Low levels of tissue factor pathway inhibitor (TFPI) increase the risk of venous thrombosis

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There is now strong experimental evidence that tissue factor pathway inhibitor (TFPI) is a critical inhibitor to modulate tissue factor–induced coagulation, but the role of TFPI as a risk factor for thrombosis is yet to be to be determined. This study investigated the role of low TFPI levels for the development of deep-vein thrombosis (DVT). We determined TFPI activity and TFPI-free and total antigen levels in the subjects enrolled in the Leiden Thrombophilia Study, which is a large population-based case-control study of 474 patients and 474 controls. The odds ratio (OR) for DVT in subjects who had TFPI-free antigen levels below the 10th percentile, as compared with those who had TFPI-free antigen levels above this cutoff, was 1.7 (95% confidence interval [CI], 1.1-2.6). The ORs for low TFPI activity and low total antigen were also mildly increased. When the 5th percentile was used as a cutoff, the ORs were 2.1 (95% CI, 1.1-4.1) for both TFPI-free antigen and TFPI total antigen. Exogenous female hormones had a profound lowering effect on TFPI levels, with lower levels in oral contraceptive users than in premenopausal nonusers, who had lower levels than men and postmenopausal women. These results indicate that low levels of TFPI, especially low TFPI-free and total antigen in plasma, constitute a risk factor for DVT. (Blood. 2003;101:4387-4392)

Introduction

Deep-vein thrombosis (DVT) is an important disease in the general population because it is frequent, often associated with secondary venous dysfunction, and occasionally complicated with fatal pulmonary embolism.[1] It is a multicausal disease, which means that more than a single risk factor needs to be present to trigger thrombosis.[1,2]

Risk factors may be classified into acquired and genetic factors.[3] Acquired risk factors for DVT include immobilization, surgery, trauma, pregnancy, puerperium, female hormones, malignant disease, and antiphospholipid antibodies.[1,4] Genetic risk factors associated with a tendency to DVT are deficiencies of the coagulation inhibitors antithrombin,[5] protein C,[6] and protein S,[7] or the presence of the factor V Leiden mutation[8] or the prothrombin gene 20210A allele.[9] However, in about 30% of patients with a family history of DVT, no underlying genetic risk factor can be found.[10] In experimental studies the balance of procoagulant and anticoagulant factors is important for net thrombin generation and potentially for a prothrombotic phenotype.[11] Several epidemiological studies have recently found that high levels of procoagulants, that is, high levels of factor VIII,[12] factor IX,[13] and factor XI,[14] are risk factors for DVT, substantiating the importance of this balance.

Tissue factor pathway inhibitor (TFPI) is a coagulation inhibitor that modulates initiation of coagulation induced by tissue factor.[15,16] In experimental models, TFPI plays a critical regulatory role in controlling the effects of tissue factor. Mice deficient in the TFPI gene died in utero owing to intrauterine coagulopathy and vascular disintegrity.[17] Immunodepletion of TFPI in rabbits dramatically lowered the threshold by which tissue factor may trigger coagulation.[18,19] Conversely, high-dose exogenous recombinant TFPI may increase this threshold and protect against disseminated intravascular coagulation[20] and venous thrombosis.[21,22]

Despite strong experimental evidence for a key role of TFPI in the regulation of coagulation, no studies have so far demonstrated a clear-cut association between plasma levels of TFPI and the risk of thrombosis, and TFPI deficiency associated with thrombotic disorders has not been detected.[23] The measurement of TFPI is made difficult by its complex inhibitory mechanism and a complex intravascular distribution, and international standards for calibration are not yet available. One possibility, therefore, is that assays of TFPI have been technically inadequate to detect an association of TFPI levels with thrombosis. Another possibility, however, is that the power of previous studies was too low to provide definite answers with regard to the role of low TFPI as a risk factor for thrombosis.

In a recent randomized, placebo-controlled study, Hoibraaten et al found that hormone replacement therapy (HRT) reduced TFPI by 30% to 50% and that this reduction was an important contributor to the increase in markers of activated coagulation found in women on such therapy.[24] This led us to investigate the role of TFPI as a risk factor for DVT in the Leiden Thrombophilia Study (LETS). Our hypothesis was that low levels of TFPI are a risk factor for DVT.

