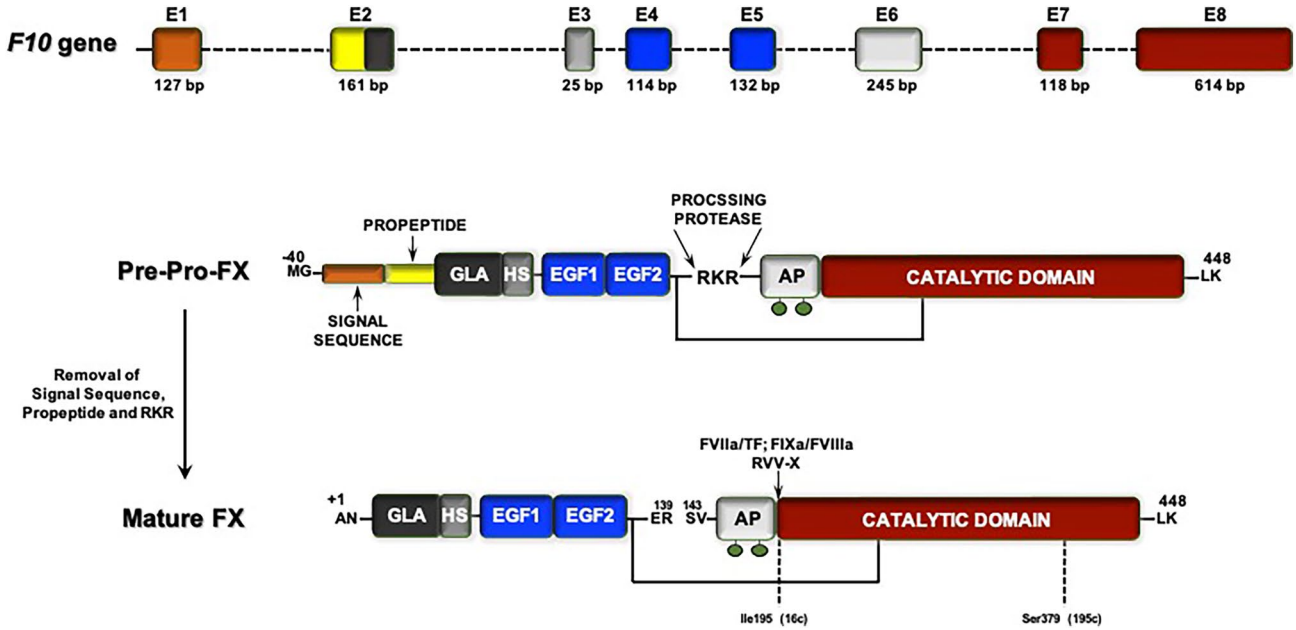

Fig. 2 Structural organization of the *F10* gene and FX protein. The *F10* gene has 8 exons and 7 introns and 22 kb long. The length of each exon is provided and are color coded for each domain they encode (see protein). Pre-pro-FX and mature FX are shown with domains annotated along with specific cleavage sites by proteases. Specific amino acids are numbered including Ile195 (16c) and Ser379 (195c) which are discussed in the text. The green spheres on the activation peptide (AP) represent N- and O-linked glycosylation sites

mature FX circulates in blood as a two-chain glycoprotein. Single chain FX has been observed following its isolation in plasma although at very low levels [9].

The *F10* gene was isolated in 1986 by the Davie laboratory and is 22 kb long and located on chromosome 13 downstream from the *F7* gene [22]. The organization and structure of the *F10* gene is very similar to the genes for protein C (*PROC*), *F9,* and *F7* highlighting their common ancestral origin. The gene contains 8 exons and 7 introns where exon 1 codes for the signal peptide; exon 2 encodes the propeptide and the gamma-carboxyglutamic acid (Gla) domain; exon 3 encodes the aromatic amino acid stack and a short linker segment; exon 4 and 5 encode for the epidermal growth factor (EGF) domains; exon 6 encodes the activation peptide region (including the tripeptide) and part of the serine protease domain; and exons 7 and 8 encode the catalytic domain (Fig. 2). The introns vary in size from 950 bp to 7.4 kb. The FX promoter driving expression in the liver has been partially characterized and lies about 280 bp upstream from the initiation start site [23].

