



# LUND UNIVERSITY

## **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.**

Strandberg, Karin; Svensson, Peter; Öhlin, Ann-Kristin

*Published in:*  
Thrombosis Research

*DOI:*  
[10.1016/j.thromres.2007.03.020](https://doi.org/10.1016/j.thromres.2007.03.020)

2007

[Link to publication](#)

### *Citation for published version (APA):*

Strandberg, K., Svensson, P., & Öhlin, A.-K. (2007). 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. *Thrombosis Research*, 121, 145-151. <https://doi.org/10.1016/j.thromres.2007.03.020>

*Total number of authors:*  
3

### **General rights**

Unless other specific re-use rights are stated the following general rights apply:  
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

Read more about Creative commons licenses: <https://creativecommons.org/licenses/>

### **Take down policy**

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

LUND UNIVERSITY

PO Box 117  
221 00 Lund  
+46 46-222 00 00



LUND UNIVERSITY  
Faculty of Medicine

---

# LU:*research*

*Institutional Repository of Lund University*

---

This is an author produced version of a paper published in Thrombosis research. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Strandberg, Karin and Svensson, Peter J  
and Ohlin, Ann-Kristin.

"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."

Thromb Res, 2007, Vol: 10 May [Epub ahead of print]

<http://dx.doi.org/10.1016/j.thromres.2007.03.020>

Access to the published version may  
require journal subscription.

Published with permission from: Elsevier

## 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**

Karin Strandberg,<sup>1</sup> Peter J Svensson<sup>2</sup>, Ann-Kristin Öhlin<sup>3</sup>

<sup>1</sup> Dept. of Clinical Chemistry, Lund University, Malmö University Hospital, Sweden

<sup>2</sup> Dept. of Coagulation Disorders, Lund University, Malmö University Hospital, Sweden

<sup>3</sup> Dept. of Clinical Chemistry, Lund University, Lund University Hospital, Sweden

Corresponding author:

Karin Strandberg, MD, PhD

Department of Clinical Chemistry

Malmö University Hospital

SE-205 02 Malmö

Sweden

Phone: +46-40-33 1459

Fax: +46-40-336286

E-mail: [karin.strandberg@med.lu.se](mailto:karin.strandberg@med.lu.se)

New word count of the abstract: 250 (250)

Word count of the text: 3033 (5000)

## **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<sup>th</sup> 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 hypercoagulation 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 –70° 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  $\mu\text{g/L}$ . Using Stabilyte tubes, the concentration in healthy individuals without medication and without a previous deep vein thrombosis, was 0.07 to 0.26  $\mu\text{g/L}$  with a mean and median of 0.13  $\mu\text{g/L}$  ( $n=80$ , median age 42 years, 28-56, 10<sup>th</sup> and 90<sup>th</sup> percentiles, Males/Females: 20/50; 28). The within-run coefficient of variation was 4.8% at 0.15  $\mu\text{g/L}$  and 3.2 % at 0.40  $\mu\text{g/L}$ , while the between-run coefficient of variation was 7.1% at 0.15  $\mu\text{g/L}$  and 5.8 % at 0.41  $\mu\text{g/L}$  ( $n=38$ ).

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  $< 0.2 \text{ mg/L}$  ( $n= 39$ ). 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  $\pm 2$  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<sup>th</sup> and 90<sup>th</sup>). 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 75<sup>th</sup> percentile ( $>0.29 \mu\text{g/L}$ , 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 95<sup>th</sup> percentile. Six of the patients had s-TM antigen concentration above the 95<sup>th</sup> percentile ( $>11.2 \text{ SEq/mL}$ ), three of these also had high concentration of APC-PCI complex ( $>0.46 \mu\text{g/L}$ ) and one had high D-dimer concentration ( $>0.44 \text{ mg/L}$ ). 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<sup>th</sup> 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<sup>R</sup> 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 75<sup>th</sup> percentile was associated with an OR of 1.9 (95% CI 0.68-5.0) and s-TM activity above the 75<sup>th</sup> 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 recurrences 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.

**Acknowledgements**

We warmly thank Dr. John Morser at Berlex Biosciences and the company PAION for generously providing us with Solulin and the monoclonal antibodies Tm43b and Tm531.

This study was supported by generous grants from the research funds of the University Hospital in Lund and the Skane county council's research and development foundation.

