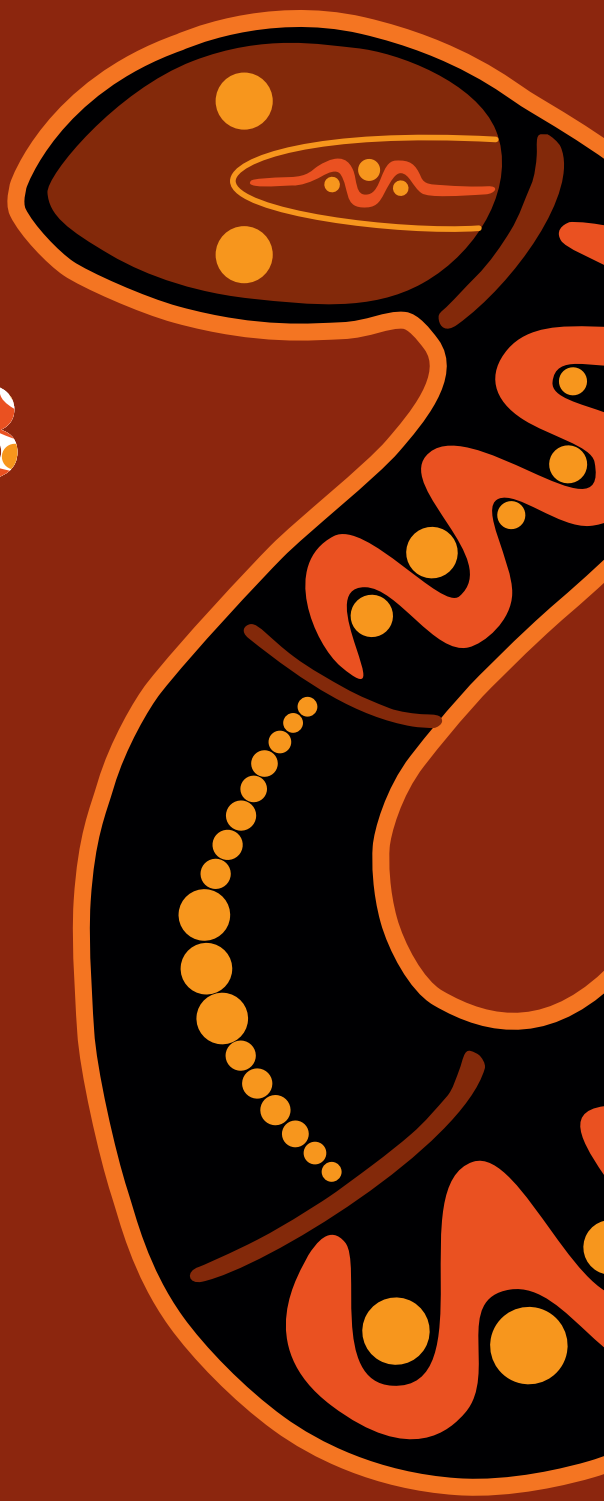
The unique procoagulant adaptations of pseudonaja textilis venom factor V and factor X

Schreuder, M.

Chapter 8

General Discussion



General Discussion

Blood coagulation is a complex system consisting of several independent biochemical and cellular processes that prevent blood loss following vascular injury. Upon vascular damage, platelets bind to the site of injury and aggregate to form a platelet plug[1]. The coagulation cascade is initiated by the exposure of tissue factor[2] and blood clotting factors are activated on the negatively-charged surface of activated platelets and endothelial cells[3]. One of the key reactions in blood coagulation is the generation of thrombin by the prothrombinase complex that assembles on anionic membranes in the presence of calcium ions and consists of factor Va (FVa) and factor Xa (FXa)[3,4]. Thrombin catalyzes the activation of platelets and is responsible for the deposition of a stabilizing fibrin network. In addition, thrombin activates several positive feedback mechanisms that are required to generate a sufficient procoagulant response. Importantly, the anticoagulant pathway consists of several anticoagulant proteins that terminate the various stages of the procoagulant reaction to prevent excessive blood clotting[5,6]. In disease settings this hemostatic balance is disturbed. Deficiencies in coagulation factors may lead to bleeding disorders such as hemophilia, whereas mutations resulting in more procoagulant coagulation factors induce a hypercoagulant state. Intriguingly, the Australian Elapid family has exploited the coagulation cascade to gain a unique selective advantage. Several evolutionary adaptations in the factor V (FV) and factor X (FX) variants that are expressed in their venom have generated clotting factors with exceptional procoagulant enhancements[7,8]. Upon envenomation, these coagulation factors generate an explosive burst of thrombin that quickly results in the circulatory collapse of the prey animal[9-11].

The aim of the research in this thesis was to gain further insights into the remarkable procoagulant adaptations of *Pseudonaja textilis* venom-derived FV (ptFV) and FX (ptFX). To do so, we have generated a panel of recombinant chimeric FV and FX proteins and studied the structure-function relationships of various structural elements. This approach has allowed us to identify several regions that are at the basis of the procoagulant enhancements and potent toxicity of these venom proteins. In the current chapter, the main findings and implications are summarized and discussed in more detail and the perspectives for further research are outlined.

The Structural Integrity of ptFVa

Unique Structural Features of ptFV

The inactivation of mammalian FVa by activated protein C (APC) is one of the key reactions that downregulates the procoagulant response. Proteolytic cleavage at the

A2 domain residues Arg306, Arg506, and Arg679 results in the dissociation of the A2 domain and FVa inactivation[12]. In contrast, ptFVa is only cleaved by human APC at a position that is homologous to human Arg506 (Lys507)[7], and despite proteolysis this FVa variant retains full cofactor activity[8]. In **chapter 3** we aimed to unravel the functional APC resistance of ptFV. Our findings showed that the absence of a homologous Arg306 cleavage site and the presence of the unique ptFV disulfide bond are not responsible for the functional resistance to APC. This implies that ptFV comprises other structural elements that prevent the loss of cofactor activity. Interestingly, human-snake chimeric FVa variants generated by us maintained structural integrity following APC-catalyzed proteolysis, demonstrating the presence of robust non-covalent interactions between the ptFVa A2 domain and A1-A3 domains. Using *in silico* analysis we identified strong and stable interactions between the ptFVa A1 and A2 domains that significantly increase the A2 domain binding affinity. We further identified the so-called A2 domain loop2 as a structural element that seems to play a major role in the association of the A2 domain with the A1-A3 domains in ptFVa.

Activated Protein C Resistance of FVa

Unraveling at least part of the mechanistic principles that are responsible for the functional resistance to APC in ptFV has identified a new concept of APC resistance. To date, APC resistance has been described as a result of mutations that either prevent certain APC-mediated cleavages or decrease the sensitivity for APC. The most common FV mutation that results in APC resistance is $FV^{Leiden\ 13}$. The molecular basis of FV^{Leiden}-dependent APC resistance is derived from a point mutation that results in an Arg506Gln substitution, leading to a ten-fold slower APC inactivation rate relative to wild-type FVa[14]. Naturally occuring FV variants with mutations at the APC cleavage site Arg306 are $FV^{Hong\ Kong}$ (Arg306Gly) and $FV^{Cambridge}$ (Arg306Thr) which display a mild APC resistant phenotype[15], presumably due to the ability of protein S to stimulate Arg306 cleavage. In addition, the Ile359Thr ($FV^{Liverpool}$) mutation introduces a glycosylation site at Asn357 that impaires Arg306 cleavage, likely by steric hindrance[16]. The His1299Arg (FVR2) mutation that has been described to result in an APC resistant phenotype[17] seems to be inducing a quantitative FV deficiency owing to impaired expression of the mutant protein[18].

Mechanistic Principles of FVa Inactivation

Factor Va is inactivated via limited proteolysis at Arg306 and Arg506 resulting in the dissociation of the A2 domain. To date, the dissociation of the A2 domain following APC-catalysis has only been demonstrated in bovine FVa[12,19], with biochemical and structural data lacking for human FVa. Molecular Dynamics simulations using a complete human FVa model have previously suggested that APC cleavage results in the release of the A2 domain[20]. In our study, we have provided biochemical evidence

that human FVa is inactivated by APC in a similar fashion as compared to bovine FVa (**Figure 3, chapter 3**). Yet, the currently available data are unable to distinguish whether cleavage at Arg306 and Arg506 or the dissociation of the A2 domain serves as the major contributor to the loss of procoagulant activity. Using an engineered FVa mutant comprising an A2-A3 interdomain disulfide bond, Gale *et al.* showed that APC-induced cleavage markedly reduced the FVa cofactor activity while A2 domain dissociation was prevented[21]. This suggests that APC cleavage rather than A2 domain dissociation is responsible for the inactivation of FVa. In contrast, Molecular Dynamics simulations using a model for FVa^{Leiden} suggested that in the absence of the Arg506 cleavage, A2 domain dissociation occurs as a result of Arg306 proteolysis[20], implying that cleavage of the cofactor at Arg506 is not required for A2 domain dissociation but is rather required to facilitate cleavage at Arg306. Our findings using ptFV support the notion that A2 domain dissociation is the main determinant for FVa inactivation (**chapter 3**). Despite cleavage at Arg306 and Lys507, ptFVa retained structural integrity and full cofactor activity. Unfortunately, we were unable to determine whether release of the A2 domain would inactivate ptFVa. However, the human FVa A2 domain comprises essential FXa and prothrombin binding sites[4] . It seems therefore reasonable to assume that similar regions are present in the ptFV A2 domain, allowing us to speculate that the dissociation of the A2 domain will result in the inactivation of ptFVa.

