


The Impact of Anticoagulant Activity of Tissue Factor Pathway Inhibitor Measured by a Novel Functional Assay for Predicting Deep Venous Thrombosis in Trauma Patients: A Prospective Nested Case-Control Study

Clinical and Applied
Thrombosis/Hemostasis
Volume 27: 1-12
© The Author(s) 2021
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/10760296211063877
journals.sagepub.com/home/cat


Xiangyu Cao¹, Yu Su², Wenjie Zhang², Huiru Zhao², Meng Wen²,
Shan Lu², Yujing Zhao², Yuying Chen³, Lei Liu⁴, Xinwei Zang¹,
and Jun Wu^{1,2} 

Abstract

Objective: Deep venous thrombosis (DVT) is a common complication in patients with traumatic injury. Tissue factor pathway inhibitor (TFPI) is a natural anticoagulant protein in the extrinsic coagulation pathway. However, the relationship between DVT after trauma and the anticoagulant activity of TFPI remains unclear. In this prospective study, we investigated the role of TFPI in trauma patients with DVT to evaluate whether the anticoagulant activity of TFPI measured by a new functional assay can be used to help predict the risk of DVT. **Patients and methods:** This prospective nested case-control study enrolled trauma patients and healthy volunteers. Forty-eight trauma patients diagnosed with DVT and forty-eight matched trauma patients without DVT were included in the study. 120 healthy volunteers were also included as controls. Blood samples and case information were collected at admission. Patients accepted angiography before surgery to diagnose DVT. The parameters examined included TFPI anticoagulant activity, free-TFPI antigen, blood cell counts, and routine clinical coagulation tests. **Results:** For the parameters of TFPI anticoagulant activity, three were markedly increased in the DVT group compared to the non-DVT group (TFPI initial anticoagulant time ratio, $P = .022$; TFPI whole anticoagulant time ratio, $P = .048$; and TFPI anticoagulant rate, $P = .034$). The free-TFPI antigen concentration also showed a significant increasing trend in trauma patients with DVT compared with trauma patients without DVT ($P = .035$). Multivariate logistic regression analysis identified four independent factors for the development of DVT (TFPI initial anticoagulant time ratio, free-TFPI antigen, prothrombin time, and red blood cell count). We calculated the TFPI correlation coefficient and found that the area under the receiver operating characteristic curve was .821. **Conclusions:** A novel functional assay was developed to measure the anticoagulant activity of TFPI. The anticoagulant activity of TFPI can be used as a potential biomarker for diagnosing DVT in trauma patients.

Keywords

tissue factor pathway inhibitor, anticoagulant activity, trauma, deep venous thrombosis

Date received: 4 October 2021; revised: 31 October 2021; accepted: 11 November 2021.

¹ Peking University Fourth School of Clinical Medicine, Beijing, China

² Beijing Jishuitan Hospital, Beijing, China

³ Beijing Chao-Yang Hospital, Capital Medical University, Beijing, China

⁴ Wuhan Third Hospital, Hubei, China

Corresponding Author:

Jun Wu, Peking University Fourth School of Clinical Medicine, Beijing Jishuitan Hospital, Xicheng District, Beijing 100035, China.

Email: wujunpostbox@sina.com



Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (<https://creativecommons.org/licenses/by-nc/4.0/>) which permits non-commercial use,

reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access page (<https://us.sagepub.com/en-us/nam/open-access-at-sage>).

1. Introduction

Deep venous thrombosis (DVT) and pulmonary embolism are the main causes of hospital-related morbidity and mortality and are also recognized as important complications in trauma patients.¹ Clinical data have shown that the incidence of DVT in patients with a traumatic fracture is almost 30%.^{2,3} The morbidity and mortality related to DVT caused by trauma is very high, although its mechanism remains unclear. Therefore, it is important to gain a better understanding of the pathophysiology of DVT caused by trauma in order to diagnose and prevent this serious condition.

Tissue factor pathway inhibitor (TFPI), also known as exogenous coagulation pathway inhibitor, is a very important natural anticoagulant *in vivo*. TFPI is produced by microvascular endothelial cells and pooled in the endothelium (80%), plasma (10%-20%), and platelets (<2.5%).^{4,5} The concentration of TFPI in plasma is very low, with the major component (80%) bound to lipoproteins (ie, high-density lipoprotein [HDL]),^{6,7} while the full length, minor component [20%], called free TFPI, is unbound and therefore has the main anticoagulant effect. The anticoagulant effect of TFPI can be attributed to two mechanisms: inhibition of tissue factor-activated coagulation factor VII [TF-FVIIa] and activated coagulation factor X [FXa] via the tissue factor-activated coagulation factor VII-tissue factor pathway inhibitor-activated coagulation factor X [TF-FVIIa-TFPI-FXa] complex,⁸ and inhibition of prothrombinase as reported in a recent study.⁹

There is convincing evidence from *in vitro* and *in vivo* experiments that TFPI plays an important role in regulating the initiation of blood clotting.^{10,11} However, to date no study has shown a clear relationship between thrombosis and TFPI. While studies have reported that the antigen level of TFPI is related to venous thrombosis and the low antigen levels are a risk factor for venous thrombosis,¹² patients with trauma contrastingly have high levels of the antigen.^{13,14} The reason for this increase in TFPI levels in trauma patients with DVT is not clear. At present, the majority of studies on TFPI focus on antigen levels,^{15,16} a parameter, which for a number of reasons, does not provide a measure of TFPI anticoagulant activity *in vivo*. The anticoagulant activity of TFPI may more accurately reflect the effect of TFPI on blood coagulation, and therefore it is very important to evaluate this anticoagulant activity in patients.

The objective of our study was to develop a functional assay for measuring the anticoagulant activity of TFPI in plasma from normal and trauma patients with or without DVT, and evaluate biomarkers of DVT risk and the relationship between TFPI and DVT.

2. Patients and Methods

2.1. Study Design and Patients

A total of 475 traumatic patients were recruited for this prospective nested case-control study at the Traumatic Orthopedics

Emergency Department of Beijing Jishuitan Hospital from November 2019 to December 2020. The inclusion criteria were: adults with traumatic lower extremity fractures of femoral shaft, distal femoral, patella, tibial plateau, tibiofibular shaft, or ankle. The exclusion criteria were: patients with diseases of the immune system, cancer, inflammation, a history of thrombosis and anticoagulant therapy, and a history of COVID19 infection. All the patients had angiographic results to estimate whether they had suffered from DVT before surgery. The trauma patients with DVT were defined as the DVT group (n = 48), while those without DVT were matched according to age and sex and called the non-DVT group (n = 48). The study also included a control group of 120 healthy individuals free from any acute or chronic disease. The study protocol is shown in Figure 1. Ethical approval for the study was given by the medical ethics committee of Beijing Jishuitan Hospital.

2.2. Blood Collection and Preparation

Blood samples were collected from the trauma patients the first time they were admitted to the emergency department after being injured. The samples were collected into tubes containing either ethylene diamine tetra-acetic acid (EDTA) or 3.2% trisodium citrate. Both tubes were centrifuged for 15 min at 2500 × g to produce platelet-poor plasma (PPP) that was used to measure routine clinical laboratory parameters. The citrated plasma sample was then centrifuged twice for 15 min at 2500 × g to produce platelet-free plasma (PFP) that was stored at -80°C until analysis. The frozen-PFP samples were thawed at 37°C for 5 min before use. All the samples from each patient were tested in the same series of analyses.

