CHAPTER 11

A NEW SCREENING TEST FOR THE PROTEIN C ANTICOAGULANT PATHWAY: THE PCP TEST

Johan R. Meinardi, Wim van der Schaaf, Ferrand H. Bom, and Jan van der Meer

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SUMMARY

**Background:** The factor V (FV) Leiden and prothrombin mutation, and deficiencies of protein C (PC) and S (PS) compromise most inherited forms of thrombophilia. Detection of these disorders may be facilitated by a new Protein C anticoagulant Pathway screening test (PCP™ test).

**Methods:** In the PCP™ test, the ratio of two phospholipid rich Russel viper venom (PRVV)-initiated clotting times, measured with and without a protein C (PC) activator, respectively, is calculated. The test was performed on a STA coagulation analyser and evaluated in healthy controls (n=42), carriers of the FV Leiden (20 heterozygotes, 11 homozygotes) or prothrombin mutation (10 heterozygotes, 2 homozygotes), patients with hereditary deficiencies of PC (n=20), PS (n=17) or antithrombin (AT) (n=4), patients with a combination of these defects (n=7), healthy women using oral contraceptives (n=37) and healthy women in the third trimester of pregnancy (n=21). PCP ratios are expressed as normalised PCP sensitivity ratios (n-PCP-SR). The sensitivity of the PCP™ test (percentage of n-PCP-SR values below the lower limit of the normal range) was assessed.

**Results:** Normal range (mean ± 2SD) of n-PCP-SR was 0.90-1.52. Sensitivity of the PCP test for detection of FV Leiden (n-PCP-SR 0.32-0.71), PC deficiency (0.37-0.99), PS deficiency (0.73-1.40), prothrombin mutation (0.77-1.03), or combined defects (0.40-0.58), was 100%, 95%, 65%, 50%, and 100%, respectively. Homozygous FV Leiden carriers and patients with combined defects of the PC pathway showed lower n-PCP-SR values than the other groups. AT did not influence the n-PCP-SR results. In women using oral contraceptives and in pregnant women, lowered n-PCP-SR values were found in 62% (n-PCP-SR 0.52-1.24) and 100% (0.38-0.88) of the women, respectively.

**Conclusion:** The PCP™ test showed a high sensitivity for FV Leiden and PC deficiency, but was less sensitive for PS deficiency and the prothrombin mutation. Homozygous FV Leiden carriers and patients with combined defects of the PC pathway, showed the lowest n-PCP-SR values, maybe reflecting their high thrombotic risk. The lowered n-PCP-SR values, frequently found during oral contraception and in pregnancy, indicate that the test results should be interpreted cautiously in these conditions.
The PCP test

11.1 INTRODUCTION

The protein C (PC) anticoagulant pathway is an important down regulating mechanism of the blood coagulation cascade. In this pathway, activated protein C (APC) degrades the factors Va and VIIIa by selective proteolytic cleavage. Protein S (PS) acts as non-enzymatic cofactor in these reactions. It has been demonstrated that mutations of genes that encode the proteins that are involved in the PC anticoagulant pathway play a major role in the predisposition to venous thrombosis. These defects include deficiencies of PC and PS, resistance to APC based on the FV Leiden mutation, and the recently discovered prothrombin (20210 G\rightarrow A) mutation. In Caucasian patients presenting with venous thrombosis, deficiencies of PC or PS are found in 6-15%, FV Leiden in 20-50%, and the prothrombin mutation in 6-18% [1, 2]. Overall, any inherited defect of the PC anticoagulant pathway can be demonstrated in 30-75% of these patients.

Disturbances of the PC anticoagulant pathway may also be acquired. Acquired APC resistance [3, 4] and PS deficiency [5] have been found in women who use oral contraceptives or during pregnancy. Furthermore, antiphospholipid antibodies may induce APC resistance and inhibition of PS [6].

Laboratory evaluation of the PC anticoagulant pathway includes measurement of PC and PS antigen levels and/or activity, activated partial thromboplastin time (aPTT) tests with and without added APC to detect APC resistance, and DNA-analysis to identify the FV Leiden and the prothrombin mutations. These tests are laborious and expensive, whereas many hospitals lack the facilities to perform them all. Therefore, there is a need for screening tests that facilitate the detection of defects in the PC anticoagulant pathway. As far, screening tests are based on activation of endogenous PC by the addition of a PC activator, and measurement of its effect on thrombin generation [7] or prolongation of aPTT [8, 9] or prothrombin time (PT) [10, 11].

