Reliability and Relevance of a Protein C Pathway Screening Assay in Patients Evaluated for Hypercoagulability

Verlässlichkeit und Relevanz eines „Protein C-Pathway“-Screening-Testes bei Hyperkoagulabilitäts-Abklärungen

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Abstract: We have evaluated the ProC® Global assay in 102 plasma samples from consecutive patients referred for hypercoagulability work-up. Of 14 samples with no clot formation detected by the analyzer, 10 were from patients on oral anticoagulation; high titer antiphospholipid antibodies were found in 2 samples; one patient had protein S deficiency (this patient displayed an abnormal ProC, Global result when enrolled a second time); one was without obvious abnormalities. In 88 remaining samples, changes in assay performance due to different reference ranges for APC resistance, proteins C and S were evaluated. Using the manufacturer's cut-off value for the ProC® Global assay, overall sensitivity, specificity, positive-predictive value and negative-predictive value were 91%, 87%, 82% and 93% for the reference range provided by the manufacturers and 100%, 85%, 78% and 100% for the locally generated reference ranges. Mixing studies with protein C and S deficient or APC resistant plasma suggest high sensitivities to changes of protein C and F. V properties. If the analyzer is unable to detect a clot and patients are not on anticoagulants, they should be regarded as potentially protein C or S deficient or APC resistant. This assay seems an overall reliable, additional tool for evaluating a possible hypercoagulability if the limitations of the assay are recognized. Since some patients referred for possible thrombophilia with no abnormalities found still displayed a pathologic result, the question arises whether the assay might be a marker of thrombophilia by itself rather than a screening test for the three distinct abnormalities.

Keywords: Blood Coagulation Disorders/diagnosis; Thrombosis; Factor V; Protein C; Protein S; Sensitivity

ty and Specificity; Fibrin Fibrinogen Degradation Products.

Zusammenfassung: Wir haben den ProC® Global Test in 102 Plasmaproben von Patienten, die uns zur Hyperkoagulabilitätsuntersuchung zugewiesen wurden, evaluiert. Bei 14 Proben konnte das Analysegerät keine Gerinnungsbildung nachweisen. Zehn dieser Proben stammten von Patienten unter oraler Antikoagulation, in 2 Proben fanden sich hochtitrige Antiphospholipid-Antikörper, bei einem Patienten fand sich ein Protein S Mangel (bei einer Kontrolluntersuchung fand sich bei diesem Patienten ein pathologisches Ergebnis im ProC® Global Test) und in einer Probe fanden sich keine Abnormalitäten. In den verbleibenden 88 Plasmaproben wurde der Einfluß verschiedener Referenzintervalle für die APC Ratio, Protein C und Protein S untersucht. Wurde der vom Hersteller angegebene Grenzwert für den ProC® Global Test herangezogen, so beliefen sich Sensitivität, Spezifität, positiv prädiktiver Wert und negativ prädiktiver Wert auf 91%, 87%, 82% und 93% (alle drei Abnormalitäten kombiniert) unter Verwendung der von den Herstellern vorgeschlagenen Referenzintervalle. Die Werte betrugen 100%, 85%, 78% und 100% unter Verwendung lokal erstellter Referenzintervalle. Mischversuche mit Protein C- und Protein S-defizienten oder APC resistenten Plasmen legen eine starke Abhängigkeit des Assays von Protein C und Faktor V Eigenschaften nahe. Kann der Analyzer bei nicht antikoagulierten Patienten keine Gerinnungsbildung feststellen, so sollte der Befund bis zum Beweis des Gegenteils als pathologisch gelten. Der Test scheint ein verlässliches zusätzliches Instrument für die Thrombophilie-Abklärung zu sein, wenn seine Limitationen berücksichtigt werden. Die Ergebnisse unserer Studie legen auch die Frage nahe, ob dieser Test nicht eher ein „Marker“ für Thrombophilie als ein Suchtest für APC Resistenz, Protein C oder Protein S Mangel sein könnte.

Schlüsselwörter: Blutgerinnungsstörungen/Diagnostik; Thrombose; Faktor V; Protein C; Protein S; Sensitivität und Spezifität; Fibrin-/Fibrinogenspaltprodukte.

