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Resistance to activated protein C and impaired TFPI activity in women with previous hormone-induced venous thromboembolism

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ABSTRACT

Introduction: Hormonal contraception is a well-known risk factor for venous thromboembolism (VTE). APC resistance and impaired functions of protein S and TFPI are thought to play an important role in the pathogenesis of hormone-related VTE. It is unknown, whether women, who develop VTE during hormonal contraception possess a vulnerability in these pathways, making them susceptible to thrombosis.
Materials and methods: Plasma samples were obtained from 57 premenopausal women in average 15.3 years after hormone-associated VTE and from 31 healthy controls. Thrombin generation at high tissue factor (TF) in the absence and in the presence of activated protein C (APC) and at low TF without and with inhibiting anti-protein S- and anti-TFPI-antibodies was measured *via* calibrated automated thrombography.
Results: Women with previous hormone-related thrombosis had higher thrombin generation at low TF, higher APC resistance, protein S- and TFPI ratios, differences: 219.9 nM IIa.min (95%CI:90.4 to 349.3); 1.88 (95% CI:0.71 to 3.05); 0.13 (95%CI:0.01 to 0.26) and 0.19 (95%CI:0.08 to 0.30), respectively. Thrombin generation at high TF without APC did not differ between the groups. Smoking decreased thrombin generation at low TF by −222.6 nM IIa.min (95%CI: −381.1 to −64.1), the APC sensitivity ratio by −2.20 (95%CI: −3.63 to −0.77) and the TFPI ratio by −0.16 (95%CI: −0.29 to −0.03), but did not influence thrombin generation at high TF.
Discussion: We demonstrated impairment of the protein S/TFPI system and increased APC resistance in women with previous hormone-induced VTE. Smoking decreased thrombin generation at assay conditions, dependent on the function of the TFPI system.

1. Introduction

Over 250 million women worldwide use hormonal contraception with combined oral contraceptives being the most popular thereof. The prevalence of hormonal contraceptive use varies between countries with different socioeconomic level and cultural background, reaching 50% of women of reproductive age in some countries. In general, hormonal contraceptives are safe drugs and severe side effects are rare. Venous thromboembolism (VTE) is one of the most feared side effects of hormonal contraceptives. The risk of VTE depends on the dose of estrogens, type of progestogen and the way of the hormone application, varying from two- to six-fold (for review see [1]). Given a low thrombosis risk in the general population of young women of reproductive age, estimated to be 0.8 cases per 10,000 women-years [2] and multiple advantages of hormonal contraception, such as childbirth control, bleeding control and reduction of endometriosis symptoms, the increase in the risk is considered to be acceptable for women without additional risk factors for thrombosis.

The incidence of VTE is greatest during the first year of contraceptive use, most likely because of the attrition of susceptibles. Hormonal contraceptives can amplify the genetically determined thrombosis risk, like that induced by factor V Leiden or the prothrombin gene mutation (G20210A) in a supra-additive manner [1]. Up to 50% of women, who develop VTE during pill use, have one or even a combination of inherited thrombophilias [3]. Nevertheless, in many cases no inherited thrombophilia can be detected. After a first thrombotic event the risk of a

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recurrent thrombosis is higher than the thrombosis risk of population without previous VTE, suggesting a persistence of hypercoagulability [4]. The recurrence rate after index events with an identifiable transient trigger is considerably lower as that of unprovoked thrombosis. Hormonal therapies are regarded to be a minor transient risk factor of VTE and the annual risk of a recurrence among women with a hormone-related VTE is about 2% [5]. Therefore, extended secondary prophylaxis is generally not recommended in this patient population, but should be considered during further hormone exposure or pregnancy. Additionally, women, who develop thrombosis while taking hormonal contraceptives, are advised to switch to a safer option of contraception, like hormone-free methods or progestogen-only preparations.

Impairment of natural anticoagulant systems, *e.g.* resistance to activated protein C (APC), and reduced function of the protein S/tissue factor pathway inhibitor (TFPI)-systems are likely to play an important role in the development of hormone-induced thrombosis and were also associated with VTE recurrence in female population [6]. APC regulates the coagulation cascade by inactivating of activated factors V(a) and VIIIa. Resistance to APC, caused either by the factor V Leiden mutation or by acquired factors, like hormone use, is associated with an increased risk of VTE. TFPI is another important natural anticoagulant that can regulate initiation of the coagulation at several levels, inhibiting factor Xa, prothrombinase [7] and down-regulating the activation of factor X by the TF/factor VIIa complex. TFPI circulates in blood in a full-length and in a variety of truncated forms, binding to lipoproteins with different affinity. Both truncation and binding to lipoproteins influence anticoagulant activity of TFPI. Protein S, circulating in plasma in a free form and bound to C4b-binding protein, acts as a non-enzymatic cofactor of APC as well as TFPI.

