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IN FOCUS

A novel anticoagulant activity assay of tissue factor pathway inhibitor I (TFPI)

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Summary. Tissue factor (TF) pathway inhibitor I (TFPI) is the physiological inhibitor of TF-induced blood coagulation. Circulating blood contains full-length TFPI and TFPI truncated at the C-terminal end. Previous studies have shown that full-length TFPI exerts a stronger anticoagulant effect on diluted prothrombin time (DPT) than truncated TFPI, and it has been suggested that full-length TFPI is biologically more important *in vivo*. The objective of this study was to develop and validate an assay of TFPI anticoagulant activity. TFPI anticoagulant activity was assayed using a modified DPT assay. Plasmas were incubated in the absence and the presence of TFPI-blocking antibodies. Results were expressed as a ratio with the clotting time in the presence of anti-TFPI as the denominator. The ratio was normalized against a ratio obtained with a reference plasma. The assay was compared with assays of TFPI free antigen, total antigen, and bound TFPI, and TFPI chromogenic substrate activity. We performed all tests in 436 healthy individuals. The normalized TFPI anticoagulant ratio was strongly associated with TFPI free antigen ($r = 0.73$) but was weakly associated with TFPI chromogenic substrate activity ($r = 0.46$), TFPI total antigen ($r = 0.48$), and bound TFPI ($r = 0.30$). TFPI chromogenic substrate activity was strongly associated with TFPI total antigen ($r = 0.73$). We have developed a novel assay of TFPI anticoagulant activity in plasma, which may be considered a functional assay of full-length TFPI. Further studies are needed to establish the role of TFPI anticoagulant activity for thrombotic disorders.

Keywords: TFPI, tissue factor, assay, coagulation.

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Introduction

Coagulation is initiated when tissue factor (TF) gains access to the circulating blood and forms a complex with zymogen coagulation factor VII (FVII) or activated FVII (FVIIa). The TF/FVIIa complex activates factor X (FX), and activated FX (FXa) catalyzes the formation of thrombin from prothrombin. TF/FVIIa catalytic activity is modulated by TF pathway inhibitor (TFPI), which in a first step binds to and inhibits FXa, the TFPI/FXa complex then binds to and inhibits the TF/FVIIa catalytic complex by forming a quaternary TFPI/FXa/TF/FVIIa complex. Thus, TFPI functions as an inhibitor of FXa as well as of TF/FVIIa [1].

There is now convincing evidence from *in vitro* and *in vivo* experiments that TFPI plays an important role in regulating the initiation of blood coagulation [2–7]. In man, some studies have found that low levels of TFPI are a weak risk factor for venous thrombosis [8–11]. However, manifest TFPI-deficiency, either associated or not with thrombotic disorders, similar to deficiencies of other coagulation inhibitors, have not yet been detected [12]. One possible explanation for this apparent discrepancy between the experimental evidence and data in humans could be that current TFPI assays are technically inadequate to detect the role of TFPI *in vivo* and thus to detect TFPI deficiency [11,12].

The complex inhibitory mechanism, as well as the complex distribution of TFPI *in vivo*, makes construction of an assay of TFPI a challenging task. A major intravascular pool of TFPI is free, full-length TFPI associated with the vascular endothelium which may be released to the circulation by heparin and other negatively charged ions. Although some free, full-length TFPI is detected in circulating blood, most circulating TFPI (80–95%) is truncated at the C-terminal and bound to lipoproteins [13,14]. Small amounts of full-length TFPI are also found in platelets.

TFPI bound to the endothelium is not readily assessed, but circulating TFPI may be detected by different assays [15]. Most commonly, TFPI is detected by commercially available enzyme linked immunosorbent (ELISA) assays [9,11], which either

detect free, full-length TFPI or lipoprotein-associated TFPI. TFPI activity assays are usually in-house methods that measure the overall capacity of TFPI to inhibit TF/FVIIa catalytic activity after prolonged incubation of TF/FVIIa with TFPI. Residual TF/FVIIa after incubation with TFPI is determined using an appropriate substrate, e.g. chromogenic substrates or tritium-labeled FIX [16–19].