Recently, a new Protein C Pathway (PCP) screening test (Kordia, Gradipore, North Ryde, Australia) has been developed, which is based on activation of coagulation by a phospholipid rich Russell viper venom (PRVV) reagent. In this test, the ratio of PRVV-initiated clotting times, measured with and without addition of a PC activator (derived from Agkistrodon Contortrix venom), respectively, is calculated. As this test only involves the common pathway, it might be more specific for defects in the anticoagulant PC pathway than those tests that are based on measurements of thrombin generation, aPTT or PT.

We evaluated the PCP test in patients with known inherited thrombophilia, in women using oral contraceptives and in pregnant women.
11.2 PATIENTS AND METHODS

11.2.1 Patients

We studied 91 patients, of whom 31 were carriers of the FV Leiden mutation (20 heterozygotes, 11 homozygotes), 12 were carriers of the prothrombin mutation (10 heterozygotes, 2 homozygotes), 41 had hereditary deficiencies of PC (n=20), PS (n=17) or antithrombin (AT) (n=4), and 7 patients with a combination of these defects. The latter group contained heterozygous FV Leiden carriers in whom also deficiencies of either PC (n=2) or PS (n=2), or the prothrombin mutation (n=3) were demonstrated. The patients neither had recently used oral contraceptives nor anticoagulant drugs. None of the women had been pregnant within the last 3 months prior to blood collection. Furthermore, the PCP test was performed in 37 women using oral contraceptives and in 21 pregnant women at 36 weeks' gestation. None of them had a personal or family history of venous thromboembolism. Normal values were determined in 42 healthy controls (26 men, 16 non-pregnant women; age 19 to 53 years), who did not use any medication and had a negative personal and family history of venous thromboembolism.

11.2.2 Laboratory assays

Citrated blood was collected by venapuncture on 1/10th volume of 0.109 M trisodium citrate. Platelet-free plasma was prepared by centrifugation at 4000 rpm (3200 x g) for 10 minutes followed by 5 minutes at 13000 rpm (12000 x g). Plasma samples were stored at -80 °C until testing.

The PCP test was performed on a STA coagulation analyser (Boehringer Mannheim, Germany). To measure the PRVV-clotting time without PC activator, 50 ml buffer of a 1:1 mix of saline and distilled water (i.e. 0.075 NaCl), was added to 50 ml of test plasma and incubated for exactly 4 minutes. After addition of 50 ml of pre-heated PRVV reagent, time till clotting was measured. The same procedure was carried out for measurement of the PRVV-clotting time with PC activator, replacing the buffer by 50 ml PC activator solution. The ratio of the PRVV-clotting time with and without PC activator, respectively, was calculated (PCP sensitivity ratio). The PCP sensitivity ratio (PCP-SR) was expressed as normalised PCP sensitivity ratio (n-PCP-SR) by dividing the PCP-SR of the test plasma by the PCP-SR of pooled normal plasma, measured in the same run. The intra-assay variation coefficient of the PCP test, assessed by 15 consecutive measurements of pooled normal plasma, was 2.9%.

PS and PC antigen levels were measured by ELISA (with reagents obtained from DAKO, Glostrup, Denmark), and PC activity (‘Berichrom’ Protein C, Behring, Marburg, Germany) and AT activity (Coatest™, Chromogenix AB, Mölndal, Sweden) were measured
The PCP test by a chromogenic substrate assay. Values of PS and PC antigen, and PC and AT activity were expressed as percentage of the levels measured in pooled normal plasma set at 100%. A hereditary deficiency was defined by a value below the lower limit of the normal range at two separate measurements in the patient and at least one family member. Normal ranges (mean ± 2SD) were 64-138% for PC activity, 63-138% for PC antigen, 67-128% for total PS antigen, and 74-113% for AT activity. Median (range) for PC activity and PC antigen in type I or II PC deficient patients were 46% (30-64%) and 50% (30-96%), respectively. Median (range) for total PS antigen and AT activity in PS (type I) and AT deficient patients, were 49% (34-65) and 47% (45-50%), respectively. FV Leiden and the prothrombin mutation were detected by polymerase chain reaction, as described previously [2, 12].