Hormone exposure during pregnancy, oral contraceptive use or hormone replacement therapy synergistically increases genetically determined APC resistance, mirroring their supra-additive effects on the VTE risk. Therefore, the prevalence of factor V Leiden is higher among women, who develop hormone-associated VTE than that in the general female population or even in the female population with VTE that was not related to hormone exposure [8]. Presently, it is not known, whether preexisting (and persisting) impairment of the protein S/TFPI system can also be amplified by contraceptive use and, therefore, similarly to APC resistance, predisposes to the development of hormone-associated thrombosis. The complexity of these pathways with varying activity of different forms of TFPI and protein S, multiple interactions and correlations between the plasma levels of TFPI, protein S and several coagulation factors, makes it is difficult to estimate the general effect of changes in these proteins on coagulation. Thrombin generation measurement is a global coagulation test that can be used to evaluate not only the endogenous thrombin potential (ETP) of plasma but also APC resistance as well as the function of TFPI and protein S. Thrombin generation-based assays demonstrate a high sensitivity to bleeding and prothrombotic disorders and were shown to predict the risk of primary [9] and recurrent VTE [10]. Yet, the predictive value of increased thrombin generation could not be reproduced in all patient populations [11] and experimental conditions of the assay, are likely to play here a crucial role.

In the current study, we investigated the thrombin generation phenotype in a selected population of women with history of a hormone-induced thrombosis as compared to a control group of healthy female volunteers. APC resistance and the function of the protein S- and TFPI-systems were evaluated in this study population using thrombin generation-based assays.

2. Materials & methods

2.1. Study population

Into the current study we included 57 premenopausal women with previous hormone-associated VTE event and 31 healthy premenopausal women without VTE, who attended Karolinska University hospital because of other reasons. Patients with active cancer or pregnancy were not eligible for this study. Blood samples were obtained at least one year after discontinuation of therapy for VTE in the follicular phase of the menstrual cycle, unless the cycle status was not known because of hysterectomy (n = 3). None of the participants was under estrogen-containing treatment at blood sampling. The first VTE as well as the recurrent event were confirmed by reviewing medical records and radiology reports. The study was approved by the Medical Ethical committee of the Karolinska Institutet, Stockholm, Sweden. All participants gave written informed consent.

Blood samples were collected into vacutainer tubes containing 3.2% sodium citrate, centrifuged twice during 15 min at 2000 ×*g* and 15 °C, shock frozen and stored at −80 °C until analysis. Pooled normal plasma was prepared by pooling plasma of 23 healthy donors free of medication (17 males and 6 females, mean age 33.3 years) as described above. All laboratory tests were performed in duplicate.

2.2. Materials and laboratory methods

The endogenous thrombin potential (ETP) was measured by calibrated automated thrombography (Stago) in reaction mixtures (125 μL) containing 80 μL plasma and 10 pM tissue factor (Dade Innovin®, Behring, Germany), defined as "high" tissue factor concentration, 16 mM added $CaCl_2$, 30 μM phospholipid vesicles, 0.32 mM fluorogenic substrate (*Z*-Gly-Gly-Arg-AMC, Bachem, Bubendorf, Switzerland) in the absence of APC (ETP_{-APC}) and in the presence of 3 nM APC (ETP_{+APC}). APC was purchased from Kordia Life Sciences, Leiden, the Netherlands and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine (DOPC), were from Avanti Polar Lipids, Alabaster, AL, USA. DOPS/DOPE/DOPC (20/20/60, M/M/M) and PS/PC (10/90, M/M) vesicles were prepared as described before [12]. The lag time, peak height, ETP, time to peak and time to start tail were calculated from thrombin generation curves using software provided by Thrombinoscope B.V. (Maastricht, The Netherlands) and normalized for day-to-day variations. The APC concentration was chosen to inhibit thrombin generation down to about 10% of the initial ETP in normal plasma. The APC sensitivity ratio (APCsr) was calculated as previously described [12] and varied from 0 to 10, where 1 would correspond the reference (normal plasma).

For measurements at "low" tissue factor concentration, thrombin generation was triggered by 1 pM tissue factor in the presence of 40 μg/ml corn trypsin inhibitor either in the absence or the presence of 67 μg/ml a monoclonal antibody against C-terminus of TFPI (Sanquin, Amsterdam, The Netherlands) or 2.8 μM an antibody directed against protein S (Dako A/S, Glostrup, Denmark). The TFPI-ratio and protein S-ratio were calculated as described elsewhere [13] and were within this study for the normal pooled plasma 0.22 and 0.68, respectively.

2.3. Statistical analysis

IBM SPSS Statistics 25 was used to perform the statistical analysis. The Kolmogorov-Smirnov test was used to test for normal distribution. Normally distributed data were expressed as mean with 95% confidence interval (95%CI), not normally distributed parameters- as median and range. Direct comparisons were performed using the Mann-Whitney *U* test. Associations of categorical values (smoking: no = 0 or current:=1; factor V Leiden: no = 0, present = 1 (no homozygote carriers of the factor V Leiden mutation were in the present study)) were analyzed by Fisher exact test and correlations between thrombin generation parameters and previous thrombosis were evaluated by multiple linear regression analysis after correction for age, BMI, smoking and factor V Leiden. A p-value ≤ 0.05 was considered to be statistically significant and Bonferroni correction was used for multiple comparisons.

