Tissue factor pathway inhibitor

T.M. Hackeng

Department of Biochemistry, Cardiovascular Research Institute Maastricht (CARIM), University Maastricht, the Netherlands

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The tissue factor pathway inhibitor (TFPI) pathway represents an essential negative regulatory mechanism on thrombin generation in plasma. TFPI is a multimodular Kunitz-type inhibitor that first needs to interact with activated factor X before inhibiting the complex of tissue factor and factor VIIa, the physiological activator complex of blood coagulation. More recently, it has become clear that vitamin K-dependent protein S acts as a cofactor for TFPI in stimulating the initial interaction with factor Xa. TFPI and protein S form a complex in plasma, which likely stabilizes TFPI, and as a result, protein S and TFPI covariate in plasma in health and disease. As TFPI and protein S are constitutively active in plasma, an effective anticoagulant barrier against procoagulant activity is provided. Both protein S and TFPI deficiencies are associated with an increased risk for venous thrombosis, and part of the thrombotic risk associated with protein S deficiency might well be explained by accompanying low levels of TFPI.

Blood coagulation

Blood coagulation proceeds through a tightly regulated cascade system that is triggered by a small stimulus causing activation of a coagulation zymogen into an active enzyme by selective peptide bond cleavages. Subsequently, the activated enzyme with its designated cofactor will activate the next coagulation factor, which again assembles with a cofactor and activates the following coagulation factor and so on, until by sequential amplification at each step, a burst of thrombin is generated.

The initiation event occurs when tissue factor comes into contact with blood. Traces of activated factor VII (FVIIa) in blood plasma bind to tissue factor generating the extrinsic tenase complex (TF/FVIIa), which subsequently activates zymogens factor X (FX) and IX (FIX). FXa and its cofactor FVa then form the prothrombinase complex (FVa/FXa) that is the central prothrombin converting complex of the blood coagulation (Figure 1). The initial thrombin generation by prothrombinase, however, is not enough to polymerize fibrinogen effectively but governs major positive feedback reactions through activation of platelets and factors XI, VIII, and V. The activation of FXI and FVIII by thrombin together with FIX activation by FXIa and extrinsic tenase marks the propagation of coagulation, leading to additional prothrombinase and thrombin formation sufficient to generate a fibrin clot (Figure 1).

Natural anticoagulants: restoring the balance

Procoagulant responses are limited to the site of injury by the presence of several inhibitors and negative feedback systems. Inhibition of active coagulation enzymes by the serine protease inhibitors (serpins) antithrombin (AT),[1] heparin cofactor II,[2] and α_1-antitrypsin (α_1AT)[3] eliminates free enzymes from plasma, preventing downstream activation of coagulation (Figure 1). In addition, the tissue factor pathway inhibitor (TFPI) regulates the initiation of coagulation by inhibiting both factor Xa (FXa) and the phospholipid-bound complex of tissue factor and factor VIIa (TF-FVIIa).[4] Lastly, traces of thrombin bound to endothelial cell receptor thrombomodulin loose their procoagulant properties and can activate (endothelial cell protein C receptor-bound) protein C. Subsequently, APC will inactivate cofactors FVIIIa[5] and FVa[6] of blood coagulation with the help of its non-enzymatic cofactor protein S,[7] shutting down the intrinsic tenase complex and the prothrombinase complex, calling a halt to thrombin generation, and restoring the haemostatic balance.[8–10]

Tissue factor pathway inhibitor TFPI

TFPI is a 276 amino acid glycoprotein from the family of Kunitz-type inhibitors. It consists of a negatively charged N-terminus, three consecutive Kunitz-domains, and a positively charged C-terminal tail (Figure 2).[11] TFPI contains several N-linked and O-linked carbohydrate chains, which add approximately 10 kDa to the average amino acid backbone mass of 31,932 Da. TFPI is mainly synthesized in endothelial cells,[12] and each of its structural elements have separate functions in the mechanism of anticoagulant action of TFPI. It was reported that Ser2 in the positively-charged N-terminus is involved in phosphorylation of TFPI,[13] that the first Kunitz-domain binds to