Factor X protein structure

Factor X is synthesized in the liver as a pre-pro protein of 488 amino acids. Prior to secretion, the signal sequence and propeptide are removed as is the Arg-Lys-Arg tripeptide sequence separating the heavy and light chains (Fig. 2). The mature protein has an N-terminal light chain of 139 amino acids comprised of the vitamin K-dependent Gla domain (10 Gla residues) and two EGF domains. The heavy chain (306 amino acids) is comprised of the glycosylated activation peptide (52 amino acids) and the serine protease domain also called the catalytic domain. The heavy and light chains are held together by a disulfide bond. The FX serine protease domain is homologous to other chymotrypsin-like enzymes with the catalytic triad residues His^{236} (57c), Asp^{282} (102c), and Ser^{379} (195c) (chymotrypsin numbering system provided in parentheses and denoted by 'c'). This domain also has calcium and sodium binding sites that are important to the function of the active enzyme. Factor X circulates in blood at a concentration of about 8–10 µg/mL (135–170 nM) [24]. It is a substrate for both the intrinsic (FVIIIa-FIXa) and extrinsic Xase (TF-FVIIa) complexes which cleave a single peptide bond at position Arg^{194}. It can also be efficiently activated by an enzyme found in the venom of *Vipera russulli* (RVV-X) which is a metalloproteinase and very useful tool in the research laboratory [9]. The crystal structure of Gla-domainless FXa was solved in 1993 [25], and numerous structures have been solved since then and are available in the PDB (https://www.rcsb.org).

Factor X deficiency

Factor X deficiency was first described in 1956 by two independent groups essentially at the same time; one in a 22-year-old women (Prower) [26] and the other a 36 year old man (Stuart) who both presented with a bleeding tendency and abnormal clotting times [27, 28]. Until the Roman numeral designation of FX in the early 1960s, FX was known as Stuart-Prower factor [29, 30]. It was evident during this period of time that Stuart-Prower factor was the same protein as thrombokinase described by Milstone. Due to its central position in the coagulation system, FX deficiency results in a severe bleeding diathesis. It is an autosomal recessive disorder and is rare with a prevalence of about 1:1 million [11–13]. According to the World Federation of Hemophilia, in 2019 there were ~ 2500 people identified with FX deficiency [31]. It is one of the most severe of the rare bleeding disorders and patients with low coagulant activity may present with central nervous system bleeding, gastrointestinal bleeding, and commonly have hemarthroses and hematomas. Treatment involves the use of fresh frozen plasma, prothrombin complex concentrates, or a freeze-dried FIX/FX concentrate. More recently, a high-purity, high potency, plasma-derived FX concentrate has become available and approved in the United States and Europe [32]. Not surprisingly, complete deficiency of FX is embryonic lethal in mice in which the gene was deleted [33].

Following the isolation of the FX gene in the mid-1980s, the molecular characterization of FX deficiency became possible. The first FX deficiency case characterized at the molecular level was $FX^{San\ Antonio}$ in 1989 [34]. The patient exhibited substantial post-operative bleeding with 14% FX coagulant activity and was found to be compound heterozygous for a Cys^{366} to Arg (182c) in the catalytic domain and the other genetic change was a 1 base pair deletion (exon 7) causing a frameshift. Shortly after this, several FX mutations were characterized [2]. To date, > 100 mutations in the FX gene have been identified most of which are missense mutations and found in the catalytic domain [12]. The Stuart and Prower pedigrees were characterized in the late 1990s. The FX mutations in the Prower pedigree are Arg^{287} to Trp (107c) and Asp^{282} to Asn (102c), the latter mutation being notable as the Asp is part of the catalytic triad [24]. The only mutation reported for the Stuart pedigree is a Val^{298} to Met (118c) which leads to defective section of the protein [24].

Recombinant expression of factor X

In order to better probe structure/function relationships and characterize naturally occurring FX mutations, recombinant expression of FX was necessary. This was first reported in 1991 by William Church's group at The University of Vermont using COS-1 monkey kidney cells [35]. A follow-up study later that year by the same group employed Chinese hamster ovary (CHO) cells [36]. While there was prior success with the expression of other vitamin K-dependent proteins (e.g., prothrombin) using a similar system, this was not the case for rFX as numerous problems were identified. Recombinant FX was secreted in a biologically active form, but the specific activity was reduced by ~ 50%. This was due to several factors including: decreased gamma-carboxylation, inefficient removal of the tripeptide sequence and propeptide, and reduced RVV-X sensitivity [36]. Additional studies revealed that human embryonic kidney (HEK) 293 cells performed better in some of these inefficiencies. It was also recognized that changing Thr^{-2} to Arg in the propeptide and co-transfecting with PACE/furin greatly facilitated removal of the propeptide and processing of the tripeptide sequence to produce two-chain FX [37]. However, inefficient gamma carboxylation remained a major problem limiting specific activity. While this could be practically overcome by the addition of a barium citrate precipitation step to the purification scheme (selectively precipitates carboxylated protein), the yield of protein remained low.

