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## ORIGINAL ARTICLE

# Major bleeding during oral anticoagulant therapy associated with factor V activation by factor Xa

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## Abstract

**Objective:** Plasma thrombin generation (TG) provides important information on coagulation status; however, current TG output parameters do not predict major bleeding of patients on anticoagulants. We recently reported that factor V (FV) activation by factor X (FX)a contributes importantly to the initiation phase of TG. Here we investigated how this pathway varies in the normal population and whether FXa-mediated activation of FV is associated with major bleeding in patients on anticoagulant therapy. **Approach:** We employed TIX-5, a specific inhibitor of FV activation by FXa, to estimate the contribution of FXa-mediated FV activation to tissue factor (TF)-initiated TG.

**Results:** We show that the contribution of this pathway to plasma TG varies considerably in the normal population, as measured by the time needed to form the first traces of thrombin (TG lag time; mean prolongation by TIX-5 40%, range 0%–116%). Comparing patients on vitamin K antagonists (VKA) of the BLEED study (263 patients

Bos and van 't Veer contributed equally to the work.

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with and 538 patients without major bleeding), showed a marked prolongation in the median TG lag time in the presence of TIX-5 in cases (12.83 versus 11.00 minutes,  $P = 0.0030$ ), while the TG lag time without TIX-5 only showed a minor although significant difference (5.83 vs. 5.67 minutes,  $P = 0.0198$ ). The TIX-5 sensitivity (lag time + TIX-5/lag time + vehicle) in the upper quartile was associated with a 1.62-fold (95% confidence interval 1.04–2.52) increased risk of major bleeding compared to the lowest quartile.

**Conclusion:** A greater dependence on FXa-mediated activation of FV of TG is associated with increased risk of major bleeding during VKA therapy.

#### KEYWORDS

anticoagulants, factor V, factor X, hemorrhage, thrombin

## 1 | INTRODUCTION

To prevent thrombotic events many patients are treated with oral anticoagulants that reduce the generation of thrombin, the key protease in coagulation. However, oral anticoagulant therapy comes with an annual risk of major bleeding in 1–3% of treated patients.<sup>1</sup> Further understanding of the regulation of thrombin generation in individuals under normal and disease conditions is therefore critical to provide a framework for personalized therapy.<sup>2–4</sup> Thrombin generation measurement, however, has failed thus far to identify patients at risk of major bleeding while on anticoagulant therapy.<sup>3</sup>

An important process in thrombin formation is the conversion of coagulation factor (F)V to activated FV (FVa) by limited proteolysis.<sup>5</sup> FVa is the cofactor for the protease FXa; both assemble on a negatively charged phospholipid surface in the presence of calcium ions to form the prothrombinase complex (FVa/FXa) that converts prothrombin to thrombin.<sup>6</sup> Thrombin activates platelets and converts fibrinogen into fibrin to form the fibrin network during hemostatic plug and thrombus formation. FXa is generated from FX by tissue factor (TF)/FVIIa activity resulting from subendothelial TF exposure in vascular lesions and by FVIIIa/FIXa activity.<sup>7</sup> Despite the general notion that thrombin was the only physiological activator of FV, it has been a longstanding enigma how the first traces of FVa are formed in a blood plasma environment.<sup>8</sup> However, by discovering the anticoagulant tick protein TIX-5 that specifically inhibits the FXa-mediated activation of FV, we were able to show that FV activation by FXa provides an essential contribution to thrombin generation in blood plasma.<sup>9</sup> Recently we elucidated the structure-function of TIX-5 with respect to its anticoagulant action.<sup>10</sup> TIX-5 consists of a rod formed by several beta sheets wrapped around a central alpha helix with on one end a phospholipid binding site and on the other end a FV binding site and a potential FXa pseudo-substrate site. Using TIX-5 we previously observed that the contribution of FV activation by FXa to thrombin generation is almost absent in fibrinogen deficient plasma,<sup>9</sup> which is in line with the inhibition of initial traces of thrombin by fibrinogen and particularly  $\gamma'$ -fibrinogen.<sup>11</sup> Furthermore, TIX-5 displayed a minor inhibitory effect in tissue factor pathway

### ESSENTIALS

- Thrombin generation provides important information on coagulation while it is currently not used to predict major bleeding.
- TIX-5, a specific inhibitor of factor V (FV) activation by factor X (FX)a is used to estimate the contribution of this pathway to thrombin generation.
- We show that while the contribution of FXa-mediated activation of FV to plasma thrombin generation varies considerably in the normal population, in patients with major bleeding a marked prolongation in the median thrombin generation lag time in the presence of TIX-5 is observed.
- A greater dependence on FXa-mediated activation of FV of thrombin generation is associated with increased risk of major bleeding during vitamin K antagonist therapy.

inhibitor (TFPI $\alpha$ ) or protein S deficient plasma,<sup>9</sup> factors that control the initiation phase of thrombin generation in plasma at low stimulus.<sup>12</sup> TFPI $\alpha$ , through its basic C-terminal region, impacts prothrombinase assembly by interacting with partially activated forms of FV(a), including FXa-cleaved FV.<sup>13,14</sup> This TFPI $\alpha$  interaction prevents intermediate forms of FV(a) from further activation and assembly with FXa into prothrombinase, thereby dampening thrombin generation.<sup>13,15</sup> Moreover, TFPI $\alpha$  also inhibits thrombin-mediated activation of FV,<sup>15,16</sup> another feature that may increase the dependence of thrombin generation on FXa-mediated FV activation. Altogether, the FV activation by FXa may play a major role in the initiation phase of thrombin generation. Of further interest is the observation that an in principle active cofactor splice variant of FV, FV-short, circulates at variable levels in association with TFPI $\alpha$  and this FV-short/TFPI $\alpha$  complex is a potent inhibitor of FXa.<sup>14,17,18</sup>

Given that a TF-triggered fibrin clot, which is meant to stop bleeding, is already formed at the end of the initiation phase of

thrombin generation,<sup>19</sup> we hypothesized that the extent to which the FV activation by FXa contributes to thrombin generation and subsequent clot formation could be at the basis of the major bleedings that occur during anticoagulant therapy. We therefore assessed the association of major bleeding events observed in the Biomarkers in the Leiden Etiology and Epidemiology of Bleeding in Vitamin K Antagonists Drug Users Study (BLEEDS, 326 cases of major bleeding)<sup>20</sup> with the contribution of FV activation by FXa to thrombin generation. For this purpose, used TIX-5 to generate a robust assay that specifically determines the contribution of FXa-mediated activation of FV to TF-initiated thrombin generation.