2.3. Detection of Routine Clinical Laboratory Parameters

An EDTA-anticoagulated whole blood sample was used to measure red blood cell (RBC), white blood cell (WBC), and platelet (PLT) counts in a Sysmex XT4000i analyzer (Sysmex, Kobe, Japan). A coagulation profile that included activated partial thromboplastin time (APTT), prothrombin time (PT), and international normalized ratio (INR) was measured in the PPP sample by an optical method using a Sysmex CS2100i analyzer (Sysmex). A Sysmex CS-5100 analyzer (Sysmex) was used to measure fibrinogen (FIB) and D-dimer (DD) levels in the PPP sample using the Clauss and immunoturbidimetry methods, respectively.

2.4. Free-TFPI Antigen Level in Plasma

The concentration of plasma-free TFPI antigen was determined by an enzyme linked immunosorbent assay (ELISA) method (Quantikine human TFPI ELISA, R&D Systems, Minneapolis, USA) using an Infinite M200_pro reader (Tecan, Männedorf Switzerland).

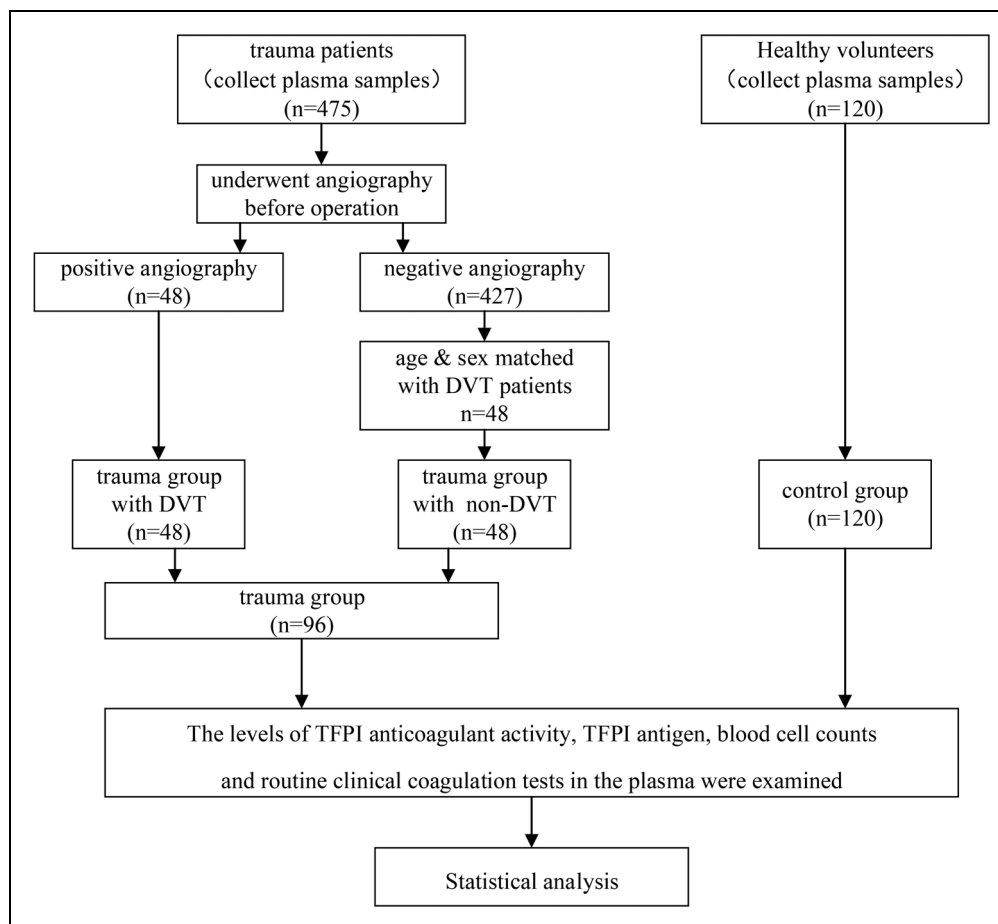


Figure 1. Study protocol.

2.5. Assay of TFPI Anticoagulant Activity

We developed an assay to evaluate TFPI anticoagulant activity based on the diluted prothrombin time (dPT) assay.¹⁷ Three reagents were used for this assay. Recombinant human tissue factor lipoprotein (rhTF) protein (PT1002a, rTF1130, assaypro, USA) at a concentration of 3000 PM was dissolved in distilled water and stored at -80°C . Anti-TFPI immunoglobulin G (anti-TFPI IgG) (AF2974, R&D Systems, Minneapolis, USA) was a purified polyclonal IgG raised in a goat and was stored at -20°C at a concentration of 0.2 mg/ml reconstituted in distilled water. Calcium chloride dihydrate crystals (HuShi, China) at a Ca^{2+} concentration of 0.5 mol/L dissolved in distilled water was stored at 4°C .

For the assay, each plasma sample was divided into two wells, one test (with TFPI antibody), and one test (without TFPI antibody) in 96-well transparent plates (E20073F9, Greiner Bio-One, Frickenhausen, Germany). In the one test well (with TFPI antibody), 48 μL PFP, 12 μL of anti-TFPI IgG (20 $\mu\text{g}/\text{ml}$), 20 μL aliquot of rhTF (25 PM), and 20 μL Ca^{2+} (0.025 M) were added to a final volume of 100 μL . In the test well (without TFPI antibody), an equal volume of distilled water replaced with anti-TFPI IgG (20 $\mu\text{g}/\text{ml}$).

The assay of TFPI anticoagulant activity was performed by initially the addition of 48 μL of PFP and then incubated for 15

min at room temperature with 12 μL distilled water or 12 μL of anti-TFPI IgG (20 $\mu\text{g}/\text{ml}$). A 20 μL aliquot of rhTF (25 PM) was then added and incubated for 10min at 37°C . Finally, 20 μL Ca^{2+} (0.025M) was added and after the mixture was shaken for 10 s the time and plasma absorbance to clotting were measured every 10 s for 2 h at 405nm and 37°C using an Infinite M200_pro reader (Tecan, Switzerland).

The reader automatically graphed two curves of time-related changes in plasma absorbance that were used to examine differences in the clotting process between plasma with or without TFPI antibodies. Another two curves were constructed using the absorbance value of the detection well subtracted from that of the blank well to examine time-related differences in absorbance difference. (Figure 2.A) We then calculated the first derivative of the two curves. (Figure 2.B&C)

Three parameters were defined to evaluate the anticoagulant activity of TFPI as follows.