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Patients, materials, and methods

Study population and blood sampling

LETS is a case-control study which has been described in detail elsewhere.[25] In short, 474 patients with an objectively diagnosed first deep-vein thrombosis (DVT) were compared with 474 sex- and age-matched controls. This study reports data from 473 patients and 473 controls, as blood samples from one subject in each group were missing. The patients were selected from 3 anticoagulation clinics in the Netherlands. The controls were acquaintances of the patients or partners of other patients. Patients with known malignancies were excluded and all patients were younger than 70 years. The study protocol was approved by the Leiden University ethics committee, and all participants gave informed consent according to the Declaration of Helsinki. Blood samples were taken from 6 to 56 months after the thrombosis was diagnosed. Whole blood (0.9 vol) was collected from the antecubital vein into Sarstedt Monovette tubes (Nümbrecht, Germany) containing 0.106 M trisodium citrate (0.1 vol). Plasma was prepared by centrifugation for 10 minutes at 2000*g* at room temperature and stored in aliquots at −70°C until assayed.

TFPI assays

TFPI-free antigen and TFPI total antigen were assayed in duplicate with commercial enzyme-linked immunosorbent assays (Asserachrom Free TFPI and Asserachrom Total TFPI, Diagnostica Stago, Asnieres, France). In both assays a mouse monoclonal antibody (T4E2) directed against the factor Xa binding site of the second Kunitz domain is used to catch TFPI. Another monoclonal antibody (HG5), which recognizes a lipoprotein-binding region between residues 161 and 240, is used to detect free TFPI. A third monoclonal antibody (2C6), directed against the first 160 amino acid residues of the TFPI molecule, is used to detect total TFPI (ie, free and bound TFPI). The detecting antibodies are both conjugated to peroxidase. The TFPI-free antigen assay is specific for free-circulating TFPI and does not detect lipid-bound TFPI. The TFPI total antigen assay is specific for the total amount of TFPI, including lipid-bound TFPI [26] (written correspondence, B. Woodhams, Diagnostica Stago, April 8, 2002). Freeze-dried human plasmas containing known amounts of TFPI were provided in the kits and were used to establish the calibration curves. Quality controls were performed using control specimens containing a high amount of TFPI supplied with the kits as well as an in-house standard containing a normal level of TFPI. Inter- and intra-assay variability, measured as coefficient of variation, were 4.4% and 2.9%, respectively, for TFPI total antigen and 4.9% and 3.8%, respectively, for TFPI-free antigen.

TFPI activity was assayed in duplicate with a 2-stage chromogenic substrate assay as described previously.[27] In this assay TFPI activity is determined by the quantification of residual factor VIIa/tissue factor catalytic activity after the incubation of diluted plasma (containing TFPI) with tissue factor, factor VIIa in excess of tissue factor binding sites, and factor Xa. Inter- and intra-assay variability were 3.5% and 1.44%, respectively.

Statistical analysis

The study involved 2 sets of analyses: one to identify the determinants of TFPI levels and the other to establish the contribution of low TFPI to the risk of thrombosis. Determinants of TFPI levels were examined in the controls, since they represent the general population. They were established by comparison of means and linear regression and are reported as means, medians, and regression coefficients, with 95% confidence intervals (CIs) for these coefficients. To compare women using oral contraceptives (OC) with nonusers, a special selection, described previously, was made to obtain comparable groups.[13] Nonmenopausal women between 15 and 49 years of age were included. Women who at the index date (date similar to date of thrombosis for patients) were pregnant, were within 30 days postpartum, had had a recent miscarriage, or had used only depot contraceptives were excluded. A total of 153 control subjects were included in this analysis.

Second, we investigated whether a low level of TFPI is a risk factor for DVT by calculating the odds ratio (OR) and its 95% CI as an estimate of relative risk. Low levels of TFPI were arbitrarily defined as levels at or below the 10th, 5th, or 2nd percentiles in the control group.[13] In all these analyses, the group with highest TFPI levels (ie, those above the 10th percentile, or above the 5th percentile, or above the 2nd percentile) served as the reference category for calculation of the OR. Logistic regression was used to adjust for possible confounders, for example, age, sex, and hormonal status. We also used restriction to obtain unconfounded risk estimates. Oral contraceptives (OC) proved to be an important confounding factor in our data. Analysis of the controls showed that OC had a strong lowering effect on TFPI levels. Most of the patients who used OC at the time of DVT were advised to stop taking them. Consequently, most of them were not using OC at the time of blood sampling, 6 to 56 months after DVT. These women most probably had lower levels of TFPI at the time of DVT than at the time of blood sampling. OC also affected the 10th-percentile limit that was used to define low levels of TFPI. Thus, most of the subjects with low levels of TFPI were OC users. To avoid confounding effects of OC use due to an imbalance between OC use in patients and OC use in controls at the time of blood sampling, women who were using OC at the time of blood sampling were excluded from several analyses.