## 2 | MATERIALS AND METHODS

### 2.1 | Healthy individual and normal pool plasma

Normal human pooled plasma (NHP) and individual plasmas of healthy volunteers were kindly provided by the Experimental Vascular Medicine Department, Amsterdam University Medical Center (UMC). Citrated blood from individual donors (30 males and 15 females without and 15 on oral contraceptives [OC]) was centrifuged twice to generate platelet poor plasma, upon which it was immediately stored at  $-80^{\circ}\text{C}$ . Collection of plasma from healthy individuals was approved by the medical ethical committee of the Amsterdam UMC.

### 2.2 | BLEEDS study

The BLEEDS is a cohort study with 17,613 years of follow-up in 16,570 patients who initiated treatment with vitamin K antagonists (VKAs) at the following anticoagulation clinics: Leiden, Hoofddorp, and the Hague (the Netherlands). The plasma samples of the BLEEDS study were collected as reported earlier<sup>20</sup> and described in more detail in the supporting information. Briefly, plasma samples were collected 3–4 weeks after initiation of the VKA treatment and routine international normalized ratio (INR)-based adjustment of the VKA dosage. Major bleeding events occurred at  $224 \pm 207$  days (mean  $\pm$  standard deviation) after initiation of VKA therapy; occasionally bleeding occurred before the sample was obtained. For the case-cohort study, all 326 patients with a major bleed (cases) were included, of whom plasma was available for 263 cases. In addition, we performed a subgroup analysis on intracranial bleeds because these are most serious and often fatal;<sup>21</sup> this involved 76 cases with plasma available for 51 cases. We used a case-cohort design because the source study (BLEEDS) was designed to study multiple outcomes.<sup>22</sup> In a case-cohort study the selection of the subcohort, here referred to as controls, was a random sample of the whole BLEEDS cohort. We selected 4% (652 patients) with plasma available for 538 patients. As such, each patient in the cohort, including the cases, has the same probability of being selected to the control group. As a result, six cases were also included in the control group of 538

patients. The BLEEDS was approved by the medical ethical committee of the Leiden University Medical Center.

### 2.3 | Thrombin generation

Thrombin generation (TG) in plasma samples was performed as described previously<sup>23</sup> (see supporting information) using the calibrated automated thrombogram (CAT) method with the Thromboscope reagents (Stago). Briefly, TF (5 pM)-initiated CAT was performed in plasma of healthy individuals or NHP. CAT in plasma of patients on anticoagulants was initiated with 20 pM TF to overcome the anticoagulant-induced effect.<sup>24</sup> TIX-5, purified to homogeneity (see supporting information), was added in 10  $\mu\text{L}$  phosphate buffered saline (PBS) in a final concentration of 4  $\mu\text{M}$  to the TG mixture, or 10  $\mu\text{L}$  PBS was added as vehicle control. Addition of 4  $\mu\text{M}$  TIX-5 to NHP extended the TG lag time by 40% when initiated by 5 pM TF (Figure S1 in supporting information).

The effect of varying levels of TFPI $\alpha$  and FV-short on thrombin generation was assessed using a purified constitutively active partial B-domainless recombinant form of human FV (FV-810).<sup>17,25</sup> As such, FV-810 lacks the basic autoinhibitory region in the B-domain similar to FV-short.<sup>17,25</sup> NHP was incubated with 5 pM TF, FV-810 (1 nM) corresponding to 5–10% the plasma level of full-length FV, either in the absence or presence of equimolar amounts of TFPI $\alpha$  (rh-TFPI, Tifacogin<sup>®</sup>, Novartis; 1–2 nM) with either TIX-5 or PBS vehicle.

### 2.4 | TFPI $\alpha$ and $\gamma'$ -fibrinogen ELISAs

TFPI $\alpha$  was measured in plasma as described previously.<sup>26</sup> The GammaCoeur<sup>™</sup>  $\gamma'$ -fibrinogen ELISA was purchased from ZEUS Scientific and performed according to the manufacturer's instructions.

### 2.5 | Statistical analysis

Analyses were performed employing either GraphPad Prism version 5.01 (GraphPad Software) or R 2.15.2 (Foundation for Statistical Computing, [www.r-project.org](http://www.r-project.org); see Methods S1 in supporting information). *P*-values < 0.05 were considered statistically significant.