1. TFPI initial anticoagulant time ratio (%) = (time AB - time DE) / time AB \times 100. (time AB represents the time about the coagulation without TFPI antibody from initial velocity to maximum velocity; time DE represents the time about the coagulation with TFPI antibody from initial velocity to maximum velocity)

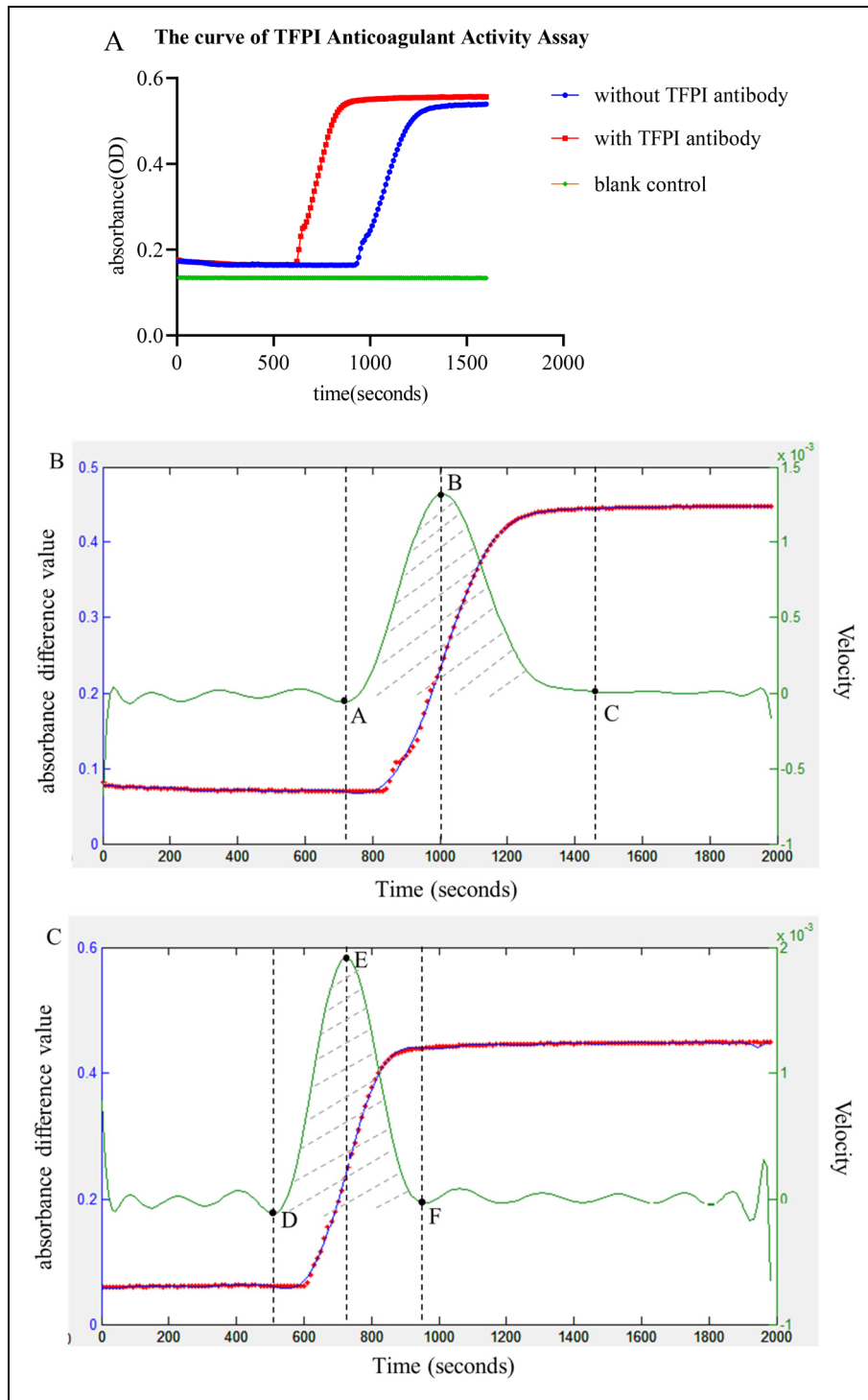


Figure 2. The curve of TFPI anticoagulant activity.

A. The curve of TFPI anticoagulant activity. The blue line was used as a curve to show the plasma coagulation process with time-dependent absorbance without TFPI antibody. The red line was used as a curve to show the plasma coagulation process with time-dependent absorbance with TFPI antibody. The green line was used as the blank control to represent the baseline.

B. The curve of TFPI anticoagulant activity (without TFPI antibody). The red dots represent the difference in absorbance, which is obtained by subtracting the absorbance value of the control hole from the absorbance value of the test hole (without TFPI antibody). The blue line represents the fitting curve. The green line represents the first derivative of the fitted curve.

C. The curve of TFPI anticoagulant activity (with TFPI antibody). The red dots represent the difference in absorbance, which is obtained by subtracting the absorbance value of the control hole from the absorbance value of the test hole (with TFPI antibody). The blue line represents the fitting curve. The green line represents the first derivative of the fitted curve.

Table 1. Characteristics of Studied Groups

Variables	Trauma (n = 96)	Trauma DVT (n = 48)	Trauma non-DVT (n = 48)	Control (n = 120)
Age (years)	63.5 (33.8)	61.0 (33.3)	65.0 (33.3)	39.0 (23.8)
Gender	N (%)			
Male	44 (45.8%)	22 (45.8%)	22(45.8%)	56 (46.7%)
Female	52 (54.2%)	26 (54.2%)	26 (54.2%)	64 (53.3%)
Fracture position	N (%)			
Femur	60 (62.5%)	33 (68.8%)	27 (56.3%)	
Tibia	21 (21.8%)	10 (20.8%)	11 (22.9%)	
Ankle joint	7 (7.3%)	5 (10.4%)	2 (62.5%)	
Patella	4 (4.2%)	0 (0.0%)	4 (62.5%)	
Calcaneus	4 (4.2%)	0 (0.0%)	4 (62.5%)	
Trauma reason	N (%)			
Tumble	71 (73.9%)	37 (77.1%)	34 (70.8%)	
Falling from height	4 (4.2%)	2 (4.2%)	2 (4.2%)	
Car accident	19 (19.8%)	8 (16.6%)	11(22.9)	
Other	2 (2.1%)	1 (2.1%)	1(2.1%)	
Time from injury to admission (hour)	N (%)			
0 to 24	54 (56.3%)	25 (52.1%)	29 (60.4%)	
25 to 72	20 (20.8%)	10 (20.8%)	10 (20.8%)	
>72	22 (22.9%)	13 (27.1%)	9 (18.8%)	
Time from injury to angiography(hour)	N (%)			
0 to 72	38 (39.6%)	16 (33.3%)	22 (45.8%)	
73 to 120	24 (25.0%)	13 (27.1%)	11 (22.9%)	
>120	34 (35.4%)	19 (39.6%)	15 (31.3%)	

2.TFPI whole anticoagulant time ratio (%) = (time AC-time DF)/ time AC × 100. (time AC represents the time about the coagulation without TFPI antibody from initial velocity to final velocity; time DF represents the time about the coagulation with TFPI antibody from initial velocity to final velocity)

3.TFPI anticoagulant rate (%) = (area DF-area AC)/ area AC × 100. (area DF represents the area under curve about the coagulation without TFPI antibody from initial velocity to final velocity; area AC represents the area under curve about the coagulation with TFPI antibody from initial velocity to final velocity)

To verify the coefficient of variation (CV), NPP was used in the assay of TFPI anticoagulant activity, three times a day for three consecutive days, with each experiment conducted independently.

2.6. Statistical Analysis

SPSS 23.0 and GraphPad Prism 7.0 were used for the statistical analyses. The data with a normal distribution were expressed as means ± standard and non-normal data were presented as median (interquartile range). The values of RBC, WBC, PLT, PT, APTT, INR, FIB, DD, free-TFPI antigen, and TFPI anticoagulant activity were compared between healthy people and trauma patients, and between three groups including healthy people and trauma patients with or without DVT before surgery. We used univariate analysis to identify variables which showed statistically significant differences between two groups. The Kruskal–Wallis test was used to assess whether

the data had normal distribution. Differences between data with an abnormal distribution were analyzed by the Mann–Whitney U test, and data with a normal distribution by the Students T test. Differences in variables between three groups were examined using analysis of variance. Multivariate logistic regression analysis incorporating a backward stepwise model was used to generate adjusted odds ratios (OR) and 95% confidence intervals (CI). To estimate the predictive value of the various indices receiver operating characteristic (ROC) curves were constructed for area under the curve (AUC) analysis with 95% CI. The correlation between variables was examined by calculating Pearson coefficients (r). For all box plots shown, the horizontal bars represent medians and the vertical bars represent the 2.fifth and 97.fifth percentiles. *P* values <0.05 were regarded as statistically significant.

