

# Natural anticoagulants: A missing link in mild to moderate bleeding tendencies

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## Abstract

**Introduction:** There is a growing interest in natural anticoagulants as a cause of mild to moderate bleeding disorders (MBDs), particularly in patients with bleeding of unknown cause (BUC), which is defined as having a mild to moderate bleeding phenotype without a definite diagnosis despite exhaustive and repeated laboratory investigations. Recently, abnormalities in two natural anticoagulant pathways, thrombomodulin (TM), and tissue factor pathway inhibitor (TFPI), were identified in single patients or families as the underlying cause for a bleeding tendency.

**Aim:** The objective of this review is to discuss the current understanding of the role of natural anticoagulants in MBDs using available clinical and translational data.

**Methods:** A Cochrane Library and PubMed (MEDLINE) search focusing on selected natural anticoagulants and their role in MBDs was conducted.

**Results:** Data on the influence of natural anticoagulants including protein C, protein S, antithrombin, TM, and TFPI or factors with anticoagulant properties like fibrinogen gamma prime ( $\gamma'$ ) on MBDs are scarce. Observations from sepsis treatment and from translational research highlight their importance as regulators of the haemostatic balance, especially via the activated protein C-related pathway, and suggest a role in some MBDs.

**Conclusion:** Similar to the distinct genetic variants of natural anticoagulants linked to thrombosis, we hypothesize that novel variants may be associated with a bleeding tendency and could be identified using next generation sequencing.

## KEYWORDS

natural anticoagulants, mild bleeding disorders, bleeding of unknown cause thrombomodulin, TFPI

## 1 | INTRODUCTION

Knowledge on the complex role of natural anticoagulants as key regulators of the haemostatic balance is increasing. Stable clot formation is the result of the complex interplay between platelets, red blood cells, and coagulation factors. This process is negatively regulated by the natural anticoagulation system.<sup>1</sup>

Mild to moderate bleeding disorders (MBDs) manifest with clinical symptoms like epistaxis, easy bruising and menorrhagia, but are also associated with severe bleeding complications such as post-operative and post-partum hemorrhage.<sup>2</sup>

A large number of patients with a mild-to-moderate bleeding tendency are classified as having bleeding of unknown cause (BUC) because no diagnosis or explanation for their clinical phenotype can

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be established, despite exhaustive, and repeated laboratory investigations of conventional coagulation tests and platelet function.<sup>2,3</sup> Although no cause can be identified, neither the bleeding severity nor the clinical phenotype of patients with BUC differs from those of patients with established diagnoses, such as von Willebrand disease (VWD) or platelet function defects (PFD),<sup>2,4,5</sup> which suggests that current laboratory assessments are inadequate at identifying all aetiologies of bleeding in BUC patients. Recently, impaired thrombin generation and plasma clot formation properties in BUC patients with otherwise normal results in coagulation tests were identified.<sup>6</sup> The mechanisms underlying these alterations in thrombin formation and clot properties are unknown, however dysregulation of the natural anticoagulant system could be contributing.<sup>4,7</sup>

While most established MBDs are defined by a clotting factor deficiency or impaired platelet function,<sup>8</sup> an up-regulation of natural inhibitors of coagulation is responsible for mild to moderate bleeding tendencies in single patients or families.<sup>9,10</sup> Although hereditary deficiencies of the natural inhibitors protein C and protein S and the prothrombotic factor V Leiden mutation are well-established risk factors for thromboembolic events in humans,<sup>11</sup> a more comprehensive understanding of a potential contribution of natural anticoagulants in patients with MBDs is needed.