3. Results

3.1. Population baseline characteristics

Table 1 summarizes the baseline characteristics of the participants of the study. Women with a venous thrombosis in the past (n = 57) were older than controls (n = 31), but had similar BMI. The mean age at the first thrombotic event was 22.8 years (95%CI: 21.5 to 24.1). Thirty-three participants (58%) were diagnosed with a deep vein thrombosis, including 4 pelvic VTE (7%), 12 with pulmonary artery embolism (21%) and 12 with cerebral vein thrombosis (21%). All women in the thrombosis group had been taking hormonal contraceptives during the first VTE for periods varying from one month to 10 years (median one year). The majority of them (27 or 47%) were using second generation OC containing levonorgesterel, 14 (25%) third generation OC containing gestodene or dienogest, one (2%) drosperinone-containing OC and two (4%) a progestogen-only contraceptive and for 13 (23%) data on the pill composition was missing. An additional transient risk, such as immobilization, long airplane flight or surgery could be identified in 25 (44%) cases and 15 women (26%) had a positive family history for thromboembolic events. Nine women (16%) developed a recurrence of thrombosis in average 9.2 years after the first event (95%CI: 4.8 to 13.7 years). Seven recurrences were deep vein thrombosis and two pulmonary artery embolism, resulting in a recurrence rate of 21% and 17% in the subgroups with DVT and pulmonary embolism, respectively. No recurrences of cerebral vein thrombosis were registered within the study. Eight recurrences were related to pregnancy and one was diagnosed after a prolonged air travel. Four women with a recurrent VTE and one woman with combined thrombophilia without a VTE recurrence received warfarin and were excluded from the analysis of the thrombin generation parameters because of the known pronounced effect of vitamin K antagonists on coagulation. The blood samples for the current study were obtained in average 15.3 years (95%CI: 13.1 to 17.5 years) after the first event. Ten women in the thrombosis group were smoking (17.5%), whereas there were no smoking women in the control group. There were more smoking women among those with a recurrence of VTE (30% *vs.* 14.3%), although the difference did not reach statistical significance in the chi-square test (p = 0.18). Twenty-two women (39%) in the thrombosis group had an acquired thrombophilia or antiphospholipid syndrome and of two patients the data were missing. Twelve patients (21%) had factor V Leiden, 5 (9%) prothrombin mutation and 3 (5.3%) were deficient for protein C, one (1.8%) for protein S and 4 (7%) patients had antiphospholipid syndrome. Three patients (5.3%) had a combined thrombophilia, two of whom (66.7%) developed a recurrent VTE (Fisher exact test p = 0.052). The presence of a genetic thrombophilia in general was not predictive for the thrombosis recurrence. Five women in the control group (18.5%) were carrier of the factor V Leiden mutation and data on 14 patients were missing.

Table 1
Baseline characteristics.

Characteristic	Thrombosis in the anamnesis (N = 57)	Controls (N = 31)	p-value
Age, years	38 (23–48)	26 (21–45)	p < 0.0001
BMI, kg/m^2	22.6 (16.9–34.6)	21.3 (19.7–29.7)	p = 0.277
Smoking	10 (17.5%)	0 (0%)	p = 0.014
Thrombophilia	22 (39%)	5 (16,1%)	p = 0.919
Factor V Leiden	12 (21%)	5 (16,1%)	p = 0.553
Prothrombin G20210A mutation	3 (5.3%)	0/12[a]	

The data are presented as a median with range in parenthesis.
[a] Data were available only on 12 patients.

3.2. Direct comparison of the thrombosis group with controls

Women with previous VTE had significantly higher ETP and peak height at a low TF concentration as compared to the controls (Table 2, Fig. 1). In the presence of an inhibiting anti-protein S or anti-TFPI antibody the difference in the ETP disappeared. The difference in the peak height between the VTE patients and controls slightly decreased (from 37% to 26%) after addition of an anti-PS antibody and disappeared in the presence of an anti-TFPI antibody. The PS and TFPI ratios were significantly higher in the group with previous thrombosis than in the controls, indicating lower PS and TFPI activity in plasma of women with previous VTE.

No differences in the thrombin generation parameters between the groups were found at high TF concentration without APC (Table 3, Fig. 1). In the presence of APC, the peak height and the ETP were significantly higher in the thrombosis group as compared to the controls. The differences in the APCsr demonstrated a trend (p = 0.123) to increased APC resistance in the group with previous VTE, but did not reach statistical significance. It is important to note, that carriers of the factor V Leiden mutation were included in this analysis.