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In recent years, resistance to activated protein C (APC resistance, APC-R) due to factor V mutation (F. V Q⁵⁰⁶, F. V Leiden) has emerged as the most common risk factor for venous thromboembolic disease [1, 2]. APC-R is responsible for 30 to 40% of unexplained thromboses; other relatively common risk factors for venous thromboses are protein C and protein S deficiencies. However, these two deficiencies taken together are less common than APC-R and are thought to be responsible for up to 10% of unexplained thromboses. Therefore, roughly 45% of all unexplained thromboses are thought to be due to a defect or deficiency in the protein C system [2-4]. Protein C, protein S, F. V and - to a lesser extent - F. VIII take part in the anticoagulatory response of this system. This is important since it entails that the protein C system as a whole is affected through the defect or deficiency of one particular component. F. V Q⁵⁰⁶ and thus APC-R is a common finding (estimated gene frequency up to 1:75) in Caucasians. Thus, the question arises whether the high frequency of this mutation would justify screening patient populations at high risk for thromboembolic events and if the results of such screening would influence patient management. First data suggest that screening for APC-R in the setting of hip replacement surgery is relevant [6]. In situations where screening for possible thrombophilia is warranted, it would be of advantage to detect as many risk factors as possible in a fast, reliable and inexpensive way.

Recently, an aPTT based assay has been launched ("ProC® Global", Dade Behring, Marburg, Germany) that uses the normalized ratio (NR) of clotting times of the patients plasma in the presence and absence of a protein C activator as an indicator for possible defects or deficiencies in the protein C system (see below). Therefore, this assay should be a screening tool for the presence of a F. V Q⁵⁰⁶ mutation or a Protein C or S deficiency [7]. We wanted to evaluate the reliability of this assay in patients referred for hypercoagulability work-up. Also, we wanted to evaluate to what extent the use of different reference intervals for protein C, protein S or APC-R (manufacturer's specifications vs. locally generated reference intervals) would influence the performance of the assay. We also wanted to find out what the relative importance of protein C or S deficiencies or APC resistance on the results generated by the assay was.

Materials and methods

Samples were collected from consecutive patients who were referred for hypercoagulability work-up or fol-

low up in patients with known hypercoagulability. The diagnoses leading to referral of the patients are given in Table 1. Blood (3.6 ml) was collected into 0.125 M sodium citrate (0.4 ml) using vacuum systems (Vacutainer, Becton Dickinson). Platelet poor plasma was prepared by centrifugation (10 minutes, 1600 g, 22 °C) and samples were immediately assayed for protein C activity in a chromogenic assay system (Berichrom; Dade Behring, Marburg, Germany) on a Behring Coagulation System (BCS, Dade Behring, Marburg, Germany). The remaining sample volume was aliquoted into plastic tubes, frozen and stored at -80 °C for later batch analysis with the ProC Global assay on a BCS, APC-R with F. V deficient plasma (Chromogenix/Endotell, Basel, Switzerland), functional protein S (Boehringer Mannheim, Rotkreuz, Switzerland) on a KC-10 (Amelung, Lemgo, Germany) and total protein S (ELISA, Boehringer Mannheim, Rotkreuz, Switzerland).

The principle of the ProC Global assay is based on the measurement of a clotting time after protein C activation in comparison to the clotting time without protein C activation. For this purpose, a direct protein C activator from the venom of *Agkistrodon contortrix* is incubated with the patient plasma together with an aPTT reagent and coagulation is initiated by the addition of calcium. Since the activation of protein C in the presence of protein S leads to rapid inactivation of activated F. V and F. VIII, the resulting clotting time is accordingly prolonged. The inactivation of F. V or F. VIII can be diminished in various ways, in which case the resulting clotting time is relatively less prolonged (Protein C Activity-dependent Clotting Time, "PCAT"). Examples for decreased inactivation of F. V and VIII includes deficiencies or dysfunctional proteins of protein C or S, increased F. VIII levels or a F. V protein, in which the cleavage site for activated protein C is mutated (F. V Q⁵⁰⁶ or F. V Leiden). Since clotting times can be prolonged due to clotting factor deficiencies or anticoagulants independently of abnormalities in the protein C system, a control experiment is needed for each ProC Global determination in which the protein C activator is replaced by buffer solution only ("PCAT/0"). A PCAT/0 that is already prolonged (≥ 60 s) indicates the likely presence of abnormalities in the "intrinsic" cascade. In this case, the ProC Global assay can not be reliably used since a pathologic result could occur even if the protein C system was intact. To correct for possible differences in the PCAT/0 (if < 60 s) between different patients, the result of the the ProC Global assay is given as a ratio of PCAT and PCAT/0. To improve inter-laboratory comparability, this PCAT to PCAT/0 ratio in the patient's plasma can be normalized for the PCAT to PCAT/0 ratio generated in a control plasma available to all laboratories, which yields the so called "normalized ratio" (NR).