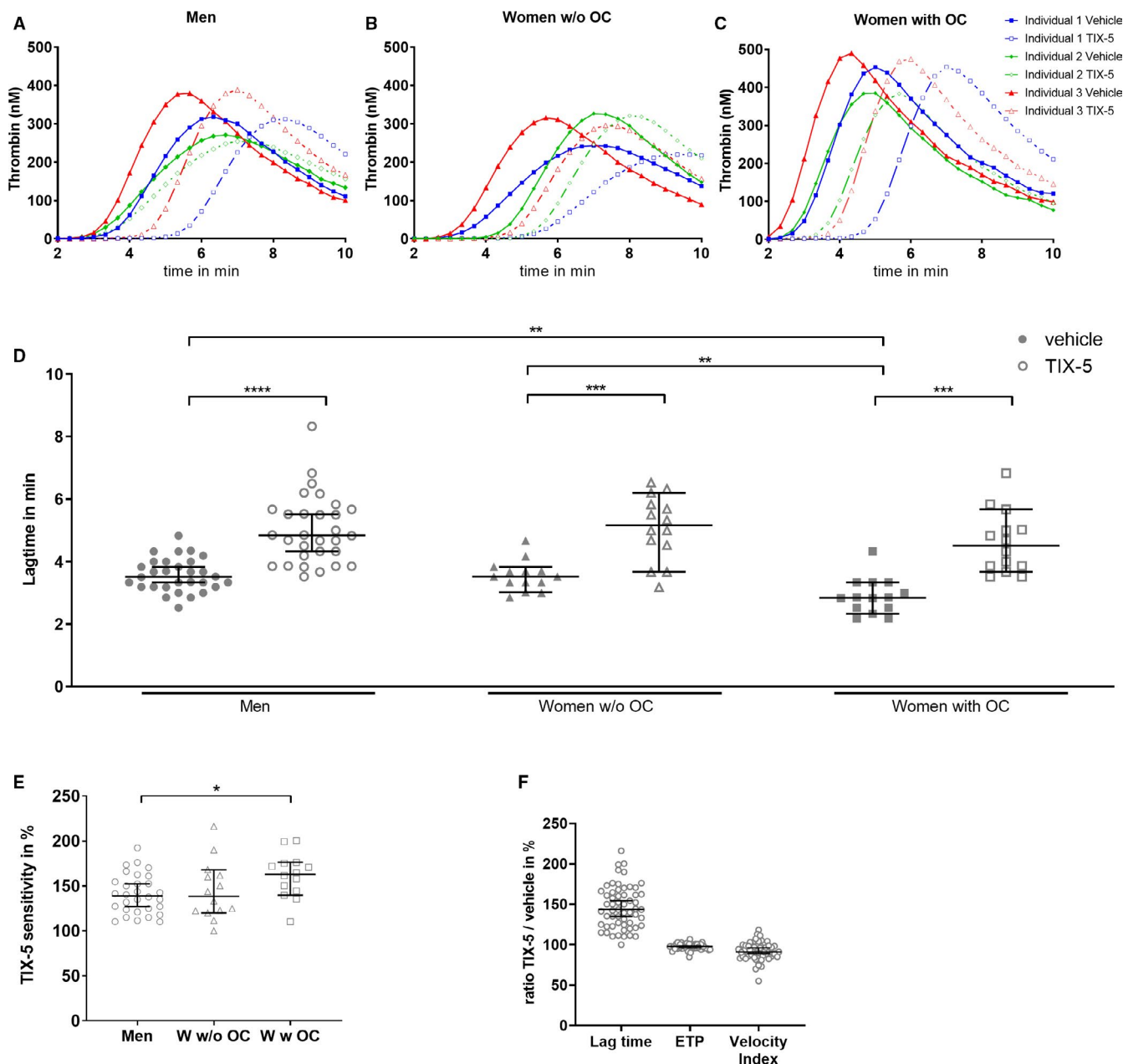
## 3 | RESULTS

### 3.1 | TIX-5 prolongs the lag time of thrombin generation in healthy individuals to a different extent

To determine the contribution of the FXa-mediated FV activation pathway to TG in the normal population, we assessed TF-triggered TG in 60 healthy individuals in the absence and presence of TIX-5. TG can be quantified by determining the lag time (the time needed to form the first traces of thrombin), the endogenous thrombin

potential (ETP, represents the total amount of active thrombin formed), and the peak height (the maximal amount of thrombin formed). As shown in selected thrombograms (Figure 1A–C), the effect of TIX-5 on TG in individual plasmas varied considerably. For some individual plasmas a major prolongation of the lag time in the presence of TIX-5 was observed (individuals 1, 3), whereas for others hardly any, or a smaller shift in lag time was observed (individual 2). However, TG curve shape, peak height, ETP, and velocity

index were maintained in the presence of TIX-5 (Figure 1A–C, Figure S3 in supporting information). TIX-5 prolonged the TG lag time significantly in men and women (Figure 1D). Interestingly, women using oral contraceptives displayed a shorter lag time in the absence of TIX-5 compared to men and women without oral contraceptives use (Figure 1D, Figure S3), corroborating previous findings.<sup>27</sup> This difference in lag time was absent in the presence of TIX-5 (Figure 1D). As a result, the TIX-5 sensitivity,



**FIGURE 1** Marked differences in TIX-5 sensitivity of the thrombin generation lag time in plasma of healthy individuals. Thrombin generation was initiated with 5 pM tissue factor in plasma of 60 healthy individuals in the absence or presence of 4  $\mu$ M TIX-5. Representative curves are shown of healthy individuals in the category men ( $n = 15$ ; A), women without (w/o) oral contraceptives (OC) use ( $n = 15$ ; B), or women with OC use ( $n = 15$ ; C). D, Lag times of thrombin generation in the absence or presence of TIX-5 or (E) expressed as TIX-5 sensitivity by the lag time + TIX-5/lag time + vehicle ratio with the vehicle condition set at 100% per category. F, TIX-5 effect on the thrombin generation parameters lag time, endogenous thrombin potential (ETP), and velocity index in plasma of all 60 healthy individuals shown as TIX-5/vehicle ratio, with the vehicle conditions set at 100%. All data are depicted as median  $\pm$  95% confidence interval. Statistical significance: \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$

defined as the ratio of the lag time with TIX-5/lag time without TIX-5, was significantly higher in women using oral contraceptives (Figure 1E). The average lag time extension was 40% but varied considerably from 0% up to 116% in healthy individuals, whereas the ETP and velocity index in healthy controls were not sensitive to TIX-5 (Figure 1F). Overall, these data indicate large differences in the contribution of FXa-mediated FV activation to thrombin generation between healthy individuals. Because of the specific effect of TIX-5 on the TG lag time in healthy subjects, we defined "TIX-5 sensitivity" as the ratio of the TG lag time observed with and without TIX-5. This TIX-5 sensitivity is rather stable in healthy individuals over time and with different work-up of the plasma sample (Figure S2 in supporting information).

### 3.2 | Association of thrombin generation parameters in the presence of TIX-5 with major bleeding in patients on anticoagulants

We next studied the association of TF-initiated TG with major bleeding during anticoagulant treatment in the BLEEDS study (for patient characteristics see Table S1 in supporting information). In the whole cohort, the incidence rate for major bleeding was 1.85 per 100 patient years and the average follow-up time per patient was 11 months.<sup>20</sup> The characteristics of the subcohort were similar to the whole cohort (Table S1). Cases with major bleeding were slightly older than controls (mean 75 years vs. 70 years, respectively). The most common indications for anticoagulant therapy were atrial fibrillation (cases 76% vs. controls 70%) and venous thrombosis (cases 15% vs. controls 21%). TIX-5 prolonged the TG lag time in plasma of patients on anticoagulants to varying extents similar to our observations in healthy individuals, although the overall thrombin generation was lower (Figure 2A), consistent with the anticoagulated status of these patients.

While a minor but significant difference was observed between the median vehicle TG lag time of cases with major bleeding and controls (5.83 vs. 5.67 minutes, respectively;  $P = 0.0198$ ), a marked difference in the median lag time was observed in the presence of TIX-5 between cases and controls (12.83 vs. 11.00 minutes, respectively;  $P = 0.0030$ ; Figure 2C). Interestingly, the lag time in the presence of TIX-5 in the highest quartile versus the lowest was associated with an increased risk of major bleeding (hazard ratio [HR] 2.10, 95% confidence interval [CI] 1.34–3.32; Table 1). A significant association was also found for the lag time in the absence of TIX-5 in the highest quartile versus the lowest (HR 2.10, 95% CI 1.32–3.31; Table 1). Although the median TIX-5 sensitivity (lag time + TIX-5/lag time + vehicle) was not significantly different between cases and controls (Figure S4B in supporting information), the TIX-5 sensitivity in the highest quartile was associated with an increased risk of major bleeding (HR 1.62, 95% CI 1.04–2.52; Table 1), indicating that a relatively large increase in lag time by TIX-5 associates with major bleeding. Importantly, this TIX-5 sensitivity does not correlate to the lag time observed

without TIX-5 (Figure 2B), confirming that TIX-5 not just prolongs extended lag times, but affects a specific TG activation pathway during the initiation phase.