Results

The mean age of patients and controls at the time of thrombosis was 45 years, and the ratio of males to females was approximately 3:4 (Table 1). Approximately one third (127 of 474) of the patients were OC users at time of thrombosis, as compared with only 69 of 474 in the control group. Among patients, most OC users had stopped using OC at the time of blood sampling.

Determinants of TFPI levels in healthy controls

TFPI-free and total antigen and TFPI activity levels were normally distributed as judged by histograms. The means and 95% CIs are listed in Table 2. Exogenous female hormones had a pronounced lowering effect on the levels of TFPI and the effect was strongest for TFPI-free antigen. Premenopausal OC users had lower TFPI values than nonusers, and nonusers had lower levels than postmenopausal women. Postmenopausal women had TFPI levels similar to levels in men. Most subjects with low TFPI values were OC users. As many as 41% of the OC users had TFPI-free antigen levels below the lowest free TFPI value detected in nonusers.

In a simple linear regression model all 3 TFPI parameters apparently increased with age. We then removed OC users at time of blood sampling from the material and performed linear regression with age separately for men, postmenopausal women, and premenopausal women not using OC. The effect of age on TFPI became virtually absent, except for a very weak effect on TFPI-free antigen in men.

Table 1. Characteristics of patients with venous thrombosis and control subjects

	Patients, n = 473	Controls, n = 473
Age, y		
Mean	45.0	44.7
Range	14-69	14-72
Sex, no.		
Male	202	201
Female	271	272
Oral contraceptive use, no.		
At index date	127	69
At blood sampling	37	59
Factor V Leiden, no.	92	14

Table 2. TFPI levels in control subjects

	OC users, n = 54	OC nonusers, n = 99	Postmenopausal women, n = 90	Men, n = 201	All, n = 473
TFPI-free antigen, ng/mL	6.17 (5.6-6.7)	11.0 (10.2-11.9)	14.5 (13.7-15.3)	15.0 (14.4-15.6)	12.7 (12.3-13.2)
TFPI total antigen, ng/mL	50.1 (47.4-52.9)	63.2 (60.7-65.6)	74.5 (71.6-77.4)	73.7 (71.7-75.7)	68.1 (66.7-69.5)
TFPI activity, %	93 (90-97)	114 (112-117)	123 (120-126)	124 (122-126)	118 (116-119)

Entries are mean TFPI levels; parenthetical entries are 95% CIs.

Including all individuals in the analysis, linear regression revealed that TFPI was either very weakly or not at all associated with plasma levels of prothrombin; factors VII, VIII, IX, X, or von Willebrand factor (VWF); or ABO blood type. Since OC use had a profound differential effect on TFPI levels, OC users were excluded in a separate analysis. TFPI now showed positive associations with all procoagulant factors (II, V, VIII, IX, X, XI), which was most noticeable for TFPI total antigen levels (Table 3). There was no association between TFPI and VWF or ABO blood type.

TFPI as a risk factor for DVT

To analyze the effect of low TFPI as a risk factor for DVT, data were excluded from 96 women using OC at the time of blood sampling and from 27 women for whom this information was missing (see "Materials and methods"). Comparison of means in patients and controls revealed similar values for TFPI-free antigen (13.6 ng/mL vs 13.8 ng/mL, respectively) and TFPI total antigen (70.1 ng/mL vs 71.1 ng/mL), whereas mean TFPI activity was slightly higher in patients (124 U/dL vs 121 U/dL).