A2 Domain Binding and Stability in the Homologous Cofactors FVIIIa and FVa

The residues that positively or negatively contribute to the FVa A2 domain binding affinity remain largely unknown. Characterization of the naturally occurring FV variants comprising an Ala221Val or Arg1698Trp mutation revealed a reduced FVa stability due to an increased dissociation rate of the heavy (A1-A2 domains) and light (A3-C1-C2 domains) chains[22,23]. Arg1698 was predicted to be located at the A2-A3 domain interface[22] and the Ala221Val mutation was found to induce structural changes toward the A2-A3 domain interface[23]. Using Molecular Dynamics simulations, we have identified the key hydrogen bond interaction pairs between the A2 domain and the A1 or A3 domain (**Figure 5, chapter 3**). Furthermore, we have shown that A2 domain dissociation upon APC cleavage is induced by a loss of A1-A2 domain affinity, indicating that the residues at the A1-A2 domain interface are crucial for A2 domain binding.

In contrast to FVa, the inactivation of the homologous factor VIIIa (FVIIIa) proceeds either via APC-mediated proteolysis[24,25] or via the spontaneous release of the A2 domain[26,27]. Whereas our understanding of the residues that contribute to productive interactions between the A2 and A1-A3 domains is lacking for FVa, biochemical and mutagenesis studies have identified multiple residues that support A2 domain binding in FVIIIa[28-35]. The FVIIIa A2 domain subunit weakly associates with the A1-A3 domains,

primarily through electrostatic interactions, and readily dissociates at physiologic pH wheareas FVIIIa stability is enhanced at pH 6.0[26]. The observation that increasing NaCl concentrations significantly and dose-dependently reduced the FVIIIa activity[27] could not be replicated for APC-proteolyzed ptFVa-h306-SS nor did we observe differences in the thermostability of our APC-treated ptFVa variants relative to wild-type ptFVa (**Figure S4, chapter 3**). Whether the unique interactions facilitated by the A2 domain loop2 in ptFVa could explain these differences remains to be determined by future biochemical studies that further characterize the functional consequences of the ptFV A2 domain loop2.

The Lipid Independent Activity and APC Resistance of ptFVa

Functional Role of the A2 Domain C-terminal Region

The FV A2 domain C-terminus (A2T) is a long, negatively charged region that is liberated upon release of the B-domain (**Figure S1, chapter 4**). Although X-ray structures have not been able to fully resolve the A2T, this structural element has been modelled as a highly flexible region with little if any conformational constraints[36,37]. From a functional perspective, the A2T has been implicated in the assembly of the prothrombinase complex and engagement of the substrate prothrombin[38-42]. However, its exact role remains poorly understood as contradictory results have been reported[43,44]. In **chapter 4** we studied the functional implications of the ptFV A2T that comprises a significant extended region relative to other known FV species. Our results revealed that the catalytic efficiency towards prothrombin was slighty reduced in FV variants comprising the ptFV A2T, indicating that the ptFV A2T engages prothrombin in a somewhat different manner. These observations are in accordance with previous reports suggesting that the FVa A2T does not provide major productive interactions with FXa and/or prothrombin, but rather plays a role in the positioning of the prothrombin activation sites towards the catalytic triad of FXa[39,42,44]. Collectively, the role of the A2T seems to be minimal in the presence of saturating amounts of phospholipid membranes, indicating that binding of FVa to FXa and prothrombin is mainly dependent on productive interactions facilitated by the regions surrounding Arg306, Arg506, and part of the A3 domain (reviewed in **chapter 2**).

The Lipid Independent Activity of ptFVa and ptFXa

Contrarily to our findings in the presence of anionic membranes, we demonstrated that the ptFV A2T plays a major functional role in the absence of membranes by facilitating prothrombin conversion via a lipid-independent mechanism. Interestingly, hFVa-ptA2T converted prethrombin-1, a prothrombin derivative that lacks the γ-carboxyglutamic

acid domain (GLA) domain and as such the membrane-binding ability, with similar rates relative to ptFVa (**Figure 4D, chapter 4**). Yet, this effect was only observed when hFV-ptA2T was complexed with ptFXa. This provides additional evidence that the lipid-independent activity of ptFV is entirely regulated by the A2T. Our findings further demonstrated that ptFXa comprises an essential element that is required to bypass the need for phospholipid binding. Currently, we can only speculate which unique region or regions are responsible. Our data excludes a role for the extended 99-loop in ptFXa, which extends towards, and possibly interacts with, FVa[45]. Another region of interest is the lipid-binding GLA domain. Binding of FXa to the lipid surface induces a conformational change that increases the affinity for FVa[46]. For ptFXa, structural differences in the GLA domain could potentially instigate similar conformational changes in the absence of anionic membrances and expose a FVa interactive site. Binding of FVa might promote further conformational changes that result in a functional FVa-ptFXa complex in solution. In our study, we used several peptides that comprise part of the ptFV A2T in an attempt to pinpoint the region that is responsible for the lipid-independent activity. These studies suggested a crucial role for the N-terminal region of the A2T (**Figure 4, S5, chapter 4**). The ptFV-ptFXa (Pseutarin C) X-ray structure revealed that this region interacts with the highly basic heparin binding site of ptFXa[45]. Based on these findings, we speculate that evolutionary changes in the ptFXa heparin binding site support the lipid-independent activity of the Pseutarin C complex. However, an initial comparison of the human ternary model and Pseutarin C structure did not reveal major structural differences[37,45]. Moreover, we can not exclude that our peptides interfere with prothrombinase activity in a non-specific manner nor can we conclude that the C-terminal region of the ptFV A2T is not involved in this procoagulant enhancement. To confirm our findings, new FV and FX variants would need to be generated and characterized that specifically substitute or truncate part of the ptFV A2T, or mutate potential important residues in the ptFXa A2T binding site, respectively.

Role of the ptFV A2T in the Functional Resistance to APC

Our findings further revealed that the ptFV A2T plays a major role in the functional resistance to APC. Substitution of the ptFV A2T for the corresponding human region significantly decreased the cofactor activity upon APC-mediated proteolysis. Intriguingly, the inactivation of ptFVa-hA2T was markedly slower compared to hFVa and retained ~25% cofactor activity following a two-hour incubation with APC (**Figure 5, chapter 4**). It is currently unclear whether prolonged incubations with APC would completely inactivate ptFVa-hA2T, but the residual cofactor activity might be explained by an enhanced stability of regions that are essential for the interaction with FXa and prothrombin. This is supported by our observation that the partial loss of ptFVa-hA2T cofactor activity was not a result of A2 domain dissociation (**Figure 6 chapter**

4). Moreover, a comparable loss of cofactor activity was observed when ptFVa was treated with the fibrinolytic serine protease plasmin (**Figure S5, chapter 3**). Similar to FVa^{leiden}, selective proteolysis of ptFVa could destabilize the interactions with ptFXa while cofactor activity is at least partially maintained by impeding A2 domain dissociation[14]. As described in previous sections, this could be the result of the increased A2 domain binding affinity facilitated by the productive interactions between the A2 domain loop2 and A1 domain. Taken together, our findings indicate that the conformational rigidity of loop2 together with the unique functional characteristics of the ptFV A2T provide a rationale for ptFVa's functional resistance to APC.

Unfortunately, we were unable to accurately determine the specific region of the A2T that contributes to the APC resistance. However, mutation of the Arg679 cleavage site in ptFVa-hA2T (**Figure S1, chapter 4**) resulted in a ptFVa variant that retained stable cofactor activity following an initial ~35% loss of activity (**Figure 5, chapter 4**). Considering the presumed high flexibility of the A2T[37,45,47], it is unlikely that cleavage at Arg679 significantly impairs FVa stability. Therefore, these results suggest that truncation of the A2T as a result of Arg679 cleavage decreases the ptFVa cofactor activity, providing evidence for a role of the A2T C-terminal region in the functional resistance to APC. In addition, the modest loss of ptFVa-hA2T-R679Q cofactor activity (~35%) following Arg679 mutation seems to indicate that the A2T N-terminal region is, besides its crucial role in the lipid-independent activity, also to some extent involved in the APC resistance of ptFV.

We further demonstrated that introduction of the ptFV A2T, the key structural element responsible for ptFVa's APC resistance (**chapter 4**), in human FVa would generate a human FVa variant with a similar gain-of-function. However, an additional mutation of the human Arg306 cleavage site was required, presumably to prevent the dissociation of the Asn307-Arg506 region. Furthermore, this hFVa-ptA2T-pt306 variant only retained full cofactor activity when in complex with ptFXa. This demonstrates that ptFXa comprises an essential element that is required for this gain-of-function feature, similar to that observed for the lipid-independent conversion of prothrombin. As discussed above, the ptFXa region that is responsible for these unique procoagulant enhancements is currently unknown, but likely involves a FVa interactive region. Collectively, our findings indicate that the ptFV A2T circumvents negative regulatory conditions by providing stable interactions with ptFXa and prothrombin in the absence of membranes or upon APC-mediated proteolysis.

Evolutionary Changes in the ptFX Activation Peptide

Role of the FX Activation Peptide in the Interaction with Intrinsic Tenase

The activation peptide of the venom-derived ptFX is remarkably shorter in amino acid length compared to that of various other FX species, including that of the liver-derived ptFX[48,49]. In **chapter 5** we aimed to unravel the functional implications of this evolutionairy adaptation by characterizing several chimeric FX variants. Assessment of the activation by the extrinsic tenase complex revealed identical kinetic constants between hFX and hFX-ptAP. This could suggest that the extrinsic tenase complex is able to interact with the venom-derived activation peptide in a similar manner as the human counterpart. Alternatively, it is possible that the FX activation peptide does not comprise a binding site for the extrinsic tenase complex. The latter is supported by previous studies revealing only modest alterations in kinetic parameters of extrinsic tenase-dependent FX activation following truncation of the activation peptide[50,51]. Our findings therefore provide further evidence that the substrate-specificity of the extrinsic tenase complex is completely dependent on the interactions with pro-exosite regions in FX. The exact interactive regions are not fully defined but previous studies have reported a tissue factor-factor VIIa binding site in the FX GLA[52,53], endothelial growth factor 1[53], and serine protease domain[54].