The authors are also grateful to Jonas Björk at RSKC for statistical help; Gun-Britt Eriksson, Kerstin Håkansson and Maria Hansson for performing analyses; Camilla Nilsson for collecting data on VTE recurrences; and professor Johan Stenflo for reviewing the manuscript.

## References

1. Toschi V, Gallo R, Lettino M, Fallon J, Gertz S, Fernandez-Ortiz A, et al. Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* 1997; **95**: 594-9.
2. Dahlbäck B. Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases. *J Int Med* 2005; **257**: 209-23.
3. Stenflo J, Dahlbäck B. Vitamin K-dependent proteins in blood coagulation. In: Stamatopolous G, Majerus PW, Perlmutter RM, Varmus H, editors. *The molecular basis of blood coagulation*. Third ed: W.B. Saunders; 2001. p. 579-613.
4. Esmon CT. The endothelial protein C receptor. *Thromb Haemost* 2000; **83**: 639-43.
5. Suzuki K, Deyashiki Y, Nishioka J, Toma K. Protein C inhibitor: structure and function. *Thromb Haemost* 1989; **61**: 337-42.
6. Huntington JA, Kjellberg M, Stenflo J. Crystal structure of protein C inhibitor provides insights into hormone binding and heparin activation. *Structure* 2003; **11**: 205-15.
7. Marlar RA, Kressin DC, Madden RM. Contribution of plasma proteinase inhibitors to the regulation of activated protein C in plasma. *Thromb Haemost* 1993; **69**: 16-20.
8. Scully MF, Toh CH, Hoogendoorn H, Manuel RP, Nesheim ME, Solymoss S, et al. Activation of protein C and its distribution between its inhibitors, protein C inhibitor,  $\alpha_1$ -antitrypsin and  $\alpha_2$ -macroglobulin in patients with disseminated intravascular coagulation. *Thromb Haemost* 1993; **69**: 448-53.
9. Espana F, Gruber A, Heeb MJ, Hansson SR, Harker LA, Griffin JH. In vivo and in vitro complexes of activated protein C with two inhibitors in baboons. *Blood* 1991; **77**: 1754-60.



10. Laurell M, Stenflo J, Carlsson TH. Turnover of I-protein C inhibitor and I-alfa-1-antritrypsin and their complexes with activated protein C. *Blood* 1990; **76**: 2290-5.
11. Strandberg K, Bhiladvala P, Holm J, Stenflo J. A new method to measure plasma levels of activated protein C in complex with protein C inhibitor in patients with acute coronary syndromes. *Blood Coagul Fibrinol* 2001; **12**: 503-10.
12. Strandberg K, Astermark J, Björgell O, Becker C, Nilsson PE, Stenflo J. Complexes between activated protein C and protein C inhibitor measured with a new method: Comparison of performance with other markers of hypercoagulability in the diagnosis of deep vein thrombosis. *Thromb Haemost* 2001; **86**:1400-8.
13. Tanigawa M, Wada H, Minamikawa K, Wakita Y, Nagaya A, Mori T, et al. Decreased protein C inhibitor after percutaneous transluminal coronary angioplasty in patients with acute myocardial infarction. *Am J Hematol* 1999; **49**: 1-5.
14. Espana F, Vicente V, Tabernero D, Sharrer I, Griffin JH. Determination of plasma protein C inhibitor and of two activated protein C inhibitor complexes in normals and in patients with intravascular coagulation and thrombotic disease. *Thromb Res* 1990; **59**: 593-608.
15. Minamikawa K, Wada H, Wakita Y, Ohiwa M, Tanigawa M, Deguchi J, et al. Increased activated protein C-protein C inhibitor complex levels in patients with pulmonary embolism. *Thromb Haemost* 1994; **71**: 192-4.
16. Strandberg K, Kjellberg M, Erb EM, Persson U, Mosher DF, Villoutreix BO, et al. Activated protein C - protein C inhibitor Complex formation: Characterisation of a neoepitope provides evidence for extensive insertion of a reactive center loop. *Biochemistry* 2000; **39**: 15713-20.