In contrast, TFPI activity may be determined as the ability of TFPI to prolong clotting after the addition of a low concentration of TF to plasma, i.e. assays based on the diluted prothrombin time (DPT) [15]. Several *in vitro* studies have shown that free, full-length TFPI has a much stronger ability to prolong the clotting time than truncated TFPI or lipoprotein-associated TFPI, even though their functional chromogenic substrate activities are similar [20–23]. Since the ability to prolong the clotting time might be considered physiologically more important, it has been hypothesized that free, full-length TFPI with strong anticoagulant activity is more relevant for the function of TFPI *in vivo* [12,24]. Therefore, an assay for TFPI anticoagulant activity could be a better assay of TFPI activity *in vivo*, and might potentially be able to reveal TFPI deficiency states that other TFPI assays cannot capture. However, methods for the assay of plasma TFPI anticoagulant activity have not yet been established.

In the present study we have established and validated a method for the assay of TFPI anticoagulant activity based on a modified DPT assay. The assay was validated against ELISAs for TFPI free and total antigen, and against TFPI chromogenic substrate activity, all recorded in plasma samples from the Leiden Thrombophilia Study (LETS) [11].

Materials and methods

Reagents

Tris-buffered saline (TBS) was made to contain 0.05 mol L^{-1} Tris–HCl, 0.15 mol L^{-1} NaCl, at pH 7.4. Calcium chloride ($CaCl_2$) was diluted from 500 to 35 m mol L^{-1} in TBS. TF was human recombinant, relipidated TF (Dade®Innovin®, Baxter Diagnostics Inc., Miami, FL, USA), which was diluted 1/200 in TBS from stock solution. Anti-TFPI immunoglobulin (anti-TFPI IgG) was purified polyclonal IgG raised against human recombinant $TFPI_{1-161}$ in a goat and was a kind gift from Dr Per Østergaard, Novo-Nordisk, Gentofte, Denmark. Normal, non-immune goat-IgG (sc-2028) was purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. Full-length, recombinant TFPI (rTFPI, Cat#4900ACT, recombinant active human TFPI) was obtained from Amercian Diagnostica Inc, Stamford, CT, USA. Truncated, $rTFPI_{1-161}$ was a kind gift from Dr Ole Nordfang, Novo-Nordisk, Denmark.

All blood samples were collected in Sarstedt Monovette tubes (Sarstedt, Numbrecht, Germany) containing 0.106 mol L^{-1} trisodium citrate (0.1 vol), centrifuged for 10 min at 2000 × *g* at room temperature and stored in aliquots at − 70 °C until assayed. Reference plasma was collected from one healthy individual and a single batch was used for the study.

Assay of TFPI anticoagulant activity

We used a DPT assay essentially as described previously by other investigators [20,25,26] but modified as follows: 32 μL plasma was incubated with 8 μL TBS or 8 μL anti-TFPI IgG and incubated for 4–6 min at room temperature. Then, 30 μL TF was added and the mixture was incubated for 3 min at 37 °C. Finally, 30 μL 35 mmol L^{-1} $CaCl_2$ was added and the time to clotting was measured with a coagulometer (Amelung KC4, Trinity Biotech, Bray, Ireland). To obtain a reasonable difference in clotting time between plasmas with and without anti-TFPI antibodies, we aimed to achieve a clotting time of the reference plasma of approximately 75 s in the absence of anti-TFPI IgG. This was achieved at 1/200-fold dilution of TF, i.e. final dilution 3/2000 in the assay. The concentration of anti-TFPI IgG that completely inhibited the ability of TFPI to prolong clotting was established by adding increasing concentrations of anti-TFPI IgG until the clotting time was no longer shortened. The final concentration used in the assay was double the maximum inhibiting concentration. Addition of anti-TFPI IgG reduced clotting times by approximately 7–8 s in the reference plasma. This shortening of clotting times after blocking TFPI may be considered a measure of the ability of TFPI in the sample to inhibit coagulation. The ability of anti-TFPI IgG to neutralize TFPI was also tested using a chromogenic substrate assay [19], in which these antibodies were able to neutralize > 95% of the chromogenic substrate activity.