Somewhat surprising, kinetic analyses demonstrated that incorporation of the venom-derived activation peptide into human FX almost completely abrogated the activation by the intrinsic tenase complex. Furthermore, we showed that the variant hFX-ptAP is a poor competitor of FX activation by the intrinsic tenase complex relative to hFX-R15Q. Therefore our results indicate that activation by the intrinsic tenase complex is mainly dependent on the recognition and binding of the FX activation peptide, as has been suggested in earlier reports[51,55]. The evolutionairy advantage of the shortened activation peptide is unclear. It is possible that the venom-derived activation peptide has specifically evolved towards recognition by the intrinsic tenase complex of prey animals and may interact in a different manner relative to the human system. On the other hand, various reports have suggested that the intrinsic coagulation pathway may have only been fully evolved after the reptile lineage[56-60]. Although speculative, this could imply that the activation of FX by the intrinsic tenase complex does not play a significant role in the coagulation cascade of reptiles. Characterization of the liver-derived ptFX will gain more insight into the intrinsic tenase-mediated activation of ptFX and the role of the activation peptide.

The Zymogen Activity of ptFX

The evolutionary intermediate ptFX (isoform ptFX[49]) was reported to display zymogen activity towards the macromolecular substrate prothrombin when in complex with venom-derived ptFV[45]. In our study, we have unraveled the molecular basis for the zymogen activity of ptFX and identified that the venom-derived activation peptide is responsible for this remarkable procoagulant characteristic (**chapter 5**). Interestingly, substitution of the human activation peptide for that of ptFX generated a human chimeric FX variant with similar zymogen activity relative to ptFX. Furthermore, we observed that the zymogen activity is completely dependent on the presence of the cofactor, in contrast to previously reported data showing that human FV is not able to bind to zymogen FX[61]. This is in line with our kinetic data revealing that the apparent human FV-FX binding affinity could not be accurately assessed. In contrast, both venom ptFX and hFX-ptAP displayed a markedly increased apparent binding affinity for their respective cofactor, relative to human FX. Moreover, kinetic evaluation showed that the FX-cofactor complexes bind prothrombin with a similar affinity relative to prothrombinase-assembled FXa[62-65]. As such, our findings indicate that the venom-derived activation peptide allows exposure of a FVa binding site and formation of an initial FVa-FX complex. The productive interactions with FVa then facilitate conformational changes in the serine protease domain of FX that are required for catalytic activity.

Functionally Important Regions in the Venom-derived Activation Peptide

The exact structural features of the ptFX activation peptide that are responsible for the resistance to the intrinsic tenase complex or for the zymogen activity are currently unknown. Compared to various FX species, including the liver-expressed ptFX, the venom-derived activation peptide lacks the central acidic region[49]. Although no studies have reported a functional role for this region, our data might suggest that it comprises a binding site for the intrinsic tenase complex. Alternatively, the acidic region could prevent potential interactions with FVa in order to avoid catalytic activity of the FX zymogen. Other structural elements that play a role in FX activation are the carbohydrate chains that are present in the FX activation peptide[66]. Whereas the human FX activation peptide comprises two O-linked and two N-linked glycosylation sites, no experimental data is available on the glycosylation status of the venom FX activation peptide. Artificial mapping of glycosylation sites predicts that ptFX contains one N-linked and one O-linked carbohydrate[66,67]. In human FX, these O-linked carbohydrates have been shown to play a key role in the catalytic efficiency of the intrinsic tenase complex, indicating that both glycans make productive interactions with the complex[51,68-70]. In order to fully understand the mechanisms that are at the basis of these functional

characteristics, mutagenesis studies are required that specifically assess the role of the carbohydrates or the acidic region in the activation peptides.

Zymogenicity in Serine Proteases

Although serine proteases are kept in a zymogen state to prevent unwanted catalytic activity, substrate conversion has been reported by various zymogens. The 'molecular sexuality' principle relies on the insertion of a peptide that is sequentially similar to the strictly conserved Ile16 N-terminus into the preformed active site[71,72]. Such peptides have been shown to induce trypsinogen to trypsin transition[71]. In addition, bacterial proteins belonging to the zymogen-activator and adhesion proteins (ZAAP) family bind to serine proteases such as plasminogen[73] and prothrombin[74,75] and insert their N-terminus into the Ile16 pocket to form the characteristic salt bridge with Asp194. Formation of this salt bridge results in conformational changes of the activation domain and activation of the zymogen in a non-proteolytic manner.

Zymogen activity has also been reported in the absence of a cofactor. It is hypothesized that both zymogen and protease exist in different conformational states within a dynamic equilibrium[76-78]. In this case, zymogens may acquire a conformation that allows for some level of catalytic activity (protease-like), whereas proteases adopt conformations with limited activity (zymogen-like). The ratio between the activity of the mature protease and the activity of the zymogen is defined as the 'zymogenicity' of the protein. In general, the zymogenicity of serine proteases is high, which results in a minimal or absence of proteolytic activity in zymogens. Tissue-type plasminogen activator (tPA) is a unique exemption since its catalytic activity increases only 5-10-fold upon activation[79-81]. The low zymogenicity of tPA was shown to be the result of an intrinsic Lys156-Asp194 salt bridge which induced conformational changes leading to the maturation and stabilization of an active trypsin-like conformation[82,83]. More recently, the zymogen forms of prekallikrein and factor XII were shown to express physiological relevant activity[84,85]. Although their zymogen activity was several orders of magnitude lower than that of the activated proteases, non-cleavable forms of prekallikrein[84] and factor XII[85] were able to initiate the contact activation pathway. Our study has identified a novel mechanism by which the zymogenicity of a serine protease can be modified. However, the exact structural requirements to achieve zymogen activity remain unclear. It has previously been suggested that the residues surrounding Lys156 have a critical role in determining the potential formation of the Lys156-Asp194 salt bridge. For instance, in bovine trypsinogen Thr21 engages in a hydrogen bond with Lys156, effectively sequestering Lys156 from interacting with Asp194[86]. Similarly, in FXa and urokinase Lys156 interacts with Thr144 and Glu144, respectively[86]. In contrast, tPA comprises Phe21 and His144 that lack the potential

to sequester Lys156 and consequently allow Lys156-Asp194 interaction[86]. These observations led to the hypothesis that hydroxyl or acid groups at positions 21 and 144 interact with Lys156 to prevent formation of the Lys156-Asp194 salt bridge and stabilize an inactive conformation. Yet, the potential to form a Lys156-Asp198 salt bridge can not fully explain the zymogen activity of serine proteases since factor XII and prekalikrein display physiologically relevant catalytic activity despite comprising Glu156. In the case of factor XII, a crystal structure revealed a direct interaction between residues Arg73 and Asp194 which may act as an alternative salt bridge inducing catalytic activity[87]. Furthermore, productive interactions with macromolecular substrates might contribute to the stabilization of the protease state, provided that the substrate-binding exosites are exposed. In accordance to this hypothesis, the shortened venom FX activation peptide allows for productive interactions with the cofactor. As such, the zymogen activity of ptFX and hFX-ptAP could be a result of FVa binding that induces conformational changes in the protease domain allowing for Lys156-Asp194 salt bridge formation and active site maturation.

Procoagulant Enhancements of ptFV and ptFX

Collectively, our studies have uncovered several regions that are at the basis of the potent procoagulant effects of ptFV and ptFX. Previously, ptFV was shown to be synthesized in an constitutively active state as a result of the shortened B domain and the lack of the basic and acidic regions that keep human FV in an inactive procofactor state[8,88]. Here, we discovered two distinct mechanisms underlying the functional resistance to APC. First, our data indicate that stable hydrogen bond formation between the A1 and A2 domain of ptFV increases the A1-A2 domain binding affinity upon APC-mediated proteolysis which presumably prevents A2 domain dissociation. This increase in binding affinity was predominantly induced by the enhanced rigidity of the so-called A2 domain loop2 which supported stable interactions with the A1 domain. Following proteolysis, the extended ptFV A2T plays a pivotal role in the formation of the prothrombinase-like complex and conversion of prothrombin. In addition, we demonstrated that the A2T allows ptFV to circumvent the need for membrane binding. Based on the available data, we speculate that the A2T is able to provide productive interactions with ptFXa and prothrombin despite APC-mediated proteolysis or in the absence of anionic membranes. Our findings also revealed that the shortened activation peptide of ptFX induces zymogen activity and resistance to the intrinsic tenase complex. Although it remains to be determined whether venom ptFX is injected into the prey in a zymogen or protease form, the zymogen activity could elicit a procoagulant response by generating small amounts of thrombin to activate the feedback mechanisms of the coagulation cascade. Whether the resistance for

activation by the intrinsic tenase complex provides a selective advantage is unclear. Finally, a prior study revealed that the extended 99-loop in ptFX reduces the sensitivity of ptFXa for the active site inhibitors antithrombin and tissue factor pathway inhibitor (TFPI)[45,63]. Collectively, these unique structural elements provide the molecular basis for the procoagulant enhancements of ptFV and ptFX and are summarized by the schematic overview in **figure 1**.

Hemostatic System of the Elapid Snakes

Sequence analysis showed that liver-expressed and venom-expressed ptFV comprise ~96% sequence homology[89]. Intriguingly, functional characterization of the liver-expressed ptFV revealed that this FV form shares the enhanced procoagulant characteristics of venom-derived ptFV[90]. As such, these findings raise questions on the regulatory mechanisms of the coagulation cascade in Elapidae. Unfortunately, it is currently unclear whether elapidae express protein C. However, it would be of interest to evaluate the effects of snake protein C on ptFV. In our study, we observed that plasmin significantly reduced the cofactor activity of ptFVa. This might suggest that plasmin plays a physiological role in the regulation of liver-expressed ptFVa. The putative plasmin-mediated inactivation of ptFVa may occur in a later stage of hemostasis relative to the action of APC. Moreover, it has been suggested that reptiles have a primitive contact activation pathway[56-60]. We therefore speculate that FV plays a more procoagulant role in the coagulation cascade of elapidae relative to the mammalian hemostatic system in order to generate sufficient amounts of thrombin.