3.3. Multiple regression analysis

Because baseline differences between two study groups could influence the results of the analysis, we analyzed association of selected thrombin generation parameters (ETP at low and high TF, APCsr, PS and TFPI ratio) with a previous thrombosis event using a multiple regression model with a correction for possible confounders, *i.e.* age, BMI and smoking status. As thrombin generation in the presence of APC is considerably influenced by the presence of factor V Leiden, we also corrected for the factor V Leiden mutation. Addition of factor V Leiden as an independent parameter in the analysis of the ETP without APC at low or high tissue factor, and the PS and TFPI ratios did not considerably influence the results. Therefore, we used a fixed model with thrombin generation parameters as dependent and thrombosis, age, BMI, smoking status and factor V Leiden as independent variables (Table 4).

In this model the ETP at high tissue factor was not influenced by

Table 2
Thrombin generation parameters at low tissue factor.

Parameters	Thrombosis group N = 52	Control group N = 31	p-value
Lag time, min	4.00 (2.72 to 9.15)	4.09 (3.02 to 6.50)	p = 0.408
ETP, nM IIa*min	776.0 (299–1464)	670.9 (175–1211)	p < 0.05
Peak height, nM IIa	112.5 (31.0–225.0)	82.4 (15.0–301.0)	p < 0.05
Time to peak, min	7.19 (5.51–12.97)	7.47 (5.01–0.60)	p = 0.713
Lag time $_{+aPS}$, min	3.59 (2.74–7.65)	3.66 (2.95–5.38)	p = 0.713
ETP$_{+aPS}$, nM IIa*min	799.0 (510.0–1331.0)	739.2 (503.0–1282.0)	p = 0.251
Peak height$_{+aPS}$, nM IIa	131.5 (58.0–223.0)	104.6 (28.0–229.0)	p < 0.05
Time to peak$_{+aPS}$, min	6.69 (5.34–11.97)	6.75 (5.03 to 9.71)	p = 0.600
Lag time $_{+aTFPI}$, min	2.85 (2.37–6.34)	2.98 (2.77–3.51)	p < 0.05
ETP$_{+aTFPI}$, nM IIa*min	808.5 (550.0–1237.0)	789.0 (611.0–1247.0)	p = 0.750
Peak height$_{+aTFPI}$, nM IIa	204.5 (86.0–272.0)	189.7 (114.-309.0)	p = 0.474
Time to peak$_{+aTFPI}$, min	5.10 (4.56 to 10.10)	5.20 (4.57 to 6.00)	p = 0.294
PS ratio	0.89 (0.54 to 1.42)	0.75 (0.42 to 1.41)	p = 0.061
TFPI ratio	0.55 (0.24–1.01)	0.42 (0.13–0.9)	p < 0.05

Because the majority of the parameters in one of the groups were not normally distributed according to the Kolmogorov-Smirnov test, all data are presented as a median with range in parenthesis.