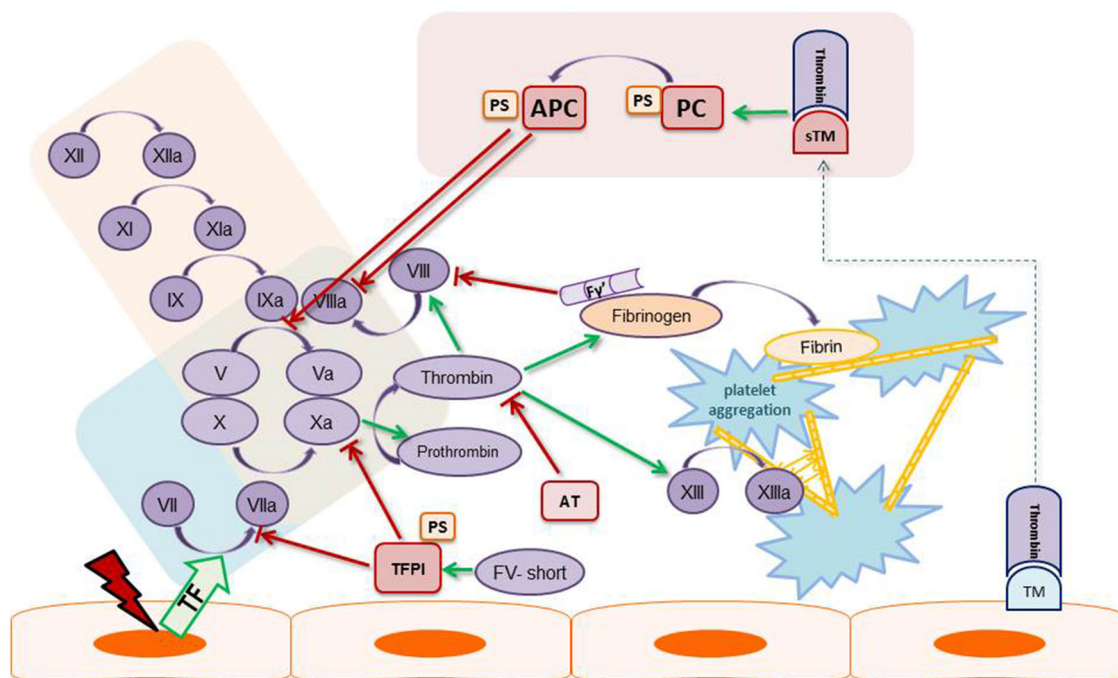
In this review, we discuss the current understanding of the role of natural anticoagulants in MBDs using available clinical and translational data. We propose that a more holistic approach to analysis of patients with BUC including characterization of natural anticoagulants may contribute to a better understanding of mechanisms underlying mild bleeding tendencies of yet undefined cause.

## 2 | PHYSIOLOGY OF NATURAL ANTICOAGULANTS

An imbalance between pro- and anticoagulant processes can lead to both pathologic thrombosis and bleeding in patients. In order to maintain the haemostatic balance and allow formation of clots at sites of injury without spontaneous activation or uncontrolled thrombin generation upon activation, coagulation factors are constantly inhibited via numerous constitutively active and activation-dependent pathways. We will focus on the natural anticoagulants protein C, protein S, thrombomodulin (TM), tissue factor pathway inhibitor (TFPI), and antithrombin (Figure 1, Table 1) as well as discuss possible contributions from the coagulation proteins factor (F) V and fibrinogen. Some of these natural anticoagulants themselves interact with each other, serving as essential co-factors.<sup>1</sup> Aforementioned, recent research efforts have led to a better understanding of the pathophysiology in MBDs and the identification of new bleeding disorders that result from abnormal natural anticoagulants function. Unfortunately, systematic investigations of natural anticoagulants in bleeding cohorts are scarce.

## 3 | THE APC PATHWAY

One key enzyme that prevents uncontrolled activation of coagulation is the vitamin-K dependent protein C pathway, which is activated to activated protein C (APC) by the TM-thrombin complex.<sup>12</sup> Protein C exerts its anticoagulant effect through inhibition of the activated coagulation FV and FVIII.<sup>12</sup> Protein S is an essential co-factor to APC in the



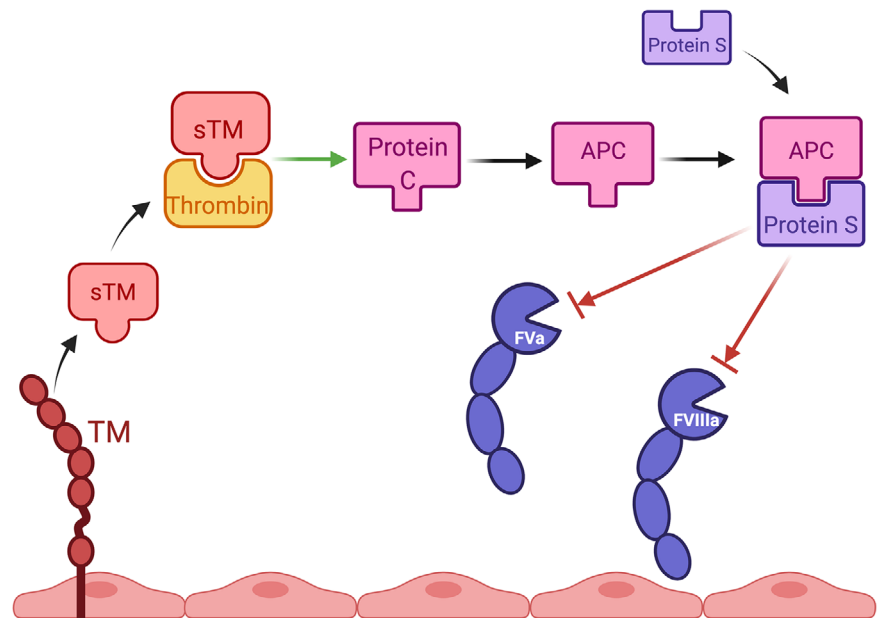
**Figure 1** Schematic illustration of interactions of natural anticoagulants in the coagulation cascade. Figure legend: TF, tissue factor; FV, factor V; APC, activated protein C; PS, protein S; (s)TM, (soluble) thrombomodulin; AT, antithrombin; TFPI, tissue factor pathway inhibitor; F $\gamma$ , fibrinogen gamma prime; red arrow: inhibition; green arrow: activation