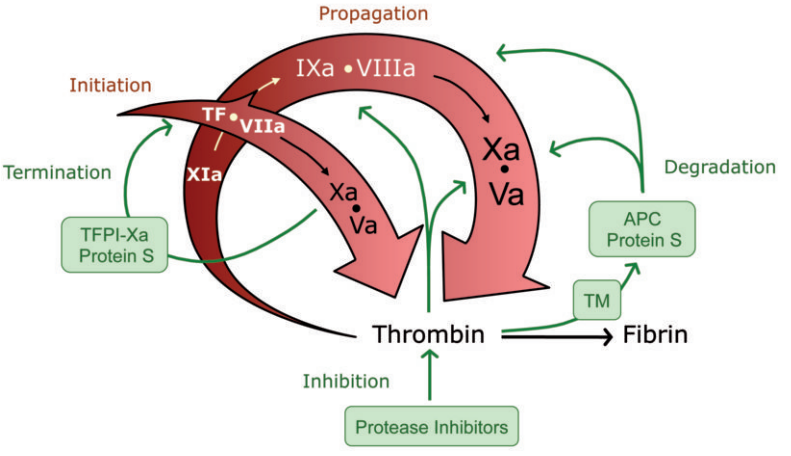


Figure 1. Blood coagulation and its regulation. *Initiation*: Clotting is initiated by a tissue factor exposed to plasma that subsequently recruits factor VIIa (VIIa) from the circulation. The TF/FVIIa complex activates factor X (Xa) and factor IX (IXa). Xa and factor Va form the prothrombinase complex that generates the first traces of thrombin. *Propagation*: A small amount of thrombin is not yet sufficient to generate fibrin but instead offers a positive feedback by activation of platelets, factors XI (XIa); more V (Va), and VIII (VIIIa). XIa generates more IXa that together with VIIIa, form the intrinsic tenase. The intrinsic tenase IXa/VIIIa generates more Xa, which together with Va, form more prothrombinase, resulting in a burst of thrombin generation and a subsequent fibrin clot. *Termination*: As soon as traces of Xa are generated, extrinsic initiator TF/VIIa is shut down by a TFPI/Xa/protein S-dependent process. As a result, clotting now becomes dependent on the propagation loop. *Inhibition*: Downstream enzyme activity is inhibited by protease inhibitors to prevent systemic coagulation activation. *Degradation*: Thrombin/thrombomodulin (TM) activates protein C that proteolytically inactivates Va and VIIIa in a protein S-dependent manner, restoring the hemostatic balance.

