Original article

Venous thromboembolism in carriers of the Factor V Leiden mutation and in patients without known thrombophilic risk factor; prediction of recurrence and APC-PCI complex concentration and/or soluble Thrombomodulin antigen and activity

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Abstract

Background The complex between activated protein C (APC) and the protein C inhibitor (PCI) is a sensitive indicator of the degree of activation of blood coagulation and higher concentrations have been measured in carriers of the FV Leiden mutation who were in the recovery phase after treatment for venous thromboembolism (VTE).

Objectives The main purpose of this study was to correlate the APC-PCI complex concentration to thrombomodulin activity and antigen concentration in the same group of patients. We also add a prospective clinical follow-up of the VTE recurrence after one year to investigate if the markers can predict the risk for a new VTE.

Patients/Methods Blood samples were collected from 50 patients with the FV Leiden mutation and 132 without any known risk factor for thrombophilia after finished treatment.

Results The APC-PCI complex**,** s-TM activity and the quotient (s-TM activity)/ (s-TM antigen) were higher in VTE patients with FV Leiden. In total, there were 19 VTE recurrences (10%) after one year. The OR for recurrence was 1.9 (95%CI 0.68-5.0) in all VTE patients with elevated APC-PCI complex (above 75^{th} percentile) and 3.6 (95%CI 1.1-12) in VTE patients without any known risk factor for thrombophilia and with elevated s-TM activity.

Conclusion The APC-PCI complex concentration**,** s-TM activity and the quotient (s-TM activity)/ (s-TM antigen) were higher in VTE patients with FV Leiden. The s-TM activity showed higher OR for recurrence of VTE in patients without known thrombophilic risk factor. Both methods could be sensitive markers of increased risk for venous thrombosis.

Key-words APC-PCI complex concentration**,** s-TM activity, s-TM antigen, VTE patients, FV Leiden, VTE recurrence.

Abbreviations APC, activated protein C, PCI, protein C inhibitor, VTE, venous thromboembolism, s-TM, soluble thrombomodulin, DVT, deep vein thrombosis, FV, Factor V.

Introduction

Blood coagulation is initiated *in vivo* when tissue factor binds Factor VII/VIIa, leading to thrombin generation and platelet activation with the ultimate formation of a fibrin network [1]. This process is regulated by the protein C anticoagulant system. Thrombin binds to thrombomodulin (TM), an integral membrane protein on endothelial cells, and changes its specificity from a pro-coagulant to an anticoagulant enzyme that activates protein C [2, 3]. This process is enhanced by the endothelial protein C receptor in large blood vessels [4]. Activated protein C (APC) serves as a circulating anticoagulant by inactivating Factors Va and VIIIa. APC is slowly inactivated in plasma by forming a 1:1 complex with the protein C inhibitor (PCI), a serpin that is the prime inhibitor of APC [5-8]. The half-life of this complex in the circulation is about 20 minutes in rabbits and 40 minutes in baboons [9, 10]. Due to the activation of protein C by thrombin bound to TM, and the subsequent inactivation of APC by PCI, the concentration of the APC-PCI complex is a sensitive indicator of the degree of activation of blood coagulation. Increased levels of APC-PCI have been observed in hyper-coagulation states, or thrombophilia, affecting both the venous and arterial systems; for example, deep vein thrombosis (DVT), pulmonary embolism, disseminated intravascular coagulation and myocardial infarction [11-15].

A new sensitive sandwich immunofluorimetric assay for measurements of the APC-PCI complex has been devised [16, 17]. The method employs a monoclonal antibody that is specific for the loop-inserted form of the inhibitor, i.e. PCI that is in complex with APC. Native PCI that is not in complex with APC exists in a 10^4-fold molar excess over the APC-PCI complex and has no affinity for the antibody. As a reporter antibody a monoclonal antibody against protein C is used.

Resistance to activated protein C is a major risk factor for venous thrombosis that is usually caused by a point-mutation in the gene of coagulation Factor V (FV) [18, 19].

This mutation, FV Leiden, which renders the FV molecule less susceptible to inactivation by APC, and is the most common hereditary risk factor for venous thromboembolism (VTE) in the Caucasian population and is found in 20-44% of patients with VTE [2]. There is a need for more specific laboratory markers that can be used to predict an elevated risk for recurrent VTE, especially in patients without diagnosed hereditary risk factors for thrombophilia.

We have previously demonstrated higher APC-PCI complex concentrations, indicating increased thrombin generation in carriers of the FV Leiden mutation who were in the recovery phase after finished treatment for VTE [20]. Of the 182 patients in the study, 50 had the FV Leiden mutation while the remaining 132 did not have any known thrombophilic risk factor.