**TABLE 1** Overview of natural anticoagulants

Protein	Gen	Physiological anticoagulation properties
Activated Protein C	PROC	Protein C is proteolytically activated by the TM-thrombin <sup>12</sup> Inactivation of FVa and FVIIIa <sup>12</sup> Enhancement of fibrinolytic activity via plasmin up-regulation (by consuming plasminogen activator inhibitor (PAI 1 and 2)) <sup>13</sup>
Protein S	PROS 1	Co-factor of APC: involved in the inhibition of FVa and FVIIIa <sup>10,14</sup> Co-factor of TFPI $\alpha$ <sup>14</sup>
Thrombomodulin	THBD	Co-factor of the protein C-based anticoagulant system <sup>10</sup> Delay of fibrinolysis by activation of TAFI independently of APC <sup>15</sup>
Tissue factor pathway inhibitor	TFPI	Free TFPI $\alpha$ is the active anticoagulatory isoform in plasma <sup>16</sup> Free TFPI $\alpha$ is found in platelets and leads to a local inhibition of thrombus development, independent of free TFPI $\alpha$ levels in plasma <sup>17</sup> Free TFPI $\alpha$ inhibits TF on endothelial cells or monocytes and the prothrombinase complex <sup>17</sup>
Antithrombin	SERPINC1	Inhibition of thrombin and FXa <sup>18</sup> Inactivation FIXa, FXIa, FXIIa, tissue plasminogen activator (tPA), urokinase, trypsin, plasmin and kallikrein <sup>18</sup>

Abbreviations: TFPI, tissue factor pathway inhibitor; TM, thrombomodulin; APC, activated protein C; F, factor;  $\gamma'$ , gamma prime; TAFI, thrombin-activatable fibrinolysis inhibitor.

**Figure 2** Pathophysiological mechanisms in TM-associated coagulopathy Figure legend: FVa, activated factor V; FVIIIa, activated factor FVIII; APC, activated protein C; PS, protein S; (s)TM, (soluble) thrombomodulin; red arrow: inhibition; green arrow: activation



degradation of activated factor V (FVa) via cleavage of peptide bonds that are sensitive to APC and protein S.<sup>12,19</sup> Hereditary deficiencies of both protein C<sup>20,21</sup> and protein S,<sup>19</sup> as well as the APC resistance due to a mutation of the APC cleavage site of FV (FV Leiden, Arg506Gln) are well-established risk factors for thromboembolic events.<sup>22,23</sup> Now, the APC pathway, specifically gain-of-function mutations in TM, is understood to play an important role in the pathophysiology of some patients with MBDs.

Several groups have recently described a TM-associated coagulopathy that leads to severe trauma- and surgery related bleeding (Figure 2, Table 2).<sup>10,24,25</sup> In these studies, significantly elevated soluble TM (sTM) levels (more than 100-fold higher than normal controls) and consequently systemic activation of protein C were found as the underlying cause for the bleeding tendency. Increased levels of sTM were also measured in family members, who retrospectively reported surgery- or trauma related bleeding. In all reported cases, the

same heterozygous mutation (c.1611C > A) in the TM-encoding gene (THBD) was identified. This mutation codes for a premature stop codon (p.Cys537Stop), leading to loss of the C-terminal cytoplasmic domain of TM,<sup>24</sup> which results in shedding of TM from the endothelium into the plasma due to a negative charged C-terminus and loss of interaction with the endothelium.<sup>10</sup> Clinically, all affected individuals had postsurgical and/or posttraumatic bleeding, and in two subjects spontaneous abdominal bleeding was reported. In all patients with this mutation, coagulation screening tests, levels of clotting factors, and platelet function tests were within normal limits; however, all had reduced thrombin generation.<sup>10,24,25</sup> Notably, the activated partial thromboplastin time (APTT) assay is usually not affected by high sTM levels because in this assay thrombin is generated rapidly and the anticoagulation properties of TM cannot come into effect.<sup>10</sup>