17. Strandberg K, Kjellberg M, Knebel R, Lilja H, Stenflo J. A sensitive immunochemical assay for measuring the concentration of the activated protein C - protein C inhibitor complex i plasma. *Thromb Haemost* 2001; **86**: 604-10.
18. Dahlbäck B, Carlsson M, Svensson PJ. Familial thrombophilia due to a previously unrecognized mechanism characterized by poor anticoagulant response to activated protein C. *PNAS*; 1993; **90**: 1004-8.
19. Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, De Ronde H, et al. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64-7.
20. Strandberg K, Stenflo J, Nilsson C, Svensson PJ. APC-PCI concentration is higher in patients with previous venous thromboembolism with Factor V Leiden, *J Thromb Haemost* 2005; **3**:2578-601.
21. Takano S, Kimura S, Ohdama S, Aoki N. Plasma thrombomodulin in health and diseases. *Blood* 1990; **76**: 2024-9.
22. Ishii H, Uchiyama H, Kazama M. Soluble thrombomodulin antigen in conditioned medium is increased by damage of endothelial cells. *Thromb Haemost* 1991; **65**:618-23.
23. Boehme MW, Galle P, Stremmel W. Kinetics of thrombomodulin release and endothelial cell injury by neutrophil-derived proteases and oxygen radicals. *Immunology* 2002;**107**:340-9.
24. Salomaa V, Matei C, Aleksic N, Sansores-Garcia L, Folsom AR, Juneja H, et al. Soluble thrombomodulin as a predictor of incident coronary heart disease and symptomless carotid artery atherosclerosis in the Atherosclerosis Risk in Communities (ARIC) Study: a case-cohort study. *Lancet* 1999; **353**: 1729-34.

25. Konstantoulas CJ, Öhlin A-K, Humphries SE, Goodall AH, Toh C-H, Mather H, Ireland H. Measurements of Soluble Thrombomodulin Activity and Antigen: Factors Contributing to Variance and Potential for Identification of Protein Variants. *Thromb Haemost* 2007; **97**: 161-4.
26. Öhlin AK, Larsson K, Hansson M. Soluble Thrombomodulin activity and soluble Thrombomodulin antigen in plasma. *J Thromb Haemost* 2005; **3**: 976-82.
27. Öhlin AK, Morser J, Ohlin H. Soluble Thrombomodulin antigen in plasma is increased in patients with acute myocardial infarction treated with thrombolytic therapy. *Thromb Res* 1996; **82**:313-22.
28. Strandberg K, Svensson A, Stenflo J. Stabilyte tubes that contain strongly acidic citrate prevents in vitro complex formation between activated protein C and protein C inhibitor. *Thromb Haemost* 2003; **89**: 947-49.
29. Faioni EM, Franchi F, Asti D, Mannucci PM. Activation of the protein C pathway in hereditary thrombophilia. *Thromb Haemost* 1998; **80**:557-60.
30. Espana F, Vaya A, Mira Y, Medina P, Estelles A, Villa P, et al. Low level of circulating activated protein C is a risk factor for venous thromboembolism. *Thromb Haemost* 2001; **86**:1368-73.
31. Hron G, Kollars M, Binder BR, Eichinger S, Kyrle PA. Identification of patients at low risk for recurrent venous thromboembolism by measuring thrombin generation. *JAMA* 2006; 296:397-402.
32. Nowak-Gottl U, Vielhaber H. Elevated levels of soluble thrombomodulin in plasma from children with Arg 506 to Gln mutation in the factor V gene. *Eur J Haematol* 1997;**58**:51-5.
33. Aleksic N, Folsom AR, Cushman M, Heckbert SR, Tsai MY, Wu KK. Prospective study of the A455V polymorphism in the thrombomodulin gene, plasma

thrombomodulin, and incidence of venous thromboembolism: the LITE Study. *J*

*Thromb Haemost* 2003; **1**: 88-94.

34. Shrivastava S, Ridker PM, Glynn RJ, Goldhaber SZ, Moll S, Bounameaux H, et al. D-dimer, factor VIII coagulant activity, low-intensity warfarin and the risk of recurrent venous thromboembolism. *J Thromb Haemost* 2006; **4**: 1208-14.

**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 <sup>€</sup>
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<sup>th</sup> and 90<sup>th</sup> 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 <sup>€</sup>	With recurrence (n=6)	Without recurrence (n=44)	p- value <sup>€</sup>	With recurrence (n=13)	Without recurrence (n=119)	p-value <sup>€</sup>
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<sup>th</sup> and 90<sup>th</sup> percentiles).

<sup>€</sup> 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 <sup>th</sup> 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 <sup>th</sup> 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 <sup>th</sup> 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.