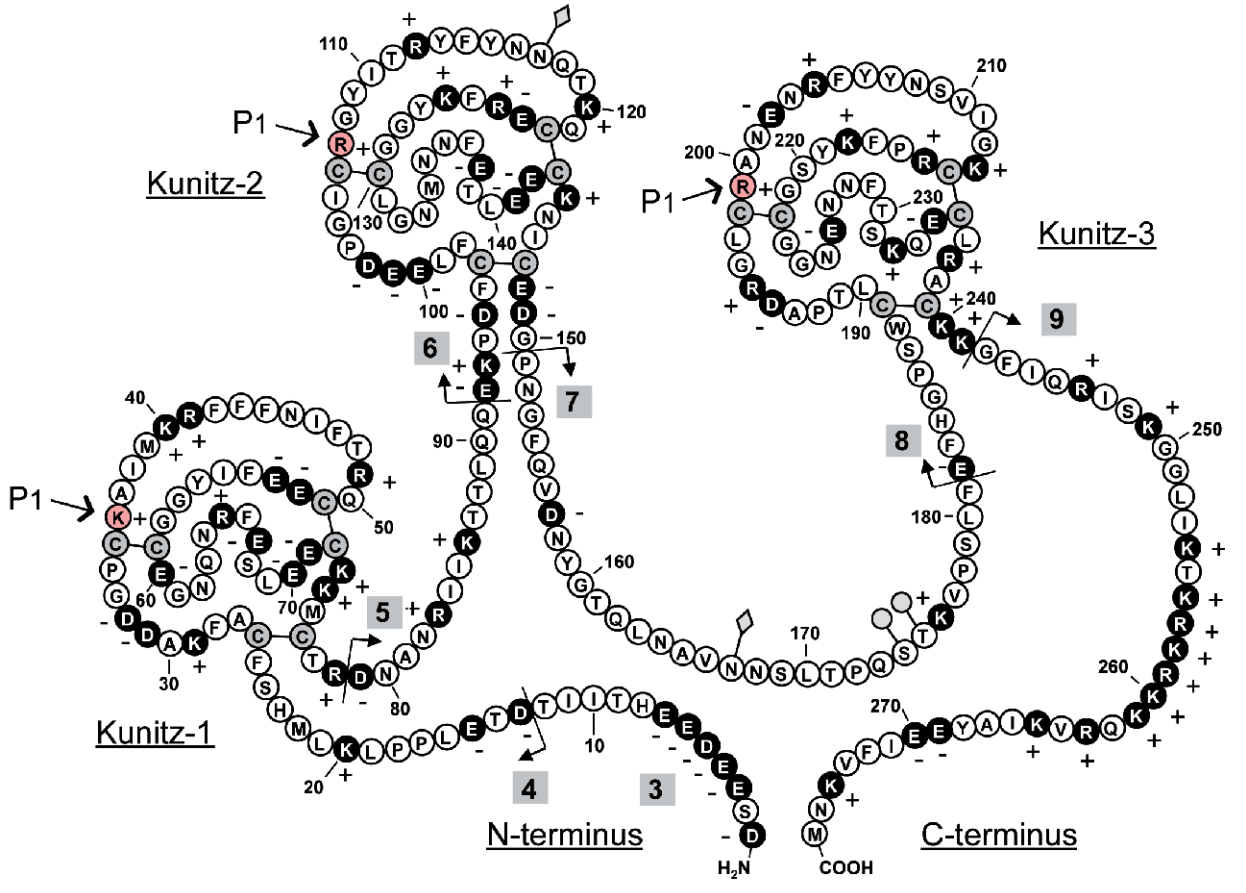


Figure 2. Primary structure of tissue factor pathway inhibitor (TFPI). TFPI is a 276-amino acid glycoprotein. Charged amino acids are indicated in black, P1 residues in red, cysteines in disulfide bonds in grey. Grey diamonds indicate N-linked glycosylation sites; grey circles indicate O-linked glycosylation sites. Backbone arrows and grey boxed numbers indicate the exon map. Adapted from Girard *et al.* Nature 1989, 338, 518-20.

and inhibits FVIIa, and that the second Kunitz-domain binds to and inhibits FXa.[4] The function of Kunitz-3 initially was less clear although it was reported that Kunitz-3 contained a heparin binding site[14] and that it was implicated in cell surface binding.[15] In addition, Kunitz-3 is involved in cross-disulfide linkages between various truncated forms of TFPI and low density lipoprotein.[16] More recently, however, it became apparent that Kunitz-3 is crucial for the TFPI-cofactor activity of protein S.[17,18] The basic C-terminal tail of TFPI was shown to interact with anionic membrane surfaces and heparin-like structures on the vessel wall.[14,19] The C-terminus of TFPI is required for optimal inhibition of FXa[4,20,21] and for the TFPI-cofactor activity of protein S[17] and therefore, full length (free) TFPI is now considered to be the only relevant anticoagulant TFPI form in the circulation.

TFPI: variants and distribution

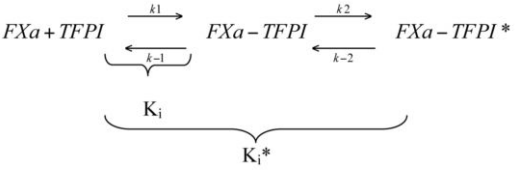
TFPI is produced and stored in endothelial cells, and on secretion, most of TFPI is bound to the endothelial cell surface through proteoglycans or GPI-linked proteins. In all, 80% of TFPI remains associated with the endothelium (Figure 1). The remainder of TFPI (20%) circulates in plasma at a concentration of approximately 2.5 nM.[22,23] The majority of circulating TFPI (70–80%) is truncated and bound to low-density lipoproteins through disulfide bonds with the Kunitz-3 domain.[16] Only 10% of plasma TFPI (2% of total TFPI: ~0.25 nM) circulates as free full length TFPI. (Figure 3). An alternatively spliced variant of TFPI (TFPIβ) has been identified, which lacks Kunitz-3 and the C-terminal domain but has a GPI-anchor by which it is directly bound to cell surfaces.[24,25] Administration of heparin releases the TFPI pool attached to endothelial cell surface proteoglycans, as well as intracellular stores of TFPI, resulting in a several-fold increase in plasma levels of full length TFPI.[26]