Recently, Westbury et al. reported another novel THBD variant (c.1487delCM; p.Pro496Argfs\*10) in a family with bleeding after

**TABLE 2** Comparison of TM-associated coagulopathy reported in four different families

	Langdown et al. <sup>10</sup>	Dargaud et al. <sup>24</sup>	Burley et al. <sup>25</sup>	Westbury et al. <sup>15</sup>
Number of affected patients	3	3	2	6
Clinical manifestations	Posttraumatic bleeding	Intra- and postoperative bleeding	Muscle and joint bleeding after minor trauma	Bleeding after dental procedures, surgery and childbirth
Genetic mutation in the THBD gene	c.1611C > A (p.Cys537Stop)	c.1611C > A (p.Cys537Stop)	c.1611C > A (p.Cys537Stop)	c.1487delC (p.Pro496Argfs*10)
sTM level $\pm$ SD (ng/mL) reference range: 2–8 ng/mL	845 $\pm$ 44	1120	640.7 $\pm$ 21.2 (n = 4, including 2 non-affected family members)	400
sTM level in affected family members $\pm$ SD (ng/mL) reference range: 2–8 ng/mL <sup>10</sup>	433 $\pm$ 55; 498 $\pm$ 124	Na	640.7 $\pm$ 21.2 (n = 4, including 2 non-affected family members)	535.6 $\pm$ 122.1
PT	Normal	Na	Na	Na
APTT	Normal	Na	Na	Na
Platelet function	normal	Na	Na	Na
Endogenous thrombin potential	Reduced	Reduced	Reduced	Reduced
Plasma clot lysis	Na	Na	Delayed time to 90% lysis	Na

Abbreviations: sTM, soluble thrombomodulin; PT, prothrombin time; APTT, activated partial thromboplastin time; na, not available.

tooth extraction, surgery, and childbirth, similar to the above mentioned cases (Table 2).<sup>15</sup> This frameshift mutation also leads to a truncated protein chain close to the transmembrane domain, resulting in increased levels of sTM in plasma. These patients also had impaired thrombin generation and delayed fibrinolysis on laboratory analysis.

In TM-associated bleeding, a thrombin activatable fibrinolysis inhibitor (TAFI)-dependent delay in fibrinolysis was demonstrated despite reduced thrombin generation. This seems counterintuitive, but may be explained by the fact that TM rather than thrombin is the main determinant of TAFI activation,<sup>25</sup> which is consistent with the finding that TAFI is activated at 1250-fold higher rate by the thrombin-TM complex compared to thrombin alone.<sup>26</sup> Activated TAFI reduces plasmin-mediated fibrinolysis by cleaving C-terminal lysine residues from fibrin and thus preventing localization of plasminogen to the fibrin surface. Interestingly, in the study by Westbury et al., a few patients with TM-associated bleeding due to the *THBD* variant had concomitant TAFI deficiency (due to an additional stop-gain variant in the CPB2 gene: c.340G > A; p.Arg114\*). These patients had less significant delays in fibrinolysis due to premature truncation of the TAFI protein and decreased TAFI generation through TM-thrombin.<sup>15</sup> These data further emphasize the central role of TM in coagulation, not only through APC activation, but also through interactions with TAFI and the fibrinolytic system.<sup>25</sup> Along the same lines, paradoxically increased levels of TAFI were found in the analyses of fibrinolysis parameters in patients with menorrhagia, hereditary mucocutaneous bleeding, and BUC.<sup>27</sup> Increased TAFI-levels have also been associated with a more severe bleeding phenotype in haemophilia patients.<sup>28</sup> To what extent

increased levels of TM underlie increased TAFI levels found in independent bleeding cohorts needs to be elucidated in further studies. Overall, these observations highlight the complex regulation of the haemostatic system by activation and inactivation of factors and the limitations of interpreting measurements of a single factor in the pathway.