The endothelial cell surface is considered to be the major physiological site for TM function and s-TM is traditionally thought to serve as a marker of endothelial cell damage [21, 22]. It has been confirmed *in vitro* that s-TM is an early marker of initial endothelial cell membrane changes that are induced by neutrophil derived proteases and oxygen radicals [23]. Although s-TM might provide some degree of protection against thrombosis due to its proposed anti-inflammatory and anti-coagulant properties, reduced TM on the endothelial cell surface would seriously compromise the anti-thrombotic and anti-inflammatory potential and add to an elevated risk for VTE and VTE recurrence [24, 25].

The aim of this study was to correlate APC-PCI complex concentrations to thrombomodulin activity (s-TM activity) and thrombomodulin antigen concentrations (s-TM antigen), measured with newly developed assays in the same group of patients [26, 27]. We also report from a prospective follow-up of VTE recurrence after one year.

Materials and methods

Subjects

The patients studied were consecutive patients recruited to the "Malmö Thrombophilia Study" which is a prospective study. The criteria for including a patient is that the patient is over 18 years, can communicate in Swedish and that the thrombosis is diagnosed at Malmö University Hospital, with an objective method. Patient history and acquired and genetic risk factors for VTE are available for each patient. Ultrasound or phlebography was used for DVT diagnosis and helical computed tomography for diagnosis of pulmonary embolism (PE).

For this particular study, we started to include consecutive patients in February 2003 and continued until September 2004. Blood samples were collected from 198 patients in the recovery phase 2-4 weeks after cessation of anticoagulant treatment for VTE. Of these, 13 patients with prothrombin 20210 mutation, one patient with protein S-deficiency and two patients with lupus anticoagulans were excluded. The subjects comprised 50 patients with FV Leiden and 132 patients without any of the risk factors: G20210A prothrombin mutation, antithrombin deficiency, protein C deficiency protein S deficiency or lupus anticoagulant; **Table I**). Venous blood was drawn after informed written consent. It was collected in 5 mL vacuum tubes (Stabilyte®, Trinity Biotech, Bray Ireland) containing citrate with a low pH, which precludes *in vitro* formation of the APC-PCI complex [28]. The blood was centrifuged at 3600xg for 10 minutes within 30 minutes of collection and the plasma frozen in aliquots at –70º C for later analysis. VTE recurrence was recorded one year after blood-sampling. The study was approved by the Ethics Committee, Lund University.

Methods

APC-PCI complex concentration was measured using the previously-described DELFIA assay, which has a functional sensitivity in Stabilyte-plasma of 0.032 μg/L. Using Stabilyte tubes, the concentration in healthy individuals without medication and without a previous deep vein thrombosis, was 0.07 to 0.26 μg/L with a mean and median of 0.13 μg/l (n=80, median age 42 years, 28-56,10^{th} and 90^{th} percentiles, Males/Females: 20/50; 28). The within-run coefficient of variation was 4.8% at 0.15 μg/L and 3.2 % at 0.40 μg/L, while the between-run coefficient of variation was 7.1% at 0.15 μg/L and 5.8 % at 0.41 μg/L (n=38).

For comparison with an established marker of coagulation activation, D-dimer was measured with Auto D-dimer (Biopool AB, Umeå, Sweden) on samples that were frozen and thawed once more. The concentration in healthy individuals was < 0.2 mg/L (n= 39). As a marker of the inflammatory state of the patients, CRP was measured with hsCRP (Tinaquant a CRP, Roche).

Soluble TM antigen and activity were measured using Stabilyte plasma [26, 27]. In citrate plasma, the reference interval (mean ±2 SD) for s-TM antigen is 3.5-8.3 SEq/mL for men and 3.2-7.2 SEq/mL for women; the reference interval for s-TM activity is 2.1-5.7 SEq/mL for men and 1.9-5.3 SEq/mL for women; and the reference interval for the quotient s-TM activity /s-TM antigen is 0.45-0.89 for men and 0.38-0.98 for women (1 ng Solulin = 1 SEq; [26]) These reference intervals were established from measurements on 50 men and 50 women without medication**.** The reference intervals for s-TM antigen and activity were verified in Stabilyte plasma in healthy individuals. In 36 healthy females, median age 44 years (range 26-61), the s-TM antigen was 3.6–7.0 SEq/mL with a mean of 5.0, and the s-TM activity was 2.1–3.9 SEq/mL with a mean of 3.0 SEq/mL. In 14 healthy males, median age 36 years (range 29-50), the reference intervals in Stabilyte plasma of both s-TM antigen and s-TM activity were also within the range observed for men in citrate plasma.