TFPI: mode of anticoagulant action

TFPI is a slow, tight binding inhibitor that regulates TF-activity through multiple mechanisms. TFPI inhibits TF/FVIIa via a two step feed-back mechanism, which involves formation of a bimolecular FXa/TFPI complex that subsequently interacts with TF/FVIIa, yielding an inactive quaternary complex and resulting in termination of TF/FVIIa-catalyzed FX activation.[4,27] In this negative feedback mechanism, the initial formation of a binary TFPI-FXa complex is a prerequisite for the final inhibition of TF/FVIIa by TFPI.[4] Alternatively, a more likely mechanism was proposed in which TFPI directly inhibits the trimolecular TF/FVIIa/FXa complex through primary Kunitz-2-FXa and secondary Kunitz-1-FVIIa interactions.[28]

The binding and inhibition of FXa by TFPI proceed through a two-step mechanism. First, a rapid encounter complex is being formed between FXa and Kunitz-2 of TFPI, in which the P1 residue Arg107 occupies the active site of FXa. Subsequently the encounter complex rearranges slowly into the tightly bound enzyme-inhibitor complex. During this process, the peptide bond between Arg107 and Gly108 is not hydrolyzed, making the final tight enzyme-inhibitor complex reversible.

During the first step of FXa inibition by TFPI, a small amount of loose complex between Kunitz-2 of TFPI and FXa is rapidly formed (*FXa-TFPI, equation a*), which results in quick inactivation of part of circulating FXa. During the second step, a slow rearrangement of this initial complex results in formation of the final tight FXa-TFPI*-complex[4] (*equation a*).



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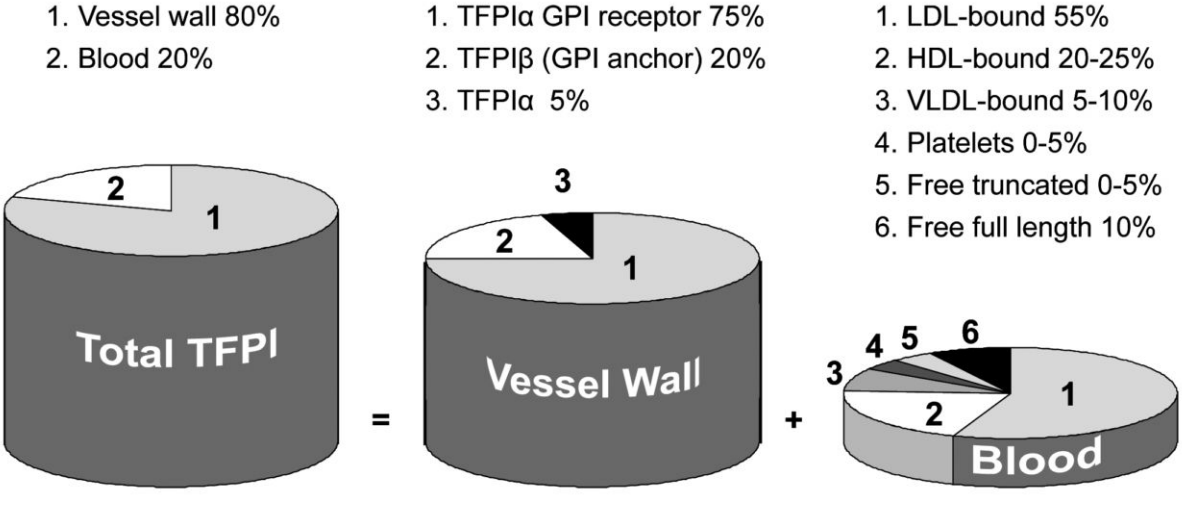

Figure 3. Distribution of TFPI variants and forms. From total TFPI, 80% is associated with endothelial cells and the vessel wall. Only 20% of TFPI circulates in plasma. From the circulating TFPI, 10% (2% of total TFPI) represents full length TFPI, the active anticoagulant form in plasma.