Clinically, experience with increased APC activity is primarily from research on the therapeutic use of human recombinant APC, predominantly in sepsis. Although initial studies suggested recombinant APC may reduce mortality in sepsis, subsequent studies found no survival benefit but an increased risk of bleeding with the use of APC.<sup>29–31</sup> This increased risk of bleeding lends credence to the hypothesis that increased APC activity is responsible for bleeding observed in patients with elevated levels of sTM.

There is also evidence that protein C levels may modulate bleeding severity in patients with severe haemophilia. In patients with severe haemophilia, protein C levels were lower than in healthy controls<sup>32</sup> and lower protein C levels were associated with a less severe bleeding phenotype, suggesting a protective mechanism of low protein C in patients with haemophilia. This raised the question of whether protein C and APC could be a potential target for haemophilia treatment. In haemophilia mouse models, a recombinant serine protease inhibitor of APC restored the haemostatic balance.<sup>33</sup> However, given the diversity of roles of APC including its anti-inflammatory functions, there are concerns with the effect of its long-term administration in patients.

However, not all studies have found that elevated protein C levels or activity are associated with bleeding. Rojnuckarin et al. performed a population-based study to investigate the role of natural anti-coagulants and their association with cardiovascular risk factors and

bleeding in 5196 healthy people, of whom 785 (15.1%) had a bleeding score over 0.<sup>34</sup> In this study, high protein C activity was associated with a higher BMI, female sex, and atherosclerosis risk factors, and was independently associated with low bleeding scores. One hypothesis is that higher protein C activity may result from a counter-regulatory increase in activation to compensate for the pro-inflammatory and hypercoagulable state.<sup>34</sup>

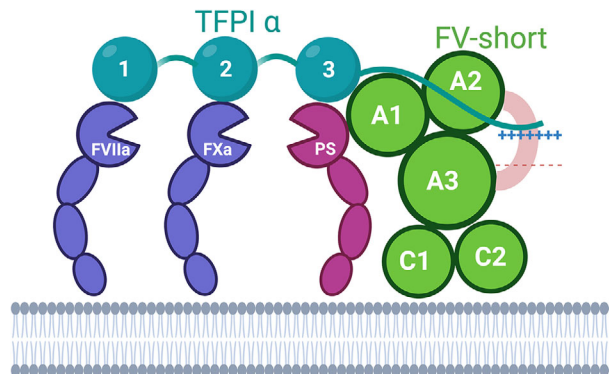
Furthermore, this study by Rojnuckarin et al. also found an association between high protein S levels and increased bleeding severity.<sup>34</sup> Interestingly, there was no correlation between protein S and protein C activity, which may be explained by the APC-independent function of protein S as a TFPI $\alpha$  co-factor.<sup>34</sup> Protein S binding to TFPI in the presence of phospholipids results in a 10-fold increase in inhibition of Xa.<sup>35</sup>

The role of protein S in haemostasis is further supported by the findings that in vitro inactivation by antibodies restored thrombin generation in plasma from patients with haemophilia.<sup>36</sup> This strategy could be applied in haemophilia treatment. In mice with haemophilia, a protein S-silencing RNA approach was used and resulted in a longer half-life and fewer injections of clotting factor concentrates.<sup>36</sup>

Another important component of the APC pathway in regulation of natural anticoagulant activity is the endothelial cell protein C receptor (EPCR). EPCR enhances activation of protein C, but also has distinct anti-inflammatory, cytoprotective and barrier stabilizing roles. An interaction of recent interest in patients with haemophilia is the binding of FVIIa to EPCR, which reduces protein C activation, promotes anti-inflammatory and vascular barrier integrity pathways, and appears to modulate severity of haemophilic arthropathy after hemarthrosis.<sup>37</sup> However, surprisingly, a recent study of mice with haemophilia A found that mice with concomitant EPCR-deficiency were protected against haemophilic arthropathy and inflammation and more responsive to recombinant FVIIa therapy. The authors hypothesized that due to loss of protein C activation, the EPCR-deficient mice were able to generate sufficient thrombin to prevent recurrent microbleeds. This loss of anticoagulant effect dominated over the loss of anti-inflammatory functions in this context and thus, EPCR could be another therapeutic target in haemophilia A that aims at reducing intrinsic anticoagulant activity.<sup>37</sup>