Liver- and venom-expressed ptFX comprise ~76% sequence homology[49], which could indicate that venom ptFX underwent evolutionary adaptations in order to circumvent negative regulatory mechanisms. This is supported by functional studies demonstrating that the extended 99-loop, unique to venom and isoform FX species in Elapidae, reduces the inhibition of FXa by antithrombin and TFPI[45,63], although inhibition experiments have not yet been performed with liver-expressed ptFX. It is possible that the activity of the liver-expressed ptFVa-ptFXa complex is mainly regulated by inhibition of ptFXa via inhibitors such as antithrombin, α2-macroglobulin, or TFPI. Furthermore, the observation that liver ptFV shares the procoagulant properties of venom ptFV[90] suggests that the activity of the liver-expressed ptFVa-ptFXa complex is confined to anionic membranes via the membrane binding requirement of liver ptFXa. As such, we hypothesize that in Elapidae, FV plays a more procoagulant role in order to overcome the premature and inefficient contact activation pathway. However, the enhanced procoagulant functions of FV might in part be averted by the regulation of liver ptFXa activity and the requirement of liver ptFXa for membrane binding.

Potential Therapeutic Implications

Our studies have at least in part uncovered the molecular mechanisms that enhance the activity of ptFV. The responsible functional regions could be implemented in novel FV variants for potential therapeutic use in bleeding disorders such as hemophilia. Hemophilia A and B are the result of a congenital defect in blood coagulation FVIII or factor IX (FIX), respectively. These bleeding discorders are effectively treated by protein replacement therapy. Unfortunately, replacement therapy may induce neutralizing inhibitory antibodies against the administered product, rendering therapy ineffective and increasing the risk of severe hemorrhagic events[91]. Disease management of inhibitor patients therefore includes the use of hemostatic bypassing agents[92]. The most commonly used bypassing agents used to treat major bleeding in hemophilia A patients with inhibitors include recombinant factors or plasma concentrates (i.e. activated prothrombin complex concentrate and recombinant activated factor VII). However, in the last decade a new class of subcutaneous non-factor products have emerged that are longer-acting relative to conventional bypassing agents[93]. Of these novel agents emicizumab has recently been approved for routine prophylaxis in hemophilia A patients with or without FVIII inhibitors[94,95]. Emicizumab is a humanized bispecific antibody that binds to both FIX(a) and FX(a) to promote FX activation in a FVIIIa-independent manner[96]. As an alternative strategy, we hypothesize that procoagulant enhancement of FV could generate novel variants that may significantly stimulate coagulation. In **chapter 3**, we demonstrated that the ptFV A2 domain loop2 engages in stable interactions with the A1 domain following APC-mediated proteolysis. As such, substitution of human loop2 for the corresponding ptFV loop2 might generate a human FV variant in which A2 domain dissociation is prevented. Previous *in vitro* and *in vivo* studies have provided evidence for a disease-modifier effect of APC-resistant FV mutations in hemophilia[97-100]. For instance, FV^{Leiden} and $FV^{Cambridge}$ showed enhanced thrombin generation in hemophilic plasma compared with wild-type FV[101,102], and FV^{leiden} was found to improve hemostasis in a murine hemophilia model[100]. Furthermore, an engineered FVa variant (^{super}FVa) comprising an A2-A3 domain disulfide bond and mutations at the APC cleavage sites Arg306, Arg506, and Arg697 was able to reduce hemophilia-related bleeding[97-99].

The ptFV A2T is an other structural element that might enhance the procoagulant activity of novel therapeutic FV variants. We demonstrated that this region is responsible for the lipid independent activity of ptFV and plays a major role in the functional resistance to APC. Importantly, these remarkable characteristics could be translated to human FV. However, the procoagulant enhancements relied on the presence of ptFXa, which likely limits the potential therapeutic use of the ptFV A2T. Alternatively,

targeting the ptFV A2T could be a viable strategy to inhibit the extreme procoagulant effects of the ptFVa-ptFXa complex upon envenomation. This would generate the first specific antidote for envenomation by brown snakes[103]. Venom-induced consumptive coagulopathy (VICC) is the most commonly observed clinical envenoming phenotype in Australia and is characterized by the activation and consumption of various coagulation factors, resulting in a markedly increased risk for bleeding[104-107]. Novel antidotes that specifically target the venom-induced procoagulant response could have a significantly enhanced potency compared to the conventional non-specific antidotes.

A potential therapeutic application for the venom-derived activation peptide is currently uncertain. Although the zymogen activity of our FX variants is several orders of magnitude lower than that of FXa, the kinetics for prothrombin conversion demonstrated that the catalytic activity can generate significant amounts of thrombin. The generation of thrombin can in turn elicit a procoagulant response by activating the feedback mechanisms of coagulation. In this way, the FX variants act in a similar fashion as factor XII and prekalikrein, which initiate the contact activation pathway in zymogen state[84,85]. Based on this principle, our chimeric FX variant could potentially be used as bypassing agent in the treatment of bleeding-related disorders. Unfortunately, the efficacy is hampered by the resistance to activation by the intrinsic tenase complex, which nullifies crucial amplication loops. Future studies could focus on the modification of the venom-derived activation peptide in order to allow intrinsic tenase-mediated FX activation while maintaining the unique zymogen activity.

Phe174-substituted FX Variants as Bypassing agent for the Direct FXa Inhibitors

Mechanistic Principles of Phe174-substituted FX Variants

In **chapter 7**, we presented Phe174-substituted FX variants that are able to counteract the anticoagulant effects of the direct FXa inhibitors. We observed that disruption of the S4 subsite significantly affected the FXa binding affinity for the inhibitors. In accordance with previous reports[63,108,109], this study confirmed the important regulatory role of the FXa S4 subsite in inhibitor binding. The crystal structure of FXa in complex with apixaban or rivaroxaban demonstrated that the P4 ring of apixaban and the morpholinone ring of rivaroxaban are sandwiched between the S4 subsite residues Tyr99 and Phe174[108,109]. These residues were shown to be crucial for the high affinity interaction and inhibitor binding stability. Furthermore, we recently demonstrated that specific modification of the S4 subsite disrupts the high affinity binding of apixaban[63]. In concordance, our kinetic data and Molecular Dynamics simulations provided additional evidence that the bypassing effect of FXa-F174 variants is mediated by a

disrupted inhibitor binding stability. Substitution of the large hydrophobic Phe174 for the smaller alanine or serine enlarges the S4 subpocket and provides a rationale for the increased inhibitor mobility. Further support for our observations comes from the Molecular Dynamics simulations performed by Qu *et al*. on the FXa-rivaroxaban X-ray structure using a modelled Phe174Ala FXa variant[110]. These Molecular Dynamics simulations revealed that the binding of rivaroxaban was affected to a greater extent by the Phe174 substitution compared to similar simulations using the FXa-apixaban structure[110]. However, in our study kinetic assessment did not reveal any differences in FXa-F174 inhibition between apixaban or rivaroxaban. This might be explained by the alternative binding mode of rivaroxaban that may occur upon disruption of the S4 subsite. In this binding mode, the morpholinone moiety of rivaroxaban shifts into the shallow S1′ pocket and forms strong interactions with Phe41, Gln61, and Gln192[110]. As a consequence, rivaroxaban occupies the essential S1 subsite in a relatively stable manner. These different rivaroxaban binding modes may ultimately result in a loss of FXa sensitivity that is similar to that observed for apixaban.

Interestingly, we observed that the ability of the FX-F174 variants to overcome the anticoagulant effects of the direct FXa inhibitors was enhanced in plasma. Our findings demonstrated that these additional effects were mediated by a decreased sensitivity for TFPI. As such, the Phe174-substituted FX variants bypass the direct FXa inhibitors via two independent mechanisms, i.e. a disrupted inhibitor binding and in part via resistance to TFPI. These results also indicate that the FXa S4 subsite is an important regulator for TFPI binding, supporting earlier reports suggesting that the hydrophobic Kunitz 2 residues Ile13 and Leu39 engage the S4 subsite residues Tyr99 and Phe174[111].

Therapeutic Potential and Comparison of Reversal Agents

Rapid reversal of the anticoagulant DOAC activity may be required in case of life-threatening hemorrhage, major trauma, emergency surgery, or DOAC overdose. Over the last years, several specific reversal agents have been generated that either act as stoichiometric bait antidotes or initiate coagulation via a bypassing mechanism. To date, andexanet alfa (andexanet) is the only FDA- and EMA-approved specific reversal agent for major bleeding events induced by the direct FXa inhibitors. Other specific reversal agents, summarized in **chapter 6**, are still in clinical or preclinical development.

A major advantage of the FX-F174 variants resides in the limited amount of protein that is needed to correct inhibitor-induced bleeding relative to the bait antidotes. Based on an average individual, 880-1760 mg andexanet is required to restore hemostasis compared to an estimated 40-130 mg of the FX-F174 variant, a 14-22-fold lower dose. This can be explained as follows. First, the mechanism of bait antidotes relies on the

sequesteration of inhibitors in order to generate free endogenous FXa. In theory, the bait antidotes sequester the inhibitors in a stoichiometric manner. However, the binding between the direct FXa inhibitors and bait antidotes is reversible and the affinity of bait antidotes for the inhibitors is usually similar, if not slightly weaker, relative to endogenous FXa. This creates an equilibrium between inhibitor-bound antidote, free inhibitor, and inhibitor-bound endogenous FXa, resulting in a persistant pool of free FXa inhibitor and inhibitor-bound FXa. As a consequence, an increased ratio between antidote and inhibitor may be required to sufficiently restore hemostasis. Conversely, the prohemostatic agents do not rely on the activity of endogenous FXa, but rather intiate coagulation in the presence of the inhibitors via a bypassing mechanism. Therefore only small amounts of inhibitor-resistant FXa would be required to restore hemostasis. Secondly, an antidote should ideally neutralize and clear the inhibitor from the circulation. Pharmacokinetics studies revealed that andexanet exhibits a terminal elimination half-life of 4 to 8 hours[112]. The relatively slow clearance of andexanet-inhibitor complexes might potentially explain the necessity for a continuous infusion of andexanet in addition to the initial bolus injection.

