

This is an author produced version of a paper published in Journal of thrombosis and haemostasis : JTH. This paper has been peer-reviewed but does not include the final publisher proof-corrections or journal pagination.

Citation for the published paper:

Strandberg, K and Stenflo, J and Nilsson, C and Svensson, P J
"APC-PCI complex concentration is higher in patients with previous
venous thromboembolism with Factor V Leiden."
J Thromb Haemost. 2005 Nov;3(11):2578-80.
<http://dx.doi.org/10.1111/j.1538-7836.2005.01617.x>

Access to the published version may require journal subscription.
Published with permission from: Blackwell Synergy

APC-PCI complex concentration is higher in patients with previous venous thromboembolism with Factor V Leiden

Strandberg, K¹, Stenflo, J¹, Nilsson, C², Svensson, PJ²

¹ Dept. of Clinical Chemistry, Lund University, Malmö University Hospital, Sweden

² Dept. of Coagulation Disorders, Lund University, Malmö University Hospital, Sweden

Corresponding author: Strandberg, K, Dept. of Clinical Chemistry, Lund University, Malmö University Hospital, S-205 02 Malmö, Sweden

e-mail: Karin.Strandberg@klkemi.mas.lu.se

Phone: +46-40-331459

Fax: +46-40-336255

Resistance to activated protein C (APC) is a major risk factor for venous thrombosis that is caused by a point-mutation in the gene of blood coagulation factor V [1, 2]. This mutation (FV Leiden) renders the FV molecule less susceptible to inactivation by APC, and is seen in 20-40% of patients with VTE [3]. It is thus the most common hereditary risk factor for VTE among Caucasians. An increased resistance to APC determined with a thrombin generation-based test in patients with the FV Leiden mutation has shown a good correlation between APC sensitivity ratios (APCsr) and the risk of venous thrombosis in carriers of FV Leiden [4, 5]. D-dimer measurements have also been used for predicting recurrent VTE and measured after withdrawal of oral anticoagulant treatment D-dimer had some predictive value with regard to recurrence, but the overall discriminative power was quite poor (AUC 0.59 ± 0.03) [6-10]. The risks of DVT with the joint presence of high D-dimer and FV Leiden was increased 12.4 –fold and were thus supra-additive in a prospective case-control study [11]. 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 hereditary risk factors for thrombophilia.

A new sensitive sandwich immunofluorimetric assay for measurements of the complex between APC and the protein C inhibitor (APC-PCI complex) has been devised [12,13]. This 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. Un-complexed native PCI, which exists in a 10^4 -fold molar excess over the APC-PCI complex has no affinity for the antibody. As a so-called reporter antibody a monoclonal antibody against protein C is used. Due to the activation of protein C by thrombin bound to thrombomodulin, 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 both affecting the venous and arterial systems such

as deep vein thrombosis (DVT), pulmonary embolism, disseminated intravascular coagulation and myocardial infarction (MI) [14-16]. Measured with our method in patients with VTE and MI, ROC-analysis demonstrated an equal or better discriminative capacity than other markers of hypercoagulability such as F1+2, TAT, SFM and D-dimer [17-18]. The aim of the present study is to find out if there is a difference in APC-PCI concentration indicating different degrees of thrombin generation between carriers and non-carriers of the FV Leiden mutation in patients with a previous history of VTE.

Venous blood was drawn after information and written consent. It was collected in 5 mL vacuum tubes (Stabilyte, Biopool, Umeå, Sweden) containing citrate with a low pH, which precludes *in vitro* formation of the APC-PCI complex [19]. The 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 was 0.07 to 0.26 µg/L with a mean and median of 0.13 µg/L [19]. The within-run coefficient of variation was 4.8% at 0.15 µg/L and 3.2 % at 0.40 µg/L [19]. The between-run coefficient of variation was 7.1% at 0.15 µg/L and 5.8% at 0.41 µg/L (n=38). Warfarin treatment reduces the APC-PCI concentration [13]. APC-PCI measurements were made on one occasion on samples stored at -70° C. The samples were frozen and then thawed once more for measurement of D-dimer with Auto D-dimer (Biopool AB, Umeå, Sweden). Statistical analyses were performed with SPSS version 11.5 for Windows (SPSS Inc. Chicago, IL, USA). Non-normally distributed data were expressed as median and percentiles (10th and 90th). The Mann-Whitney U test was used for bivariate analysis of continuous variables, the Chi square test for nominal variables and Spearman rank correlation test for correlations. P-values < 0.05 were considered significant.