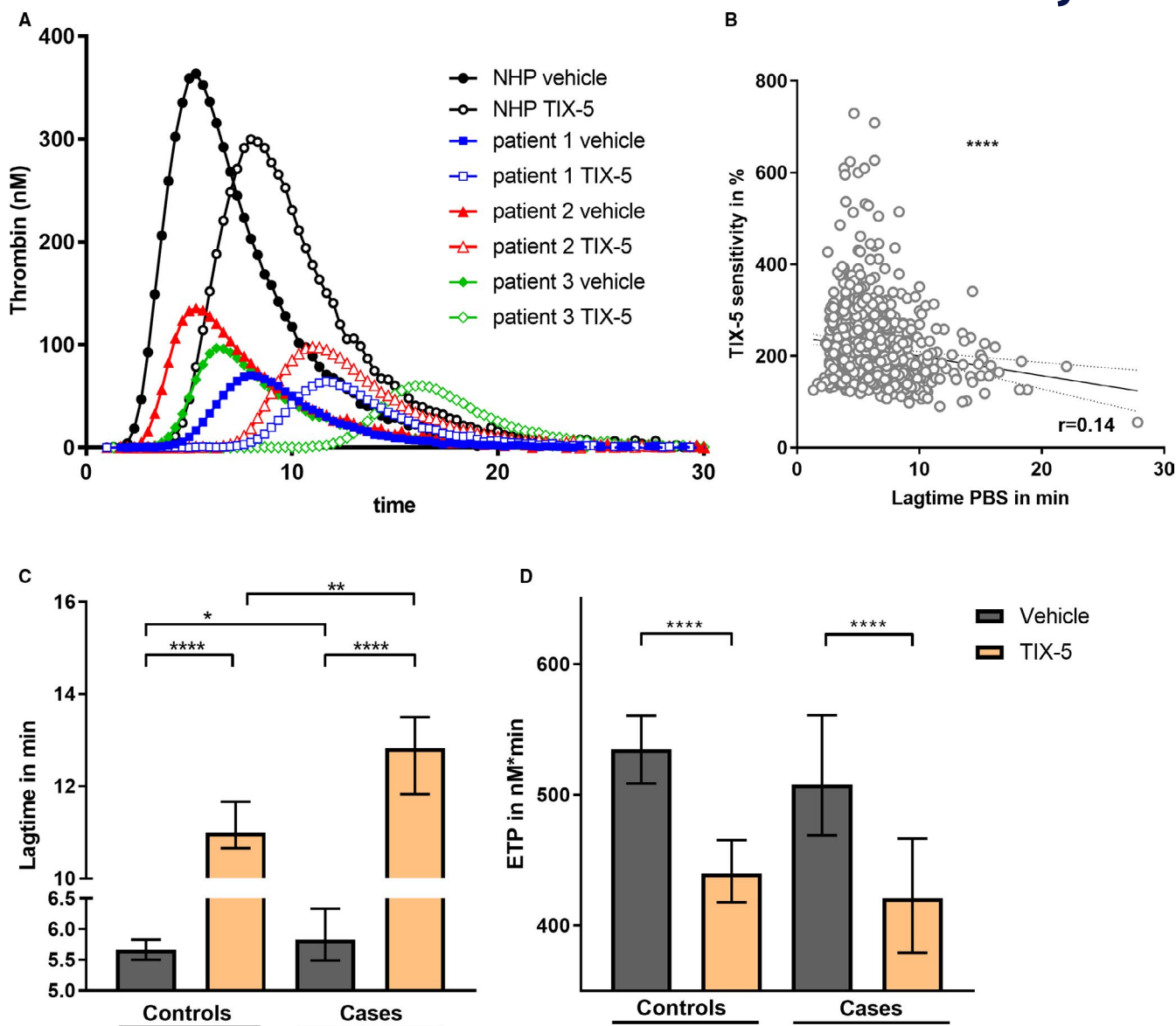
While TIX-5 significantly reduced the ETP in anticoagulated patient plasma for both cases and controls (Figure 2D and Figure S4C), the median ETPs with and without TIX-5 did not differ significantly between cases and controls (Figure 2C). However, the lowest three quartiles of ETP in the presence of TIX-5 were associated with major bleeding (range HR 1.59–1.69, range 95% CI 1.00–2.68; Table 1). To summarize, an increased lag time and decreased ETP in the presence of TIX-5 is associated with major bleeding during anticoagulant treatment. This implies that the FXa-mediated activation of FV contributes substantially more to TG in patients on anticoagulants who will develop major bleeding.

### 3.3 | Major bleeds are associated with elevated levels of $\gamma'$ -fibrinogen, and $\gamma'$ -fibrinogen correlates with TIX-5 sensitivity

Previously, our group has shown that the anticoagulant effect of TIX-5 depends on factors that regulate the initiation phase of thrombin generation including fibrinogen.<sup>9</sup> Interestingly, a splice variant of fibrinogen known as  $\gamma'$ -fibrinogen comprises a specific thrombin binding site that inhibits thrombin activation of FV.<sup>11</sup> To assess the association of  $\gamma'$ -fibrinogen with TIX-5 sensitivity and with the risk of major bleeding, we determined the  $\gamma'$ -fibrinogen levels in the BLEEDS study. The  $\gamma'$ -fibrinogen levels were significantly higher in cases than controls (Figure 3A), translating into a 1.62-fold (95% CI 1.06–2.48) increased risk of major bleeding in the >75th percentile compared with the lowest quartile (Table 2). In line with our previous observation of the lack of effect of TIX-5 in fibrinogen-deficient plasma,<sup>9</sup>  $\gamma'$ -fibrinogen levels displayed a significant, although subtle, correlation with TIX-5 sensitivity when evaluated in all patient plasmas (cases and controls combined; Figure 3B). The correlation of  $\gamma'$ -fibrinogen with both major bleeding and TIX-5 sensitivity appears consistent with the inhibitory effect of this fibrinogen splice variant on thrombin activity.

