Venous thromboembolism during pregnancy is not associated with persistent elevated activated protein C (APC) sensitivity ratio based on the endogenous thrombin potential

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Summary

Women who are using oral contraceptives can acquire APC resistance, measured by the effect of APC on the endogenous thrombin potential (ETP). The objective of our study was to examine whether persistent APC resistance determined with an ETP-based normalized APC sensitivity ratio (nAPCsr) is a risk marker for venous thromboembolism in women with pregnancy-associated thromboembolism. We determined the activities of antithrombin, protein C, protein S, and performed a genetic analysis of factor V Leiden G1691A, prothrombin mutation G20210A, and methylenetetrahydrofolate reductase mutation (MTHFR C677T) in 65 women with venous thromboembolism during pregnancy or the puerperium and in 114 normal women. A significantly (p<0.05) higher nAPCsr was present in normal women using hormones, in younger women (≤ 45 yrs), and in women with carrier status of factor V Leiden. In normal women without factor V Leiden a significant (p< 0.05) negative correlation of nAPCsr with age (r=−0.39), antithrombin activity (r=−0.38), protein S activity (r=−0.26), and a significant positive correlation with hormone intake (r=0.36) was present. nAPCsr is influenced by several coagulation parameters, which are modified by the use of oral contraceptives. Consequently, a multivariate analysis of our data did not show a significant association of nAPCsr to venous thromboembolism, neither as a continuous variable (odds ratio 0.8, 95% CI 0.6–1.1, p=0.10) nor using a cut-off value (nAPCsr cut-off 3.1: odds ratio 1.2, 95% CI 0.3–5.3, p=0.77). Our study demonstrates that nAPCsr is not a risk marker for pregnancy-associated venous thromboembolism.

Keywords

Endogenous thrombin potential (ETP), APC sensitivity ratio (nAPCsr), venous thromboembolism, pregnancy

Introduction

Activated protein C resistance (APCR) has been used to describe the phenomenon by which plasma fails to be anticoagulated by the addition of APC in vitro, even in the presence of normal protein S function (1). In most cases, APC resistance results from the substitution of adenin for guanin at nucleotide 1691 of the factor V gene (G1691A), which causes the arginine in residue 505 of the factor V protein to be replaced by glutamine (Arg506Gln). The resulting protein is so called factor V Leiden (2). Activated protein C resistance was found in 46% to 78% of women investigated for venous thrombosis in pregnancy (3–5).

In previous studies it could be demonstrated that a specific method for the screening of the sensitivity to activated protein C (APC) using the anticoagulant effect of APC on the endogenous thrombin potential (ETP) (the time integral of thrombin generated in clotting plasma) is considerably affected by oral contraceptives (6). Women who use oral contraceptives acquire a lower sensitivity to APC and this phenomenon is more pronounced in women using third-generation oral contraceptives than in women using second-generation oral contraceptives (7–9).

Whether acquired APC resistance induced by oral contraceptives marks an increased risk of venous thromboembolism remains to be established.

The objective of our study was to examine whether reduced sensitivity to APC determined with an ETP-based normalized APC sensitivity ratio (nAPCsr) is a risk determinant for venous thromboembolism in women with pregnancy-associated thromboembolism.
**Materials and methods**

**Women with thromboembolism and normal women**

We studied 65 consecutive women with a history of venous thromboembolism during pregnancy and the puerperium (6 weeks postpartum) and 114 normal women. The women with a history of thromboembolism were referred for treatment of venous thromboembolism or because of consultation for prophylaxis from local hospitals to the Düsseldorf University Medical Center between January 1990 and December 1998 and were seen again during 1999 for laboratory investigation of thrombophilia. None of the women had tested positive for lupus anticoagulant or had overt evidence of autoimmune or neoplastic disease. In all participants of this study, blood samples for the determination of coagulation parameters were collected in our institution at least 3 months post partum or 3 months after the cessation of lactation to exclude any pregnancy-related alterations of coagulation and fibrinolysis. In addition, in women with a history of thromboembolism, the blood samples were obtained at least 6 months after the thromboembolic event to exclude any thrombosis- and/or inflammation-related effects on laboratory test results. None of the patients received antithrombotic treatment at the time of blood sampling.

All the women with a history of thromboembolism have had an objectively diagnosed episode of deep venous thrombosis or pulmonary embolism. Deep venous thrombosis was diagnosed by Doppler ultrasonography, impedance plethysmography, computed tomography, or nuclear magnetic resonance tomography during pregnancy and by Doppler ultrasonography or venography after delivery. Pulmonary embolism during pregnancy was diagnosed by computed tomography or nuclear magnetic resonance tomography and in the postpartum period by computed tomography, ventilation-perfusion scanning, or pulmonary angiography.