We compared the APC-PCI complex concentration, in 182 consecutive patients who were in the recovery phase after cessation of anticoagulant treatment for VTE, with D-

dimer. At the time for blood sampling, all patients had normal protein C values. Only five patients included in the study had had more than one thrombotic event, which gives a low recurrence frequency of 2.75%. The reason for this is that patients with more than one VTE-episode, are usually treated with longterm anticoagulation and were not included in the study. Exclusion of these five patients did not change the obtained results. Blood samples were collected two to four weeks after cessation of treatment. The purpose of this study was to collect samples from 50 patients with heterozygosity for FV Leiden as only risk factor for thrombosis. All other patients without FV Leiden or any other thrombophilia (G20210A prothrombin mutation, antithrombin-, protein C- or protein S- deficiency, lupus anticoagulant) constituted the control group (n=132; **Table 1**).

The APC-PCI complex concentration was higher in FV Leiden patients with a previous VTE episode than in patients without hereditary thrombophilia ($p < 0.001$, Mann-Whitney U test; **Table 1, Fig. 1**). There was no difference in D-dimer concentrations between the groups ($p = 0.13$). For APC-PCI concentrations above the 75th percentile ($> 0.29 \mu\text{g/L}$), the OR for FV Leiden was 3.83 (95% CI 1.88-7.79). In the group of patients without hereditary thrombophilia, there were several with concentrations of both markers above the 95th percentile, 12 patients with APC-PCI complex $> 0.46 \mu\text{g/L}$ and 16 patients with D-dimer $> 0.44 \text{ mg/L}$. Seven of these patients had high concentrations of both markers. To assess whether these very high levels influenced the findings, we excluded patients with concentrations above the 95th percentile. After exclusion of these patients, there was a difference also in D-dimer concentrations between the groups ($p = 0.002$), and for concentrations above the 75th percentile the OR for FV Leiden was 5.90 (95% CI 2.63-13.1) for APC-PCI and 1.64 (95% CI 0.77-3.5) for D-dimer ($> 0.18 \text{ mg/L}$).

In FV Leiden patients a significant positive correlation was found between the concentrations of APC-PCI and D-dimer ($r = 0.53$, $p < 0.001$; Spearman's rank test, $r^2 = 0.28$).

We also observed positive correlations between the concentrations of APC-PCI complex and D-dimer ($r=0.54$, $p<0.001$, $r^2=0.29$) in the whole study group. Positive correlations were also observed between age and D-dimer ($r=0.42$, $p<0.001$, $r^2=0.18$) and between APC-PCI and age ($r=0.30$, $p<0.001$, $r^2=0.09$).

The capacity of the methods to discriminate VTE-patients with FV-Leiden from VTE-patients without, was compared in a ROC-analysis (receiver operating characteristics; not shown). The AUC (area under curve) for the APC-PCI complex concentration was higher than for the D-dimer method (0.71, SD=0.039 vs. 0.57, SD=0.042), but the confidence intervals for the methods were overlapping and thus not significantly different. After exclusion of extreme and outlier values among patients without thrombophilia, the AUCs increased to 0.79, SD=0.037 for APC-PCI and to 0.67, SD=0.043 for D-dimer, still with overlapping confidence intervals and no significant difference. The capacity of the APC-PCI method to discriminate VTE-patients ($n=182$) from healthy individuals of the reference population ($n=80$)[19] was also compared and gave an AUC of 0.87, SD=0.02, significantly larger than for APC-PCI determination among VTE patients.

This study shows a higher APC-PCI concentration in FV Leiden patients with a previous VTE episode than in those with a previous VTE but without hereditary thrombophilia, which indicates an increased thrombin generation. ROC-analysis resulted in a higher AUC for the APC-PCI method than for D-dimer and thus better discrimination between the two groups although not significantly different. Some patients without known hereditary thrombophilia had extreme values for D-dimer and for the APC-PCI complex and we assume that among these patients those with an increased risk for future VTE can be found. There was an almost 4-fold increased presence of FV Leiden in patients with APC-PCI concentration $> 0.29 \mu\text{g/L}$ (75th percentile), which further supports the hypothesis that high APC-PCI concentrations is a marker of increased thrombin generation. There were also higher

APC-PCI concentrations in patients with a previous history of VTE without hereditary risk factors for thrombosis than in healthy individuals. The reason for this could be the presence of unknown risk factors in these patients, of which the APC-PCI complex concentration may be a marker. The risks of VTE with the joint presence of high D-dimer and FV Leiden was shown to be increased 12.4 –fold and were thus supra-additive in a prospective case-control study [11]. Our study suggests that the APC-PCI complex concentration, which in contrast to D-dimer is a well defined analyte, could be a marker of an increased risk for future VTE. However, clinical intervention studies need to be performed to study if withdrawal of oral anticoagulant treatment can be based on APC-PCI complex determinations.