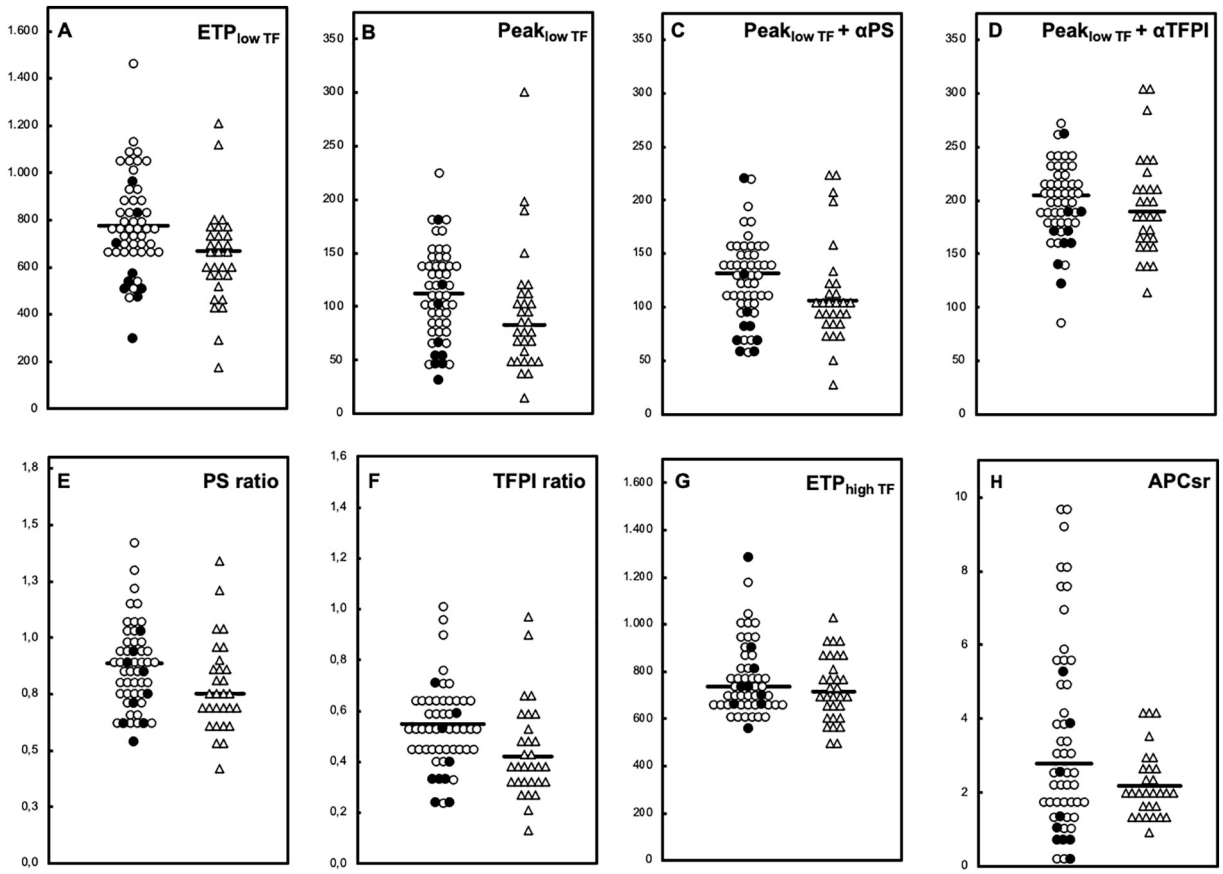

Fig. 1. Selected thrombin generation parameters in women with and without previous venous thromboembolism.
A: The endogenous thrombin potential (ETP) measured at 1 pM tissue factor (TF) ($p < 0.05$); B: the peak height measured at 1 pM TF ($p < 0.05$); C: the peak height measured at 1 pM TF in the presence of anti-protein S antibody ($p < 0.05$); D: the peak height measured at 1 pM TF in the presence of anti-TFPI antibody ($p = 0.750$); E: the protein S ratio ($p = 0.061$); F: the TFPI ratio, ($p < 0.05$); G: the ETP measured at 10 pM TF, in the absence of activated protein C (APC), ($p = 0.503$) and H: the APC sensitivity ratio (APCsr) ($p = 0.123$) are depicted for 43 non-smoking (○) and 9 smoking (●) women with previous venous thromboembolism and for 31women without previous VTE (△).

Table 3
Thrombin generation parameters at high tissue factor.

Parameters	Thrombosis group N = 52	Control group N = 31	p-value
Lag time, min	1.65 (1.28–3.98)	1.61 (1.28–3.74)	p = 0.178
ETP, nM IIa*min	737.5 (561.0–1285.0)	715.0 (496.0–1031.0)	p = 0.503
Peak height, nM IIa	254.0 (206.0–385.0)	265.2 (51.0–515.0)	p = 0.851
Time to peak, min	3.12 (2.66–5.60)	3.04 (2.69–8.61)	p = 0.236
Lag time$_{+APC}$, min	2.05 (1.54–5.54)	2.18 (1.17–2.63)	p = 0.578
ETP$_{+APC}$, nM IIa*min	219.0 (13–780)	175.0 (60–703)	p < 0.05
Peak height$_{+APC}$, nM IIa	77.5 (3.0–342.0)	53.3 (16.0–243.0)	p < 0.05
Time to peak$_{+APC}$, min	3.74 (3.05 to 7.01)	3.71 (3.00 to 10.38)	p = 0.284
APCsr	2.79 (0.2 to 9.97)	2.18 (0.92 to 9.99)	p = 0.123

Because the majority of the parameters in one of the groups were not normally distributed according to the Kolmogorov-Smirnov test, all data are presented as a median with range in parenthesis.

previous VTE, whereas women with history of thrombosis had higher thrombin generation at low tissue factor (ETP difference: 219.9, 95%CI: 90.4 to 349.3, $p < 0.05$), APC resistance (APCsr difference: 1.88, 95%CI: 0.71 to 3.05, $p < 0.05$), protein S ratio (difference 0.13, 95%CI: 0.01 to 0.26, $p < 0.05$) and TFPI ratio (difference 0.19, 95%CI: 0.08 to 0.30, $p < 0.05$). Factor V Leiden was a strong determinant of the APCsr, increasing it by 3.72 (95%CI: 2.61 to 4.82 $p < 0.0001$), if present. As expected, factor V Leiden did not influence thrombin generation without APC at different TF concentrations. Interestingly, other covariates also had impact on thrombin generation in this model. With increasing BMI by 10 kg/m^2, the ETP measured at high tissue factor increased by 197 nM IIa*min and the protein S ratio by 0.15. APC resistance reduced with advancing age, with a decrease of 0.77 in the APCsr every 10 years, but age did not significantly affect the other parameters.