To overcome the problem of inefficient gamma-carboxylation, it was found that swapping the FX propeptide for the prothrombin propeptide greatly enhanced the total amount of protein that was fully gamma-carboxylated [38]. This work was done during my post-doctoral studies in Dr. Katherine High's laboratory (University of Pennsylvania) in collaboration with Dr. Darrel Stafford (University of North Carolina-Chapel Hill), who was at the time working on understanding propeptide affinity for the carboxylase. The rationale for this approach was based on findings from the Stafford laboratory indicating that the FX propeptide has the greatest affinity for the carboxylase enzyme while prothrombin had to lowest [39]. This, with the fact that production of recombinant prothrombin in HEK cells results in fully gamma-carboxylated protein suggested the swapping approach may be helpful. Overall, these modifications and adjustments were a significant technical advance and now allow for high-level expression of biologically active rFX in HEK293 cells in milligram quantities.

Factor X/Xa therapeutics

Since FX/FXa is uniquely positioned in the coagulation cascade and a key driver of thrombin generation, there has been significant interest in developing ways to modulate its activity. In the context of anticoagulation, FXa-specific inhibitors have been investigated for over 30 years. Over the past decade, highly effective, small molecule direct FXa inhibitors (DOACs) were approved to treat venous thromboembolism [40]. For hemophilia, approaches to enhance FXa production (e.g., recombinant FVIIa or activated prothrombin complex concentrates, aPCC) are very successful in patients with neutralizing antibodies to either FVIII or FIX [41]. Further, approaches that effectively protect existing FXa activity by reducing natural plasma inhibitors (e.g. anti-TFPI or reduction of antithrombin levels) also show promise in clinical trials [42]. In the remaining sections, I will review the only two rFXa proteins that have been evaluated clinically; one to treat acute bleeding and the other to reverse the effects of FXa inhibitors.

Zymogen-like FXa variants

Prior to the development of emicizumab, the mainstay of hemophilia therapy for patients with neutralizing inhibitory antibodies to infused FVIII or FIX was recombinant FVIIa and aPCCs [41, 42]. Both products work by enhancing FXa production. In principle, infusion of wild-type (wt)-FXa should also be effective in this context, however, wt-FXa is rapidly inactivated by plasma protease inhibitors, resulting in a very short half-life. Previous attempts using wt-FXa either alone or in combination with phospholipids in a hemophilic dog model were unsuccessful [43].

To circumvent this, my laboratory bioengineered FXa variants that could overcome protective inhibitory mechanisms without compromising hemostatic benefit by targeting the FX zymogen to protease transition pathway [44, 45]. The zymogen to protease transition, shared by all serine proteases, involves cleavage of FX at a highly conserved site (e.g., Arg^{194} (15c)-Ile^{195} (16c)) [46] which liberates a new N-terminus (I^{195}-VGG). The new N-terminus inserts into a hydrophobic pocket within the catalytic domain forming a salt-bridge with Asp^{378} (194c) [47–49], and induces a conformational change that yields the mature protease. To alter this process, we changed the amino acid at position 195 (16c) or 196 (17c) to make the ensuing conformational change sub-optimal. Biochemical characterization of a range of zymogen-like variants revealed they have varying degrees of catalytic impairment (5 to 1000-fold) [44, 50], and partial resistance to circulating inhibitors (e.g. antithrombin and TFPI) leading to a prolonged half-life in plasma [44, 45, 50]. Importantly, the biologic activity of the FXa variants could be rescued by binding FVa, such that assembly in prothrombinase resulted in near normal procoagulant activity.

Based on these features, we surmised that zymogen-like forms of FXa should act as long-lived proteases in circulation that are otherwise functionally inert in the absence of FVa but retain the ability to catalyze thrombin formation upon FVa binding on an activated cellular surface. In vitro and ex vivo studies of increasing complexity showed that $FXa^{I195L\ (16c)}$ (herein refer to as FXa^{I16L}), a prototypical zymogen-like FXa species, was highly effective in promoting thrombin generation in hemophilic plasma/blood with or without inhibitors [45, 50, 51]. Initial preclinical studies in hemophilic mice indicate that FXa^{I16L} appears safe and effective after a single dose when administered either before or after injury in multiple injury models [50, 51]. Repeated infusions at the effective dose of FXa^{I16L} did not result in systemic activation of coagulation. The zymogen-like variant used in these models, FXa^{I16L}, has a relatively short half-life, limiting its use as a prophylactic agent [52, 53]. However, we found that FXa^{I16L} was very effective for use in acute bleeding settings, where the desired effect is to stop bleeding quickly without prolonged activation of coagulation [50, 51]. In addition to its assessment in models such as hemophilia, FXa^{I16L} shows promise reversing the effects of FXa DOACs in vitro and mitigating DOAC associated bleeding in vivo in mice [54].