We then determined the effect of low TFPI as a risk factor for DVT. Ten percent (n = 39) of healthy controls had TFPI-free antigen levels below 9.0 ng/mL (10th percentile = 9 ng/mL). Nineteen percent of the patients had TFPI-free antigen levels below this cutoff, which means that individuals with a TFPI-free antigen below 9 ng/mL had an approximately 2-fold increased risk of developing DVT compared with individuals having TFPI levels above this cutoff value. Similar results were obtained for TFPI total antigen and TFPI activity. Further analyses revealed that lower TFPI levels, (eg, cutoff at the 5th and 2nd percentiles) were associated with higher ORs for DVT (Table 4). This dose-response relationship of higher risk with lower TFPI levels suggests a threshold level for the protective effect of TFPI against DVT.

Adjusting for hormonal state (male, premenopausal female not using OC, postmenopausal female) did not materially change any of the ORs. Restriction analysis—that is, removal from the analysis of individuals with antithrombin deficiency (< 0.80 U/mL), protein C deficiency (< 0.67 U/mL), protein S deficiency (< 0.67 U/mL), the factor V Leiden mutation, or the prothrombin 20210A allele—only marginally changed the ORs, with a slight decrease in the OR for TFPI-free antigen and a slight increase in the ORs for TFPI total antigen and TFPI activity.

Table 3. Regression coefficients showing increase in TFPI per unit increase in the coagulation factor studied

Factor, U/dL	Free TFPI, ng/mL	Total TFPI, ng/mL	TFPI activity, U/dL
Prothrombin	0.06 (0.03-0.09)	0.17 (0.08-0.3)	−0.03 (−0.1-0.7)
Factor V	0.05 (0.03-0.06)	0.16 (0.1-0.2)	0.11 (0.07-0.2)
Factor VII	0.05 (0.03-0.07)	0.15 (0.08-0.2)	0.04 (−0.03-0.1)
Factor IX	0.09 (0.07-0.1)	0.21 (0.1-0.3)	0.10 (0.03-0.2)
Factor X	0.11 (0.08-0.1)	0.25 (0.2-0.3)	−0.03 (−0.1-0.07)

Parenthetical entries are 95% CIs. Oral contraceptive users at blood sampling were excluded.

We performed subgroup analyses of men, pre- and postmenopausal women, and younger and older subgroups (with the median age as the dividing line). The ORs were all increased in the same range, although the confidence intervals were wide owing to the smaller group sizes (Table 5). When we completely eliminated the effect of OC use by excluding all women who used OC either at the index date (time of thrombosis) or at venipuncture, the power of the study was reduced, as we ended up with 336 patients (90 exclusions) and 387 controls (10 exclusions) and the ORs for thrombosis at low levels of TFPI were attenuated. For low TFPI-free antigen, for example, the OR was 1.2 (95% CI, 0.8-2.0) for cutoff at the 10th percentile, increasing to 2.1 (95% CI, 0.8-5.9) for cutoff at the 2nd percentile.

Discussion

We have found that low TFPI, defined as a value below the 10th percentile of the distribution of values in control subjects, was a weak risk factor for DVT. The relative risk was 1.1 to 1.7 for the

Table 4. Crude odds ratios for DVT, by TFPI level

	Patients, n = 426	Controls, n = 397	OR (95% CI)
TFPI-free antigen			
10th percentile			1.7 (1.1-2.6)
Above	359	358	—
At or below	67	39	—
5th percentile			2.1 (1.1-4.1)
Above	384	378	—
At or below	42	19	—
2nd percentile			2.2 (0.89-5.3)
Above 2nd	410	390	—
At or below 2nd	16	7	—
TFPI total antigen			
10th percentile			1.5 (0.98-2.3)
Above	366	358	—
At or below	60	39	—
5th percentile			2.1 (1.1-4.1)
Above	392	378	—
At or below	34	19	—
2nd percentile			3.0 (1.3-7.2)
Above	404	390	—
At or below	22	7	—
TFPI activity			
10th percentile			1.1 (0.73-1.8)
Above	377	356	—
At or below	49	41	—
5th percentile			1.6 (0.87-2.8)
Above	395	378	—
At or below	31	19	—
2nd percentile			2.4 (1.1-5.1)
Above	401	387	—
At or below	25	10	—

Oral contraceptive users at blood sampling were excluded.