3.4. Effect of smoking on thrombin generation parameters

Interestingly, smoking appeared to be a significant determinant of the ETP at low tissue factor, the APCsr and the TFPI ratio but did not influence thrombin generation at high tissue factor (Fig. 1). Smoking women had a lower ETP at low tissue factor (−222.6, 95%CI: −381.1 to −64.1, $p < 0.05$), lower APCsr (−2.20, 95%CI: −3.63 to −0.77, $p < 0.005$) and TFPI ratio (−0.16, 95%CI: −0.29 to −0.03, $p < 0.05$). Additionally, a direct comparison of thrombin generation parameters between non-smoking and smoking women with previous VTE using the Mann-Whitney *U* test showed significantly lower peak height and ETP at low tissue factor and at high tissue factor in the presence of APC. The APCsr and TFPI ratio were also lower in smoking women. The ETP at high tissue factor and the protein S ratio did not differ between smoking and non-smoking women with previous VTE in this analysis.

Table 4
Results of multiple regression analysis.

Determinants	$ETP_{high\ TF}$ $R = 0.51$	$ETP_{low\ TF}$ $R = 0.47$	APCsr $R = 0.68$	PS ratio $R = 0.41$	TFPI ratio $R = 0.46$
Previous thrombosis	53.4 (−32.0 to 138.8)	219.9 (90.4 to 349.3)*	1.88 (0.71 to 3.05)*	0.13 (0.01 to 0.26)*	0.19 (0.08 to 0.30)*
Age, years§	−0.18 (−0.46 to 0.10)	−0.18 (−0.47 to 0.11)	−0.25 (−0.49 to −0.01)*	−0.14 (−0.50 to 0.18)	−0.28 (−0.60 to 0.13)
Smoking	85.6 (−19.0 to 190.2)	−222.6 (−381.1 to −64.1)*	−2.20 (−3.63 to −0.77)*	−0.09 (−0.24 to 0.06)	−0.16 (−0.29 to −0.03)*
BMI§	0.47 (0.25 to 0.69)*	0.07 (−0.16 to 0.29)	−0.04 (−0.22 to 0.15)	0.26 (0.03 to 0.48)*	0.06 (−0.17 to 0.32)
FVL	54.0 (−26.9 to 135.0)	2.3 (−120.3 to 124.9)	3.72 (2.61 to 4.82)*	0.06 (−0.06 to 0.17)	0.06 (−0.05 to 0.16)

The data are presented as unstandardized B coefficient for categorical and standardized coefficient β for continuous values§ with 95% confidence interval in parenthesis. The standardized coefficient β refers to how many standard deviations thrombin generation parameters change, if the independent variable changes by one standard deviation. Standard deviations were for age 7.62 years; BMI 3.47 kg/m^2; $ETP_{high\ TF}$ 145.68 nM IIa.min; $ETP_{low\ TF}$ 214.8 nM IIa.min; the APCsr 2.34; the protein S ratio (PS ratio) 0.196 and the TFPI ratio 0.177.
R: multiple correlation coefficient, corresponding the proportion of variance in the thrombin generation parameters that can be explained by the regression model including previous thrombosis, age, smoking, BMI and factor V Leiden (FVL).
* $p < 0.05$.

3.5. Thrombosis recurrence and thrombin generation

No association between the studied thrombin generation parameters and occurrence of a VTE- recurrence was observed. However, it should be noted that the number of women with a recurrence, who did not take vitamin K antagonists and therefore were available for analysis was low (n = 5) and the study was underpowered to detect such correlation.

4. Discussion

Our data demonstrate a prothrombotic state in the population of women after previous hormone-induced VTE that can predispose to thrombosis during hormonal contraception and, among others, could explain the risk of thrombosis recurrence under continuing or new exposure of exogenous or increased levels of endogenous hormones. These changes were more pronounced in thrombin generation assays, probing the TFPI/protein S system and APC resistance that suggest an impairment in the function of these natural anticoagulants.

The present study cohort was representative for the patient collective with hormone-induced thrombosis with respect to the proportion of carriers of factor V Leiden and the prothrombin mutation (G20210A) or a positive family history for thrombosis [3]. In our analysis women were younger at the time of the index event as compared to other studies on hormone-related VTE. This can be explained by the fact that our study group only consisted of women, using hormonal contraception at the index event. In contrast, other studies also included patients, obtaining other hormonal treatments, like hormone replacement therapy, which is generally administered in an older postmenopausal population [14]. In line with previous reports, more than 50% of first VTEs occurred within the first year of contraceptive use (data not shown). Interestingly, there was a relatively large proportion of women with cerebral vein thrombosis in our study. On the other hand, venous thrombosis at this location was demonstrated to occur 3 times more frequently in women as in men, and oral contraceptives were shown to be the major risk factor for cerebral vein thrombosis in women, with 54% to be associated with use of oral contraceptives [15]. Additionally, a high awareness among medical practitioners in Sweden can also account for the enrichment of our study population by this form of VTE. In agreement with published data for young women, particularly after a thrombosis event associated to reproductive factors, the recurrence rate was low in our study [14]. Interestingly, the recurrences occurred much later in our population as compared to other studies, possibly due to the younger age of our participants, as age is considered as an independent risk factor for recurrent thrombosis.