According to pilot trials [7], the manufacturer of ProC Global suggests a cut-off value of 0.8 for the ratio (NR) obtained with the assay; NR values below 0.8 should be indicative of possible APC-R and pro-

Nonstandard abbreviations: APC-R, resistance to activated protein C; aPTT, activated partial prothrombin time; CRP, C-reactive protein; CV, coefficient of variation; NPV, negative predictive value; NR, normalized ratio; PAI, plasminogen activator inhibitor; PCAT, Protein C activity-dependent clotting time; ROC, receiver operating curve.

Table 1. The reason for and relative frequencies of patient referral as well as the numbers of patients with a NR < 0.8, factor V Leiden or protein C or S deficiency in the respective referral group (n=79)

Reason for referral; patient history	Number of patients (frequencies)	Numbers of abnormalities in the respective group				
		ProC	Global NR < 0.8	F. V Q ⁵⁰⁶	Protein C deficiency	Protein S deficiency
DVT and/or PE	26 (33%)	13		5	2	1
High risk patients ^a	18 (23%)	8		6	0	2
Cerebrovascular events ^b	15 (19%)	5		3	0	1
CNS thromboses ^c	8 (10%)	2		2	0	0
Others ^d	12 (15%)	3		1	0	0
Total	79 (100%)	31		17	2	4

^a Patients referred for work-up due to episodes of thromboembolic complications in relatives but not the patients themselves.

^b Patients with stroke, transitory ischaemic attacks (TIA) or prolonged but regressive ischaemic neurologic deficits (PRIND).

^c Patients with cerebral vein thromboses as well as retinal vein thromboses.

^d Other manifestations of thromboembolism or possible thromboembolism such as perfusion deficits in peripheral arteries, subclavian thrombosis, portal vein thrombosis, myocardial infarction etc.

tein C- and protein S deficiencies. Intra- and inter-assay reproducibility of the ProC Global assay was tested by determination of the coefficient of variation (n=10). Also, quantitative D-Dimer measurements were immediately performed in all patients using a quantitative, immunoturbidimetric D-Dimer assay (D-Dimer Tinaquant, Boehringer Mannheim, Rotkreuz, Switzerland) on a Hitachi 917 analyzer. All patients with an APC-R ratio ≤ 2.1 had genotyping for the F. V Q⁵⁰⁶ mutation performed (allele specific hybridization assay, ViennaLab, Vienna, Austria). To test for linearity and the relative influence of a singular defect, plasma of single patients with APC-R, protein C or S deficiencies (but not combined defects) were mixed with different volumes of normal plasma (Standard Human Plasma, Dade Behring, Marburg, Germany), yielding mixtures with 100%, 75%, 50%, 25% and 0% of patient plasma; in these mixtures, the NR and the respective protein C, protein S or APC-R values were measured (Figure 2). Regarding the generation of the reference ranges and cut-off values, the following informations were obtained from the manufacturers: the cut-off for the ProC Global assay was generated using data from a cohort of 243 healthy blood donors, 161 volunteers with unimpaired protein C system, 85 patients with heterozygous F. V Q⁵⁰⁶ mutation, 4 patients with homozygous F. V Q⁵⁰⁶ mutation, 99 patients with protein C deficiency and 87 patients with protein S deficiency; the reference range used for the Berichrom protein C assay (0.80 - 1.20) is a well established reference range that had been validated against the international WHO standard (personal communication, Dr. C. Wagner, Dade Behring). The cut-off for the APC-R assay was selected to be sensitive to all F. V Q⁵⁰⁶ mutations, based on a multicenter trial that evaluated the assay without the use of F. V deficient plasma [7a]. No