Table 5. Odds ratios for DVT among subgroups with low TFPI-free antigen

	TFPI-free antigen at or below 10th percentile	TFPI-free antigen above 10th percentile	OR (95% CI)
Men, n = 403			1.9 (0.80-4.7)
Patients	15	187	—
Controls	8	193	—
Women, n = 420			1.6 (0.98-2.6)
Patients	52	172	—
Controls	31	165	—
Postmenopausal women, n = 172			1.9 (0.44-8.1)
Patients	5	77	—
Controls	3	87	—
Older than 45 y			1.5 (0.87-2.6)
Patients	23	238	—
Controls	12	247	—
Younger than 45 y			2.0 (0.97-4.1)
Patients	44	121	—
Controls	27	111	—

Oral contraceptive users at blood sampling were excluded.

3 types of TFPI investigated. Lower cutoff values for TFPI, (ie, below the 5th or the 2nd percentile) were associated with slightly higher risk of DVT, which suggests a threshold effect for the ability of TFPI to protect against thrombosis. The findings were not due to the effects of hormonal status, the factor V Leiden mutation, the prothrombin 20210GA gene mutation, antithrombin deficiency, or protein C or S deficiency. Our finding is contradictory to those of numerous clinical and epidemiological studies, which have failed to detect a clear association between low TFPI levels and the risk of any type of thrombosis,[23,28] although some recent studies have suggested that patients with venous thrombosis may have slightly lower mean TFPI levels than controls.[29-31]

The findings are, however, consistent with experimental evidence on the critical role of TFPI in the regulation of tissue factor–induced coagulation. This evidence is based on gene knockout experiments and the effects of immunodepletion of TFPI. Disruption of the TFPI gene was not compatible with normal embryonic development in mice,[17] whereas severe immunodepletion of TFPI in a rabbit model dramatically altered the threshold by which tissue factor may activate coagulation.[18,19] In rabbits immunodepleted of TFPI by 80% to 90%, severe disseminated intravascular coagulation was triggered by low doses of tissue factor that were without effect in rabbits with normal TFPI levels. In the present study, we have investigated the effects of much less reduction in TFPI levels, that is, levels of TFPI essentially within the low normal range of TFPI in controls. Mean TFPI was not lower in patients with DVT, but TFPI levels below the 10th percentile proved to be a weak risk factor for DVT. Our findings therefore support the hypothesis of a threshold effect in which moderately reduced TFPI levels, which could be encountered in heterozygous TFPI deficiencies, may slightly increase the risk of thrombosis, whereas a much stronger reduction in TFPI, which could be encountered in homozygous deficiencies, may cause early-onset severe thrombotic disease and be incompatible with life.

Exogenous female hormones had profound lowering effects on TFPI levels. The levels were related to the burden of female hormones in the following order: TFPI levels in OC users were lower than levels in OC nonusers, which were lower than levels in postmenopausal women and in men. In our data mean free TFPI in OC users was almost half that in nonusers. Forty-one percent of OC users had lower TFPI values than the lowest TFPI value observed in premenopausal nonusers, and as many as 24 of 25 individuals with free TFPI values below 5 ng/mL used OC. Similar effects of OC on TFPI have also been reported by other investigators.[26,32] This lowering effect of OC on TFPI is probably the strongest biological effect of OC on any coagulation factor known to date.[33] Postmenopausal hormonal replacement therapy (HRT) may have similar effects.[24]

An important question that remains open is whether the marked reduction in TFPI induced by OC (and HRT) may explain the increased risk of venous thrombosis associated with such treatment. In the present study, blood samples were collected several months after the acute event, and most of the women had followed the advice not to continue with OC. This introduced a great imbalance in the number of OC users among patients and controls at time of blood sampling. The control group thus contained a large excess number of OC users with low TFPI secondary to OC use, which would have falsely eliminated the effect of low TFPI if they had been included in the analyses.

The risk associated with low TFPI levels decreased when the effect of OC was completely excluded by analyzing only women who had never used OC. For the lowest levels (< 2nd percentile), an elevated risk clearly remained. This attenuation may indicate a confounding effect of OC, that is, that because of the TFPI-lowering effect of OC, the observed association of TFPI levels with risk is, at least in part, the result of the effect of OC use on risk. However, it is equally possible that lowering of TFPI is one of the mechanisms by which OC exert their risk-enhancing effect for thrombosis. Obviously, if that were the mechanism, adjustment would not be justified. We believe that this latter explanation—a true causal role for low TFPI levels in the etiology of thrombosis—is plausible, since we also observed an effect in men.