### 3.4 | Major bleeds are associated with elevated levels of TFPI $\alpha$ , but TFPI $\alpha$ levels do not correlate with TIX-5 sensitivity

Previously, we obtained evidence indicating that the anticoagulant effect of TIX-5 also depends on TFPI $\alpha$ , as hardly any effect of TIX-5 was observed in plasma depleted of TFPI $\alpha$ .<sup>9</sup> To assess the association of TFPI $\alpha$  with TIX-5 sensitivity and with the risk of major bleeding, we determined the TFPI $\alpha$  levels in the BLEEDS cohort. The TFPI $\alpha$  levels were significantly higher in cases than controls (Figure 3C), and a 2.29-fold (95% CI 1.46–3.61) increased risk to develop a major bleeding was observed for the highest quartile of TFPI $\alpha$  plasma levels versus the lowest (Table 2). TFPI $\alpha$  levels



**FIGURE 2** Substantial prolongation of the thrombin generation (TG) lag time by TIX-5 in plasma of vitamin K antagonist (VKA)-treated patients with major bleeding. Thrombin generation was initiated with 20 pM tissue factor in the absence or presence of 4  $\mu$ M TIX-5 in plasma of patients on VKA therapy without (controls,  $n = 538$ ) or with major bleeding (cases,  $n = 263$ ). A, Representative TG curves for normal human pooled plasma (NHP) and individual patients on VKAs. B, Vehicle lag time of all patients on VKA without or with major bleeding were plotted against the TIX-5 sensitivity of the lag time of TG of these patients. C, The lag time and (D) endogenous thrombin potential (ETP) of TG in the absence or presence of TIX-5 in plasmas of controls and cases. Data are depicted as median  $\pm$  95% confidence interval. Statistical significance: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$

did not correlate with TIX-5 sensitivity when evaluated in all patients on anticoagulants (cases and controls combined; Figure 3D), and the highest TIX-5 sensitivity appeared to coincide with normal levels of TFPI $\alpha$  in plasma ( $\sim 0.25$  nM; Figure S5 in supporting information). Interestingly, TFPI $\alpha$  levels that were approximately 9-fold higher than normal in two control patients ( $\sim 2.2$  nM; Figure S5A) were associated with a rather low TIX-5 sensitivity (Figure S5), but an average lag time without TIX-5 (not shown). In summary, major bleedings in patients on anticoagulants were associated with elevated plasma levels of TFPI $\alpha$ , while TFPI $\alpha$  levels did not correlate with TIX-5 sensitivity.

### 3.5 | High levels of TFPI $\alpha$ in the presence of FV-short lead to decreased TIX-5 sensitivity

TFPI $\alpha$  is known to interact with splice variants of FV that lack the basic regulatory region in their B-domains.<sup>17,28</sup> As a result of alternative splicing, FV-short comprises a stretch of acidic residues that are available for interaction with the basic C-terminal tail of TFPI $\alpha$ . High FV-short levels are associated with high TFPI $\alpha$  levels, presumably because the interaction with FV-short prevents the rapid clearance of TFPI $\alpha$  from plasma.<sup>14,17,28</sup> To investigate how FV-short levels affect thrombin generation and TIX-5 sensitivity in

**TABLE 1** Hazard ratio of major bleeding associated with quartiles of thrombin generation lag time and ETP

	Cases	HR (95% CI)
Lag time vehicle (min)		
<25 (<4.32)	44	Reference
25–50 (4.32–5.67)	64	1.56 (0.97–2.50)
50–75 (5.67–7.24)	51	1.14 (0.70–1.86)
>75 (>7.24)	85	<b>2.10 (1.32–3.31)</b>
Lag time TIX-5 (min)		
<25 (<8.00)	45	Reference
25–50 (8.00–11.00)	50	1.21 (0.74–1.97)
50–75 (11.00–15.67)	64	1.47 (0.92–2.35)
>75 (>15.67)	85	<b>2.10 (1.34–3.32)</b>
TIX-5 sensitivity (ratio) <sup>a</sup>		
<25 (<1.56)	56	Reference
25–50 (1.56–1.97)	61	1.09 (0.69–1.72)
50–75 (1.97–2.50)	51	1.01 (0.63–1.62)
>75 (>2.50)	76	<b>1.62 (1.04–2.52)</b>
ETP vehicle (nM*min)		
<25 (<383)	74	1.35 (0.87–2.11)
25–50 (383–531)	59	1.07 (0.68–1.68)
50–75 (531–730)	54	0.92 (0.58–1.47)
>75 (>730)	57	Reference
ETP TIX-5 (nM*min)		
<25 (<305)	69	<b>1.69 (1.07–2.68)</b>
25–50 (305–439)	64	1.59 (1.00–2.53)
50–75 (439–638)	64	<b>1.65 (1.04–2.64)</b>
>75 (>638)	47	Reference
TIX-5 ETP ratio		
<25 (<0.76)	67	1.41 (0.89–2.21)
25–50 (0.76–0.85)	49	0.94 (0.59–1.51)
50–75 (0.85–0.94)	73	1.27 (0.82–1.98)
>75 (>0.94)	55	Reference

Abbreviations: CI, confidence interval; HR, hazard ratio; ETP, endogenous thrombin potential.

Bold values indicate statistical significance.

<sup>a</sup>TIX-5 sensitivity defined as the lag time + TIX-5/lag time + vehicle ratio.

the absence and presence of TFPI $\alpha$ , we made use of the FV mutant FV-810 that lacks the basic autoinhibitory region in the B-domain similar to FV-short and as a consequence displays FVa cofactor activity.<sup>17,25,28</sup> Adding 1 nM of FV-810 to normal plasma led to a shortened lag time and a reduced TIX-5 effect (Figure 4A–C), which is consistent with the fact that FV-810 does not require proteolytic activation to function as a cofactor of FXa. An equimolar amount of TFPI $\alpha$  (1 nM) apparently neutralized the cofactor activity of FV-810 because TIX-5 regained some of its inhibitory effect, indicating that FXa activation of FV is a limiting factor under these conditions. Addition of a moderate excess of 2 nM TFPI $\alpha$  resulted in a prolongation of the lag time, a considerably lower velocity

index, and absence of any effect of TIX-5 on thrombin generation (Figure 4), indicating that TFPI $\alpha$  itself is a limiting factor for free FXa to activate FV in this set-up. These results indicate that TFPI $\alpha$  and FV-short differentially affect the contribution of FXa-mediated activation of FV to TG in plasma, dependent on their relative concentrations.