3. Results

3.1. Study Population

A total of 475 trauma patients who satisfied the inclusion and exclusion criteria were enrolled in the study between November 2019 and December 2020. 48 patients were diagnosed with DVT and were age- and sex-matched to 48 patients without DVT. In addition, 120 healthy controls (males 56 and females 64) aged 26 to 93 years were enrolled in the study. The demographics and clinical characteristics of the healthy controls and patients are summarized in Table 1. During the study, the incidence of DVT before surgery in the trauma patients was 10.1% (48/475).

Table 2. Parameters of TFPI anticoagulant activity in Healthy Population

Parameters	Gender			Age, y					P
	Male (n = 64)	Female (n = 56)	P	18 to 30 (n = 25)	31 to 40 (n = 42)	41 to 50 (n = 18)	51 to 60 (n = 18)	≥61 (n = 17)	
TFPI initial anticoagulant time ratio (%)	35.14 ± 11.35	34.03 ± 10.85	0.586	37.01 ± 9.33	36.65 ± 11.88	29.98 ± 5.58	36.24 ± 11.32	33.80 ± 9.39	0.062
TFPI whole anticoagulant time ratio (%)	11.40 ± 5.03	10.36 (29.15)	0.119	11.10 ± 5.43	10.90 ± 5.26	10.82 ± 4.16	10.86 ± 5.69	11.08 ± 9.61	0.947
TFPI anticoagulant rate (%)	28.41 ± 8.56	28.70 ± 10.16	0.862	28.22 ± 7.08	30.70 ± 9.68	26.04 ± 5.51	29.65 ± 10.50	26.76 ± 9.15	0.064

*Normally distributed data are presented as mean ± standard, and non-normally distributed data are showed as median (interquartile range).

3.2. Determination of the Reference Range for TFPI Anticoagulant Activity in Healthy Population

The descriptive statistics of the three parameters of TFPI anticoagulant activity was established in 120 healthy individuals. TFPI initial anticoagulant time ratio, mean $33.81 \pm 10.48\%$; TFPI whole anticoagulant time ratio, mean $10.45 \pm 5.10\%$; and TFPI anticoagulant rate, mean $28.18 \pm 9.22\%$. The three parameters' values were also not significantly different at different ages and sexes (Table 2).

The between-day coefficient of variation (CV) for TFPI initial anticoagulant time ratio, TFPI whole anticoagulant time ratio, and TFPI anticoagulant rate were 2.8%, 18.5%, and 9.1%, respectively, while the corresponding within-day CVs were 6.4%, 19.9%, and 3.4%.

Table 3. Comparison of Parameters in Healthy Controls and Trauma Patients

Parameters	Healthy group (n = 120)	Trauma group (n = 96)	P
TFPI initial anticoagulant time ratio (%)	33.81 ± 10.48	37.51 ± 12.19	0.049
TFPI whole anticoagulant time ratio (%)	10.45 ± 5.10	12.77 ± 6.77	0.045
TFPI anticoagulant rate (%)	28.18 ± 9.22	28.58 (17.35)	0.825
Prothrombin time (s)	12.00 (0.70)	12.43 ± 0.85	<0.001
International normalized ratio	1.00 ± 0.06	1.02 ± 0.09	0.021
Activated partial thromboplastin time (s)	27.07 ± 2.24	24.98 ± 3.09	<0.001
Fibrinogen (mg/dL)	247.90 (43.08)	346.50 (187.60)	<0.001
D-dimer (mg/L)	0.19 (0.17)	5.93 (8.66)	<0.001
White blood cell ($\times 10^9/L$)	5.87 (1.65)	9.72 (4.64)	<0.001
Red blood cell ($\times 10^9/L$)	4.62 ± 0.51	4.14 (0.81)	<0.001
Platelet ($\times 10^9/L$)	229.23 ± 51.42	213.00 (75.50)	0.053

*Normally distributed data are presented as mean ± standard, and non-normally distributed data are showed as median (interquartile range).

3.3. Comparison of TFPI Anticoagulant Activity and Other Parameters Between Healthy People and Trauma Patients

All the parameters measured in the healthy controls and trauma patients are shown in Table 3.

For TFPI anticoagulant activity, there were significant increases in the levels of TFPI initial anticoagulant time ratio and TFPI whole anticoagulant time ratio in the trauma group ($37.51 \pm 12.19\%$; $12.77 \pm 6.77\%$) compared to the healthy group ($33.81 \pm 10.48\%$, $P = .049$; $10.45 \pm 5.10\%$, $P = .045$), respectively. The TFPI anticoagulant rate was not significantly different between the two groups ($P = .825$).

3.4. Comparison of TFPI Anticoagulant Activity and Other Parameters in Healthy People and Trauma Patients with or Without DVT

All the parameters measured in the healthy controls and trauma patients with DVT or non-DVT are shown in Table 4.

For the parameters of TFPI anticoagulant activity, the levels of TFPI initial anticoagulant time ratio, TFPI whole anticoagulant time ratio, and TFPI anticoagulant rate were higher in the trauma group with DVT ($39.91 \pm 13.44\%$; $13.65 \pm 7.96\%$; and $32.61 \pm 15.43\%$) compared with the healthy group ($33.81 \pm 10.48\%$, $P = .013$; $10.45 \pm 5.10\%$, $P = .016$; and $28.18 \pm 9.22\%$, $P = .029$, respectively). In addition, there was no significant difference in these three parameters between the healthy group and the trauma group without DVT.

3.5. Biomarkers of DVT Risk

3.5.1. Comparison of parameters between DVT and non-DVT patients. The univariate analyses shown in Figure 3 indicated that TFPI initial anticoagulant time ratio, TFPI whole anticoagulant time ratio, TFPI anticoagulant rate, free-TFPI antigen, PT, and D-dimer were significantly associated with the occurrence of DVT in trauma patients.

For the parameters of TFPI anticoagulant activity, all three parameters were increased markedly in the DVT group compared with the non-DVT group. The level of TFPI initial

Table 4. Comparison of Parameters in Healthy Controls, and Trauma Patients with or without DVT

Parameters	Healthy controls (n = 120)	Trauma & Non-DVT (n = 481)	Trauma & DVT (n = 48)	P value
TFPI initial anticoagulant time ratio (%)	33.81 ± 10.48	32.19 ± 10.28	39.91 ± 13.44 ^{a, b}	0.013
TFPI whole anticoagulant time ratio (%)	10.45 ± 5.10	9.77 ± 4.91	13.65 ± 7.96 ^{a, b}	0.016
TFPI anticoagulant rate (%)	28.18 ± 9.22	25.87 ± 10.32	32.61 ± 15.43 ^{a, b}	0.029
Prothrombin time (s)	12.00 (0.70)	12.23 ± 0.76 ^a	12.63 ± 0.89 ^a	<0.001
International normalized ratio	1.00 ± 0.06	1.01 ± 0.07	1.03 ± 0.09 ^a	0.028
Activated partial thromboplastin time (s)	27.07 ± 2.24	25.28 ± 3.51 ^a	24.76 ± 3.03 ^a	<0.001
Fibrinogen (mg/dL)	247.90 (43.08)	358.05 ± 152.88 ^a	362.70 ± 117.83 ^a	<0.001
D-dimer (mg/L)	0.19 (0.17)	3.31 (10.68) ^a	7.30 (5.85) ^a	<0.001
White blood cell (×10 ⁹ /L)	5.87 (1.63)	10.19 ± 3.72 ^a	10.58 (4.41) ^a	<0.001
Red blood cell (×10 ⁹ /L)	4.62 ± 0.51	4.22 ± 0.63 ^a	4.07 (1.07) ^a	<0.001
Platelet (×10 ⁹ /L)	229.23 ± 51.42	223.85 ± 59.60	215.00 (94.00)	0.149