Statistics

Statistical analyses were performed with SPSS version 14 for Windows (SPSS Inc. Chicago, IL, USA). Non-normally distributed data were expressed as median and percentiles (10^{th} and 90^{th}). The Mann-Whitney U test was used for bivariate analysis of continuous variables, and the Spearman rank correlation test for correlations. P-values < 0.05 were considered significant. A chi-squared test was used for nominal variables. Logistic regression analysis was performed to calculate the odds ratios for categorical variables and predictive values for the combined effect of the APC-PCI and D-dimer or s-TM activity methods in discriminating VTE patients with FV Leiden and without known thrombophilic risk factor.

Results

The APC-PCI complex concentration was higher in VTE patients with FV Leiden than in patients without known thrombophilic risk factors (**Table II;** [20]). There was no difference in D-dimer, s-TM antigen or hsCRP concentrations between the groups, whereas also s-TM activity and the quotient (s-TM activity)/ (s-TM antigen) were both higher in patients with FV Leiden (**Table II, Fig. 1**). For APC-PCI concentration above the 75th percentile (>0.29 μg/L, defined from the whole study group), the OR for FV Leiden was 3.8 (95% CI 1.9-7.8).

The group of patients without known thrombophilic risk factor had higher APC-PCI complex concentrations than the reference population [28]. There were a number of patients with concentrations of APC-PCI complex and D-dimer above the 95th percentile. Six of the patients had s-TM antigen concentration above the 95th percentile (>11.2 SEq/mL), three of these also had high concentration of APC-PCI complex (>0.46 μg/L) and one had high D-dimer concentration (>0.44 mg/L). Exclusion of these patients did not affect s-TM antigen concentrations between the groups (p=0.55).

In the whole study group, we observed positive correlations between the concentrations of APC-PCI complex concentration and D-dimer (r=0.54, p<0.001, Spearman´s rank test; r^2 =29%), APC-PCI complex and s-TM activity (r=0.30, p<0.001, r^2 =9%) and APC-PCI and s-TM antigen (r=0.21, p=0.004, r^2 =4%). Significant positive correlations were also found between APC-PCI complex concentration and hsCRP (r=0.16, p<0.05, r^2 =3%) and APC-PCI complex and age (r=0.30, p<0.01, r^2 =9%). In FV Leiden patients a significant positive correlation was found between the concentrations of APC-PCI and D-dimer (r=0.53, p<0.001, r^2 =28%) and APC-PCI levels and s-TM activity (r=0.43, p=0.002, r^2 =18%).

In total, 19 VTE recurrences (10%) were observed after one year**.** None of the laboratory markers demonstrated a difference in concentration in FV Leiden patients with or without VTE recurrence, whereas in patients without hereditary thrombophilia, a significant increase was seen in s-TM activity and the s-TM activity/antigen quotient (**Table III**). The OR for VTE recurrence was 1.9 (95% CI 0.68-5.0) in VTE patients with elevated APC-PCI complex (above the 75^{th} percentile), 2.1 (95% CI 0.78-5.8) in patients with elevated s-TM activity and 1.1 (95% CI 0.38-3.3) in patients with elevated D-dimer (**Table IV)**. In FV Leiden-patients with elevated APC-PCI complex the OR for VTE recurrence was 3.2 (95%CI 0.52-19), whereas elevated s-TM activity in patients without FV Leiden gave an OR for VTE recurrence of 3.6 (95%CI 1.1-12).

Discussion

The aim of this study was to search for more specific laboratory markers that can be used to predict an elevated risk for VTE and to investigate whether there is an association between APC-PCI complex concentration and s-TM antigen and activity in carriers and non-carriers of the FV Leiden mutation in patients with a previous history of VTE. In addition, in a clinical follow-up after one year we investigated the ability of the methods to predict VTE recurrence. In the whole study group, both carriers and non-carriers of the FV Leiden mutation, correlations were observed between the APC-PCI concentration and D-dimer (r^2 =29%) as well as between APC-PCI concentration and s-TM activity (r^2 =9%) and antigen (r^2 =4%). All these measurements have been suggested as reflecting an on-going prothrombotic state.