Test results were expressed as a TFPI anticoagulant ratio with DPT in the absence of anti-TFPI IgG as the numerator and DPT in the presence of TFPI blocking antibodies as the denominator. To minimize the effects of variations in assay conditions, e.g. day-to-day and batch-to-batch (of Innovin©) variations, both the anticoagulant ratios (and individual DPTs) were normalized against the corresponding ratios and DPTs obtained with the reference plasma. The normalized ratio was called the normalized TFPI anticoagulant (n-TFPIac) ratio. A low normalized ratio (< 1.0) indicates low TFPI anticoagulant activity, whereas a high ratio (> 1.0) indicates high TFPI anticoagulant activity. As a result of the limited amounts of plasma, most blood samples were only assayed in a single run. The between-day coefficient of variation (CV) was 2.8% and within-day CV was between 0.5% and 1.5%.

Control experiments revealed that rapid freezing and thawing for up to six cycles had a negligible effect on the test results. Moreover, normal, non-specific goat-IgG had no effect on clotting times.

Other TFPI assays

TFPI free antigen and TFPI total antigen were assayed in duplicates with commercial ELISAs (Asserachrom® Free TFPI and Asserachrom® Total TFPI, Diagnostica Stago, Asnière,

France) as described in detail elsewhere [11]. In both assays a monoclonal antibody was used to catch TFPI. Two other monoclonal antibodies conjugated with peroxidase were used to detect either free, i.e. full-length TFPI, or total, i.e. full-length and truncated and lipoprotein-associated TFPI. The TFPI free antigen assay was specific for free, circulating, full-length TFPI, and did not detect lipid-bound TFPI. The TFPI total antigen assay detected the total amount of TFPI, including full-length and truncated TFPI as well as lipid-bound TFPI. Freeze-dried human plasmas containing known amounts of TFPI that were provided in the kits were used for calibration. Quality controls were performed using a control specimen containing a high amount of TFPI as well as a sample with a normal level of TFPI. Inter- and intra-assay variability, measured as coefficients of variation, was 4.4% and 2.9% for TFPI total antigen, and 4.9% and 3.8% for TFPI free antigen, respectively.

Bound TFPI was calculated as the difference between TFPI total antigen and TFPI free antigen.

TFPI chromogenic substrate activity was assayed in duplicate with a two-stage chromogenic substrate assay as described earlier [19]. In this assay the TFPI activity was determined by the quantification of residual TF/FVIIa catalytic activity after the incubation of diluted plasma (containing TFPI) with TF, FVIIa in excess of TF binding sites, and FXa. Inter- and intra-assay variabilities were 3.5% and 1.4%, respectively.

Study population

In this study, we assayed blood samples collected from control subjects in The Leiden Thrombophilia Study (LETS). Details of study design and blood collection have been described in detail elsewhere [27]. In short, LETS was a case–control study with 474 patients with an objectively diagnosed first-time deep vein thrombosis and 474 sex- and age-matched controls. Residual plasma samples were available from 436 individuals (183 men and 253 women). We have previously reported that low levels of TFPI in this study, either of TFPI free antigen, TFPI total antigen, or TFPI chromogenic substrate activity, were associated with an increased risk of deep vein thrombosis [11]. The patients were selected from three anticoagulation clinics in the Netherlands, and the controls were acquaintances of the patients or partners of other patients. The Leiden University ethics committee approved the study protocol, and all participants gave informed consent to participate according to the Declaration of Helsinki.

Statistical analysis

The association between the different TFPI assays and the n-TFPIac ratio was studied in the controls because they represent the general population. Three individuals with extremely high n-TFPIac ratios were excluded from all analyses, but are reported separately.

Data are reported as mean and standard deviation (SD). Simple linear regression with TFPI chromogenic substrate activity and n-TFPIac ratio as dependent variables was used to estimate the association with the other TFPI assays and with other coagulation factors. Normalized regression coefficients (z-score of the independent variable, i.e. the independent variable expressed as a number of SD, negative or positive, from the mean) were calculated for comparison of the different regression coefficients. Extreme outliers were excluded from the regression analysis. All statistical analyses were executed using the STATISTICAL PACKAGE FOR SOCIAL SCIENCES version 10 (SPSS Inc, Illinois, USA).