Given the promising in vivo animal studies establishing FXa^{I16L} as a rapid pro-hemostatic, its clinical development was pursued with Pfizer as PF-05230907. In preclinical pharmacokinetic studies in cynomolgus monkeys, its half-life was found to be ~ 3 min. A dose-dependent decrease in the activated partial thromboplastin time (aPTT) was also observed, consistent with FXa^{I16L} functioning as a pro-hemostatic [55]. In a first-in-human phase 1 dose escalation study in 49 healthy adults (NCT01897142), FXa^{I16L} was well-tolerated up to 5 µg/kg, with a half-life of about 4 min [56]. There were no serious adverse events, and no dose-limiting toxicity up to the maximal dose. aPTT and thrombin generation assay parameters changed in a dose-dependent manner, as did prothrombin fragment 1.2 and D-dimer levels. One patient developed a weakly positive, transient, non-neutralizing antibody to FXa^{I16L} that did not cross-react with FX or FXa; no other patients exhibited an immune response [56]. A second phase 1b study (NCT02687191) was initiated to determine the maximum tolerated dose of FXa^{I16L} administered to subjects with intracerebral hemorrhage. Factor Xa^{I16L} was found to be well tolerated in these patients with thrombotic events only observed at the highest dose level tested. However, recruitment within the therapeutic window

of opportunity, where there was still ongoing intracerebral hemorrhage, remained a significant challenge [57].

Andexanet alpha

Over the past decade, several orally administered, active-site directed FXa inhibitors, including rivaroxaban, apixaban, edoxaban, and betrixaban, were approved for the prevention of stroke in patients with nonvalvular atrial fibrillation, and prevention and treatment of venous thromboembolism [58–60]. Like all anticoagulants, DOACs carry a risk of bleeding. The effects of older anticoagulants like warfarin and heparin can be reversed pharmacologically in the event of bleeding or in the event that patients require urgent or emergent surgery. In contrast, when they were developed, DOACs lacked a reversal agent.

To address this, andexanet alfa (Gla-domainless $FXa^{S379A\ (195c)}$, herein referred to as GD-FXa^{S195A}) was developed, a recombinant FXa molecule produced in CHO cells that lacks catalytic activity as well as the ability to bind membranes, making it a scavenger for all direct FXa inhibitors [61]. The removal of the Gla-domain eliminates protein binding to anionic membranes, and this also greatly diminishes binding to FVa which is effectively a membrane-dependent interaction. The Ser to Ala mutation at position 379 (195c), which is one of the catalytic triad residues, eliminates proteolytic activity, but importantly retains an open active site. These combined modifications make GD-FXa^{S195A} ideally suited to bind free plasma FXa active site inhibitors and eliminate their anticoagulant potential. However, binding exosites on the catalytic domain of GD-FXa^{S195A} can still interact with physiologic ligands and TFPI can still interact with GD-FXa^{S195A}. The binding of GD-FXa^{S195A} to circulating TFPI has caused some concern as this can lead to increased thrombin generation.

Due to its structural modification and mechanism of action, GD-FXa^{S195A} effectively acts as a decoy protein that binds FXa active site inhibitors (as well as low molecular weight heparin) with high affinity to rapidly diminish anticoagulation. Pre-clinical studies in animals documented its effectiveness in rapidly reducing the activity of FXa inhibitors [61]. After its clinical assessment, in 2018 the FDA granted andexanet alfa accelerated approval for patients treated with rivaroxaban or apixaban who require reversal of the anticoagulant effects in life-threatening or uncontrolled bleeding. Approval was largely based on two trials: ANNEXA-A and ANNEXA R which investigated the safety and efficacy of andexanet alfa in reducing anti-FXa activity [62]. Despite its short-half-life (~ 1 h), there is some evidence from the trials that andexanet alfa can temporarily correct hemostasis and control bleeding. In the ANNEXA-4 trail, andexanet alfa was associated with a reduction in anti-FXa activity as expected and 82% of patients enrolled achieved excellent or good hemostasis after infusion [63]. However, there was not a control group for comparison. Additional clinical assessment of andexanet alfa is ongoing.

Concluding remarks and future developments

Over the past five decades of research, remarkable advances in our understanding of the biochemistry and molecular genetics of FX have been made. In addition to its well-established role in coagulation as the serine protease component of prothrombinase, it is also now well-established that FXa is also an important signaling molecule working through PAR receptors [64]. Due to its critical role in the generation of thrombin, it is an attractive target for drug development and FXa-specific DOACs are remarkably successful. Going forward, targeting FXa to control bleeding or diminish thrombosis by taking advantage of critical binding surfaces (exosites) or through allosteric modulation has tremendous promise. Several biochemical insights over the past two decades point to multiple approaches in which this could be achieved. In fact, creative research laboratories are pursuing these efforts now with a new generation of rFX/FXa mutant proteins with therapeutic potential [65–67].

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Declarations

Conflict of interest RMC is a consultant for and receives research support from Pfizer and Bayer. RMC also receives licensing fees for technology related to zymogen-like FXa.

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