Although the relationship between the effect of OC on TFPI levels and the risk of DVT may not be answered by this study, a randomized study on the effects of HRT found that a reduction in free TFPI was the most important factor for an increase in markers of activated coagulation.[24] Both OC use and HRT induce acquired resistance to activated protein C (APC). This is especially obvious when an endogenous thrombin potential (ETP)–based APC sensitivity test is used.[34,35] It is thus of interest that free TFPI played an important role for the APC-resistant phenotype in women randomized to HRT.[35]

Several studies have reported that TFPI increases somewhat with age.[36] We also found an age-dependent increase in TFPI, but this increase was mainly explained by hormonal state. It is thus important that future studies consider hormone state when measuring TFPI in humans. The difference in TFPI total antigen between cases and controls in the study of Amini-Nekoo et al[31] could, for instance, be explained by an imbalance of female hormonal influence in the 2 groups.

We assayed TFPI in plasma by well-established methods, but it has not yet been confirmed that these assays are relevant for the biological activity of TFPI in vivo. The biology of TFPI is rather complex with regard to both mechanism of action and distribution of TFPI. TFPI is both an inhibitor of factor Xa and tissue factor/factor VIIa catalytic activity, but the contributory role of factor Xa inhibition for the antithrombotic action of TFPI is still controversial.[15,16] We assayed TFPI activity with a chromogenic substrate assay, which essentially provides information on the capacity to inhibit tissue factor/factor VIIa catalytic activity, but not on the ability to inhibit factor Xa or on reaction kinetics.

Another problem relates to the complex intravascular distribution of TFPI. A major proportion (60%-80%) of TFPI in the blood

vessels is normally bound to the vascular endothelium, but this pool may be mobilized to circulating blood after the injection of heparins and other negatively charged ions.[37] Plasma contains only a minor fraction (20%-30%) of intravascular TFPI, and it is to a great extent associated with lipoproteins.[38] Our study provides information on the role of plasma TFPI, but TFPI bound to the vascular endothelium could hypothetically be the relevant TFPI fraction in vivo.

It is of interest that different forms of TFPI have markedly different abilities to prolong clotting in diluted prothrombin time assays. At the same level of TFPI chromogenic substrate activity (or level of TFPI total antigen), full-length TFPI—that is, TFPI bound to the vascular endothelium—has a much stronger anticoagulant effect than truncated forms of TFPI—that is, TFPI found in lipoproteins.[39,40] It might be argued that TFPI with strong anticoagulant potential is biologically more active than TFPI with low anticoagulant activity.[16,23] Assay of TFPI anticoagulant activity would therefore potentially be of great interest, but to our knowledge, no laboratories have so far been able to standardize assays for plasma TFPI anticoagulant activity. However, TFPI-free antigen in plasma has been shown to exert a strong anticoagulant effect in such assays[16,23] and may be considered as a marker of TFPI anticoagulant activity. The current data show that TFPI-free and total antigen were the most important risk factors, while the effect of TFPI activity was weaker.

The molecular basis for low TFPI is still unknown. Six polymorphisms have been reported in the TFPI gene: a −399C→T transition and a −287T→C transition in the promotor region of the TFPI gene,[41,42] a −33C→T transition in intron 7,[43] a 384T→C transition in exon 4 which does not change Tyr56,[31,43] a 536C→T transition in exon 7 which gives a Pro151Leu exchange,[44] and a 874G→A transition in exon 9 which predicts a Val264Met exchange.[43] Although some studies have found associations of these polymorphisms with levels of TFPI or the risk of DVT, convincing evidence for a clear relationship between genotype, TFPI levels, and the risk of DVT is still missing. However, Ameziane et al[45] found the −33T→C polymorphism in intron 7 was associated with higher levels of TFPI total antigen and with a protective effect on the risk of DVT, but they failed to show that low TFPI total antigen was associated with the risk of DVT.

We conclude that plasma TFPI below the 10th percentile is likely to be a weak risk factor for DVT. Our findings corroborate experimental evidence of a critical role of TFPI in the regulation of tissue factor–induced coagulation. Female hormones have a strong lowering effect on plasma levels of TFPI, but it remains open whether the reduction of TFPI on OC can explain the increased risk of venous thrombosis associated with OC use.

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