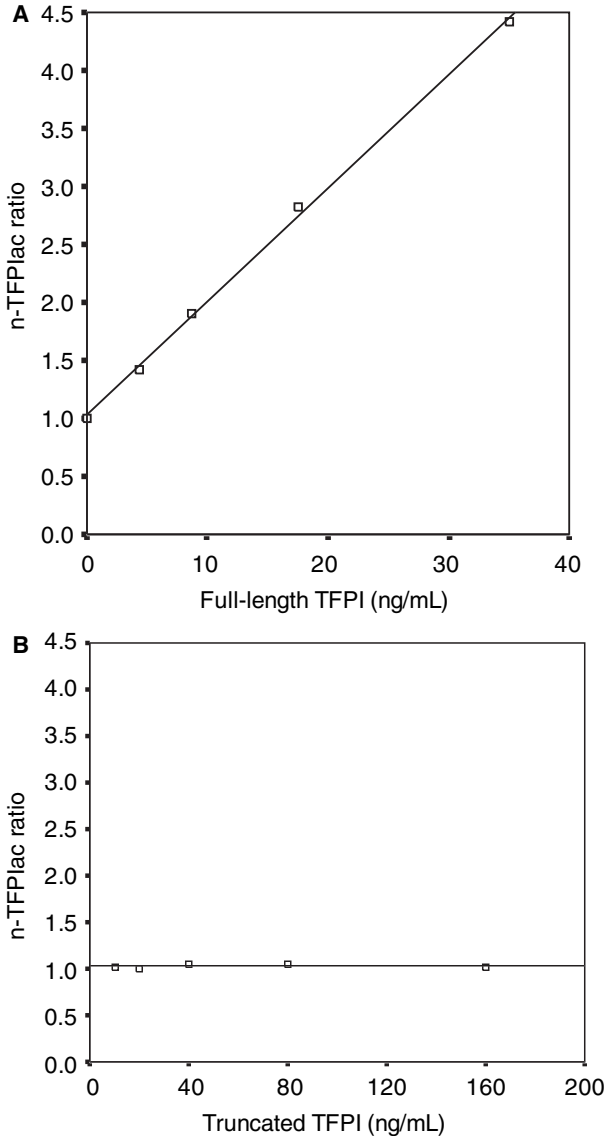

Fig. 1. Effect of full-length, recombinant TFPI (A) and recombinant $TFPI_{1-161}$ (B) on the normalized TFPI anticoagulant (n-TFPIac) ratio. The x-axis shows the final concentration of added recombinant TFPI in a carrier-plasma.

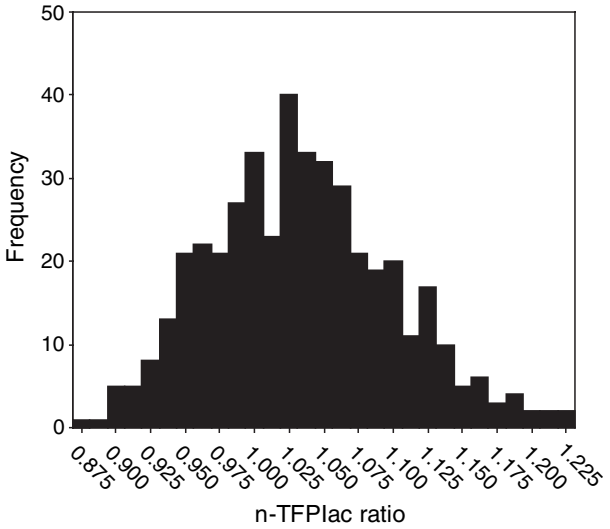

Fig. 2. Distribution of normalized TFPI anticoagulant (n-TFPIac) ratio in 436 individuals. Three individuals with TFPI anticoagulant ratios of 1.37, 1.41, and 1.85 were excluded.