### 3.6 | Intracranial bleeding is associated with low ETP measured in the presence of TIX-5

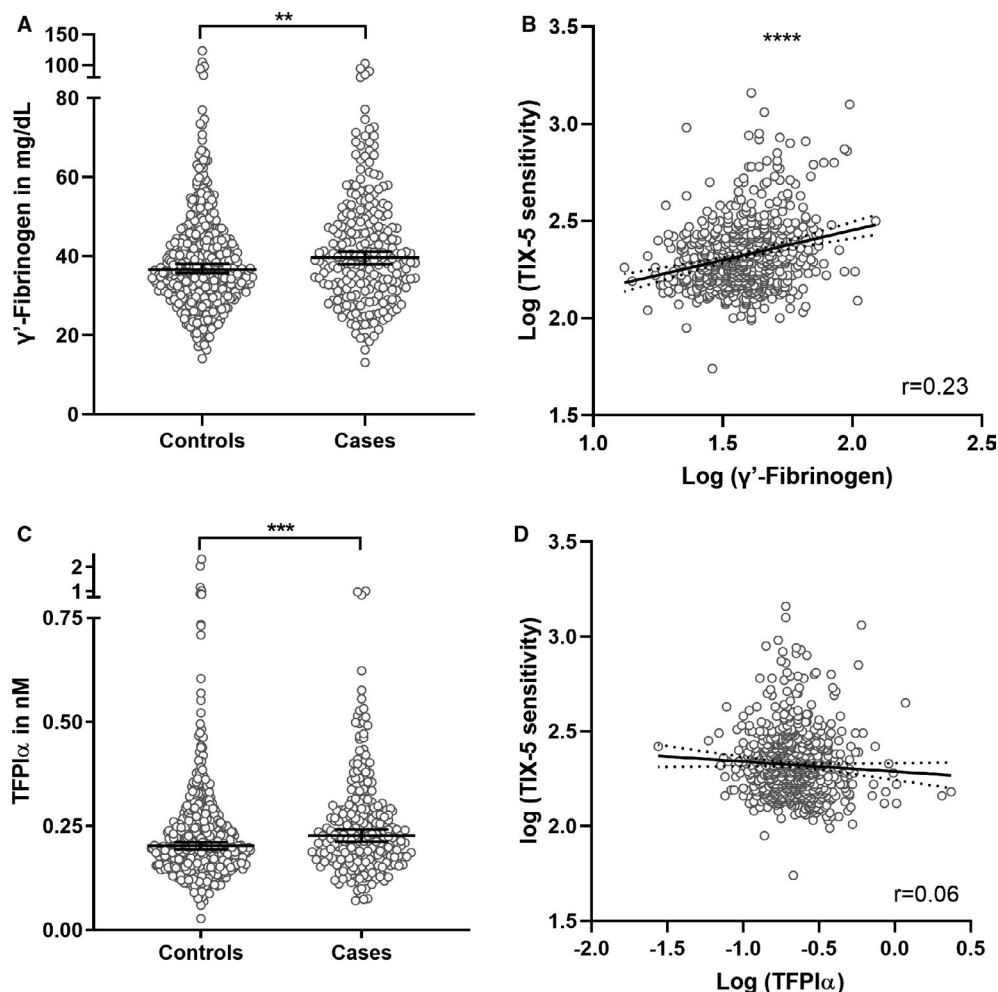
Because intracranial bleedings are most serious and often fatal, we determined the HRs of the TG parameters,  $\gamma'$ -fibrinogen, and TFPI $\alpha$  levels for intracranial bleeding separately (Table S4 in supporting information). Of these values, the ETP obtained in the presence of TIX-5 demonstrated an increased risk of intracranial bleeding in the lowest quartile (HR 2.14, 95% CI 1.00–4.62). This suggests a specific association of the TIX-5 inhibited ETP in TG and intracranial bleeding.

## 4 | DISCUSSION

Here we discovered that TG parameters measured in the presence of TIX-5 associated with major bleeding in the BLEEDS study with >16,000 patients on anticoagulant therapy, indicative of a role for FV activation by FXa in major bleeding. In particular, a marked prolongation of the median TG lag time in the presence of TIX-5 and a higher lag time-based TIX-5 sensitivity were associated with an increased risk of major bleeding. Moreover, a low ETP with TIX-5 present was also associated with major bleeding. Importantly, low ETP measured in the presence of TIX-5 was found to be associated with intracranial bleeding, the most severe and often fatal type of bleeding. These results suggest that a greater contribution of FXa-mediated activation of FV to TG is associated with an increased risk of major bleeding during anticoagulant therapy.

To our knowledge, this is the first demonstration of an association of plasma TG parameters with major bleeding during anticoagulant treatment.<sup>3,29</sup> Our study corroborates with the report of men et al. of CAT analysis in a cohort of 129 patients who received VKA therapy, from whom 26 patients were diagnosed with a less severe but clinically relevant bleeding,<sup>3</sup> and did not show a difference in plasma TG parameters compared to controls.

In line with the specific inhibitory action of TIX-5 on FXa mediated FV activation during the initiation phase of thrombin generation,<sup>9</sup> only the lag time of TG was sensitive to the effect of TIX-5 in plasma of healthy volunteers (Figure 1). The sensitivity to lag time prolongation by TIX-5 of individual donor plasma varied considerably, from no effect to a more than 2-fold prolonged lag time, which implies a large variation in the contribution of FV activation by FXa to thrombin generation between individuals (Figure 1). The TIX-5 sensitivity of the lag time appears stable over time in individuals and with different plasma handling (Figure S2). Using deficient



**FIGURE 3** Association of  $\gamma'$ -fibrinogen and tissue factor pathway inhibitor (TFPI $\alpha$ ) levels with major bleeding and TIX-5 sensitivity in cases and controls on vitamin K antagonist (VKA) therapy. Plasma levels of  $\gamma'$ -fibrinogen (A) or TFPI $\alpha$  (C) were determined in VKA patients without ( $n = 538$ ; controls) and with major bleeding ( $n = 263$ ; cases). The data are depicted as median  $\pm$  95% confidence interval. The TIX-5 sensitivity (lag time + TIX-5/lag time + vehicle ratio) was plotted versus the plasma levels of  $\gamma'$ -fibrinogen (B) or TFPI $\alpha$  (D) in all cases and controls combined. Correlations were analyzed by linear regression analysis. Statistical significance: \*\* $P < 0.01$ , \*\*\* $P < 0.005$ , \*\*\*\* $P < 0.001$