We found a higher APC-PCI concentration in VTE patients with FV Leiden than in VTE patients without known risk factor for thrombophilia, indicating an increased thrombin generation and presumably individuals at higher risk for recurrent VTE. In both FV Leiden patients and VTE patients without known risk factor for thrombophilia the APC-PCI method detected a higher degree of hypercoagulability than in healthy individuals. Two earlier case-control studies of patients with previous VTE without thrombophilic defects reported contradictory results, showing either increased or decreased APC levels as a risk factor for thrombosis [29, 30]. In contrast to these methods, we have a very sensitive method that indirectly measures activation of the protein C system and thus *in vivo* thrombin generation. Increased thrombin generation has been measured recently in patients with recurrent VTE [31]. With that method, no difference in peak thrombin generation was seen between patients with and without FV Leiden, although FV Leiden was detected in more patients with recurrent than without recurrent VTE (38 vs. 27%, p=0.03).

Our results also show that s-TM activity is higher in patients with FV Leiden than in non-carriers, despite the lack of difference between the groups regarding s-TM antigen concentration. The finding regarding s-TM antigen concentration is, however, in disagreement with the results of two previous studies — a small study of children with and without FV Leiden, and a large study of patients suffering from venous thromboembolism — both of which reported that FV Leiden carriers had higher levels of s-TM antigen concentration than patients without this mutation [32, 33]. One explanation for this discrepancy might be the method used to measure antigen concentration. The earlier studies used a commercially available enzyme-linked immunosorbent assay kit (Asserachrom^{R} Thrombomodulin, Diagnostica Stago, France), while we used an in-house enzyme-linked immunosorbent assay. S-TM is present in plasma as fragments of different sizes, and the different results obtained by these two methods might indicate that they measure, at least partly, different fragments. A statistically-significant correlation has been observed between soluble TM antigen levels and soluble TM cofactor activity [26]. This notwithstanding, one possibility is that measurement of s-TM activity is more sensitive than our in-house method of measuring the antigen concentration to changes in plasma concentration of s-TM fragments in this group of patients. If this is true, our measurements of s-TM activity do agree with earlier published results on FV Leiden and s-TM, even though our antigen concentration measurement method was not sensitive enough to detect the small differences between the groups.

It has been suggested that s-TM is degraded from the endothelial cell surface as a consequence of endothelial damage [21]. High concentration and activity of circulating s-TM may imply less TM on the endothelium and an ongoing prothrombotic state for example in patients carrying FV Leiden. On the other hand, a high plasma concentration of s-TM has been associated with a lower risk of developing coronary heart disease, suggesting that s-TM

may reflect the level of endothelial expression, at least in patients not carrying TM mutations that affect this expression [24]. The relationship of s-TM antigen concentration and s-TM activity with venous as well as arterial thrombosis is complex, and it remains to be seen whether one or both could be clinically useful.

Although the small sample size limits the possibility for evaluating the ability of the methods to predict VTE recurrence in the different patient groups, an APC-PCI concentration above the 75th percentile was associated with an OR of 1.9 (95% CI 0.68-5.0) and s-TM activity above the 75th percentile with an OR of 2.1 (95% CI 0.78-5.8) for VTE recurrence. This is the same order of magnitude as in a recent management study, where a twofold increased risk for recurrent events was observed in patients with elevated D-dimer seven weeks after the end of anticoagulant treatment [34]. In patients without FV Leiden and with VTE recurrence, a significant increase was seen in s-TM activity and the s-TM activity/antigen quotient. The OR for VTE recurrence in patients without FV Leiden and with elevated s-TM activity was 3.6 (95% CI 1.1-12**)**. Since the number of recurrencies is small, our study only indicates that the APC-PCI and the s-TM activity analytes could be sensitive markers of an increased risk for VTE recurrence. In contrast to D-dimer methods, these methods measure well-defined analytes. The patients included in this study are part of a prospective study and we will thus have the opportunity to further evaluate the correlation of the described methods with thrombotic events.

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Table I Patient demographics

	Pat. with FV Leiden (n=50)	Pat. without known thrombophilic risk factor (n= 132)	P-values*
Distal thrombosis	13 (26%)	42 (32%)	0.47
Proximal thrombosis	13 (26%)	45 (34%)	0.38
Pulmonary embolism	18 (36%)	32 (24%)	0.21
Warfarin treatment time (median)	6 months (range 2-12)	6 months (range 2-24)	0.89
Age (median)	66 years (range 24-90)	66 years (range 22-91)	0.57
Males	25 (50%)	64 (48%)	0.95
VTE recurrence	6 (12%)	13 (9.8%)	0.11

*Mann-Whitney U test was used for the comparison of patients with FV Leiden and in patients without known thrombophilic risk factor.
Chi-squared test was used for nominal variables.

Table II. APC-PCI complex, D-dimer and soluble Thrombomodulin activity and antigen concentrations in patients with FV Leiden and in patients without known thrombophilic risk factor .