The prohemostatic bypassing agents that are currently in preclinical development are recombinantly-generated variants of endogenous coagulation factors. As such, these factor-based products could potentially elicit an immune response which would limit their efficacy of repeated use. However, the immunogenic potential of FXa-I16L and the FX-F174 variants might be minimal since they comprise only a single amino acid substitution. In contrast, FX-C contains an unique nine-residue insertion and substitution of surrounding residues derived from the *P. textilis* isoform FX[63] that could potentially be targeted by the immune response. Whether these variants would elicit an immune response upon administration is unpredictable. Thus far, non-neutralizing antibodies against andexanet have been detected in patients albeit with low antibody titers[113,114]. Importantly, neutralizing antibody responses against andexanet have not been reported and neither have antibodies with cross-reactivity to factor X(a) been detected[114]. Moreover, only a few patients with acquired inhibitors against FX have been reported[115], which might suggest that the immunogenic potential of FX is low.

Thrombotic complications associated with FXa inhibitor reversal remain a major concern in clinical practice. Thrombotic events occurred in approximately 10% of the patients within 30 days following andexanet treatment[113]. This is relatively high compared to the percentage of thrombotic events observed during idarucizumab-mediated reversal of dabigatran (4.8%)[116] or other anticoagulant reversal studies (3.9-7%)[117,118]. The relatively high incidence of thrombosis might be explained by the interaction of andexanet with the natural anticoagulant TFPI. Previous reports have shown that andexanet binds to

TFPI with high affinity and dose-dependently increases the thrombin peak height in normal and hemophilic plasma[119-121]. Whether andexanet is able to interact with TFPI when andexanet is bound to the direct FXa inhibitors is unlikely. However, considering the high administrated dose of andexanet, we speculate that significant amounts of the antidote circulate in free form and thus could interact with TFPI. Additionally, due to their reversible binding nature, the direct FXa inhibitors and TFPI compete for binding to andexenet and establish an equilibrium between andexanet-inhibitor and andexanet-TFPI complexes. Since the inhibitory constants of the FXa inhibitors are similar to that of TFPI[63,122,123], significant amounts of andexanet might circulate in complex with TFPI. Jourdi *et al.* circumvented these potential macromolecular interactions by generating a GLA-domainless FXa in complex with α2-macroglobulin[63,124]. Although this antidote could potentially be safer, it might prove difficult to large-scale purify or recombinantly produce α2-macroglobulin for therapeutic use.

Even though clinical data for the prohemostatic bypassing agents remain to be obtained, these agents may potentially induce thrombotic complications. Patients could be at risk for overdosis since it might prove difficult to determine adequate dosing as a consequence of individual variation in blood clotting. In addition, the therapeutic window of these reversal agents is likely narrow because small differences in dosing may significantly impact the hemostatic outcome. From a safety perspective, FXa-I16L requires FVa to induce catalytic activity towards prothrombin[62,125]. Furthermore, FXa-I16L is unlikely to interact appreciably with plasma proteins, most notably the endogenous protease inhibitors, due to its disordered active site[125] which could limit the risk for reversal agent-induced thromboembolisms. In our studies, we have generated FX variants in zymogen form. When coagulation is triggered, these factors will be activated through the intrinsic and extrinsic pathways in a similar manner as endogenous FX. Moreover, by making use of the zymogen form of FX-F174 variants, productive interactions or sequesteration by plasma proteins might be prevented. As such, these reversal agents have the potential to restore hemostasis in case of life-threatening bleeding events that are induced by the direct FXa inhibitors and might minimize the risk for thrombotic complications.

Concluding Remarks

The aim of the research in this thesis was to gain further insights into the remarkable procoagulant adaptations of *Pseudonaja textilis* venom-derived FV and FX. To achieve this goal we examined the structure-function relationships of several structural elements unique to ptFV and ptFX. A better understanding of the procoagulant enhancements of ptFV and ptFX could provide significant opportunities for the generation of novel

therapeutic agents. The FX-C and FX-F174 variants originated from research on the extended 99-loop in ptFX and provide important examples of how unique structural elements can be used for therapeutic purposes or how the exceptional functionalities increase our knowledge of important physiological processes.

Our findings in **chapters 3** and **4** strongly suggest that the ptFV A2 domain loop2 and the uniquely extended ptFV A2T are responsible for the functional resistance of ptFV to APC. Furthermore, we revealed that the ptFV A2T facilitates prothrombin conversion in the absence of membranes by enabling productive interactions with ptFXa and prothrombin. By making use of the ptFX activation peptide we showed in **chapter 5** that the human FX activation peptide comprises an essential binding site for the intrinsic tenase complex. In addition, the venom-derived activation peptide allows FVa binding, inducing structural rearrangements that allow for the maturation of the active site and thereby enable catalytic activity while in zymogen state. Finally, in **chapter 7** we generated FX variants that have the potential to serve as rescue therapeutic agents to overcome the effect of the direct FXa-inhibitors. Collectively, these studies have identified the mechanistic principles by which ptFV and ptFX have circumvented the normal regulatory mechanisms of blood clotting. Translation of these procoagulant enhancements to human coagulation proteins might provide unique opportunities towards the generation of novel therapeutic agents for bleeding disorders.

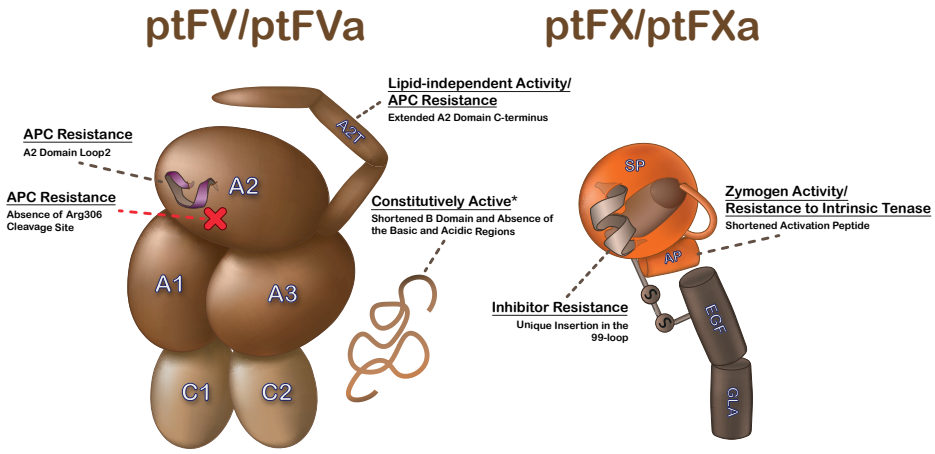


Figure 1. Schematic summary of the unique procoagulant enhancements of venom-derived ptFV and ptFX. Venom-expressed ptFV comprises several unique structural modifications that are responsible for ptFV's constitutive cofactor activity, the functional resistance to APC, and the lipid-independent cofactor activity. Furthermore, venom-expressed ptFX comprises a shortened activation peptide and insertion in the 99-loop that facilitate zymogen activity and resistance to activation by the human intrinsic tenase complex, and a reduced sensitivity for active site inhibitors, respectively. *Upon activation to ptFVa, the ptFV B domain that covalently connects the C-terminal A2 domain (A2T) with the N-terminal A3 domain is proteolyically removed. Abbreviations: A2T, A2 domain C-terminus; GLA, γ-carboxyglutamic acid domain; EGF, endothelial growth factor 1 and 2 domains; AP, activation peptide; SP, serine protease domain. S-S indicates the disulfide bond covalently linking the EGF-2 and serine protease domains.