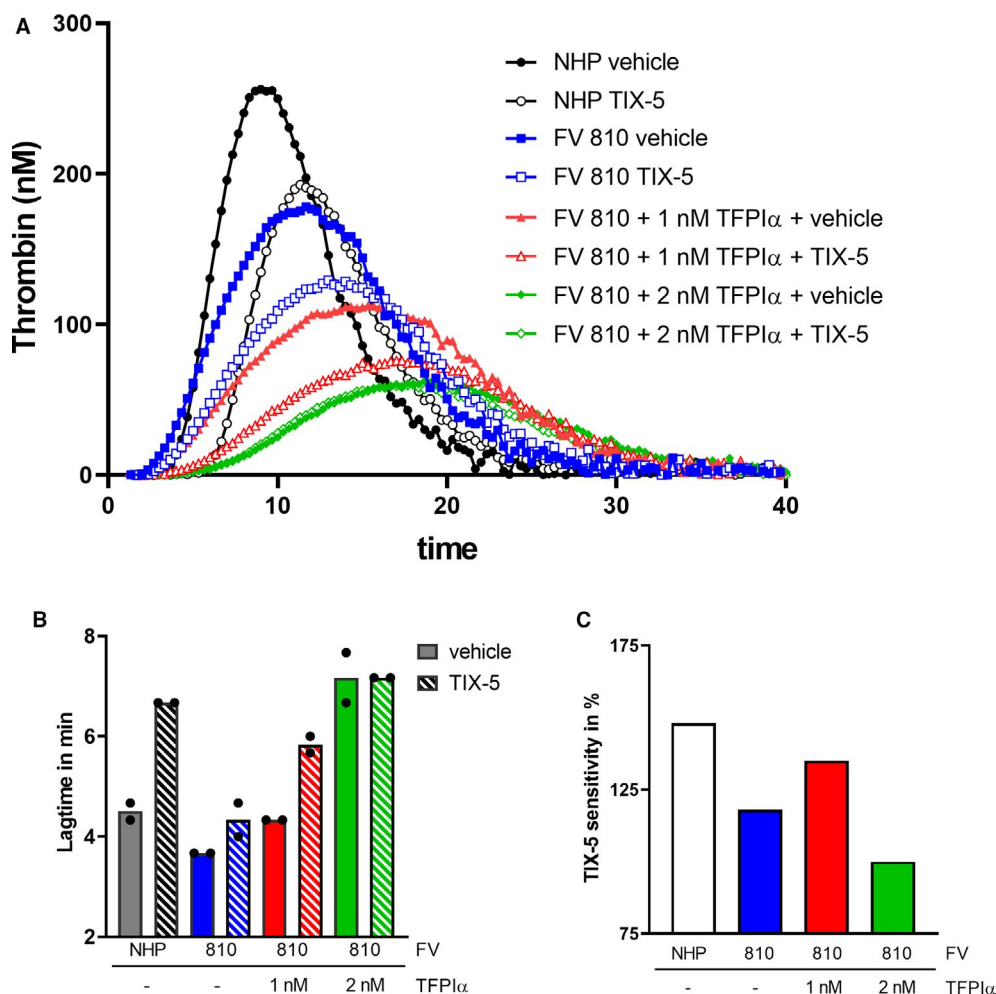
**TABLE 2** Hazard ratio of major bleeding associated with quartiles of  $\gamma'$ -fibrinogen and TFPI

	Cases	HR (95% CI)
$\gamma'$ -fibrinogen (mg/dL)		
<25 (<30.9)	51	Reference
25–50 (30.9–36.7)	40	0.70 (0.43–1.12)
50–75 (36.8–44.1)	56	0.97 (0.62–1.52)
>75 (>44.1)	87	<b>1.62 (1.06–2.48)</b>
TFPI (nM)		
<25 (<0.16)	45	Reference
25–50 (0.16–0.20)	50	1.13 (0.69–1.84)
50–75 (0.20–0.26)	54	1.20 (0.74–1.94)
>75 (>0.26)	91	<b>2.29 (1.46–3.61)</b>

Abbreviations: CI, confidence interval; HR, hazard ratio; TFPI, tissue factor pathway inhibitor.

Bold values indicate statistical significance.

plasmas we previously showed that TFPI and fibrinogen pose major restraints on thrombin generation and both contribute to the importance of the FXa-mediated FV activation pathway.<sup>9</sup> Here we show that TFPI $\alpha$  levels associate with risk of major bleeding in the BLEEDS study, but TFPI $\alpha$  levels do not correlate with TIX-5 sensitivity. TFPI $\alpha$  levels are regulated by interaction with FV-short, a FV splice variant that normally circulates at subnanomolar concentrations, but at nanomolar levels in case of mutation at specific FV splice sites. FV-short lacks a part of the B-domain including a so-called autoinhibitory basic region.<sup>17,28</sup> As such FV-short-like FV variants are active cofactors in the prothrombinase complex.<sup>25</sup> However, without this basic region, the acidic region in the B-domain of FV-short is available for interaction with TFPI $\alpha$ , which greatly reduces the clearance of TFPI $\alpha$  from the circulation and results in high circulating TFPI $\alpha$  plasma levels in complex with FV-short.<sup>17</sup> In complex with TFPI $\alpha$ , FV-short acts as a cofactor for TFPI $\alpha$  in the inhibition of FXa.<sup>18</sup> We validated the effect of FV-short alone and in combination with

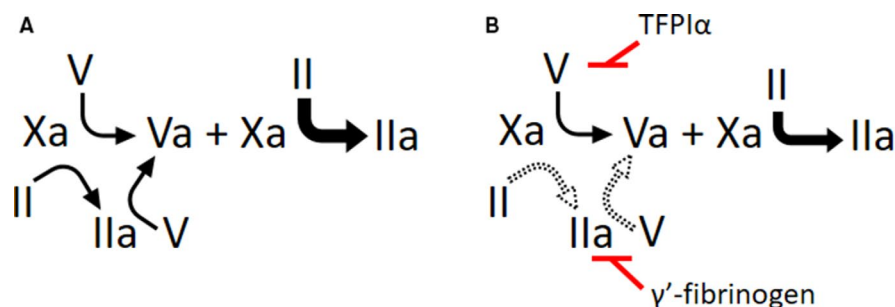


**FIGURE 4** Ablation of TIX-5 mediated lag time prolongation by FV-short like FV-810 is modulated by tissue factor pathway inhibitor (TFPI $\alpha$ ). Thrombin generation was initiated with 5 pM tissue factor in normal human pooled plasma (NHP) in the absence or presence of 4  $\mu$ M TIX-5 and with 1 nM FV-810 as indicated. A, Thrombograms representing NHP (black), NHP + FV810 (blue), NHP + FV810 + 1 nM TFPI $\alpha$  (red), or NHP + FV810 + 2 nM TFPI $\alpha$ . B, Lag times of thrombin generation in the absence (filled) or presence (striped) of TIX-5 depicted as mean ( $n = 2$ ), and (C) the TIX-5 sensitivity (lag time + TIX-5/lag time + vehicle ratio) of the curves in (A)