* Normally distributed data are presented as mean ± standard, and non-normally distributed data are showed as median (interquartile range).

a P < 0.050, and the difference is statistically significant when compared with healthy controls.

b P < 0.050, and the difference is statistically significant when compared with trauma patients with non-DVT.

anticoagulant time ratio was higher in the DVT group (39.91 ± 13.44%) than in the non-DVT group (32.19 ± 10.28%, $P = .022$). The level of TFPI whole anticoagulant time ratio was markedly elevated in the group with DVT (13.65 ± 7.96%) compared with those in the group with non-DVT (9.77 ± 4.91%, $P = .048$). The level of TFPI anticoagulant rate in DVT patients (32.61 ± 15.43%) also tended to be increased compared with that in non-DVT patients (25.87 ± 10.32%, $P = .034$).

For free-TFPI antigen concentration, the level of free-TFPI antigen showed a similar significant increasing trend in trauma patients with DVT (33.43 [16.15] ng/ml) compared with those without DVT (29.43 ± 9.93 ng/ml, $P = .035$).

For the routine clinical coagulation parameters, a significant difference in the level of PT was observed between the DVT (12.63 ± .89 s) and non-DVT groups (12.23 ± .76 s, $P = .030$). The level of DD was also increased in the DVT group (7.30 [5.85] mg/L) compared with the non-DVT group (3.31 [10.68] mg/L, $P = .039$).

3.5.2. Risk factors for DVT. Factors significant in the univariate analyses were included in the multivariate logistic regression analysis (Table 5).

In trauma patients, the TFPI initial anticoagulant time ratio (OR = .902, 95% CI 0.814-0.998, $P = .046$), free-TFPI antigen (OR = 0.941, 95% CI 0.890-0.995, $P = .033$), and PT (OR = 0.364, 95% CI 0.174-0.764, $P = .008$) were protective factors for DVT, while RBC (OR = 2.748, 95% CI 1.085-6.957, $P = .033$) was a risk factor of DVT.

We then created a formula for the TFPI correlation coefficient that fitted the model and the new indicator as: $(-0.104) \times \text{TFPI initial anticoagulant time ratio (\%)} + (-0.061) \times \text{free-TFPI antigen (ng/ml)} + (-1.010) \times \text{PT (s)} + (1.001) \times \text{RBC (} \times 10^9/\text{L)} + (7.239)$.

3.5.3. Diagnostic value of TFPI anticoagulant activity. To investigate the usefulness of the parameters (TFPI initial anticoagulant

time ratio, TFPI whole anticoagulant time ratio, TFPI anticoagulant rate, free-TFPI antigen, PT, DD, and TFPI correlation coefficient) for diagnosing DVT in trauma patients, a ROC curve analysis was conducted for the DVT and non-DVT groups (Table 6 and Figure 4).

For the TFPI initial anticoagulant time ratio, the optimal cut-off value was 33.53% with a sensitivity of 71.11% and specificity of 55.32% (AUC 0.646, 95% CI 0.533-0.759, $P = .016$). For the free-TFPI antigen, the optimal cut-off value was 37.65 ng/mL with a sensitivity of 40.43% and specificity of 82.61% (AUC 0.627, 95% CI 0.513-0.740, $P = .035$). For the PT, the optimal cut-off value was 12.05s with a sensitivity of 72.92% and specificity of 47.92% (AUC 0.617, 95% CI 0.505-0.730, $P = .048$). For the DD, the optimal cut-off value was 3.825 mg/L with a sensitivity of 85.42% and specificity of 51.11% (AUC 0.624, 95% CI 0.505-0.744, $P = .039$). For the TFPI correlation coefficient, the optimal cut-off value was 0.370 with a sensitivity of 64.44% and specificity of 89.36% (AUC 0.821, 95% CI 0.734-0.907, $P < .001$).

4. Discussion

In this study, we developed a method to quantify and evaluate the anticoagulant activity of TFPI in plasma based on the dPT experiment that extends the clotting time. This method uses plasma turbidimetry to monitor and graph, in real time, changes in plasma absorbance over time. The graphs were used to compare differences between the plasma coagulation curve with and without TFPI antibody and calculate the relevant parameters of the first derivative curve to evaluate TFPI anticoagulation ability and quantify the anticoagulant effect produced by TFPI in plasma. We also established the reference range for this method in healthy people and found that the parameters were not significantly different in age and gender groups.

Research findings in recent years on the anticoagulant effect of TFPI can be divided into two parts.^{8,9} The first part is inhibition of FXa and TF-FVIIa which is considered to occur in two