References

1 Broos, K., Feys, H. B., De Meyer, S. F., Vanhoorelbeke, K. & Deckmyn, H. Platelets at work in primary hemostasis. *Blood Rev* **25**, 155-167, doi:10.1016/j.blre.2011.03.002 (2011).
2 Versteeg, H. H., Heemskerk, J. W., Levi, M. & Reitsma, P. H. New fundamentals in hemostasis. *Physiol Rev* **93**, 327-358, doi:10.1152/physrev.00016.2011 (2013).
3 Mann, K. G., Nesheim, M. E., Church, W. R., Haley, P. & Krishnaswamy, S. Surface-dependent reactions of the vitamin K-dependent enzyme complexes. *Blood* **76**, 1-16 (1990).
4 Schreuder, M., Reitsma, P. H. & Bos, M. H. A. Blood coagulation factor Va's key interactive residues and regions for prothrombinase assembly and prothrombin binding. *J Thromb Haemost* **17**, 1229-1239, doi:10.1111/jth.14487 (2019).
5 Dahlback, B. & Villoutreix, B. O. The anticoagulant protein C pathway. *FEBS Lett* **579**, 3310-3316, doi:10.1016/j.febslet.2005.03.001 (2005).
6 Mast, A. E. Tissue Factor Pathway Inhibitor: Multiple Anticoagulant Activities for a Single Protein. *Arterioscler Thromb Vasc Biol* **36**, 9-14, doi:10.1161/ATVBAHA.115.305996 (2016).
7 Rao, V. S., Swarup, S. & Kini, R. M. The nonenzymatic subunit of pseutarin C, a prothrombin activator from eastern brown snake (Pseudonaja textilis) venom, shows structural similarity to mammalian coagulation factor V. *Blood* **102**, 1347-1354, doi:10.1182/blood-2002-12-3839 (2003).
8 Bos, M. H. *et al.* Venom factor V from the common brown snake escapes hemostatic regulation through procoagulant adaptations. *Blood* **114**, 686-692, doi:10.1182/blood-2009-02-202663 (2009).
9 Fry, B. G. Structure-function properties of venom components from Australian elapids. *Toxicon* **37**, 11-32, doi:10.1016/s0041-0101(98)00125-1 (1999).
10 Rao, V. S. & Kini, R. M. Pseutarin C, a prothrombin activator from Pseudonaja textilis venom: its structural and functional similarity to mammalian coagulation factor Xa-Va complex. *Thrombosis and haemostasis* **88**, 611-619 (2002).
11 Kini, R. M. The intriguing world of prothrombin activators from snake venom. *Toxicon* **45**, 1133-1145, doi:10.1016/j.toxicon.2005.02.019 (2005).
12 Mann, K. G., Hockin, M. F., Begin, K. J. & Kalafatis, M. Activated protein C cleavage of factor Va leads to dissociation of the A2 domain. *J Biol Chem* **272**, 20678-20683 (1997).
13 Bertina, R. M. *et al.* Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* **369**, 64-67, doi:10.1038/369064a0 (1994).
14 Griffin, J. H. *et al.* Activated protein C resistance: molecular mechanisms. *Thrombosis and haemostasis* **74**, 444-448 (1995).
15 Norstrom, E., Thorelli, E. & Dahlback, B. Functional characterization of recombinant FV Hong Kong and FV Cambridge. *Blood* **100**, 524-530, doi:10.1182/blood-2002-02-0343 (2002).
16 Steen, M. *et al.* Functional characterization of factor V-Ile359Thr: a novel mutation associated with thrombosis. *Blood* **103**, 3381-3387, doi:10.1182/blood-2003-06-2092 (2004).
17 Castaman, G., Lunghi, B., Missiaglia, E., Bernardi, F. & Rodeghiero, F. Phenotypic homozygous activated protein C resistance associated with compound heterozygosity for Arg506Gln (factor V Leiden) and His1299Arg substitutions in factor V. *Br J Haematol* **99**, 257-261, doi:10.1046/j.1365-2141.1997.3993213.x (1997).
18 Yamazaki, T., Nicolaes, G. A., Sorensen, K. W. & Dahlback, B. Molecular basis of quantitative factor V deficiency associated with factor V R2 haplotype. *Blood* **100**, 2515-2521, doi:10.1182/blood.V100.7.2515 (2002).
19 Adams, T. E., Hockin, M. F., Mann, K. G. & Everse, S. J. The crystal structure of activated protein C-inactivated bovine factor Va: Implications for cofactor function. *Proc Natl Acad Sci U S A* **101**, 8918-8923, doi:10.1073/pnas.0403072101 (2004).

20 Orban, T., Kalafatis, M. & Gogonea, V. Completed three-dimensional model of human coagulation factor va. Molecular dynamics simulations and structural analyses. *Biochemistry* **44**, 13082-13090, doi:10.1021/bi050891t (2005).
21 Gale, A. J., Xu, X., Pellequer, J. L., Getzoff, E. D. & Griffin, J. H. Interdomain engineered disulfide bond permitting elucidation of mechanisms of inactivation of coagulation factor Va by activated protein C. *Protein Sci* **11**, 2091-2101, doi:10.1110/ps.0210002 (2002).
22 Calzavarini, S. *et al.* Molecular basis of coagulation factor V deficiency caused by the R1698W inter-domain mutation. *Thrombosis and haemostasis* **110**, 31-38, doi:10.1160/TH12-10-0780 (2013).
23 Steen, M., Miteva, M., Villoutreix, B. O., Yamazaki, T. & Dahlback, B. Factor V New Brunswick: Ala221Val associated with FV deficiency reproduced in vitro and functionally characterized. *Blood* **102**, 1316-1322, doi:10.1182/blood-2003-01-0116 (2003).
24 Wilhelm, A. R. *et al.* Activated Protein C has a Regulatory Role in Factor VIII Function. *Blood* **137**, 2532-2543, doi:10.1182/blood.2020007562 (2021).
25 Fay, P. J., Smudzin, T. M. & Walker, F. J. Activated protein C-catalyzed inactivation of human factor VIII and factor VIIIa. Identification of cleavage sites and correlation of proteolysis with cofactor activity. *J Biol Chem* **266**, 20139-20145 (1991).
26 Lollar, P. & Parker, C. G. pH-dependent denaturation of thrombin-activated porcine factor VIII. *J Biol Chem* **265**, 1688-1692 (1990).
27 Fay, P. J., Haidaris, P. J. & Smudzin, T. M. Human factor VIIIa subunit structure. Reconstruction of factor VIIIa from the isolated A1/A3-C1-C2 dimer and A2 subunit. *J Biol Chem* **266**, 8957-8962 (1991).
28 Bloem, E. *et al.* Mass spectrometry-assisted study reveals that lysine residues 1967 and 1968 have opposite contribution to stability of activated factor VIII. *J Biol Chem* **287**, 5775-5783, doi:10.1074/jbc.M111.308627 (2012).
29 Wakabayashi, H., Monaghan, M. & Fay, P. J. Cofactor activity in factor VIIIa of the blood clotting pathway is stabilized by an interdomain bond between His281 and Ser524 formed in factor VIII. *J Biol Chem* **289**, 14020-14029, doi:10.1074/jbc.M114.550566 (2014).
30 Wakabayashi, H., Griffiths, A. E. & Fay, P. J. Combining mutations of charged residues at the A2 domain interface enhances factor VIII stability over single point mutations. *J Thromb Haemost* **7**, 438-444, doi:10.1111/j.1538-7836.2008.03256.x (2009).
31 Monaghan, M., Wakabayashi, H., Griffiths, A., Wintermute, J. & Fay, P. J. Enhanced factor VIIIa stability of A2 domain interface variants results from an increased apparent affinity for the A2 subunit. Results from an increased apparent affinity for the A2 subunit. *Thrombosis and haemostasis* **112**, 495-502, doi:10.1160/TH14-01-0086 (2014).
32 Celie, P. H. *et al.* Substitution of Arg527 and Arg531 in factor VIII associated with mild haemophilia A: characterization in terms of subunit interaction and cofactor function. *Br J Haematol* **106**, 792-800, doi:10.1046/j.1365-2141.1999.01590.x (1999).
33 Monaghan, M., Wakabayashi, H., Griffiths, A. E. & Fay, P. J. Stabilizing interactions between D666-S1787 and T657-Y1792 at the A2-A3 interface support factor VIIIa stability in the blood clotting pathway. *J Thromb Haemost* **14**, 1021-1030, doi:10.1111/jth.13292 (2016).
34 Pipe, S. W., Saenko, E. L., Eickhorst, A. N., Kemball-Cook, G. & Kaufman, R. J. Hemophilia A mutations associated with 1-stage/2-stage activity discrepancy disrupt protein-protein interactions within the triplicated A domains of thrombin-activated factor VIIIa. *Blood* **97**, 685-691, doi:10.1182/blood.v97.3.685 (2001).
35 Bloem, E., Meems, H., van den Biggelaar, M., Mertens, K. & Meijer, A. B. A3 domain region 1803-1818 contributes to the stability of activated factor VIII and includes a binding site for activated factor IX. *J Biol Chem* **288**, 26105-26111, doi:10.1074/jbc.M113.500884 (2013).

36 Pomowski, A., Ustok, F. I. & Huntington, J. A. Homology model of human prothrombinase based on the crystal structure of Pseutarin C. *Biological chemistry* **395**, 1233-1241, doi:10.1515/hsz-2014-0165 (2014).
37 Shim, J. Y., Lee, C. J., Wu, S. & Pedersen, L. G. A model for the unique role of factor Va A2 domain extension in the human ternary thrombin-generating complex. *Biophysical chemistry* **199**, 46-50, doi:10.1016/j.bpc.2015.02.003 (2015).
38 Kalafatis, M., Beck, D. O. & Mann, K. G. Structural requirements for expression of factor Va activity. *J Biol Chem* **278**, 33550-33561, doi:10.1074/jbc.M303153200 (2003).
39 Hirbawi, J., Bukys, M. A., Barhoover, M. A., Erdogan, E. & Kalafatis, M. Role of the acidic hirudin-like COOH-terminal amino acid region of factor Va heavy chain in the enhanced function of prothrombinase. *Biochemistry* **47**, 7963-7974, doi:10.1021/bi800593k (2008).
40 Bukys, M. A., Kim, P. Y., Nesheim, M. E. & Kalafatis, M. A control switch for prothrombinase: characterization of a hirudin-like pentapeptide from the COOH terminus of factor Va heavy chain that regulates the rate and pathway for prothrombin activation. *J Biol Chem* **281**, 39194-39204, doi:10.1074/jbc.M604482200 (2006).
41 Beck, D. O., Bukys, M. A., Singh, L. S., Szabo, K. A. & Kalafatis, M. The contribution of amino acid region ASP695-TYR698 of factor V to procofactor activation and factor Va function. *J Biol Chem* **279**, 3084-3095, doi:10.1074/jbc.M306850200 (2004).
42 Bakker, H. M. *et al.* Functional properties of human factor Va lacking the Asp683-Arg709 domain of the heavy chain. *J Biol Chem* **269**, 20662-20667 (1994).
43 Kalafatis, M. & Mann, K. G. The role of the membrane in the inactivation of factor va by plasmin. Amino acid region 307-348 of factor V plays a critical role in factor Va cofactor function. *J Biol Chem* **276**, 18614-18623, doi:10.1074/jbc.M007134200 (2001).
44 Toso, R. & Camire, R. M. Role of Hirudin-like factor Va heavy chain sequences in prothrombinase function. *J Biol Chem* **281**, 8773-8779, doi:10.1074/jbc.M511419200 (2006).
45 Lechtenberg, B. C. *et al.* Crystal structure of the prothrombinase complex from the venom of Pseudonaja textilis. *Blood* **122**, 2777-2783, doi:10.1182/blood-2013-06-511733 (2013).
46 Qureshi, S. H., Yang, L., Manithody, C. & Rezaie, A. R. Membrane-dependent interaction of factor Xa and prothrombin with factor Va in the prothrombinase complex. *Biochemistry* **48**, 5034-5041, doi:10.1021/bi900240g (2009).
47 Corral-Rodriguez, M. A., Bock, P. E., Hernandez-Carvajal, E., Gutierrez-Gallego, R. & Fuentes-Prior, P. Structural basis of thrombin-mediated factor V activation: the Glu666-Glu672 sequence is critical for processing at the heavy chain-B domain junction. *Blood* **117**, 7164-7173, doi:10.1182/blood-2010-10-315309 (2011).
48 Rao, V. S., Swarup, S. & Manjunatha Kini, R. The catalytic subunit of pseutarin C, a group C prothrombin activator from the venom of Pseudonaja textilis, is structurally similar to mammalian blood coagulation factor Xa. *Thrombosis and haemostasis* **92**, 509-521, doi:10.1160/TH04-03-0144 (2004).
49 Reza, M. A., Minh Le, T. N., Swarup, S. & Manjunatha Kini, R. Molecular evolution caught in action: gene duplication and evolution of molecular isoforms of prothrombin activators in Pseudonaja textilis (brown snake). *J Thromb Haemost* **4**, 1346-1353, doi:10.1111/j.1538-7836.2006.01969.x (2006).
50 Baugh, R. J. & Krishnaswamy, S. Role of the activation peptide domain in human factor X activation by the extrinsic Xase complex. *J Biol Chem* **271**, 16126-16134, doi:10.1074/jbc.271.27.16126 (1996).
51 Yang, L., Manithody, C. & Rezaie, A. R. Functional role of O-linked and N-linked glycosylation sites present on the activation peptide of factor X. *J Thromb Haemost* **7**, 1696-1702, doi:10.1111/j.1538-7836.2009.03578.x (2009).