Results

In vitro effects of TFPI on n-TFPIac ratio

In vitro recovery experiments were performed using either full-length rTFPI or truncated $rTFPI_{1-161}$. These experiments showed that increasing concentrations of full-length rTFPI (0–34,7 ng mL^{-1}) gave a corresponding linear increase in n-TFPIac ratio, while truncated $rTFPI_{1-161}$ (0–160 ng mL^{-1}) essentially had no effect on the n-TFPIac ratio (Fig. 1).

Normalized TFPI anticoagulant ratio in vivo

The mean age of the study population was 47 years (range 16–73 years). The n-TFPIac ratio was approximately normally distributed as determined by inspecting the histogram (Fig. 2). Mean n-TFPIac ratio was 1.03 (SD 0.066) with a range from 0.87 to 1.22. Three individuals had extremely high ratios (1.37, 1.41, and 1.85). These individuals also had elevated levels of TFPI antigen or chromogenic substrate activity. They were obvious outliers and consequently were excluded from the analyses. Other coagulation factors, i.e. fibrinogen, prothrombin, FV, FVII, FVIII, FIX, FX, FXI, protein S, and TAFI, and age had no or only a marginal effect on the n-TFPIac ratio.

The effects of plasma TFPI on the DPT

Normalized DPT (n-DPT), reflecting the prolongation in clotting times induced by plasma TFPI, was associated with plasma TFPI levels. This association was strongest for TFPI free antigen, and weakest for TFPI total antigen and bound TFPI (Fig. 3A–C). In the absence of TFPI, i.e. after the addition of anti-TFPI IgG, there were no clear associations between n-DPT and any of the TFPI assays (Fig. 3D–F).