The dissociation constant of the first rapid equilibrium is represented by K_i ($K_i=k_{-1}/k_1$ = [FXa].[TFPI] / [FXa-TFPI]) and the overall equilibrium constant after the slow summarization is represented by K_i^* (K_i^* = [FXa].[TFPI] / {[FXa-TFPI] + [FXa-TFPI*]}). The Ki* for the final tight complex (0.05–0.07 nM) is several orders of magnitude lower than K_i for the initial encounter complex (5–15 nM).[17,29]

The fact that TFPI is a slow inhibitor of FXa has important implications for the down-regulation of the TF pathway by TFPI in plasma. For instance, it was observed that thrombin generation initiated by relatively high amounts of TF (14 pM) could not be effectively inhibited by TFPI. Only when TF concentrations went down to approximately 1 pM, efficient down-regulation of procoagulant response by TFPI could be observed.[30] This is not easy to understand if one realizes that at 14 pM of TF, the plasma concentration of free full length TFPI (0.25 nM) is still in more than 10-fold excess of TF and 5-fold over the reported Ki* for the final tight complex of FXa/TFPI of approximately 0.06 nM.[17,9] Under these conditions, efficient inhibition of FXa and thrombin generation by TFPI is to be expected. That this is not the case is caused by the fact that TFPI is a slow inhibitor of factor Xa.[31] In other words, only when FXa formation is slow and low, TFPI gets sufficient time to block the TF pathway before thrombin generation and clotting can occur. Therefore, at high TF concentrations, the rate of FX-activation exceeds a threshold that can effectively be managed by TFPI, and FXa can escape regulation.

So although kinetic parameters of final tight FXa/TFPI* complex formation are very favorable for FXa-inhibition, the slow rearrangement of the initial FXa/TFPI complex limits the efficacy of TFPI. In this respect, the rapid FXa/TFPI encounter complex gains importance as FXa is already inhibited in this loose complex. On the other hand, since the K_i of the initial inhibitory complex between TFPI and FXa (5–15 nM) is several times higher than the concentration of full length TFPI in plasma (~0.25 nM),[22] it was difficult to understand mechanistically how TFPI could be an effective inhibitor of TF-induced thrombin generation in plasma,[17,29] until it was uncovered that protein S acts as a cofactor for TFPI.

TFPI-cofactor activity of protein S

As described above, inhibition of TF-FVIIa by TFPI is a two-step process. In the first step, Kunitz-2 of TFPI binds to and inhibits FXa, while the second step involves binding of Kunitz-1 of TFPI to FVIIa. TFPI can either first form a bimolecular complex with FXa that subsequently acts on the bimolecular FVIIa/TF, or acts directly on tertiary FVIIa/TF/FXa to form the inactive quaternary complex.[4,28] In addition, the interaction of TFPI with FXa proceeds by a two-stage reaction, rapid formation of a loose complex followed by a slow rearrangement into a tight complex. More recently, it was observed that protein S reduces the Ki of the initial loose FXa-TFPI complex 10-fold from 5 nM to 0.5 nM, thereby enhancing the first step of the reaction and increasing the amount of FXa rapidly taken from the circulation by TFPI.[17] Initially, it was suggested that protein S accelerated the total feedback inhibition of the TF-mediated coagulation pathway, but alternatively it was proposed that TFPI stimulation by protein S only affects the inhibition of free FXa, and would be less important for the final inhibition of the TF-FVIIa-(FXa) complex.[17,32]

The identification of protein S as a cofactor for TFPI solved the long-standing question of how TFPI could be an important anticoagulant as kinetic experiments indicated that at its plasma concentration of 0.25 nM, full-length TFPI would be a poor inhibitor of thrombin generation in plasma.[33] By reducing the Ki of initial bimolecular FXa-TFPI complex formation from 5 nM to 0.5 nM, protein S brings the TFPI concentration necessary for effective FXa-inhibition well within range of the full-length TFPI concentration (0.25 nM) in plasma; in other words, protein S explains the physiological relevance of the plasma concentration of full length TFPI.

The current hypothesis is that TFPI can effectively inhibit the tertiary complex of FVIIa/TF/FXa, but when FXa dissociates from the extrinsic tenase on its way to participate in prothrombinase complex and thrombin formation, protein S is crucial for inhibition of free factor Xa by TFPI (Figure 4). When applying this hypothesis we should realize that some observations, such as the inability of TFPI and protein S to regulate FX-activation by the intrinsic route of coagulation, remain unexplained.[10]