The data on the predictive value of thrombin generation test for a first or recurrent VTE remain controversial [10,16]. One of the possible explanations for this can be a great flexibility of the thrombin generation assay, where the experimental conditions can be tailored depending on the study question. Whereas this flexibility enables assessment of particular coagulation pathways or anticoagulant systems, it hampers a comparison of studies, using different assay conditions. Nonetheless, a stronger association seems to exist between the risk of a first and recurrent VTE and thrombin generation measured at lower tissue factor concentrations or determined in the presence of APC or thrombomodulin [6,17,18]. Thrombin generation triggered by lower tissue factor concentrations was suggested to provide a better estimate for the physiological situation as that measured at high tissue factor concentrations [11]. Therefore, one may hypothesize that one or more determinants of thrombin generation at these conditions are more relevant for the thrombosis risk than those of thrombin generation triggered with high tissue factor concentrations. TFPI is the most likely candidate, since it has been shown to be one of the common determinants of APC resistance as well as thrombin generation at low TF in the absence and in the presence of thrombomodulin [19]. Protein S is another important determinant of APC resistance and thrombin generation measured at low TF concentrations [19]. Low levels of protein S and TFPI were associated with increased risk of a first and recurrent VTE [20–22], although this association was weaker or even absent in several other studies [23,24]. These inconsistencies could have several explanations, among others that age, gender, obesity, hormonal treatments and possibly even smoking status may influence the protein S and/or TFPI levels and, therefore account for a great part of their variability in the general population. Hence, the results of the analyses could depend on whether adjustments for all these variables have been performed and which fractions of TFPI and protein S (*e.g.* total, free or activity) were determined. Additionally, significant covariations between TFPI and various coagulation factors that has been demonstrated by the LITE and LETS studies [20,24], could also influence the association between the TFPI levels and the risk of VTE.

In our study women, who have developed VTE while taking hormonal contraceptives were more resistant to APC as compared to healthy controls, even after correction for factor V Leiden. Furthermore, the thrombosis group had higher thrombin generation at low tissue factor and elevated protein S and TFPI ratios as compared to women without a history of VTE, indicating an impairment of the function of the protein S/TFPI system. There were no differences in thrombin generation at high tissue factor between women with and without previous hormone-induced thrombosis. The observed coagulation changes are not likely to be a consequence of the occurred thrombotic event, but rather reflect the intrinsic coagulation profile, revealing possible predisposition for VTE during hormonal exposure. The prothrombotic effect of steroid hormones was suggested to be greatly attributed to APC resistance and decreased function of the protein S/TFPI system [25]. Therefore, it is possible that women, who have a preexisting impairment of these pathways are more susceptible to the thrombogenic influence of steroid hormones. For instance, Wood et al. described a contribution of low TFPI levels to the hypercoagulable state due to the factor V Leiden mutation [26]. A recent study of Khialani et al. confirmed that increased APC resistance and low TFPI levels were associated with VTE, but failed to demonstrate a higher risk of VTE among women with this prothrombotic profile during hormone replacement therapy [27]. However, because hormone replacement therapy is considerably lower dosed as

compared to combined oral contraceptives, this issue should further be investigated in a population of pill users.

Remarkably, smoking status appeared to be a strong factor, influencing several thrombin generation parameters and APC resistance in our study. Smoking women had lower thrombin generation at low tissue factor than non-smoking women (Fig. 2). This difference was less pronounced, if the protein S function was inhibited by a specific antibody and completely reversed by addition of TFPI antibody or at high tissue factor concentrations. Altogether, this strongly suggests that smoking increases activity of the TFPI system, confirming the data of van Paridon et al., who also demonstrated lower peak height at 1 pM of tissue factor in a part of their population, consisting of smoking women [28] and later confirmed that smokers have increased TFPI activity [29]. Higher TFPI activity in smokers could possibly be a result of endothelial damage or chronical inflammation in the vessel wall with consequently increased synthesis, release or altered binding of TFPI to the endothelium by glucosaminoglycans [30]. Similarly, other cardiovascular risk factors, like insulin resistance, dyslipidemia, obesity can also increase TFPI levels, particularly in women, and were demonstrated to correlate with endothelial cell markers and fibrinogen [31,32]. The increase in the TFPI levels induced by smoking possibly counteracts the prothrombotic changes, like elevated fibrinogen, factor IX and decreased antithrombin that have been previously described in smoking population [33] and could explain controversial data on the impact of smoking on the risk of VTE [34]. Influence of smoking on the coagulation system should be further investigated and also should be taken into account while analyzing coagulation parameters in study populations, including smokers.