References

- 1 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.
- 2 Bertina RM, Koeleman BPC, Koster T, Rosendaal FR, Dirven RJ, De Ronde H, Van der Velden PA, Reitsma PH. Mutation in blood coagulation factor V associated with resistance to activated protein C. *Nature* 1994; **369**: 64-7.
- 3 Dahlbäck B. Blood coagulation and its regulation by anticoagulant pathways: genetic pathogenesis of bleeding and thrombotic diseases. *J Intern Med* 2005; **257**: 209-23.
- 4 Nicolaes GAF, Thomassen MCLGD, Tans G, Rosing J, Hemker HC. Effect of activated protein C on thrombin generation and on the thrombin potential in plasma of normal and APC-resistant individuals. *Blood Coagul Fibrinol* 1997; **8**:28-38.
- 5 Rosing J, Tans G, Nicolaes GAF, Thomassen MCLGD, van Oerle R, van der Ploeg PMEN, Heijnen P, Hamulyak K, Hemker HC. Oral contraceptives and venous thrombosis: different sensitivities to activated protein C in women using second – and third- generation oral contraceptives. *Br J Haematol* 1997; **97**: 233-8.
- 6 Palareti G, Legnani C, Cosmi B, Valdré L, Lunghi B, Bernardi F, Coccheri S. Predictive value of D-dimer test for recurrent venous thromboembolism after anticoagulant withdrawal in subjects with a previous idiopathic event and in carriers of congenital thrombophilia. *Circulation* 2003; **108**: 313-8.
- 7 Eichinger S, Minar E, Bialonczyk C, Hirschl M, Quehenberger P, Schneider B, Weltermann A, Wagner O, Kyrle PAI. D-dimer levels and risk of recurrent venous thromboembolism *JAMA* 2003; **290**: 1071-4.

- 8 Le Gal G, Bounameaux H. D-dimer testing to predict recurrence risk in venous thromboembolism: looking for a useful treshhold: a rebuttal. *J Thromb Haemost* 2004; **2**: 1670-2.
- 9 Eichinger S, Kyrle PA. D-dimer testing for predictive recurrence risk in venous thromboembolism: looking for a useful treshhold:reply to a rebuttal. *J Thromb Haemost* 2004; **2**: 1672-3.
- 10 Cosmi B, Legnani C, Palareti G. D-dimer testing for predictive recurrence risk in venous thromboembolism: looking for a useful treshhold:reply to a rebuttal. *J Thromb Haemost* 2004; **2**: 1673-5.
- 11 Andreescu ACM, Cushman M, Rosendaal FR. D-dimer as a risk factor for deep vein thrombosis: The Leiden Thrombophilia Study. *Thromb Haemost* 2002; **87**: 47-51.
- 12 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**:15713-15720.
- 13 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.
- 14 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.
- 15 Espana F, Vicente V, Taberner 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.

- 16 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**(2):192-4.
- 17 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.
- 18 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.
- 19 Strandberg K, Svensson A, Stenflo J. Stabilyte tubes that contain strongly acidic citrate prevent in vitro complex formation between activated protein C and protein C inhibitor. *Thromb Haemost* 2003;**89**:947-49.

Table 1. Patient demographics and results

	n (n=182)	Pat. with FV Leiden (n=50)	Pat. without FV Leiden (n= 132)	p-values °
Distal thrombosis	55 (30%)	13 (26%)	42 (32%)	p=0.47
Proximal thrombosis	58 (32%)	13 (26%)	45 (34%)	p=0.38
Pulmonary embolism	50 (27%)	18 (36%)	32 (24%)	p=0.21
Warfarin treatment time (median)	6 months (range 2-24)	6 (range 2-12)	6 (range 2-24)	p=0.89
Age (median)	66 years (range 22-91)	66 (range 24-90)	66 (range 22-91)	p=0.57
Males	89 (49%)	25 (50%)	64 (48%)	p=0.95
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

* median and in parenthesis: 10th and 90th percentiles.

° Chi square test was used for nominal variables, Mann Whitney U test was used for continuous variables.

Legend to figure

Fig. 1 Box-plots representing APC-PCI concentrations (dark boxes) in normals (n=80) [19] and in patients with a previous VTE episode, with the FV Leiden mutation or without any hereditary risk factor for VTE. Box-plots representing D-dimer concentrations (un-filled boxes) in patients with a previous VTE episode, with the FV Leiden mutation or without any hereditary risk factor for VTE. Patients with APC-PCI (n=12) or D-dimer (n=16) concentrations above the 95th percentile were excluded from the figure. The concentration of D-dimer in healthy individuals was < 0.2 mg/L (n= 39).