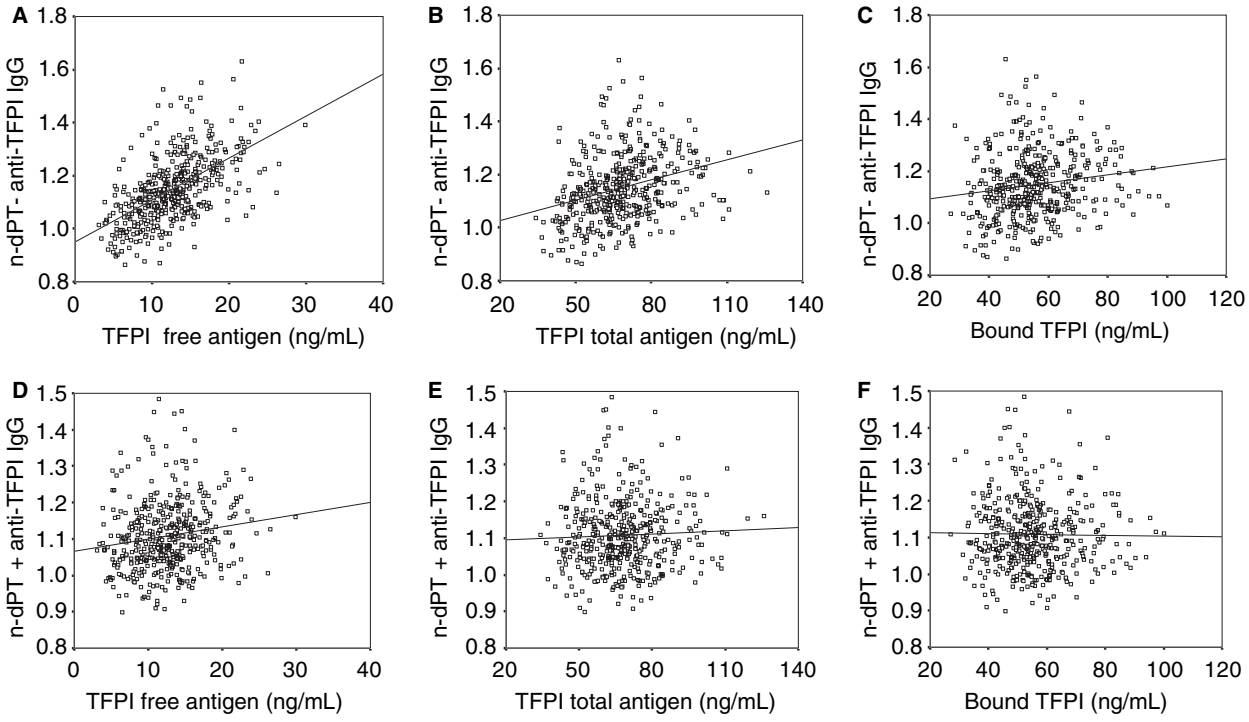

Fig. 3. Scatter plots with linear regression lines showing TFPI free antigen, TFPI total antigen, and bound TFPI as determinants of the normalized DPT (n-DPT). (A)–(C): the absence (–) of anti-TFPI IgG; (D)–(F): the presence (+) of anti-TFPI IgG.

Determinants of n-TFPIac ratio

TFPI free antigen was a stronger determinant of the n-TFPIac ratio than TFPI total antigen, TFPI chromogenic substrate activity, and bound TFPI (Fig. 4 and Table 1). Bound TFPI had the weakest association to the n-TFPIac ratio, while TFPI total antigen and TFPI chromogenic substrate activity had an intermediate and quite similar association to the n-TFPIac ratio.

Determinants of TFPI chromogenic substrate activity

TFPI total antigen and bound TFPI had approximately the same degree of association to TFPI chromogenic substrate activity. Both were stronger determinants of TFPI chromogenic substrate activity than TFPI free antigen (Fig. 5 and Table 2).

Discussion

In this study, we have tested a novel assay of TFPI anticoagulant activity in plasma. The assay is based on the ability of TFPI in plasma to prolong clotting time using a DPT assay essentially as described by other investigators [20,25,26]. However, in the present report TFPI anticoagulant activity was expressed as the ratio of DPT in plasma recorded in the presence of TFPI, i.e. after preincubation with buffer, to the DPT determined in the absence of TFPI, i.e. after preincubation with TFPI blocking antibodies. This ratio was normalized against a ratio obtained with a reference plasma to minimize variations of assay conditions. The assay was reproducible with intra- and interassay coefficients less than 3%, and showed a clear dose–response relationship for full-length rTFPI (but not $rTFPI_{1-161}$) added *in vitro*. Finally, the assay was validated against currently available antigen and functional assays.

We have found that n-TFPIac ratio is strongly related to TFPI free antigen, i.e. free, full-length TFPI, in plasma, but not with TFPI chromogenic substrate activity, TFPI total antigen or bound TFPI. These results are strongly supported by the *in vitro* recovery experiments which demonstrated that the assay was highly sensitive to full-length rTFPI, but was not influenced by truncated TFPI ($rTFPI_{1-161}$). The n-TFPIac ratio may therefore be considered a functional assay of free, full-length TFPI, and would potentially allow detection of functionally abnormal TFPI molecules. Our findings corroborate previous studies showing that free, full-length TFPI exerts a much stronger anticoagulant effect in DPT assays than truncated or lipoprotein-associated TFPI at concentrations with nearly identical chromogenic substrate activities [20–23], and that the C-terminal end is vital for the anticoagulant activity in DPT-based assays [28–30]. We have also found that TFPI chromogenic substrate activity is mainly associated with bound or lipoprotein-associated TFPI and confirmed that TFPI chromogenic activity is strongly associated with TFPI total antigen [19].