The 114 normal women with at least 1 previous pregnancy were recruited by the Heinrich Heine University Blood Donation Center. They were from the same geographic region as the women with a history of thromboembolism, but were unrelated to them. There were no statistical differences in the prevalence of current or previous oral contraceptive use (88 percent vs. 91 percent, p=0.46), age (40.4 years vs. 37.1 years, p=0.11), smoking history (45 percent vs. 46 percent, p=0.91), and body-mass-index (24.8 vs. 23.9, p=0.19) between patients and controls.

Personal histories to document the presence or absence of thromboembolic disease were obtained from all women with the use of a standardized questionnaire. The study was performed according to the Helsinki Declaration, and informed consent was given by all the women.

**Laboratory tests**

Samples of whole blood were collected in vacuum tubes containing 3.8% (wt/vol) sodium citrate in a ratio 1:9 (vol/vol) anticoagulant to blood. Platelet-poor plasma was prepared by centrifugation at 2000 x g for 10 minutes and stored at −80°C until analysis. The activities of plasma protein C and protein S were measured by a functional clotting assay (Instrumentation Laboratory, Milan, Italy). Conventional APC resistance was determined using COATEST APC Resistance (Chromogenix, Mölndal, Sweden). Antithrombin activity was measured with the use of Berichrom (Dade Behring, Liederbach, Germany). Lupus anticoagulant was determined with the DVV test and DVV confirm test (American Diagnostica, Greenwich, CT, USA).

Normalized APC sensitivity ratios were determined in a modification of a previously described method (4). Plasma was first defibrinogened by treatment with 1 U/ml Ancrod (Sigma) for 10 minutes at 37°C; the liquid was harvested after pressing the clot with a plastic spatula. Thrombin generation was induced by mixing 4 parts of the defibrinogened plasma with 3 parts of 15 mM phospholipid vesicles (dioleoyl phosphatidylserine/dioleoyl phosphatidylethanolamine/dioleoyl phosphatidylcholine, 20/20/60, M/M/M; Avanti Polar Lipids, USA), 0.1 ng/ml relipidated tissue factor (Innovin, Dade, The Netherlands) and 17 mM added CaCl₂. The thrombin generation was induced either with or without the presence of 5 nM APC (Chromogenix AB, Sweden). After 30 minutes incubation, the reaction was stopped by adding EDTA to a final concentration of 40 mM. The amidolytic activity was determined (thrombin plus alpha-2-macro-globulin – thrombin complex) with p-Phe-pipetyl-Arg-pNa (S2238; Chromogenix AB, Sweden). Background activity of non-activated plasma with added APC was recorded by replacing the lipid/tissue factor by buffer addition. For normalization, the tests were performed in a study-specific pooled sample (random, n=30), prepared from samples of the study population excluding occurrence of factor V Leiden mutation and cases of VTE. The APC ratio was determined as the ratio of activity determined in the presence and absence of APC, after subtraction of the background activity. The normalized ratio (APCsr) was determined by dividing the APCsr of an individual by the APCsr of the pooled sample.

**Genetic analysis**

DNA was extracted from peripheral blood leukocytes according to standard protocols using the Chelex system (BIO-RAD, München, Germany). The factor V G1691A mutation (factor V Leiden) was identified by allele-specific restriction enzyme analysis (10). The G20210A mutation of the prothrombin gene was identified by allele-specific restriction enzyme analysis (11). Screening for the MTHFR C677T substitution was performed as described by Froost et al. (12).

**Statistical analysis**

The SAS statistical package (version 6.12, SAS Institute, Cary, NC, USA) was used for all statistical analyses. Depending on the type of data, the Wilcoxon rank-sum test or Fisher's exact test (2-tailed) was used to compare the different groups. Multivariate analyses were performed using a logistic regression procedure. Complete data were not available for all women with thromboembolism.

**Results**

Means and percentiles of the ETP-based normalized sensitivity ratio (nAPCsr) in normal women are given in Table 1. A significantly higher nAPCsr could be demonstrated in women using hormones, in younger women, and in women who are carriers of factor V Leiden (Table 2).
In normal women without factor V Leiden a significantly negative correlation of nAPCsr with age ($r=–0.39$), antithrombin activity ($r=–0.38$), protein S activity ($r=–0.26$), and a significantly positive correlation with hormone intake ($r=0.36$) could be demonstrated. In a subgroup analysis of normal women without factor V Leiden and without hormone use, the same variables were identified as significantly correlated with nAPCsr (age: $r=–0.29$, antithrombin activity: $r=–0.30$, protein S activity: $r=–0.33$).