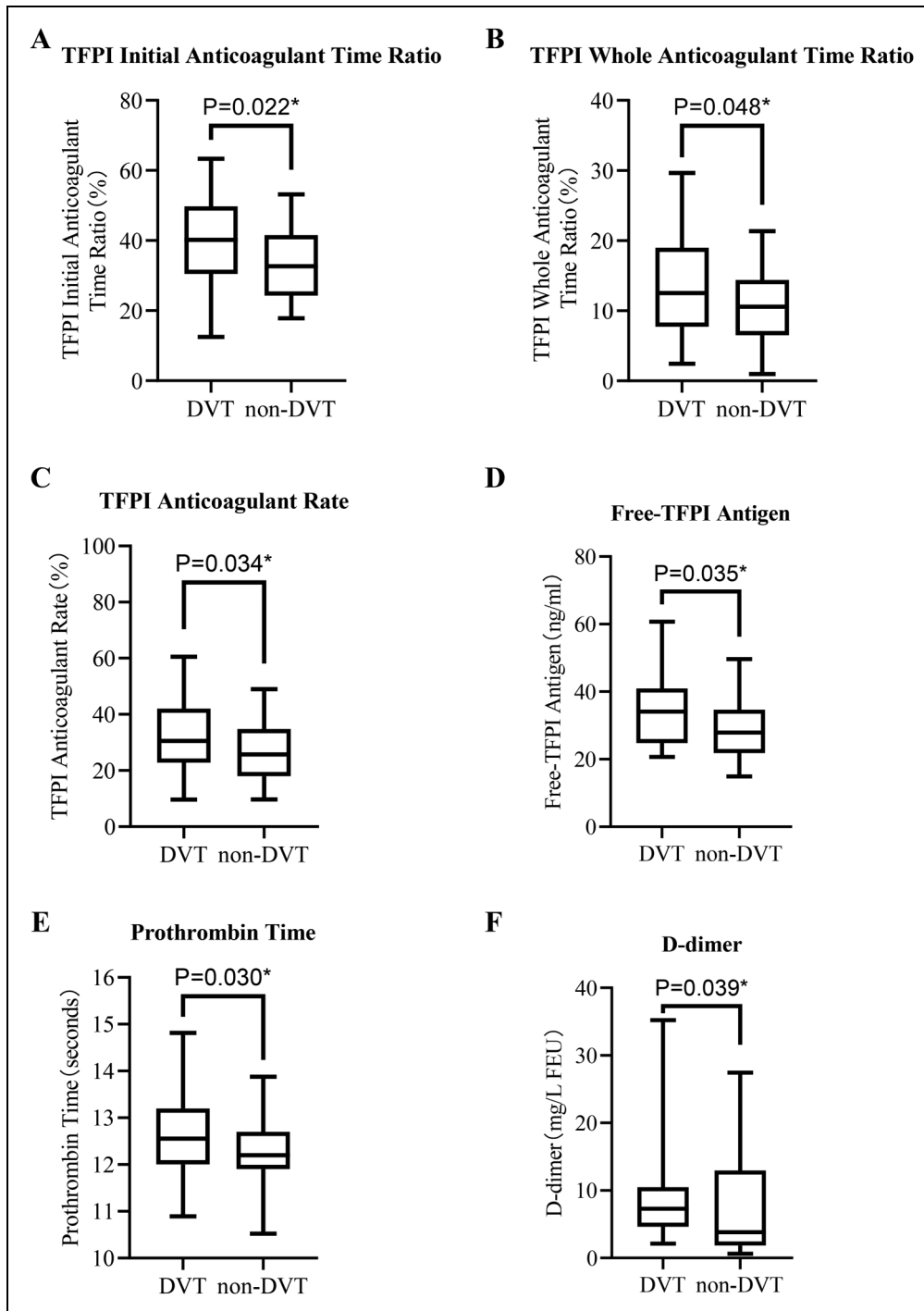


Figure 3. Comparison of parameters between the DVT group (n = 48) and the non-DVT group (n = 48), including the (A) TFPI initial anticoagulant time ratio (P = .022), (B) TFPI whole anticoagulant time ratio (P = .048), (C) TFPI anticoagulant rate (P = .034), (D) free-TFPI antigen (P = .035), (E) Prothrombin time (P = .030), and (F) D-dimer (P = .039). * P < .05, and the difference is statistically significant.

steps. The first step is the combination of TFPI- Kunitz domain 2 and FXa, leading to inhibition of FXa's coagulation function. The second step is to combine TFPI- Kunitz domain 1 in the tissue factor pathway inhibitor-activated coagulation factor X (TFPI-FXa) complex with FVIIa in the TF-FVIIa complex to form the TF-FVIIa-TFPI-FXa quadruplex complex. The

binding of TFPI and FXa is the rate limiting step. Due to the presence of the C-terminus in full-length TFPI, the rate at which it binds and inhibits FXa increases. Therefore, compared with truncated TFPI, full-length TFPI can form a TFPI-FXa complex faster, resulting in a more rapid and effective anticoagulation effect, thereby prolonging the clotting time.¹⁸ The

Table 5. Independent Risk Factors for DVT

Risk factors	β value	OR value	OR of 95% CI	P value
TFPI initial anticoagulant time ratio, %	-0.104	0.902	0.814 to 0.998	0.046
Free-TFPI antigen, ng/ml	-0.061	0.941	0.890 to 0.995	0.033
Prothrombin time, s	-1.010	0.364	0.174 to 0.764	0.008
Red blood cell, $\times 10^9/L$	1.001	2.748	1.085 to 6.957	0.033

Abbreviation: CI, confidence interval; OR, odds ratio.

second part is the inhibitory effect of TFPI on prothrombinase, which was discovered in recent years and represents the unique anticoagulant function of full-length TFPI.¹⁹ Studies have shown that the anticoagulant activity of TFPI depends mainly on full-length TFPI,^{20,21} and therefore we used full-length TFPI antibody in the test. Because the TFPI anticoagulant activity measured is due mainly to the effect of full-length TFPI, the test may not be sensitive to truncated TFPI. The anticoagulant activity of TFPI can therefore be regarded as a measurement of the anticoagulant activity of free full-length TFPI.

At present, the commonly used methods for TFPI are the double antigen sandwich method for measuring the antigen level of TFPI and the substrate color method for assessing the ability of TFPI to inhibit the TF-FVIIa complex.^{22,23} The free-TFPI antigen detection method does not evaluate the true anticoagulant effect of TFPI in the body, while the TFPI substrate color method does not distinguish between full-length and truncated TFPI. Therefore what is detected is only part of rather than the entire anticoagulant effect of TFPI.²⁴ However, the detection method has the advantage of measuring the anticoagulant effect in the whole body, which has greater biological relevance.

We then applied this test to trauma patients and compared the anticoagulant activity of TFPI between trauma patients and healthy people. We found that the anticoagulant activity of TFPI in trauma patients was significantly higher than that in healthy controls, a finding consistent with the results of Keyloun's study.¹³ The reason for the increased anticoagulant activity of TFPI in patients with trauma may be related to extensive injury of vascular endothelial cells and inflammation

caused by traumatic fractures. There is no doubt that traumatic fracture as an external force leads to endothelial cell activation and injury.²⁵ TFPI is expressed and released mainly by vascular endothelial cells,²⁶ with its main pool related to the surface of endothelial cells,²⁷ and is speculated to play a major role in regulating the function of vascular wall cells. Previous studies have shown that during the clinical course of DIC, the level of plasma free-TFPI antigen increases in conjunction with the increase in the plasma TF antigen level.^{28,29} This indicates that plasma TFPI is released from the inside or surface of vascular endothelial cells. Kato et al.²⁷ found an inverse relationship between the level of high density lipoprotein cholesterol and free TFPI before and after administration of heparin, a finding that reflects the compensatory enhancement of vascular wall antithrombotic properties to maintain normal endothelial function. The inverse relationship between lipoprotein lipase and free TFPI after heparin indicates competitive sharing of the binding sites of heparan sulfate proteoglycans on the endothelial surface. These results suggest that full-length TFPI in plasma may be a marker of endothelial cell dysfunction. Basic experiments carried out by Zhao et al.³⁰ found that TFPI can reduce endothelial cell injury by inhibiting endothelial autophagy and the class III PI3K/Beclin-1 pathway, while in clinical studies, Keyloun et al.¹³ used TFPI as a biomarker of endothelial injury and showed that the high level of TFPI in burn patients was related to a nine-fold increase in mortality (OR:9.59), and provided a predictive index of burn mortality. On the other hand, the inflammation caused by trauma has always been the focus of research attention, with studies showing that the levels of pro-inflammatory factors in plasma of post-traumatic patients were increased significantly.³¹ In recent years, the relationship between TFPI and inflammation has also been studied extensively.^{32,33} Ameri et al.³⁴ found in endothelial cell experiments that the inflammatory mediators (phorbol myristate acetate, endotoxin, interleukin-1, or tumor necrosis factor- α) stimulated endothelial cells to express high levels of TFPI mRNA and release high levels of TFPI into the supernatant. This indicated that unlike thrombomodulin and plasmin synthesized by endothelial cells, TFPI synthesis was not down-regulated, or even slightly up-regulated during inflammation. Cibor et al.³⁵ then reported that the level of free-TFPI antigen in patients with inflammatory enteritis and acute pancreatitis was significantly higher than that in healthy controls, with the concentration of plasma TFPI related to the

Table 6. Diagnostic Values of the Identified Indicators

Diagnostic Values	Cut-off value ^a	Sensitivity%	Specificity%	AUC	AUC of 95% CI	P value
TFPI initial anticoagulant time ratio	33.53	71.11	55.32	0.646	0.533 to 0.759	0.016
TFPI antigen	37.65	40.43	82.61	0.627	0.513 to 0.740	0.035
Prothrombin time	12.05	72.92	47.92	0.617	0.505 to 0.730	0.048
D-dimer	3.825	85.42	51.11	0.624	0.505 to 0.744	0.039
TFPI correlation coefficient	0.370	64.44	89.36	0.821	0.734 to 0.907	<0.001

^aAll cut-off values were determined by the Youden index.