TFPI $\alpha$  on TG TIX-5 sensitivity to determine how these factors in combination affect the contribution of FXa mediated FV activation to TG. In accordance with the notion that FV-short is a fully active cofactor in prothrombinase, TIX-5 sensitivity was greatly reduced by 1 nM FV-short addition to plasma alone. Addition of 1 nM TFPI $\alpha$  to 1 nM FV-short reinstated TIX-5 sensitivity in plasma, while a 2-fold molar excess of TFPI $\alpha$  addition with 1 nM FV-short resulted again in lack of TIX-5 sensitivity. Concentrations of 2 nM TFPI $\alpha$  reach the level that will inhibit FXa efficiently,<sup>12</sup> and this apparently reduces free FXa concentrations such that these do not contribute to the activation of FV in the presence of FV-short. Interestingly, we observed the highest TFPI $\alpha$  levels of ~2.2 nM in two control patients without major bleeding, while these TFPI $\alpha$  levels are more than 8-fold elevated relative to the normal level of 0.25 nM TFPI $\alpha$ .<sup>26</sup> Our findings suggest that the present TG assay with TIX-5 is at least sensitive and potentially indicative for the relative concentrations of FV-short and TFPI $\alpha$ . At present, FV-short levels can only be estimated by immunoprecipitation of large plasma volumes

and subsequent semiquantitative western blotting. A low volume high-throughput assay would be required to estimate FV-short in large cohorts with relatively low plasma volumes available such as the BLEEDS study.

Another plasma protein that affects the contribution of FXa-mediated activation of FV to TG is fibrinogen, and particularly the splice variant  $\gamma'$ -fibrinogen that contains a high affinity thrombin binding site and inhibits FV activation by thrombin.<sup>11</sup> In the BLEEDS study, significantly higher  $\gamma'$ -fibrinogen levels were observed in cases with major bleeding compared to controls. Although the association was significant, the correlation between TIX-5 sensitivity and  $\gamma'$ -fibrinogen was low, indicating that the contribution of the FV activation by FXa to thrombin generation is for a considerable part regulated by factors other than  $\gamma'$ -fibrinogen. In Figure 5 we schematically represented the present findings regarding the risk of major bleeding associated with the dependence of thrombin generation on FXa mediated FV activation, and increased TFPI $\alpha$  and  $\gamma'$ -fibrinogen levels.



“increased risk of major bleeding”

**FIGURE 5** Schematic representation of the observed associated risk of major bleeding during vitamin K antagonist (VKA) therapy with processes and regulators of the pathway to thrombin. A, Factor FXa (Xa) and thrombin (IIa) activate FV (V) to FVa (Va) during the initiation phase of prothrombin (II) activation to thrombin. B, The quick activation of FV by thrombin (IIa) is inhibited by  $\gamma'$ -fibrinogen, increasing the contribution of FXa-mediated activation of FV. Tissue factor pathway inhibitor (TFPI $\alpha$ ) inhibits FXa, FV activation, FV-short, and FV activation intermediates (latter two not indicated for clarity). Greater dependence of thrombin generation on FXa-mediated FV activation, and higher TFPI $\alpha$  and  $\gamma'$ -fibrinogen levels are associated with major bleeding during VKA therapy

Although our findings clearly indicate an association of TG parameters measured in the presence of TIX-5 with major bleeding in the BLEEDS cohort, the predictive power for bleeding of TG with TIX-5 for the individual patient is limited as displayed by the large spread of the TG parameters both in the control group and group with major bleeding (see Figure S4). This is consistent with the observations that TG parameters vary considerably in plasma of patients treated with VKA<sup>3</sup> and are sensitive to plasma preparation and sample handling as reviewed recently by Tripodi.<sup>30</sup> While control and case plasmas assessed in this study were prepared and handled in the same way, we cannot rule out that minor differences may have occurred that contribute to variations in TG analyses. Another limitation of our study is that the effects of platelets and other blood cells are lacking in the TG measurements. Platelets provide primary hemostasis and regulate thrombin generation by exposure of procoagulant phospholipids and secretion of FV as well as TFPI $\alpha$ .<sup>31</sup> It should be noted that alterations in platelets and/or blood cells are not considered in the present study, while these could contribute to major bleeding. Excitingly, thrombin generation evaluation is now possible in whole blood and may provide additional info on bleeding risk.<sup>3</sup> However, in a large study of more than 300 patients with unknown cause of bleeding plasma TG parameters were found to be significantly different from healthy controls, indicating the value of plasma TG assessment to understand bleeding tendencies.<sup>32</sup> At present the algorithms to predict the occurrence of a major bleeding rely on clinical parameters, such as the most commonly used HAS-BLED score.<sup>33</sup> In the BLEEDS, we observed an association with major bleeding of thrombin generation parameters in the presence of TIX-5, TFPI $\alpha$  levels, and  $\gamma'$ -fibrinogen. These may prove to be valuable predictors as part of algorithms to help identify patients at risk for major bleeding while on anticoagulant therapy.

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## CONFLICTS OF INTEREST

Anja Maag and Cornelis van 't Veer applied for a patent under the name: “Method for determining the risk of a thromboembolic event or a major bleeding,” which is currently pending. All other authors declare no conflicts of interest.

## AUTHOR CONTRIBUTIONS

Anja Maag: experimental design, laboratory analysis, interpretation, and drafting of the manuscript. Nienke van Rein: interpretation and drafting and revision of the manuscript. Tim J. Schuijt: revision of the manuscript. Wil F. Kopatz: laboratory analysis. Daniëlle Kruijswijk: laboratory analysis. Stella Thomassen: laboratory analysis. Tilman M. Hackeng: providing vital new reagents and tools and revision of the manuscript. Rodney Camire: providing vital new reagents and tools and revision of the manuscript. Tom van der Poll: revision of the manuscript. Joost C.M. Meijers: revision of the manuscript. Mettine H.A. Bos: experimental design, interpretation, and revision of the manuscript. Cornelis van 't Veer: experimental design, laboratory analysis, interpretation, drafting and revision of the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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