#### 4 | THE ROLE OF COAGULATION FACTOR V AND TFPI

The pivotal coagulation factor, FV, has both, pro- and anticoagulation properties.<sup>16</sup> Both full-length and partially cleaved FV circulates in plasma, the latter is released from alpha-granules and makes up 20% of total FV in blood.<sup>16</sup> Following the discovery of APC resistance and the increasing molecular knowledge of the FV protein, the anticoagulant role of FV has come into focus. Both FV and APC serve as co-factors with protein S within the tenase complex on negatively charged microvesicles in the process of FVIIIa degradation.<sup>16</sup> Mutations in the FV gene have been associated with both, thrombosis and bleeding tendencies.<sup>16</sup>



**Figure 3** Pathophysiological mechanisms in East Texas/FV Amsterdam bleeding disorder Adapted from Dahlbäck B. *Int J Lab Hematol.* 2016<sup>33</sup> Figure legend: FV, factor V; FVIIa, activated factor FVII; FXa, activated factor factor X; PS, protein S; TFPI, tissue factor pathway inhibitor

Recent translational studies on patients with undiagnosed bleeding diathesis led to the discovery of novel bleeding disorders and expanded our understanding of the role of FV in pathologic bleeding and its interactions with TFPI $\alpha$ .<sup>9,38,39</sup>

Increased free TFPI $\alpha$  levels were found in a large East Texas family<sup>9</sup> and in a Dutch family,<sup>39</sup> both with a mild to moderate bleeding tendency (Figure 3, Table 3). Clinical symptoms of affected individuals included menorrhagia, bruising and epistaxis.<sup>9,39</sup> In global coagulation assessments, patients had a prolonged prothrombin time (PT) and APTT. All other coagulation tests including FV activity were within the normal range, however levels of TFPI $\alpha$  were elevated.<sup>9,39</sup> The high TFPI $\alpha$  levels were each attributed to two different single nucleotide polymorphism (SNPs) in the exon 13 of the F5 gene (East Texas bleeding disorder: A2440G; FV Amsterdam: c.C2588G), which activate rare splice donor sites, resulting in an in-frame deletion of nucleotides and subsequent truncation of the basic region of the B-domain of the FV gene. The products of these transcripts have increased binding affinity for TFPI $\alpha$ , which results in increased TFPI $\alpha$  in circulation.

Another 832 base pair deletion within exon 13 of the FV gene (FV Atlanta) was reported in one patient with intramuscular hematomas and was also associated with increased TFPI $\alpha$  levels.<sup>40</sup> While the East Texas family is characterized by an increase in the naturally occurring, but previously unidentified, alternatively spliced FV-short, FV Amsterdam is a completely novel truncated FV isoform.<sup>9,39</sup> FV Atlanta, on the other hand, may result from a loss of a regulatory sequence for FV-short production within the F5 gene, leading to up-regulation of FV-short production.<sup>40</sup>

By thoroughly investigating patients with undiagnosed bleeding diathesis, researches identified new rare bleeding disorders and expanded our understanding of the role of FV and TFPI $\alpha$  co-activity (Figure 3). Under normal conditions, FV carries a very small fraction of TFPI $\alpha$  in healthy individuals.<sup>9</sup> The binding of TFPI $\alpha$  to the negatively charged C-terminus of the B-domain of FV-short increases the concentration of TFPI $\alpha$ . This binding also assists in the localization of TFPI $\alpha$  to the surface of negatively charged phospholipids where FXa inhibition takes place.<sup>41</sup> Thus, truncated FV isoforms serve both, as carriers of



**TABLE 3** Comparison of East Texas and FV Amsterdam bleeding disorder

	East Texas <sup>9</sup>	Factor V Amsterdam <sup>39</sup>
Clinical manifestations	Menorrhagia, bruising, epistaxis, and massive bleedings after trauma or surgery	
Genetic mutation in exon 13, F5	2440A > G	c.2588C > G
FV isoform	FV short	Truncated FV Amsterdam
Free TFPI $\alpha$ levels	1.25 $\pm$ 0.7 nM around 9-fold higher than normal range	149 and 109 ng/mL around 10-fold higher than normal range
PT	Prolonged	Prolonged
APTT	Prolonged	Prolonged
Endogenous thrombin potential	Reduced	Reduced

Abbreviations: TFPI, tissue factor pathway inhibitor; FV, factor V; PT, prothrombin time; APTT, activated partial thromboplastin time.