information could be obtained on how the reference ranges for the immunologic and functional protein S assays used in this study were obtained. Local reference ranges for study purposes were generated by studying 20 healthy volunteers (8 women, 12 men) and calculating the mean value ± 2 standard deviations. The 2 standard deviations above and below the mean were used as upper and lower limit of the reference range after the distribution of the data was proven to be normal. Calculation of sensitivity, specificity, negative predictive value and positive predictive value were based on the presence or absence of APC-R, protein C or protein S deficiency as defined by the reference ranges provided by the manufacturers or by the locally generated reference ranges. The positive predictive value describes the likelihood of a patient truly having APC-R, protein C or protein S deficiency if the ProC Global assay gives a result below the cut-off. The positive predictive value of an assay is defined as the ratio of the number of "true positives" divided by the number of "true positives" plus the number of "false positives" multiplied by 100, to give a result in percent. By analogy, the negative predictive value describes the likelihood of a patient truly not having APC-R, protein C or protein S deficiency if the ProC Global assay gives a result above the respective cut-off and it is defined as the ratio of the number of "true negatives" divided by the number of "true negatives" plus the number of "false negatives" multiplied by 100, to give a result in percent. Differences between median D-Dimer values of patients above or below the cut-off were evaluated by Wilcoxon Sign Rank Test, calculations were done using SigmaStat software (Jandel Scientific, San Jose, CA, USA). Receiver operating curve (ROC) characteristics for both sets of reference ranges were calculated with the BMP statistical

Figure 1 Receiver Operating Characteristic (ROC) plot using the locally generated reference ranges (open symbols, "locally generated") and the reference ranges provided by the manufacturers (closed symbols, "manufacturers").

Figure 2 Plasma from one patient each with APC resistance, protein C deficiency and protein S deficiency were mixed with increasing amounts of a standard plasma. In each mixture performed, the normalized ratio of the ProC Global assay (cut-off: 0.8) as well as the level of the respective parameter (numbers displayed close to each measurement) were determined. With the protein S deficient plasma, the undiluted sample did not yield a detectable clot formation on the BCS analyzer; thus, in this part of the curve the slope can only be estimated (dashed line).

software program (BMP, Cork, Ireland) and plotted with SigmaPlot (Jandel Scientific, San Jose, CA, USA; Figure 1).

Results

The coefficient of variation (CV) for intra-assay reproducibility was 0.9% for a high (mean NR 0.84) and 3.5% for a low control plasma (mean NR 0.47) with 10 determinations in each case. The CV for inter-assay

reproducibility was 0.6% for the same high and 3.3% for the same low control plasma. One hundred and two samples from patients evaluated for hypercoagulability were collected and examined with the "ProC Global" assay. Fourteen specimens could not be further evaluated since the analyzer (BCS) could not detect a clot formation. Ten of these 14 specimens were obtained from patients on oral anticoagulant treatment. One specimen showed a borderline protein C concentration (outside the limits of the reference range provided by manufacturer, but within the limits of the lo-

Figure 3 Box plots on the different distribution of D-Dimer values in samples below (< 0.8) and above (> 0.8) the cut-off ($p = 0.002$). Bars and box lines represent the 10th, 25th, 50th (median), 75th and 90th percentiles, respectively. The open symbols represent single measurements below the 10th and above the 90th percentiles.

cally generated reference range) and high titers of anticardiolipin- as well as antiphosphatidylserine-antibodies. One specimen was from a patient who had leukocytosis, increased Prothrombin Fragment F1+2 level, high titer of antiphosphatidylserine-antibodies (normal anticardiolipin-antibody titer), increased ALAT and a borderline ratio for APC-R (2.1); this patient refused follow-up and genotyping for F. V Q⁵⁰⁶. Another specimen was from a patient with known familial protein S deficiency, in whom a very weak Lupus Anticoagulant might have been present (but normal anticardiolipin- as well as antiphosphatidylserine-antibody titers were found) and who had had a borderline APC-R (2.1) in the past, but was F. V Q⁵⁰⁶ negative by genotyping; no other abnormalities were seen in this patient. This patient was enrolled twice, the second result yielded an NR of 0.67. Finally, one specimen was from a patient with a shortened aPTT and increased CRP, but otherwise normal laboratory results.