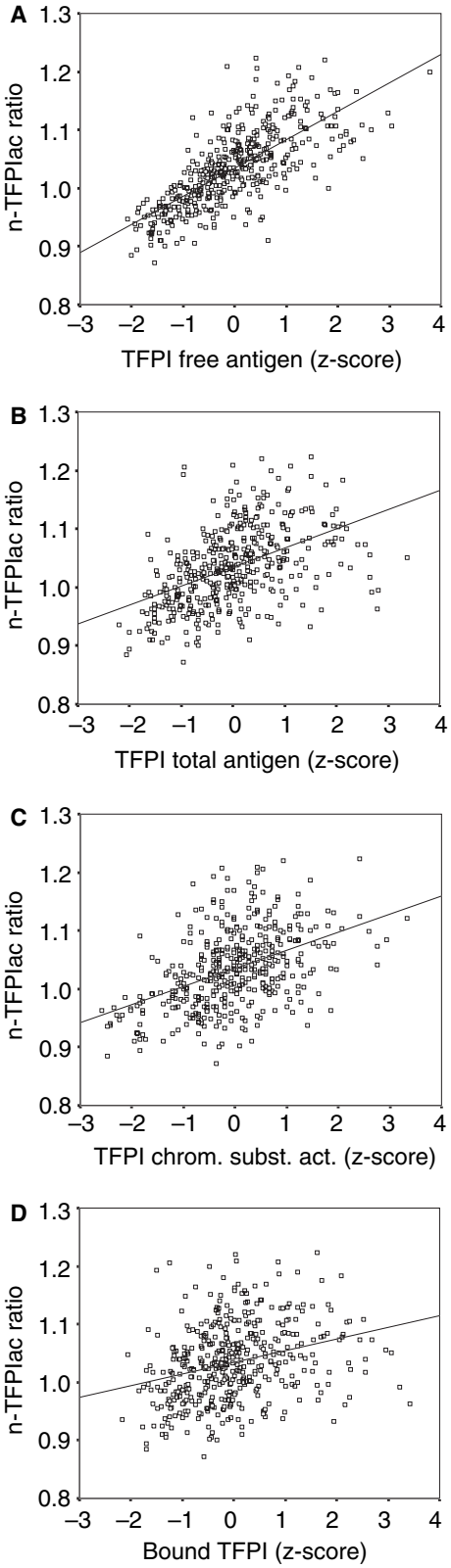

Fig. 4. Scatter plots with linear regression lines. The dependent variable (*y*-axis) is the normalized TFPI anticoagulant (n-TFPIac) ratio in all four plots. The independent variable (*x*-axis) is expressed as the number of standard deviations from the mean (*z*-score) and is TFPI free antigen (A), TFPI total antigen (B), TFPI chromogenic substrate activity (C), and bound TFPI (D).

Table 1 The association of normalized TFPI anticoagulant ratio to other TFPI assays

TFPI assay	Normalized TFPI anticoagulant ratio *b* (95% CI)	Correlation coefficient *r*
TFPI free antigen	0.049 (0.044–0.053)	0.73
Total TFPI	0.033 (0.027–0.038)	0.48
TFPI activity	0.031 (0.025–0.037)	0.46
Bound TFPI	0.020 (0.014–0.026)	0.30

Standardized regression coefficient (*b*), 95% confidence interval (CI) and correlation coefficient (*r*). (*b*) shows the change in n-TFPIac ratio per increase of one standard deviation in the TFPI assay studied.

Mechanistically, the difference between the two activity assays may be attributed to the reaction kinetics between TFPI and its target proteases. TFPI is both an inhibitor of FXa and of the TF/FVIIa catalytic complex. Inhibition is thought to occur in two steps, first the binding of the second TFPI Kunitz domain to FXa, which results in functional inhibition of FXa, and in a second step, binding of the first TFPI Kunitz domain of the TFPI/FXa complex to FVIIa of the TF/FVIIa complex [1]. This results in the formation of a catalytically inactive quaternary TFPI/FXa/TF/FVIIa complex.

Binding of TFPI to FXa is the rate-limiting step [31]. Free, full-length TFPI binds to and inhibits FXa at a several-fold higher rate than truncated or lipoprotein-associated TFPI [1,32], which is attributed to the conserved C-terminal end of full-length TFPI [1,33]. Once formed, the TFPI/FXa complex inhibits TF/FVIIa catalytic activity with similar efficiency irrespective of the nature of the TFPI, i.e. truncated or full-length TFPI, incorporated in the TFPI/FXa complex [1,31]. In the DPT assay used for assay of n-TFPIac activity, clotting occurs within approximately 75 s after the addition of a small amount of TF, and reaction velocities then play an important role for anticoagulant activity. TFPI cannot inhibit TF/VIIa until some FXa has been generated. TFPI/FXa complexes are formed more rapidly with free, full-length TFPI than with truncated and lipoprotein-associated TFPI, which allows more rapid and efficient inhibition of TF/FVIIa and consequently prolonged clotting time. TFPI anticoagulant activity may therefore be considered a kinetic assay and to a large extent dependent on the initial inhibition of FXa [34,35].