	All patients (n=182)	Patients with F V Leiden (n=50)	Patients without known thrombophilic risk factor (n= 132)	p-value€
APC-PCI (μg/L)	0.23* (0.15, 0.56)	0.29 (0.21, 0.58)	0.21 (0.14, 0.44)	p<0.001
D-dimer (mg/L)	0.12 (0.05, 0.62)	0.13 (0.08, 0.41)	0.11 (0.05, 0.83)	p=0.13
s-TM antigen (SEq/mL)	5.70 (4.35, 8.05)	5.65 (4.41, 8.63)	5.70 (4.30, 7.95)	p=0.90
s-TM activity (SEq/mL)	3.75 (2.90, 5.05)	3.90 (3.11, 5.50)	3.65 (2.80, 4.90)	p=0.032
s-TM activity/antigen	0.66 (0.53, 0.82)	0.69 (0.59, 0.84)	0.65 (0.51, 0.81)	p=0.043
hsCRP	2.0 (0.7, 16.0)	2.5 (0.73, 8.7)	2.0 (0.7, 8.6)	p=0.60

*Median (10^{th} and 90^{th} percentiles).
€ Mann-Whitney U test was used for the comparison of patients with FV Leiden and in patients without known thrombophilic risk factor.

Table III. APC-PCI complex, D-dimer and soluble Thrombomodulin activity and antigen concentrations in patients with and without VTE recurrence.

	All patients (n=182)			Patients with F V Leiden (n=50)			Patients without known thrombophilic risk factor (n= 132)		
	With recurrence (n=19)	Without recurrence (n=163)	p-value€	With recurrence (n=6)	Without recurrence (n=44)	p-value€	With recurrence (n=13)	Without recurrence (n=119)	p-value€
APC-PCI (μg/L)	0.26* (0.16, 0.67)	0.23 (0.15, 0.53)	p=0.11	0.34 (0.22, 0.41)	0.29 (0.20, 0.58)	p=0.52	0.25 (0.14, 0.78)	0.21 (0.14, 0.42)	p=0.17
D-dimer (mg/L)	0.13 (0.08, 0.96)	0.12 (0.05, 0.59)	p=0.30	0.15 (0.08, 0.35)	0.13 (0.07, 0.42)	p=0.82	0.12 (0.08, 1.20)	0.11 (0.05, 0.78)	p=0.27
s-TM antigen (SEq/mL)	6.10 (4.30, 7.70)	5.7 (4.34, 8.46)	p=0.69	5.20 (4.30, 15.4)	5.80 (4.47, 8.21)	p=0.79	6.10 (3.80, 7.66)	5.60 (4.30, 8.80)	p=0.50
s-TM activity (SEq/mL)	4.0 (3.1, 5.1)	3.70 (2.84, 5.06)	p=0.14	3.70 (3.10, 5.0)	3.90 (3.17, 5.50)	p=0.35	4.20 (3.0, 5.22)	3.60 (2.80, 4.80)	p=0.02
s-TM activity/ antigen	0.72 (0.5, 0.84)	0.65 (0.53, 0.80)	p=0.04	0.72 (0.23, 0.82)	0.67 (0.58, 0.84)	p=0.78	0.75 (0.54, 0.86)	0.65 (0.48, 0.79)	p=0.03

*Median (10^{th} and 90^{th} percentiles).

€ Mann-Whitney U test was used for the comparison of patients with and without recurrence.

Table IV. Odds ratios for VTE recurrence with elevated APC-PCI complex, and D-dimer concentrations and soluble Thrombomodulin activity.

	All patients (n=182)		Patients with FV Leiden		Patients without known thrombophilic risk factor	
	N (%)	OR for recurrence	N (%)	OR for recurrence	N (%)	OR for recurrence
APC-PCI >75 th percentile (0.29 μg/L)	46 (25)	1.9 (0.68-5.0)	21 (42)	3.2 (0.52-19)*	25 (19)	1.3 (0.34-5.2)
D-dimer >75 th percentile (0.23 mg/L)	44 (24)	1.1 (0.38-3.3)	12 (24)	0.60 (0.06-5.7)	32 (24)	1.4 (0.41-5.0)
s-TM activity> 75 th percentile (4.3 SEq/mL)	42 (23)	2.1 (0.78-5.8)	13 (25)	0.53 (0.06-5.1)	29 (22)	3.6 (1.1-12)

*95% CI

Legend to figure 1

Figure 1 Box-plots representing s-TM antigen concentrations and s-TM activity in patients with a previous VTE episode, either with FV Leiden mutation or without known thrombophilic risk factor for VTE. Medians are shown by lines, boxes show first and third quartiles, bars show minimum and maximum values, circles signify outliers, and asterisks represent extreme-values.