To exclude any influence due to the factor V Leiden status and external hormone use, the following comparisons between patients and healthy women were performed in individuals without factor V Leiden who are not using hormones. ETP-based nAPCsr were compared between patients with a history of venous thromboembolism and normal women using the continuous values for nAPCsr (Table 3) and in addition using the 90% percentile and the 95% percentile as cut-off for the upper limit of normal (Table 4). Neither the analysis with continuous nAPCsr nor the analysis using cut-off for nAPCsr showed any significant difference between patients and normal women. However, although insignificant, the odds ratio for an increased nAPCsr in women with a history of thromboembolism was approximately 1.6 using the 90% percentile cut-off and approximately 3 using the 95% percentile.

The conventional aPTT based APCR assay did not show a significant difference between patients and controls without factor V Leiden mutation ($p=0.90$). Of the 65 patients 1 had a deficiency of antithrombin (<85% activity), 9 had a deficiency of protein C (<75% activity), and 4 had a deficiency of protein S (<55% activity). Using logistic regression analysis adjusted for age and hormone intake, including the variables nAPCsr, protein C and S activity, antithrombin activity, factor V Leiden, pro-thrombin mutation G20210A, and MTHFR C677T in the same model, only factor V Leiden (odds ratio 6.5, 95% CI 1.5–28, $p=0.01$), and deficiencies of protein C (<75% activity) (odds ratio 11.7, 95% CI 1.3–107, $p=0.03$) and protein S (<55% activ-

### Table 1: Means and percentiles of nAPCsr in healthy women.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>5%</th>
<th>10%</th>
<th>50%</th>
<th>90%</th>
<th>95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>FV Leiden neg.</td>
<td>106</td>
<td>1.6</td>
<td>1.42</td>
<td>0.3</td>
<td>0.38</td>
<td>1.23</td>
<td>3.32</td>
<td>3.77</td>
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<td>FV Leiden pos.</td>
<td>8</td>
<td>6.06</td>
<td>4.48</td>
<td>1.37</td>
<td>1.37</td>
<td>5.14</td>
<td>14.88</td>
<td>14.88</td>
</tr>
<tr>
<td>FV Leiden neg &amp; no hormone use</td>
<td>59</td>
<td>1.14</td>
<td>1.31</td>
<td>0.27</td>
<td>0.35</td>
<td>0.8</td>
<td>1.85</td>
<td>3.17</td>
</tr>
<tr>
<td>FV Leiden neg &amp; hormone use</td>
<td>47</td>
<td>2.18</td>
<td>1.35</td>
<td>0.56</td>
<td>0.67</td>
<td>1.85</td>
<td>3.74</td>
<td>4.57</td>
</tr>
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</table>

### Table 2: nAPCsr in healthy women according to age, factor V Leiden, and hormone status.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean</th>
<th>SD</th>
<th>5%</th>
<th>10%</th>
<th>50%</th>
<th>90%</th>
<th>95%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≤ 45 yr</td>
<td>46</td>
<td>1.2</td>
<td>1.45</td>
<td>0.3</td>
<td>0.35</td>
<td>0.82</td>
<td>1.85</td>
<td>3.17</td>
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<tr>
<td>Hormone use</td>
<td>40</td>
<td>2.61</td>
<td>1.32</td>
<td>0.72</td>
<td>0.98</td>
<td>2.16</td>
<td>3.76</td>
<td>5.09</td>
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<tr>
<td>Age &gt; 45 yr</td>
<td>13</td>
<td>0.92</td>
<td>0.61</td>
<td>0.18</td>
<td>0.4</td>
<td>0.77</td>
<td>1.7</td>
<td>2.21</td>
</tr>
<tr>
<td>Hormone use</td>
<td>7</td>
<td>0.84</td>
<td>0.39</td>
<td>0.29</td>
<td>0.29</td>
<td>0.67</td>
<td>1.34</td>
<td>1.34</td>
</tr>
</tbody>
</table>

### Table 3: Comparison between patients with a history of venous thromboembolism in pregnancy or puerperium and healthy women.

<table>
<thead>
<tr>
<th></th>
<th>Patients*</th>
<th>Controls*</th>
<th>Wilcoxon-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≤ 45 yr</td>
<td>1.14</td>
<td>1.21</td>
<td>0.35</td>
</tr>
<tr>
<td>Hormone use</td>
<td>7.04</td>
<td>0.84</td>
<td>0.39</td>
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<tr>
<td>Wilcoxon-Test</td>
<td>0.0001</td>
<td>0.0003</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>0.2005</td>
<td>0.466</td>
<td>0.3243</td>
</tr>
</tbody>
</table>

* All individuals were factor V Leiden negative and did not use hormones.
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ity) (odds ratio 6.9, 95% CI 0.9–55, p=0.07) were identified as independent risk factors predicting thrombosis in women with thromboembolism during pregnancy and the puerperium. Results in the multivariate model showed an odds ratio for nAPCsr as continuous variable of 0.8 (95% CI 0.6–1.1, p=0.10) and of 1.2 (95% CI 0.3–5.3, p=0.77) for nAPCsr using a cut-off of 3.1.