Several limitations of our study have to be discussed. First of all, the sample size is relatively small to be compared with large national-wide studies, like LETS or LITE [20,24]. However, our study was a cross-sectional study with a relatively homogenous study population consisting of premenopausal women that allows us to address the study question on a selected patient collective, who developed thrombosis during hormonal contraception. Another limitation of the study is that our control group was younger and did not include smoking women as compared to the group with history of thrombosis. Therefore, adjustments for smoking status and age have been performed. Furthermore, our control group was by chance enriched in carriers of factor V Leiden. This could be explained by the fact that recruitment of healthy controls of reproductive age, who attended our gynaecological department and were not using hormonal contraceptives lead to an inclusion of a number of infertile women with planned assisted reproduction procedures. Some reports suggested a higher prevalence of factor V Leiden in this patient collective [35], which could possibly explain a relatively high proportion of women with factor V Leiden in our control group (16,1%). However, this selection bias would rather lead to an increase in APC resistance in the control group and, thereby to an underestimation of the difference in APC resistance between women with previous VTE and the control group. Moreover, adjustment for factor V Leiden in the statistical analysis did not considerably change our conclusions. One of the strengths of the study that the time elapsed between the index event and the blood sampling in our patient population precludes any influences of the acute phase reaction, residual thrombus or residual effect of shortly discontinued anticoagulant therapy. Women with VTE are generally discouraged to use estrogen-containing pills. Therefore, the most frequent challenging situation remains pregnancy, which also considerably reduces protein S and TFPI levels and increases APC resistance [36]. Eight from nine VTE recurrences in our study were related to pregnancy.

Altogether, our study demonstrates long-term impairment of protein S/TFPI system and increased APC resistance in women with previous hormone-induced VTE. These findings substantiate the hypothesis that these alterations predispose to thrombosis during hormonal contraception and supports recommendations to prophylactic anticoagulant therapy during pregnancy and puerperium for this collective irrespective of the time after the index event.

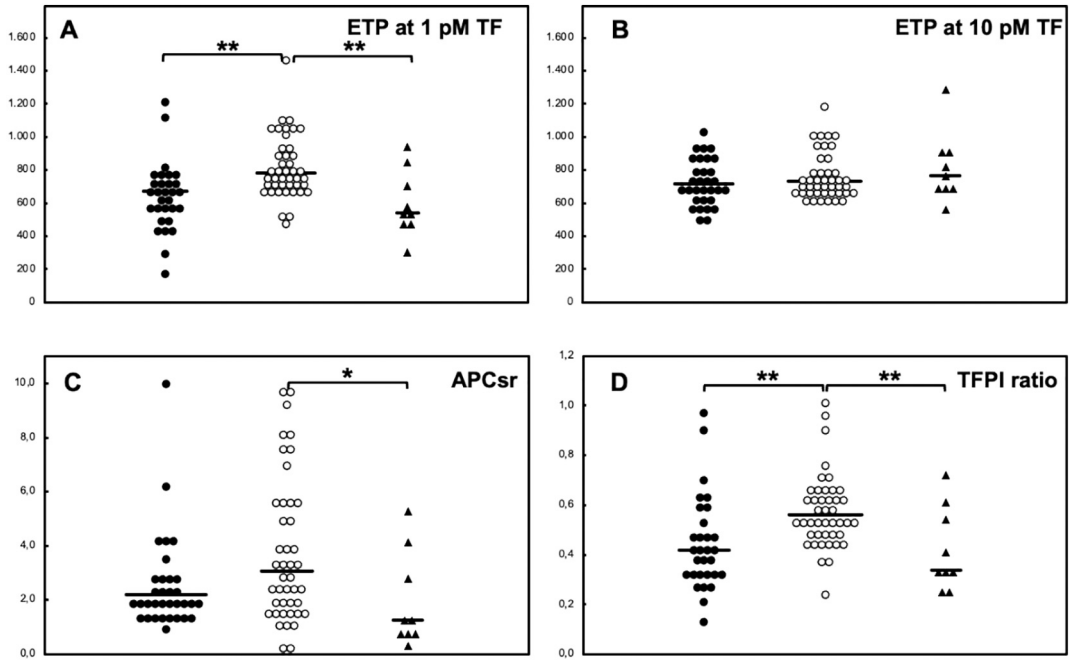

Fig. 2. Differences in thrombin generation parameters between smoking and non-smoking women.
A: The endogenous thrombin potential (ETP) measured at 1pM tissue factor (TF), B: the ETP measured at 10 pM TF, C: the APC sensitivity ratio (APCsr) and D: the TFPI ratio in plasma of 31 non-smoking controls (●), 43 non-smoking women with previous VTE (○) and 9 smoking women with previous VTE (▲), one of smoking women received vitamin K antagonists and was therefore excluded from the analysis. The groups were significantly different according to the Kruskal-Wallis test for the ETP at 1 pM tissue factor $p < 0.001$, the APCsr $p < 0.05$ and the TFPI ratio $p < 0.001$. Following Mann-Whitney *U* test to compare non-smoking women without and with previous VTE and non-smoking and smoking women with previous VTE resulted indicated differences: * $p < 0.025$; ** $p < 0.01$.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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