11.2.3 Statistical analysis

Differences between the groups were evaluated by one-way ANOVA. A Bonferroni correction was performed for multiple groups comparisons. A two-tailed p value of p<0.05 is considered significant. Analysis was performed using SAS software, version 6.12 (SAS-Institute Inc., Cary, North Carolina).

11.3 RESULTS

In the 42 healthy volunteers, the normal range (mean ± 2SD) of n-PCP-SR was 0.90 to 1.52. There was no difference between men (0.90-1.54) and women (0.89-1.47). The results in patients with hereditary thrombophilia, women using oral contraceptives, and pregnant women are outlined in Figure 11.1 and summarized in Table 11.1. The PCP test showed to be particularly sensitive for FV Leiden and PC deficiency. A lowered n-PCP-SR was found in all 31 (100%) heterozygous carriers or homozygous carriers of FV Leiden, and in 19 of 20 (95%) of PC deficient patients. The only PC deficient patient with a normal n-PCP-SR had lowered levels of PC antigen (59 and 62%), while PC activity levels (64 and 67%) and PS levels (77 and 65%) were within the normal range. Values of n-PCP-SR in heterozygous carriers of FV Leiden and PC deficient patients were comparable. A more pronounced lowering of n-PCP-SR was observed in homozygous carriers, compared with heterozygous carriers of FV Leiden (p<0.001). A strongly lowered n-PCP-SR was also found in heterozygous FV Leiden carriers with a concomitant PC deficiency (0.39, 0.40), PS deficiency (0.41, 0.43) or prothrombin mutation (0.47, 0.53, 0.58). The difference with
Figure 11.1 Distribution of n-PCP-SR in patients with established hereditary thrombophilia, women using oral contraceptives and pregnant women. The horizontal line depicts the lower limit of the normal range. Heterozygous and homozygous FV Leiden carriers are indicated by grey and black circles, respectively. Black squares denote homozygous carriers of the prothrombin mutation.
heterozygous FV Leiden carriers did not reach statistical significance, probably due to the small number of patients with combined defects. Moderate lowered n-PCP-SR values were found in PS deficient patients, of whom 11 (65%) showed an abnormal n-PCP-SR. Although the group of patients with the prothrombin mutation showed lowered n-PCP-SR values, the sensitivity of the test was only 50%. In the four AT deficient patients, the test revealed a borderline lowered n-PCP-SR in one patient (0.89), and normal values in the remaining three patients (0.94, 1.06 and 1.30). Of 37 women, who used oral contraceptives, 23 (62%) had a lowered n-PCP-SR, as was observed in all 21 pregnant women.

**Table 11.1 Normalized PCP ratios in the tested groups**

<table>
<thead>
<tr>
<th>subjects</th>
<th>n</th>
<th>median</th>
<th>range</th>
<th>sensitivity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>controls</td>
<td>42</td>
<td>1.20</td>
<td>0.81 - 1.64</td>
<td>-</td>
</tr>
<tr>
<td>factor V Leiden mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterozygous</td>
<td>20</td>
<td>0.51</td>
<td>0.40 - 0.71**</td>
<td>100%</td>
</tr>
<tr>
<td>homozygous</td>
<td>11</td>
<td>0.36</td>
<td>0.22 - 0.37***</td>
<td>100%</td>
</tr>
<tr>
<td>protein C deficiency</td>
<td>20</td>
<td>0.68</td>
<td>0.37 - 0.99**</td>
<td>65%</td>
</tr>
<tr>
<td>protein S deficiency</td>
<td>17</td>
<td>0.66</td>
<td>0.73 - 1.40**</td>
<td>65%</td>
</tr>
<tr>
<td>antithrombin deficiency</td>
<td>4</td>
<td>1.00</td>
<td>0.89 - 1.30**</td>
<td>25%</td>
</tr>
<tr>
<td>prothrombin mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>heterozygous</td>
<td>10</td>
<td>0.63</td>
<td>0.60 - 1.03**</td>
<td>40%</td>
</tr>
<tr>
<td>homozygous</td>
<td>2</td>
<td>(0.77 - 0.86)</td>
<td>(100%)</td>
<td></td>
</tr>
<tr>
<td>miscellaneous****</td>
<td>7</td>
<td>0.42</td>
<td>0.40 - 0.58**</td>
<td>100%</td>
</tr>
<tr>
<td>women with OC</td>
<td>37</td>
<td>0.86</td>
<td>0.62 - 1.24**</td>
<td>62%</td>
</tr>
<tr>
<td>pregnant women</td>
<td>21</td>
<td>0.76</td>
<td>0.36 - 0.86**</td>
<td>100%</td>
</tr>
</tbody>
</table>