52 Pinotti, M. *et al.* Reduced activation of the Gla19Ala FX variant via the extrinsic coagulation pathway results in symptomatic CRMred FX deficiency. *Thrombosis and haemostasis* **88**, 236-241 (2002).
53 Thiec, F., Cherel, G. & Christophe, O. D. Role of the Gla and first epidermal growth factor-like domains of factor X in the prothrombinase and tissue factor-factor VIIa complexes. *J Biol Chem* **278**, 10393-10399, doi:10.1074/jbc.M212144200 (2003).
54 Chattopadhyay, A. & Fair, D. S. Molecular recognition in the activation of human blood coagulation factor X. *J Biol Chem* **264**, 11035-11043 (1989).
55 Duffy, E. J. & Lollar, P. Intrinsic pathway activation of factor X and its activation peptide-deficient derivative, factor Xdes-143-191. *J Biol Chem* **267**, 7821-7827 (1992).
56 Doolittle, R. F. & Feng, D. F. Reconstructing the evolution of vertebrate blood coagulation from a consideration of the amino acid sequences of clotting proteins. *Cold Spring Harb Symp Quant Biol* **52**, 869-874 (1987).
57 Jiang, Y. & Doolittle, R. F. The evolution of vertebrate blood coagulation as viewed from a comparison of puffer fish and sea squirt genomes. *Proc Natl Acad Sci U S A* **100**, 7527-7532, doi:10.1073/pnas.0932632100 (2003).
58 Ponczek, M. B., Gailani, D. & Doolittle, R. F. Evolution of the contact phase of vertebrate blood coagulation. *J Thromb Haemost* **6**, 1876-1883, doi:10.1111/j.1538-7836.2008.03143.x (2008).
59 Davidson, C. J., Tuddenham, E. G. & McVey, J. H. 450 million years of hemostasis. *J Thromb Haemost* **1**, 1487-1494 (2003).
60 Joseph, J. S., Chung, M. C., Mirtschin, P. J. & Kini, R. M. Effect of snake venom procoagulants on snake plasma: implications for the coagulation cascade of snakes. *Toxicon* **40**, 175-183 (2002).
61 Guinto, E. R. & Esmon, C. T. Loss of prothrombin and of factor Xa-factor Va interactions upon inactivation of factor Va by activated protein C. *J Biol Chem* **259**, 13986-13992 (1984).
62 Toso, R., Zhu, H. & Camire, R. M. The conformational switch from the factor X zymogen to protease state mediates exosite expression and prothrombinase assembly. *J Biol Chem* **283**, 18627-18635, doi:10.1074/jbc.M802205200 (2008).
63 Verhoef, D. *et al.* Engineered factor Xa variants retain procoagulant activity independent of direct factor Xa inhibitors. *Nat Commun* **8**, 528, doi:10.1038/s41467-017-00647-9 (2017).
64 Pozzi, N., Chen, Z., Pelc, L. A., Shropshire, D. B. & Di Cera, E. The linker connecting the two kringles plays a key role in prothrombin activation. *Proc Natl Acad Sci U S A* **111**, 7630-7635, doi:10.1073/pnas.1403779111 (2014).
65 Ahnstrom, J., Gierula, M., Temenu, J., Laffan, M. A. & Lane, D. A. Partial rescue of naturally occurring active site factor X variants through decreased inhibition by tissue factor pathway inhibitor and antithrombin. *J Thromb Haemost* **18**, 136-150, doi:10.1111/jth.14627 (2020).
66 Gupta, R., Jung, E. & Brunak, S. Prediction of N-glycosylation Sites in Human Proteins. **Available online: http://www.cbs.dtu.dk/services/NetNGlyc/ (accessed on April 2018).** (2004).
67 Steentoft, C. *et al.* Precision mapping of the human O-GalNAc glycoproteome through SimpleCell technology. *EMBO J* **32**, 1478-1488, doi:10.1038/emboj.2013.79 (2013).
68 Iino, M. *et al.* The role of human factor X activation peptide in activation of factor X by factor IXa. *J Biochem* **116**, 335-340, doi:10.1093/oxfordjournals.jbchem.a124528 (1994).
69 Inoue, K. & Morita, T. Identification of O-linked oligosaccharide chains in the activation peptides of blood coagulation factor X. The role of the carbohydrate moieties in the activation of factor X. *Eur J Biochem* **218**, 153-163 (1993).
70 Sinha, U. & Wolf, D. L. Carbohydrate residues modulate the activation of coagulation factor X. *J Biol Chem* **268**, 3048-3051 (1993).
71 Bode, W. & Huber, R. Induction of the bovine trypsinogen-trypsin transition by peptides sequentially similar to the N-terminus of trypsin. *FEBS Lett* **68**, 231-236, doi:10.1016/0014-5793(76)80443-7 (1976).

72 Bode, W. The transition of bovine trypsinogen to a trypsin-like state upon strong ligand binding. II. The binding of the pancreatic trypsin inhibitor and of isoleucine-valine and of sequentially related peptides to trypsinogen and to p-guanidinobenzoate-trypsinogen. *J Mol Biol* **127**, 357-374, doi:10.1016/0022-2836(79)90227-4 (1979).
73 Verhamme, I. M., Panizzi, P. R. & Bock, P. E. Pathogen activators of plasminogen. *J Thromb Haemost* **13 Suppl 1**, S106-114, doi:10.1111/jth.12939 (2015).
74 Friedrich, R. *et al.* Staphylocoagulase is a prototype for the mechanism of cofactor-induced zymogen activation. *Nature* **425**, 535-539, doi:10.1038/nature01962 (2003).
75 Kroh, H. K., Panizzi, P. & Bock, P. E. Von Willebrand factor-binding protein is a hysteretic conformational activator of prothrombin. *Proc Natl Acad Sci U S A* **106**, 7786-7791, doi:10.1073/pnas.0811750106 (2009).
76 Huber, R. & Bode, W. Structural Basis of the Activation and Action of Trypsin. *Accounts of Chemical Research* **11**, 114-122 (1978).
77 Pozzi, N., Vogt, A. D., Gohara, D. W. & Di Cera, E. Conformational selection in trypsin-like proteases. *Curr Opin Struct Biol* **22**, 421-431, doi:10.1016/j.sbi.2012.05.006 (2012).
78 Chakraborty, P., Acquasaliente, L., Pelc, L. A. & Di Cera, E. Interplay between conformational selection and zymogen activation. *Sci Rep* **8**, 4080, doi:10.1038/s41598-018-21728-9 (2018).
79 Ranby, M. Studies on the kinetics of plasminogen activation by tissue plasminogen activator. *Biochim Biophys Acta* **704**, 461-469, doi:10.1016/0167-4838(82)90068-1 (1982).
80 Tate, K. M. *et al.* Functional role of proteolytic cleavage at arginine-275 of human tissue plasminogen activator as assessed by site-directed mutagenesis. *Biochemistry* **26**, 338-343, doi:10.1021/bi00376a002 (1987).
81 Ranby, M., Bergsdorf, N. & Nilsson, T. Enzymatic properties of the one- and two-chain form of tissue plasminogen activator. *Thromb Res* **27**, 175-183, doi:10.1016/0049-3848(82)90197-9 (1982).
82 Tachias, K. & Madison, E. L. Converting tissue type plasminogen activator into a zymogen. Important role of Lys156. *J Biol Chem* **272**, 28-31, doi:10.1074/jbc.272.1.28 (1997).
83 Renatus, M. *et al.* Lysine 156 promotes the anomalous proenzyme activity of tPA: X-ray crystal structure of single-chain human tPA. *EMBO J* **16**, 4797-4805, doi:10.1093/emboj/16.16.4797 (1997).
84 Ivanov, I. *et al.* Protease activity in single-chain prekallikrein. *Blood* **135**, 558-567, doi:10.1182/blood.2019002224 (2020).
85 Ivanov, I. *et al.* Proteolytic properties of single-chain factor XII: a mechanism for triggering contact activation. *Blood* **129**, 1527-1537, doi:10.1182/blood-2016-10-744110 (2017).
86 Stubbs, M. T., Renatus, M. & Bode, W. An active zymogen: unravelling the mystery of tissue-type plasminogen activator. *Biological chemistry* **379**, 95-103, doi:10.1515/bchm.1998.379.2.95 (1998).
87 Pathak, M. *et al.* Coagulation factor XII protease domain crystal structure. *J Thromb Haemost* **13**, 580-591, doi:10.1111/jth.12849 (2015).
88 Bos, M. H. & Camire, R. M. A bipartite autoinhibitory region within the B-domain suppresses function in factor V. *J Biol Chem* **287**, 26342-26351, doi:10.1074/jbc.M112.377168 (2012).
89 Minh Le, T. N., Reza, M. A., Swarup, S. & Kini, R. M. Gene duplication of coagulation factor V and origin of venom prothrombin activator in Pseudonaja textilis snake. *Thrombosis and haemostasis* **93**, 420-429, doi:10.1160/TH04-11-0707 (2005).
90 Verhoef, D. *et al.* Functional implications of the unique disulfide bond in venom factor V from the Australian common brown snake Pseudonaja textilis. *Toxin Reviews* **33**, 37-41, doi:10.3109/15569543.2013.844712 (2014).
91 Wight, J. & Paisley, S. The epidemiology of inhibitors in haemophilia A: a systematic review. *Haemophilia* **9**, 418-435, doi:10.1046/j.1365-2516.2003.00780.x (2003).