A total of 88 samples from 79 patients were evaluable. Four patients were evaluated twice, one patient

with a known APC-R was enrolled six times during and after pregnancy. The reasons for patient referral, frequencies of clinical abnormalities and frequencies of laboratory abnormalities found (using the locally generated reference ranges, see below) are given in Table 1.

The reference ranges provided by the manufacturers compared to those obtained in a pool of 20 healthy volunteers are given in Table 2. The data from the 20 healthy volunteers were normally distributed, as assessed by Kolmogorov-Smirnov test. The numbers on patient samples correctly or incorrectly identified by the ProC Global assay are given in Table 3. The corresponding results on sensitivity, specificity, positive and negative predictive value (for both types of reference ranges) are given in Table 4. All of the cases with APC-R were recognized by the "ProC Global" assay, while two of the protein C "deficiencies" (0.66 and 0.79, respectively) and one of the protein S "deficiencies" (total protein S 0.93, clotting assay 0.64) would have been missed by the assay when applying the manufacturers reference ranges for protein C and pro-

Table 2 Different reference ranges provided by the manufacturers of the APC resistance, the protein C and protein S assays and generated from 20 healthy volunteers

Reference ranges	provided by manufacturer	locally generated (range)
APC-Ratio	> 2.5; (borderline 2.0 - 2.5)	2.10 - 2.90 (2.20 - 3.00)
Protein S (total, ELISA)	0.70 - 1.40	0.73 - 1.56 (0.68 - 1.49)
Protein S (functional, clotting)	0.70 - 1.40	0.61 - 1.57 (0.66 - 1.53)
Protein C (functional, chromogenic)	0.80 - 1.20	0.59 - 1.48 (0.72 - 1.41)

tein S. As mentioned above, none of these three cases was identified as having a deficiency state when using the locally generated reference ranges.

Using the manufacturer's reference ranges for APC-R, protein C and protein S, samples of 7 patients were found to have an NR ≤ 0.8 without evidence of protein C or S deficiency or an APC-R. However, these seven patients displayed one or multiple abnormalities that might be related to a hypercoagulable state: antiphospholipid antibodies (2 patients), increased PAI-1 levels (5 patients), prolonged euglobulin lysis time after venous occlusion (3 patients) and shortened aPTTs (3 patients). When using the locally generated reference ranges, samples of 8 patients were found to have an NR ≤ 0.8 without evidence of protein C or S deficiency or an APC-R. The first seven patients were the same patients as described above, the eighth patient did not display any other abnormality. The median D-Dimer level was found to be significantly higher in the patients with a NR ≤ 0.8 (median D-Dimer 310 $\mu\text{g/l}$, range 60 - 2750 $\mu\text{g/l}$) compared to those with NR > 0.8 (median D-Dimer 230 $\mu\text{g/l}$, range 80 - 2590 $\mu\text{g/l}$; $p = 0.002$), but there was considerable overlap between the two groups (Figure 3). For the comparison of D-Dimer levels, samples from pregnant women were excluded due to the fact that D-Dimer increases occur physiologically during pregnancy.

In one patient with known APC-R enrolled six times over a period of four months in and after pregnancy, NR was found to be below the cut-off all six times with only minimal variation of the result (range:

0.49 to 0.54). Four patients evaluated twice displayed the following results: one patient evaluated for hypercoagulability during pregnancy (second trimester; no APC-R, normal protein C and S levels) had a decreased NR (0.61) at that point, but showed a normal NR (0.91) three months post partum. Another patient evaluated for hypercoagulability initially displayed a borderline functional protein S value (0.67) with an NR of 0.72. Five months later, her functional protein S had risen to 0.87 and her NR had normalized to 0.87 as well. A third patient, who was found to be APC resistant (NR 0.56), showed only little variation during a second control 6 months later (NR 0.61). The fourth patient showed an NR of 0.74 and an increased PAI-1; 4 months later, the PAI-1 level had dropped by roughly 30% and the NR was found to be 0.83.