OC = oral contraceptives; * percentage of n-PCP-SR values below 0.80; ** p < 0.0001 as compared to controls; *** p < 0.001 as compared to heterozygous factor V Leiden carriers; **** includes heterozygous factor V Leiden carriers with combined protein C deficiency (n=2), protein S deficiency (n=7) or the prothrombin mutation (n=5).

### 11.4 DISCUSSION AND CONCLUSIONS

Defects of the PC anticoagulant pathway are frequently found in patients experiencing venous thrombosis. Screening tests of the PC pathway that detect all these abnormalities might be worthwhile in clinical practice, provided that their sensitivity and specificity are high. We evaluated the recently developed, and commercially available PCP test. This is the first screening test of the common pathway. In the present study we assessed the sensitivity of the PCP test in patients with established hereditary thrombophilia. The sensitivity of the PCP test was 100% for detection of FV Leiden, 95% for PC deficiency and 65% for PS
deficiency. These results are similar to those reported for other screening tests [8-11]. The overall sensitivity of the PCP test for these abnormalities was approximately 90%. The low sensitivity for detection of PS deficiency may be due to the fact that PS has only a limited cofactor activity in the PC pathway, at least in vitro.

Homozygous carriers of FV Leiden showed a pronounced decrease of n-PCP ratios, that significantly differed from the n-PCP-SR values in heterozygous FV Leiden carriers. Furthermore, n-PCP ratios of heterozygous FV Leiden carriers with a concomitant other defect of the PC pathway, lied in between. This finding suggests that an excessively lowered n-PCP-SR may predict the high risk of thrombosis in these patients.

The results of the PCP test in patients with the prothrombin mutation are of particular interest, because this mutation is the second most prevalent thrombophilic genetic defect of the PC pathway, and is not being evaluated previously in screening tests. The sensitivity of the PCP test for this mutation, however, was low (50%). Remarkably, all n-PCP ratios were found just above or below the lower limit of the normal range. As to be expected, n-PCP-SR was not influenced by AT deficiency.

We also performed the PCP test in women using oral contraceptives and pregnant women during the third trimester of their pregnancy. A lowered n-PCP-SR was found in 62% and 100% of these women, respectively. A lowered n-PCP-SR in these conditions might be due to acquired APC resistance or PS deficiency [3-5]. As PS deficiency had a limited effect on n-PCP-SR, the lowered n-PCP-SR is more likely the result of acquired APC resistance. Acquired APC resistance has been attributed to concomitantly increased FVIII levels during use of oral contraceptives and pregnancy [3, 13]. However, as FVIII is not being involved in the PCP test, increased FVIII levels can not explain a lowered n-PCP-SR. This observation suggests that changing levels of proteins involved in the common pathway, rather than increased levels of FVIII are responsible for acquired APC resistance. Irrespective of the precise underlying mechanism, hormonal changes during oral contraception or pregnancy may lower the n-PCP-SR. This should taken into account at interpretation of the PCP test results in these conditions.

A lowered n-PCP-SR informs about the presence of any defect in the anticoagulant PC pathway. To further specify the defect, the PCP test can be modified by dilution of the test plasma with FV, PC and PS deficient plasma, respectively. We have not evaluated this possibility. An additional benefit of the PCP test is its potential insensitivity for heparin and lupus anticoagulants, due to the presence of a heparin inhibitor and phospholipids, respectively, in the assay.

In conclusion, the PCP test is the first screening test of the common pathway, that would be more specific than other thrombin generation, aPTT or PT based screening tests. The PCP test showed a high sensitivity for FV Leiden and PC deficiency, but appeared to be less sensitive for PS deficiency and the prothrombin mutation. Homozygous FV Leiden carriers and patients with combined defects of the PC pathway, showed a pronounced lowering of n-PCP ratios, maybe reflecting their high thrombotic risk. The lowered n-PCP-SR values,
frequently found during use of oral contraceptives and in pregnancy, indicate that the test results should be interpreted cautiously in these conditions.
11.5 REFERENCES