Discussion

Resistance to activated protein C (APC) is often associated with a mutation in factor V (factor V Leiden). Individuals without factor V Leiden who exhibit a response in functional APC-resistance tests similar to that of carriers of factor V Leiden are considered to be acquired APC resistant. In particular, this phenomenon was observed in women who are using oral contraceptives (6, 9). In contrast to conventional aPTT-based APC-resistance tests, a more recent assay based on the endogenous thrombin potential (nAPCsr) was shown to yield significant differences in sensitivity to APC between non-oral contraceptive users and oral contraceptive users and between users of second and third generation oral contraceptives (7–9). It has been hypothesized that the actual value of the nAPCsr of an individual, especially oral contraceptive users, is subject to differential modulation by other plasma proteins in the ETP- and aPTT-based APC-resistance tests and that oral contraceptives change the level of plasma proteins that modulate the effect of APC on thrombin formation initiated via the extrinsic coagulation pathway (8). Notably, the acquired APC-resistance is expected to be test-dependent, as also evident from the observation that the aPTT and ETP-based variants do not correlate in normal individuals (8, 13). APC-resistance assessed by the aPTT test is associated with an increased risk for VTE, both in subjects who are carriers of factor V Leiden (genetic APC resistance) and in subjects without factor V Leiden mutation (acquired APC resistance) (14). Thus, the association of the aPTT-based test and venous thromboembolism has been reported, however, the clinical significance of acquired APC resistance evaluated by ETP nAPCsr remains to be determined.

In this retrospective study, we found no significant association between a persistent increased ETP-based nAPCsr and pregnancy-associated venous thromboembolism. There are, however, some observations that need further evaluation. Neither the analysis with continuous nAPCsr nor the analysis using cut-off values for nAPCsr showed any significant difference between patients and normal women. However, although not significant, the odds ratio for an increased nAPCsr above the 95 percentile in women with a history of thromboembolism was approximately 3. Interestingly, such a phenomenon was also observed in males (15). The most likely explanation is an association of nAPCsr with known risk determinants of thrombosis. Such an association might pretend an influence of nAPCsr on thromboembolism, which in fact is mainly determined by other risk factors. In normal women a significant negative correlation of nAPCsr with age, antithrombin activity, protein S activity, and a significant positive correlation with hormone intake was present. In conclusion, nAPCsr is influenced by several coagulation parameters, which are modified by the use of oral contraceptives. Further observations on covariation of coagulation factors and APC resistance indicate involvement of protein S (16). Consequently, our multivariate analysis did not show an independent significant association of nAPCsr to venous thromboembolism, neither as a continuous variable nor using a nAPCsr cut-off. Since the ETP-based APC resistance test is a functional assay and affected by plasma levels of other coagulation proteins, some loss in the association of APCsr to venous thromboembolism using a multivariate analysis can be expected. However, the complete loss of the odd ratio in the multivariate analysis indicates that nAPCsr is in fact not an independent predictor of venous thrombosis. This does not exclude the possibility that the nAPCsr may be used as a surrogate marker of an increased thrombotic risk induced by several other known risk factors, either single or combined (17). However, the use of the nAPCsr as surrogate marker remains to be determined.

A limitation that our study has in common with most other studies is that its retrospective design does not allow to give information about possible temporary pregnancy-associated changes in the ETP-based nAPCsr prior to the thrombotic event. Prospective studies, which would allow such, are extremely difficult to conduct due to the rarity of venous thrombosis during...
pregnancy. Our data do not exclude the possibility that hormonal changes and/or decreased protein S levels result in increased ETP-based nAPCsr ratios during pregnancy. Our results are in line, however, with those of other retrospective studies, which found no association of ETP-based nAPCsr and venous thromboembolism in non-pregnant patients (16, 18). In addition, Høibraaten et al. (19) found that in women using hormone replacement therapy changes in ETP-based APCsr were not predictive for or associated with later occurring venous thrombosis.

The clinical relevance of acquired APC resistance determined by nAPCsr remains to be determined. Our study demonstrates that nAPCsr is not a risk marker for pregnancy-associated venous thromboembolism.

References