ROC characteristics showed improved performance of the assay using the locally generated reference ranges vs. the reference ranges provided by the manufacturers (area under the receiver operating curve 0.9854 vs. 0.9419). This is reflected by the left shift of the ROC curve (Figure 1). To obtain 100% sensitivity for APC-R, protein C and protein S-deficiency, the necessary NR cut-offs were found to be 0.80 for the locally generated reference ranges and 0.97 for the reference ranges provided by the manufacturers.

The evaluation for linearity and influences of the particular abnormalities showed a satisfactory linear behavior of the assay; the slopes of the curves obtained were quite different. The slope obtained with protein S deficient plasma (residual antigen level 51%)

Table 3 The numbers of correctly and incorrectly identified abnormalities using the ProC Global assay and the different reference ranges. This statistic is based on 88 plasma samples of the 79 evaluable patients

Reference ranges	provided by manufacturer			locally generated		
	median NR (range)	incorrect/ correct determination n =	total n =	median NR (range)	incorrect/ correct determination n =	total n =
APC-Resistance	0.56 (0.42 - 0.79)	0 / 23	23	0.56 (0.42 - 0.79)	0 / 23	23
Protein S deficiency	0.71 (0.66 - 0.92)	1 / 5	6	0.68 (0.66 - 0.76)	0 / 4	4
Protein C deficiency	0.73 (0.44 - 0.96)	2 / 2	4	0.51 (0.44 - 0.58)	0 / 2	2
No APC-R/deficiencies	0.92 (0.61 - 1.37)	7 / 48	55	0.92 (0.61 - 1.37)	8 / 51	59

Table 4 Overall (i.e. combined) sensitivity, specificity, positive predictive value and negative predictive value of the ProC Global assay to identify APC resistance, protein C and protein S deficiencies when using the reference ranges suggested by the manufacturers and the locally generated reference ranges

Reference ranges	provided by manufacturer	locally generated
Sensitivity	91%	100%
Specificity	87%	85%
Positive-predictive value	82%	78%
Negative-predictive value	93%	100%

was flat compared to the ones obtained with protein C deficient plasma (residual activity 49%) or plasma from a patient heterozygous for F. V Q⁵⁰⁶ (APC-R 1.6). This results in a relatively wide range of protein S concentrations that will be found close to the cut-off compared to protein C and APC-R values (Figure 2).

Discussion

Recently, the ProC Global assay has been launched as a tool to simultaneously screen for APC Resistance as well as protein C and protein S deficiencies [7]. The rationale for this assay is the fact that protein C and S deficiencies as well as the presence of an F. V Q⁵⁰⁶ mutation lead to a decreased response (i.e. decreased inactivation of F. Va and VIIIa) when a protein C activator is added [8]. Thus, relatively shorter clotting times in an aPTT based assay will result in the presence of these abnormalities and a protein C activator in comparison to plasma without the above abnormalities. Expressing the result as a ratio (i.e. the clotting time with protein C activator over the clotting time without protein C activator), the value obtained will become smaller if any of the three named abnormalities is present.

The assay is easy to perform and is fully automatable (here: on a BCS analyzer); the reproducibility was excellent in this setting. In our hands, the assay showed very good performance when used in a population of patients referred for hypercoagulability work-up or in patients with known thrombophilia followed up for various reasons. But we found that the definition of the reference ranges for APC-R, protein C and protein S has considerable influence on the performance of the ProC Global assay. This might be important since many larger hemostaseologic laboratories create their own in house reference ranges. With locally generated reference ranges (which were different from those provided by the manufacturers for the protein C and the protein S assays but essentially consistent with the one provided for APC-R), the ProC Global assay was 100% sensitive and had a 100% negative predictive value (NPV) for the targeted abnormalities. In comparison, when we used the reference ranges provided by the manufacturers to decide whether a patient was APC resistant or protein C or S deficient, there was a loss of overall sensitivity and negative predictive value of the ProC Global assay (sensitivity 91%, NPV 94%) to predict presence of the targeted abnormalities. This was due to a loss of sensitivity and negative predictive for protein C or S deficiency but not APC-R detection. It has to be kept in mind that generation of a reference range using 20 volunteers results in a relatively wide distribution due to the low number of samples used and might therefore not be a suitable approach for the routine laboratory; however, the low number of volunteers were purposely chosen to demonstrate the dependence of the assay performance on the reference ranges used. Moreover, it has