TFPI-protein S complex

Somewhere in time during the protein S-catalyzed inhibition of FXa, a tertiary complex between FXa, TFPI, and protein S is likely to exist. Considering the fact that protein S is a cofactor for full length TFPI only, and that a truncated variant (1–161) of TFPI lacking Kunitz-3 and the C-terminus is not stimulated by protein S, the interaction between TFPI and protein S is mediated through the Kunitz-3/C-terminus of TFPI.[17] Later it was observed that Kunitz-3 of TFPI is responsible for the interaction with protein S.[18] As a result, almost half the plasma TFPI circulates in a complex with protein S.[34]

TFPI and thrombosis risk

The physiological relevance of TFPI was established by the uniform lethality of TFPI knock-out mice.[35] To date, no hereditary deficiencies of TFPI have been described in humans, but plasma TFPI levels show large inter-individual variations. Although TFPI in plasma represents only a fraction of all TFPI, several studies have shown that low levels of plasma TFPI (particularly free TFPI) are associated with approximately 2-fold increased risk of venous thromboembolism.[22,36-38] In 1999, it was reported that decreased levels of heparin-releasable TFPI were associated with venous thrombosis.[39] As heparin-releasable TFPI is the full length form of TFPI, this observation emphasizes the role of full length TFPI as an anticoagulant. In addition, it suggests an anticoagulant role of endothelial cell-bound TFPI, as lower availability of this pool leads to an increased risk for venous thrombosis.

It has been reported that protein S and TFPI covariate in normal individuals[40] and that plasma levels of full-

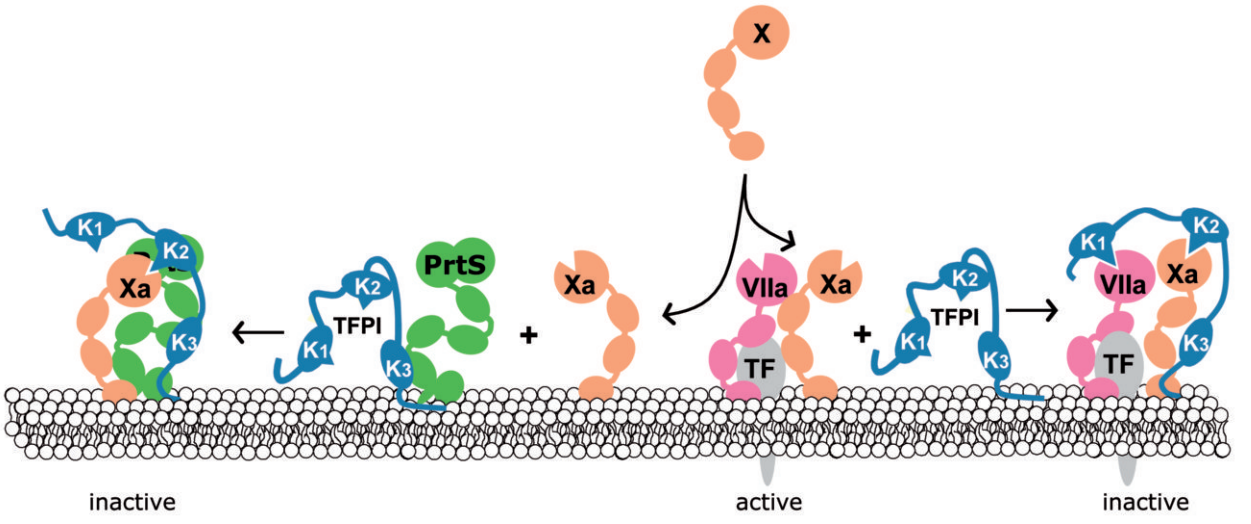

Figure 4. Regulation of coagulation by full length TFPI; state of the art. Factor X (X) is activated by tissue factor/factor VIIa (TF/VIIa). The tertiary complex of TF/VIIa/Xa reacts with TFPI to form an inactive quaternary TF/VIIa/Xa/TFPI complex. When Xa dissociates from the TF/VIIa complex to generate the prothrombinase complex, protein S acts as a cofactor to TFPI in the inactivation of Xa by stimulating Xa/TFPI complex formation.

length TFPI are low in protein S-deficient patients.[34] Since TFPI and protein S act in close collaboration to downregulate thrombin generation in plasma, combined low levels of both proteins may synergize in causing thrombosis. Both TFPI and protein S deficiencies are risk factors for development of venous thrombosis[41] and in this context, part of the thrombosis risk associated with protein S deficiency might actually be mediated by accompanying low levels of TFPI.[34]

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