In the TFPI chromogenic substrate activity assay TF, FVIIa and FXa are added to plasma containing TFPI and incubated for approximately 30 min, which allows near-equilibrium conditions for the inhibition of TF/FVIIa. This means that full-length TFPI and the different forms of TFPI are all allowed to form complexes with FXa irrespective of the reaction velocities. The TFPI chromogenic substrate assay is therefore functionally an end-point or equilibrium assay, which cannot distinguish the ability of different TFPI molecules to inhibit FXa and the TF/FVIIa. TFPI chromogenic substrate assay may also be considered a capacity assay because it correlates well with TFPI total antigen.

We conclude that the present TFPI anticoagulant activity assay primarily measures the effect of full-length TFPI and is essentially insensitive to truncated TFPI. TFPI anticoagulant activity can therefore be considered an activity-assay of free, full-length TFPI. Further studies are needed to determine the role of TFPI anticoagulant activity *in vivo*, and whether TFPI anticoagulant activity should replace one or more current assays for the measurement of TFPI in plasma samples from patients with thrombosis.

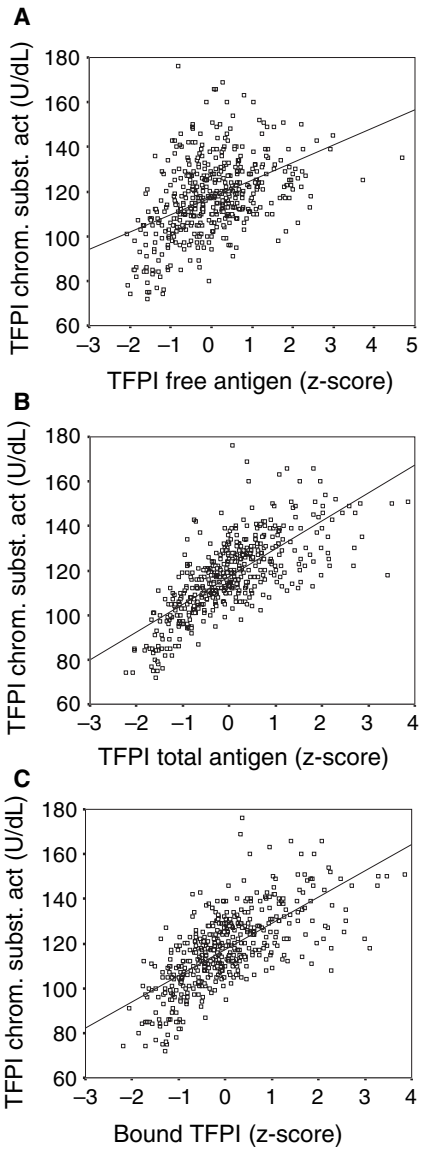

Fig. 5. Scatter plots with linear regression lines. The dependent variable (*y*-axis) is TFPI chromogenic substrate activity in all three plots. The independent variable (*x*-axis) is expressed as the number of standard deviations from the mean (*z*-score) and is TFPI free antigen (A), TFPI total antigen (B), and bound TFPI (C).

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Table 2 Association of TFPI chromogenic substrate activity to other TFPI assays

TFPI assay	TFPI chromogenic substrate activity *b* (95% CI)	Correlation coefficient *r*
Total TFPI	12.5 (11.5–13.6)	0.73
Bound TFPI	11.7 (10.6–12.9)	0.68
TFPI free antigen	7.82 (6.42–9.21)	0.45

Standardized regression coefficient (*b*), 95% confidence interval (CI) and correlation coefficient (*r*). (*b*) shows the change in chromogenic substrate activity per increase of one standard deviation in the TFPI assay studied.

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