to be kept in mind that the patient group was a selected one (referral for hypercoagulability work-up) and that therefore the frequency of abnormalities is likely to be higher than in a non-selected population: this can influence the performance of an assay in such an evaluation. Sensitivity, specificity, positive and negative predictive values were not separately assessed for the particular abnormalities since the assay is intended to be used as a "global" screening assay for abnormalities of the protein C system (although the assay can be made APC-R "specific" if F. V deficient plasma is used). The above results in combination with the mixing studies (Figure 2) suggest that the assay is mainly dependent on F. V and protein C properties. The cut-off at a NR of 0.8 results in excellent separation of APC resistant from non-APC resistant samples. However, in our mixing studies, normal protein C concentrations led to abnormal ProC Global normalized ratios (NRs), whereas abnormal low protein S levels resulted in normal NRs (Figure 2). It has to be stressed though that in their native state, the plasmas used for the mixing studies were correctly identified by the ProC Global assay.

In four specimens from patients not on oral anticoagulation, the analyzer could not detect a regular clot formation after addition of the protein C activator: two of these patients displayed high titers of antiphospholipid-antibodies, suggesting a possible interference with the assay, especially since "spurious" protein C deficiencies in clotting based assays due to antiphospholipid antibodies have been reported in the past [9]. In the sample from another patient with known protein S deficiency, the analyzer could again not detect a regular clot formation on one occasion, but (correctly) revealed a pathologic NR at a second occasion. This patient also displayed a borderline APC-R (2.1) despite normal F. V alleles. This might indicate that the assay is sensitive to summation effects of (borderline) abnormalities of the protein C system. In the fourth specimen, in which no regular clot formation was detected, no other abnormality or explanation for this phenomenon could be found.

Four patients were enrolled more than once into this evaluation study: in two patients with F. V Leiden, the ProC Global assay was performed to a total of 8 determinations at different occasions; it was found to correctly identify the presence of an abnormality in every instance. In the two other patients, the assay correctly identified dynamic changes occurring during and after pregnancy (postpartal normalization of the NR) in one patient and during normalization of a borderline protein S deficiency in the other.

Patients with a pathologic NR (< 0.8) but no F. V Leiden, protein C or S deficiency in all or in 7 of 8 cases (reference intervals provided by the manufacturers or generated locally, respectively) showed abnormalities that can be related to a hypercoagulable state (see "Results" for detailed results). One can speculate that patients with abnormalities in clotting proteins not taking part in the protein C system still might lead to

abnormalities in the protein C system through so far unknown interactions; a possible example for this is the recent observation that antiphospholipid antibodies might confer an APC resistance phenotype that is not bound to the F. V Leiden mutation or that antiphospholipid antibodies might increase the prothrombotic potential of an F. V Leiden mutation [10-14].

The fact that the D-Dimer values between the two groups of patients (below and above the NR cut-off) are statistically highly significantly different (i.e. the group with the lower NR generates more fibrin) suggests that the results obtained with the ProC Global assay indeed reflect an increased thrombophilic risk. Thus, the question arises whether a global screening assay for the protein C system is "only" a screening tool for the presence of APC resistance or protein C or S deficiencies or if such an assay itself might be a marker of thrombophilia - irrespective of the results of the above named three abnormalities.

In summary, the ProC Global assay seems to be a valuable tool to screen for APC resistance as well as for protein C and S deficiencies in patients presented for hypercoagulability work-up, but one has to recognize the limitations of the assay. In particular, this entails the fact that the sensitivity is dependent on the reference range used to define a "deficiency", as shown in this study; and that assay displays better discrimination between different levels of protein C deficiency and APC resistance as compared to protein S deficiency. Using the manufacturer's cut-off value for the ProC® Global assay, the sensitivity of the assay is not 100% for the targeted abnormalities and might be considerably lower in a non-selected patient population. Future studies will have to clarify if the assay itself, independently of the presence of the targeted abnormalities, identifies a risk for thromboembolic events. Despite the fact that this study was sponsored by the manufacturer of the assay, it is fair to state that this assay is a valuable and interesting tool in the hemostaseologic laboratory, if used with the necessary precautions.

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