92 Shapiro, A. D., Mitchell, I. S. & Nasr, S. The future of bypassing agents for hemophilia with inhibitors in the era of novel agents. *J Thromb Haemost* **16**, 2362-2374, doi:10.1111/jth.14296 (2018).
93 Nogami, K. & Shima, M. New therapies using nonfactor products for patients with hemophilia and inhibitors. *Blood* **133**, 399-406, doi:10.1182/blood-2018-07-820712 (2019).
94 Oldenburg, J. *et al.* Emicizumab Prophylaxis in Hemophilia A with Inhibitors. *N Engl J Med* **377**, 809-818, doi:10.1056/NEJMoa1703068 (2017).
95 Mahlangu, J. *et al.* Emicizumab Prophylaxis in Patients Who Have Hemophilia A without Inhibitors. *N Engl J Med* **379**, 811-822, doi:10.1056/NEJMoa1803550 (2018).
96 Kitazawa, T. *et al.* Factor VIIIa-mimetic cofactor activity of a bispecific antibody to factors IX/IXa and X/Xa, emicizumab, depends on its ability to bridge the antigens. *Thrombosis and haemostasis* **117**, 1348-1357, doi:10.1160/TH17-01-0030 (2017).
97 von Drygalski, A. *et al.* An engineered factor Va prevents bleeding induced by anticoagulant wt activated protein C. *PLoS One* **9**, e104304, doi:10.1371/journal.pone.0104304 (2014).
98 von Drygalski, A. *et al.* Improved hemostasis in hemophilia mice by means of an engineered factor Va mutant. *J Thromb Haemost* **12**, 363-372, doi:10.1111/jth.12489 (2014).
99 Bhat, V., von Drygalski, A., Gale, A. J., Griffin, J. H. & Mosnier, L. O. Improved coagulation and haemostasis in haemophilia with inhibitors by combinations of superFactor Va and Factor VIIa. *Thrombosis and haemostasis* **115**, 551-561, doi:10.1160/TH15-07-0525 (2016).
100 Schlachterman, A. *et al.* Factor V Leiden improves in vivo hemostasis in murine hemophilia models. *J Thromb Haemost* **3**, 2730-2737, doi:10.1111/j.1538-7836.2005.01639.x (2005).
101 Bos, M. H., Meijerman, D. W., van der Zwaan, C. & Mertens, K. Does activated protein C-resistant factor V contribute to thrombin generation in hemophilic plasma? *J Thromb Haemost* **3**, 522-530, doi:10.1111/j.1538-7836.2005.01181.x (2005).
102 van 't Veer, C. *et al.* An in vitro analysis of the combination of hemophilia A and factor V(LEIDEN). *Blood* **90**, 3067-3072 (1997).
103 Zdenek, C. N. *et al.* Coagulotoxic effects by brown snake (Pseudonaja) and taipan (Oxyuranus) venoms, and the efficacy of a new antivenom. *Toxicol In Vitro* **58**, 97-109, doi:10.1016/j.tiv.2019.03.031 (2019).
104 Berling, I. & Isbister, G. K. Hematologic effects and complications of snake envenoming. *Transfus Med Rev* **29**, 82-89, doi:10.1016/j.tmrv.2014.09.005 (2015).
105 Isbister, G. K. Procoagulant snake toxins: laboratory studies, diagnosis, and understanding snakebite coagulopathy. *Semin Thromb Hemost* **35**, 93-103, doi:10.1055/s-0029-1214152 (2009).
106 Johnston, C. I. *et al.* The Australian Snakebite Project, 2005-2015 (ASP-20). *Med J Aust* **207**, 119-125, doi:10.5694/mja17.00094 (2017).
107 Masci, P. P., Rowe, E. A., Whitaker, A. N. & de Jersey, J. Fibrinolysis as a feature of disseminated intravascular coagulation (DIC) after Pseudonaja textilis textilis envenomation. *Thromb Res* **59**, 859-870, doi:10.1016/0049-3848(90)90399-w (1990).
108 Pinto, D. J. *et al.* Discovery of 1-(4-methoxyphenyl)-7-oxo-6-(4-(2-oxopiperidin-1-yl)phenyl)-4,5,6,7-tetrahydro-1H -pyrazolo[3,4-c]pyridine-3-carboxamide (apixaban, BMS-562247), a highly potent, selective, efficacious, and orally bioavailable inhibitor of blood coagulation factor Xa. *J Med Chem* **50**, 5339-5356, doi:10.1021/jm070245n (2007).
109 Roehrig, S. *et al.* Discovery of the novel antithrombotic agent 5-chloro-N-({(5S)-2-oxo-3-[4-(3-oxomorpholin-4-yl)phenyl]-1,3-oxazolidin-5-yl}methyl)thiophene- 2-carboxamide (BAY 59-7939): an oral, direct factor Xa inhibitor. *J Med Chem* **48**, 5900-5908, doi:10.1021/jm050101d (2005).
110 Qu, S. Y. *et al.* An unexpected dynamic binding mode between coagulation factor X and Rivaroxaban reveals importance of flexibility in drug binding. *Chem Biol Drug Des* **94**, 1664-1671, doi:10.1111/cbdd.13568 (2019).

111 Burgering, M. J. *et al.* The second Kunitz domain of human tissue factor pathway inhibitor: cloning, structure determination and interaction with factor Xa. *J Mol Biol* **269**, 395-407, doi:10.1006/jmbi.1997.1029 (1997).
112 Siegal, D. *et al.* Safety, pharmacokinetics, and reversal of apixaban anticoagulation with andexanet alfa. *Blood Adv* **1**, 1827-1838, doi:10.1182/bloodadvances.2017007112 (2017).
113 Connolly, S. J. *et al.* Full Study Report of Andexanet Alfa for Bleeding Associated with Factor Xa Inhibitors. *N Engl J Med* **380**, 1326-1335, doi:10.1056/NEJMoa1814051 (2019).
114 Siegal, D. M. *et al.* Andexanet Alfa for the Reversal of Factor Xa Inhibitor Activity. *N Engl J Med* **373**, 2413-2424, doi:10.1056/NEJMoa1510991 (2015).
115 Menegatti, M. & Peyvandi, F. Factor X deficiency. *Semin Thromb Hemost* **35**, 407-415, doi:10.1055/s-0029-1225763 (2009).
116 Pollack, C. V., Jr. *et al.* Idarucizumab for Dabigatran Reversal - Full Cohort Analysis. *N Engl J Med* **377**, 431-441, doi:10.1056/NEJMoa1707278 (2017).
117 Sarode, R. *et al.* Efficacy and safety of a 4-factor prothrombin complex concentrate in patients on vitamin K antagonists presenting with major bleeding: a randomized, plasma-controlled, phase IIIb study. *Circulation* **128**, 1234-1243, doi:10.1161/CIRCULATIONAHA.113.002283 (2013).
118 Goldstein, J. N. *et al.* Four-factor prothrombin complex concentrate versus plasma for rapid vitamin K antagonist reversal in patients needing urgent surgical or invasive interventions: a phase 3b, open-label, non-inferiority, randomised trial. *Lancet* **385**, 2077-2087, doi:10.1016/S0140-6736(14)61685-8 (2015).
119 Ersayin, A. *et al.* Catalytically inactive Gla-domainless factor Xa binds to TFPI and restores ex vivo coagulation in hemophilia plasma. *Haematologica* **102**, e483-e485, doi:10.3324/haematol.2017.174037 (2017).
120 Marlu, R. & Polack, B. Gla-domainless factor Xa: molecular bait to bypass a blocked tenase complex. *Haematologica* **97**, 1165-1172, doi:10.3324/haematol.2011.055699 (2012).
121 Lu, G., Lin, J. P., Curnutte, J. T. & Conley, P. B. Abstract: Effect of Andexanet-TFPI Interaction on in Vitro Thrombin Formation and Coagulation Markers in the TF-Pathway. *Blood* **130**, 629 (2017).
122 Huang, Z. F., Wun, T. C. & Broze, G. J., Jr. Kinetics of factor Xa inhibition by tissue factor pathway inhibitor. *J Biol Chem* **268**, 26950-26955 (1993).
123 Hackeng, T. M., Maurissen, L. F., Castoldi, E. & Rosing, J. Regulation of TFPI function by protein S. *J Thromb Haemost* **7 Suppl 1**, 165-168, doi:10.1111/j.1538-7836.2009.03363.x (2009).
124 Jourdi, G. *et al.* FXa-alpha2-Macroglobulin Complex Neutralizes Direct Oral Anticoagulants Targeting FXa In Vitro and In Vivo. *Thrombosis and haemostasis* **118**, 1535-1544, doi:10.1055/s-0038-1667014 (2018).
125 Bunce, M. W., Toso, R. & Camire, R. M. Zymogen-like factor Xa variants restore thrombin generation and effectively bypass the intrinsic pathway in vitro. *Blood* **117**, 290-298, doi:10.1182/blood-2010-08-300756 (2011).