and co-factors to TFPI $\alpha$  in the inhibition of FXa.<sup>9,39,42</sup> In the previously described patients, the FV antigen and activity levels were within the normal range in affected individuals, but the proportion of the FV short isoform was significantly increased compared to healthy subjects. This led to a 10-fold increase of free TFPI $\alpha$  in plasma, resulting in impaired thrombin generation and a clinical bleeding tendency.<sup>9</sup>

In a large cohort of 620 patients with MBDs and BUC, we recently identified increased levels of free TFPI $\alpha$  associated with abnormal thrombin generation including a prolonged lag time and time to peak.<sup>7</sup> We did not identify relevant variants in the FV gene and while the values of free TFPI $\alpha$  in these patients were slightly higher than in healthy controls, they did not reach the high values of patients with East Texas or FV Amsterdam bleeding disorders. Nevertheless, we concluded that free TFPI $\alpha$  may contribute to unexplained bleeding, which is likely multifactorial in a majority of patients.<sup>43</sup>

Consistent with these observations, previous studies have shown that free TFPI $\alpha$  negatively affects thrombin generation in a TM-independent manner in healthy individuals.<sup>44,17</sup> Additionally, polymorphisms that are associated with altered plasma levels of TFPI $\alpha$  have been reported, but genetic data associated with TFPI-induced bleeding are in general scarce and inconsistent.<sup>17,45</sup>

On the other hand, low TFPI levels have been associated with a slightly increased risk for thrombosis, whereas total TFPI deficiency has not been described and may be associated with embryonic lethality.<sup>44</sup> The procoagulant effect of low TFPI levels has also been shown to attenuate bleeding severity in patients with FV deficiency or haemophilia, which could serve as a therapeutic target.<sup>46</sup> Anti-TFPI antibodies have been developed therapeutically and are in clinical trial. This approach might offer patients with haemophilia new treatment possibilities, which are independent of FVIII or FIX.<sup>47</sup>

## 5 | NATURAL ANTICOAGULANTS WITHOUT KNOWN ASSOCIATION TO MILD BLEEDING DISORDERS

Antithrombin (AT) is an important negative regulator of the coagulation cascade and hypothetically excess AT could result in a bleed-

ing phenotype. Antithrombin is an inhibitor of thrombin and FXa,<sup>18</sup> and its activity is enhanced 100-fold by binding to a specific domain of heparin. To a lesser extent, AT also inactivates FIXa, FXIa, FXIIa, tissue plasminogen activator (tPA), urokinase, trypsin, plasmin, and kallikrein.<sup>18</sup> A bleeding tendency due to an increased activity or levels of endogenous AT has not been reported to the best of our knowledge, however because levels of AT decrease in disseminated intravascular coagulation (DIC) and severe sepsis, AT replacement has been used therapeutically. Notably, a recent meta-analysis including 29 trials and 3882 participants with severe sepsis and DIC showed that AT therapy does not influence mortality, but increases the bleeding risk.<sup>48</sup> Lower levels of AT have been suspected to reduce the bleeding tendency in patients with haemophilia<sup>32</sup> and AT-blocking antibodies have been shown to restore thrombin generation in haemophilic plasma.<sup>49</sup> Currently fitusiran, an RNA interference therapy that suppresses AT synthesis by the liver is in advance stages of clinical trials. In a phase 1 study, fitusiran effectively lowered AT levels, improved thrombin generation, was generally well tolerated and may have reduced bleeding rates in patients with moderate and severe haemophilia and inhibitors.<sup>50</sup> More recently, in the phase 3 studies, there was a voluntary pause in recruitment due to reports of non-fatal vascular events. Trials were restarted with a dosing modification that aims for a higher residual AT level (15%-35%) in order to reduce the risk of thrombotic events.<sup>51</sup>

## 6 | FACTORS WITH POSSIBLE ANTICOAGULANT PROPERTIES: FIBRINOGEN GAMMA PRIME

Data from clinical and translational research on bleeding patients suggest that non-traditional natural anticoagulants may also contribute to MBDs.