Abbreviation: AUC, area under the curve; CI, confidence interval.

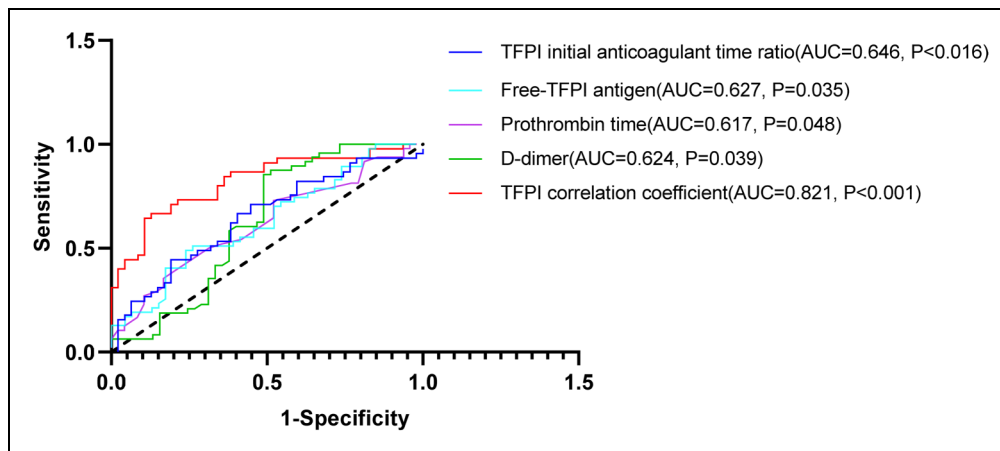


Figure 4. Receiver operating characteristic curves for indicators to predict DVT in trauma patients. The TFPI initial anticoagulant time ratio is represented by a dark blue line with an area under the curve of .646 ($P < .016$); free-TFPI antigen is represented by a light blue line with an area under the curve of 0.627 ($P = .035$); prothrombin time is represented by a purple line with an area under the curve of 0.617 ($P = .048$); D-dimer is represented by a green line with an area under the curve of .624 ($P = .039$); and TFPI correlation coefficient is represented by a red line with an area under the curve of 0.821 ($P < .001$).

severity of disease and prognosis. These results suggest that the increase in the anticoagulant activity of TFPI in patients with trauma may be related to the release of inflammatory mediators after trauma. However, further investigations are needed to determine the reasons for the increase in TFPI levels in trauma patients.

DVT is one of the most common complications of traumatic lower extremity fractures.¹ At present, the diagnosis of post-traumatic DVT relies of invasive angiography,³⁶ and there is a lack of new biomarkers for accurate assessment of coagulation status and diagnosis of DVT. We therefore divided the trauma patients into DVT and non-DVT groups. A case-control study by Dham et al.¹² showed that a decrease in the TFPI concentration was a risk factor for DVT. Interestingly, in contrast with the results of Dham et al. we observed that the anticoagulant activity and antigen level of TFPI in the DVT group were significantly higher than those in the non DVT group, consistent with the results of Sidemann et al.¹⁴ This variability in results may be due to the different pathophysiological mechanism of DVT caused by different diseases. For example, Dham et al.¹² studied the relationship between the occurrence of DVT and TFPI, Sidemann et al.¹⁴ studied acute DVT patients, while our study focused on the occurrence of DVT in trauma patients. As expected after trauma, TFPI was not depleted due to hypercoagulability, although the anticoagulant activity and antigen level of TFPI increased. We made the following assumptions to elucidate the cause of the increase of TFPI level in patients with DVT after trauma. First, we speculated whether the relationship between the occurrence of DVT and the increase of TFPI concentration after trauma was related to the injury of vascular endothelial cells. In previous studies, our team observed that endothelial dysfunction in trauma patients played an important role in the occurrence and development of DVT. Thrombomodulin (TM), as an endothelial cell membrane protein, was increased significantly in trauma

patients with DVT.³⁷ In addition, the content of endothelial tissue factor microparticles was increased significantly in post-traumatic DVT patients (results not published). On one hand, the increase of TFPI level may be a stress reaction to the hypercoagulable state in trauma patients with DVT, and on the other hand, may reflect the degree of endothelial cell damage, one of three major factors of thrombosis. We subsequently speculated on the potential impact of inflammation on the relationship between DVT and TFPI after trauma, because TFPI can be produced by over-reaction to inflammatory stimuli and it has been reported that inflammation is related to the formation of DVT after trauma. The above discussion needs to be verified by follow-up studies.

Although the current research study achieved its objectives, it had certain limitations. Because of the low DVT positive rate in trauma patients and the difficulty of sample collection, the sample size and the number of detection indicators were relatively small. In order to prevent interference from other factors such as surgery and drugs, no samples were collected at consecutive time points. Therefore, in order to verify our results, a large-sample, multicenter cohort study needs to be conducted. At present, only the potential relationship between the occurrence of DVT in trauma patients and changes of TFPI have been studied. In order to clarify the relationship between TFPI and trauma and the complication of DVT, in-depth basic research is needed in the future, such as cell experiments and study of post-traumatic mouse models of DVT. The results of the current study lay the foundation for future research directions.

6. Conclusions

In conclusion, trauma patients have a risk to suffer DVT, in which TFPI plays an important role. We consider that the risk of posttraumatic DVT may be correlated with increased

anticoagulant activity of TFPI and endothelial injury. We propose the anticoagulant activity of TFPI can be used as a potential biomarker for diagnosing DVT in trauma patients.

Authors' Note

Xiangyu Cao and Jun Wu designed and performed the research. Xiangyu Cao, Yu Su, Wenjie Zhang, Huiru Zhao, Yujing Zhao, Meng Wen, Shan Lu, Lei Liu and Xinwei Zang acquired the data. Yu Su, Huiru Zhao, Yujing Zhao, Meng Wen, Shan Lu, Wenjie Zang, Yuying Chen and Lei Liu provided valuable technical assistance. Xiangyu Cao and Jun Wu analyzed and interpreted the data and wrote the manuscript. All authors reviewed and made critical revisions and approved the final version of the manuscript. The study was conducted with approval of the Institutional Ethics Committee of Beijing Jishuitan Hospital. (NO.202004-89)

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Jun Wu  <https://orcid.org/0000-0001-5178-0906>

Supplemental Material

Supplemental material for this article is available online.

References

- Ruskin KJ. Deep vein thrombosis and venous thromboembolism in trauma. *Curr Opin Anaesthesiol*. 2018;31(2):215-218.
- Song K, Yao Y, Rong Z, Shen Y, Zheng M, Jiang Q. The preoperative incidence of deep vein thrombosis (DVT) and its correlation with postoperative DVT in patients undergoing elective surgery for femoral neck fractures. *Arch Orthop Trauma Surg*. 2016;136(10):1459-1464.
- Calder JDF, Freeman R, Domeij-Arverud E, van Dijk CN, Ackermann PW. Meta-analysis and suggested guidelines for prevention of venous thromboembolism (VTE) in foot and ankle surgery. *Knee Surg Sports Traumatol Arthrosc*. 2016;24(4):1409-1420.
- Bajaj MS, Kuppuswamy MN, Saito H, Spitzer SG, Bajaj SP. Cultured normal human hepatocytes do not synthesize lipoprotein-associated coagulation inhibitor: evidence that endothelium is the principal site of its synthesis. *Proc Natl Acad Sci U S A*. 1990;87(22):8869-8873.
- Hackeng TM, Maurissen LFA, Castoldi E, Rosing J. Regulation of TFPI function by protein S. *J Thromb Haemost*. 2009;7(Suppl 1):165-168.
- Novotny WF, Girard TJ, Miletich JP, Broze GJ. Purification and characterization of the lipoprotein-associated coagulation inhibitor from human plasma. *J Biol Chem*. 1989;264(31):18832-18837.
- Subramaniam S, Kanse SM, Kothari H, Reinhardt C, Fletcher C. Post-transcriptional, post-translational and pharmacological regulation of tissue factor pathway inhibitor. *Blood Coagul Fibrinolysis*. 2018;29(8):668-682.
- Girard TJ, Warren LA, Novotny WF, et al. Functional significance of the kunitz-type inhibitory domains of lipoprotein-associated coagulation inhibitor. *Nature*. 1989;338(6215):518-520.
- Wood JP, Bunce MW, Maroney SA, Tracy PB, Camire RM, Mast AE. Tissue factor pathway inhibitor-alpha inhibits prothrombinase during the initiation of blood coagulation. *Proc Natl Acad Sci U S A*. 2013;110(44):17838-17843.
- Mehic D, Tolios A, Hofer S, et al. Elevated levels of tissue factor pathway inhibitor in patients with mild to moderate bleeding tendency. *Blood Adv*. 2021;5(2):391-398.
- Dahm A, Rosendaal FR, Andersen TO, Sandset PM. Tissue factor pathway inhibitor anticoagulant activity: risk for venous thrombosis and effect of hormonal state. *Br J Haematol*. 2006;132(3):333-338.
- Dahm A, Van Hylckama Vlieg A, Bendz B, Rosendaal F, Bertina RM, Sandset PM. Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis. *Blood*. 2003;101(11):4387-4392.
- Keyloun JW, Le TD, Pusateri AE, et al. Circulating syndecan-1 and tissue factor pathway inhibitor, biomarkers of endothelial dysfunction, predict mortality in burn patients. *Shock*. 2021;56(2):237-244.
- Sidelmann JJ, Bladbjerg E-M, Gram J, Münster A-MB, Jespersen J. Tissue factor pathway inhibitor relates to fibrin degradation in patients with acute deep venous thrombosis. *Blood Coagul Fibrinolysis*. 2008;19(5):405-409.
- Norimatsu Y, Miyagawa T, Fukui Y, et al. Serum levels of tissue factor pathway inhibitor: potential association with Raynaud's Phenomenon and telangiectasia in patients with systemic sclerosis. *J Dermatol*. 2021;48(8):1253-1256.
- Khialani D, Vasan S, Cushman M, et al. Venous thrombosis with oral postmenopausal hormone therapy: roles of activated protein C resistance and tissue factor pathway inhibitor. *J Thromb Haemost*. 2021;19(7):1729-1737.
- Dahm AEA, Andersen TO, Rosendaal F, Sandset PM. A novel anticoagulant activity assay of tissue factor pathway inhibitor I (TFPI). *J Thromb Haemost*. 2005;3(4):651-658.
- Lindahl AK, Sandset PM, Abildgaard U. The present status of tissue factor pathway inhibitor. *Blood Coagul Fibrinolysis*. 1992;3(4):439-449.
- Wood JP, Petersen HH, Yu B, Wu X, Hilden I, Mast AE. TFPI α interacts with FVa and FXa to inhibit prothrombinase during the initiation of coagulation. *Blood Adv*. 2017;1(27):2692-2702.
- Hansen JB, Huseby KR, Huseby NE, Sandset PM, Hanssen TA, Nordøy A. Effect of cholesterol lowering on intravascular pools of TFPI and its anticoagulant potential in type II hyperlipoproteinemia. *Arterioscler Thromb Vasc Biol*. 1995;15(7):879-885.
- Lindahl AK, Jacobsen PB, Sandset PM, Abildgaard U. Tissue factor pathway inhibitor with high anticoagulant activity is increased in post-heparin plasma and in plasma from cancer patients. *Blood Coagul Fibrinolysis*. 1991;2(6):713-721.
- Ostergaard PB, Beck TC, Orsted H, et al. An enzyme linked immunosorption assay for tissue factor pathway inhibitor. *Thromb Res*. 1997;87(5):447-459.

23. Bendz B, Andersen TO, Sandset PM. A new sensitive chromogenic substrate assay of tissue factor pathway inhibitor type 1. *Thromb Res.* 2000;97(6):463-472.
24. Adams MJ, Oostryck R. A comparative study of functional assays for tissue factor pathway inhibitor using normal plasma and clinical samples. *Blood Coagul Fibrinolysis.* 2000;11(4):327-333.
25. Greven J, Pfeifer R, Zhi Q, Pape HC. Update on the role of endothelial cells in trauma. *Eur J Trauma Emerg Surg.* 2018;44(5):667-677.
26. Maroney SA, Mast AE. Expression of tissue factor pathway inhibitor by endothelial cells and platelets. *Transfus Apher Sci.* 2008;38(1):9-14. doi: 10.1016/j.transci.2007.12.001
27. Kato H. Regulation of functions of vascular wall cells by tissue factor pathway inhibitor: basic and clinical aspects. *Arterioscler Thromb Vasc Biol.* 2002;22(4):539-548.
28. Walborn A, Rondina M, Mosier M, Fareed J, Hoppensteadt D. Endothelial dysfunction is associated with mortality and severity of coagulopathy in patients with sepsis and disseminated intravascular coagulation. *Clin Appl Thromb Hemost.* 2019;25:1076029619852163.
29. Gando S, Levi M, Toh C-H. Disseminated intravascular coagulation. *Nat Rev Dis Primers.* 2016;2:16037.
30. Zhao Y, Zhu M, Chen W, et al. TFPI α alleviated vascular endothelial cell injury by inhibiting autophagy and the class III PI3 K/beclin-1 pathway. *Thromb Res.* 2020;195:151-157.
31. Lieberthal J, Sambamurthy N, Scanzello CR. Inflammation in joint injury and post-traumatic osteoarthritis. *Osteoarthritis Cartilage.* 2015;23(11):1825-1834.
32. Levi M, van der Poll T. Inflammation and coagulation. *Crit Care Med.* 2010;38(2 Suppl):S26-S34.
33. Wang BQ, Shi M, Zhang JP, et al. Knockdown of TFPI-anchored endothelial cells exacerbates lipopolysaccharide-induced acute lung injury via NF- κ B signaling pathway. *Shock.* 2019;51(2):235-246.
34. Ameri A, Kuppuswamy MN, Basu S, Bajaj SP. Expression of tissue factor pathway inhibitor by cultured endothelial cells in response to inflammatory mediators. *Blood.* 1992;79(12):3219-3226.
35. Cibor D, Szczeklik K, Mach T, Owczarek D. Levels of tissue factor pathway inhibitor in patients with inflammatory bowel disease. *Pol Arch Intern Med.* 2019;129(4):253-258.
36. Wilbur J, Shian B. Diagnosis of deep venous thrombosis and pulmonary embolism. *Am Fam Physician.* 2012;86(10):913-919.
37. Zhang W, Su Y, Liu L, et al. Fibrinolysis Index as a new predictor of deep vein thrombosis after traumatic lower extremity fractures. *Clin Chim Acta.* 2020;511:227-234.