While fibrinogen is classically defined by its terminal role in the coagulation cascade and important in stable thrombus formation, fibrinogen is now appreciated to have roles in clot formation, regulation of coagulation, inflammation, and response to infection. Fibrinogen is composed of three pairs of polypeptide chains, A $\alpha$ , B $\beta$ , and  $\gamma$ . Of interest in haemostasis is the phenotypic presentation of misbalanced

fibrinogen  $\gamma'$  levels, which has been associated with both an increased thrombotic risks and a bleeding tendency.<sup>52</sup>

Fibrinogen  $\gamma'$  was found to increase FV inactivation via APC<sup>53</sup> and also to have an anticoagulant function via high-affinity binding to exosite II of thrombin, which leads to a sequestering of thrombin in the fibrin clot and decreasing availability of active thrombin in plasma.<sup>54</sup> Further in vitro studies have reported anticoagulant effects of the fibrinogen  $\gamma'$  chain through inhibition of thrombin-mediated cleavage of FVIII and associated prolongation of APTT. Thus, some have hypothesized a potential role of fibrinogen  $\gamma'$  as a cause for increased bleeding in patients with MBDs.<sup>55</sup> In a case-control study of patients with intracerebral haemorrhage and healthy controls, fibrinogen  $\gamma'$  levels were significantly higher in bleeding patients, whereas the  $\gamma'$ /total fibrinogen ratio was not affected. This rise of fibrinogen  $\gamma'$  and fibrinogen was interpreted as an acute phase response in the bleeding cohort.<sup>56</sup>

On the other hand, increased and decreased fibrinogen  $\gamma'$  has been associated with arterial and venous thrombosis, but data are inconsistent.<sup>54</sup> SNPs have been identified that regulate levels of the fibrinogen  $\gamma'$  isoform. In the FGG gene, 10034C > T was associated with reduced fibrinogen  $\gamma'$  levels and increased risk for venous thrombosis and 9340T > C was associated with increased levels of fibrinogen  $\gamma'$  and arterial thrombosis; however, these findings could not be confirmed in a genome wide association study.<sup>54,57</sup>

## 7 | CONCLUSION AND FUTURE PERSPECTIVES

Natural anticoagulants are essential to maintaining the haemostatic balance and, alongside the fibrinolytic system, important for the prevention of a pathologic thrombosis. Specific genetic alterations that lead to an up-regulation of certain natural anticoagulants have been reported.<sup>9,39</sup> Nevertheless, natural anticoagulants have rarely been systematically investigated in large bleeding cohorts and their evaluation is not performed routinely in the assessment of MBDs.<sup>8</sup>

Patients with MBDs are challenging, as no cause of the bleeding tendency is found in a majority of patients, possibly due to an insufficient haemostatic work-up. Additionally, it is estimated that the prevalence of rare bleeding disorders, when excluding von Willebrand disease (VWD) and haemophilia A and B, is very low (< .001%) in the general population, which makes its advancing diagnostics even more challenging.<sup>58</sup>

Based on the physiological roles of coagulation inhibitors and previous reports on TM- and TFPI associated bleeding disorders, an investigation of these factors in bleeding disorders seems justified. Whereas hyperfibrinolysis is considered when investigating BUC, and at least discussed in recent recommendations on the routine work-up of patients with MBDs,<sup>8</sup> the system of natural anticoagulant has not been included in these algorithms.

In the last decades, NGS led to the discovery of numerous genes that were also associated to bleeding disorders.<sup>58</sup> Downes et al. recently reported a high-throughput screening panel within the ThromboGenomics project for patients with bleeding or thrombotic disorders.

However, the success rate for a molecular diagnosis in patients with an unclassified bleeding, however, was only 3.2%.<sup>58</sup> In the future, whole exome sequencing or broader analyses may enable the identification of bleeding-associated mutations and could lead to a better understanding of pathways underlying MBDs.<sup>58</sup> In these analyses, we believe the role of natural anticoagulants needs to be investigated, both, on a laboratory and genetic level. Polygenic risk scores, as they are already available for several disease like diabetes mellitus or coronary artery disease,<sup>58-60</sup> could be a novel approach for better diagnostics of patients with MBDs, especially those that are lacking a definite diagnosis.<sup>58</sup>

Our comprehensive investigations on BUC patients have found evidence of impaired haemostatic potential, possible due to hyperfibrinolysis and the ABO blood group influence. With additional investigation, we may also find natural anticoagulants to play a role.

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## AUTHOR'S CONTRIBUTIONS

Dino Mehic, Meaghan Colling, and Johanna Gebhart performed a literature research and wrote the manuscript. Ingrid Pabinger gave input and revised the intellectual content of the manuscript. All authors approved the content of the manuscript.

## CONFLICT OF INTEREST

Data derived from public domain resources. DM received honoraria for participation in advisory board meetings from CSL Behring. IP received honoraria for occasional lectures and advisory board meetings from Bayer, Daiichi-Sancho, Pfizer, and Sanofi. JG received honoraria for occasional lectures and advisory board meetings from Novartis, Amgen, CSL Behring, and Sobi.

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