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Original Report

ProC Global: A New Automated Screening Assay for the Evaluation of Total Function of the Protein C System

*Francesco Dragoni, M.D., †Daniela Tormene, M.D., †Paolo Simioni, M.D., Ph.D., *Paolo Arcieri, M.D., *Giuseppe Avvisati, M.D., Ph.D., and †Antonio Girolami, M.D.

**Thrombosis Center, Department of "Biotecnologie Cellulari ed Ematologia," University "La Sapienza," Rome; and †Department of Medical and Surgical Sciences, Second Chair of Medicine, University of Padua Medical School, Padua, Italy*

Summary: Protein C (PC) pathway represents a major physiologic inhibitory mechanism regulating the coagulation cascade. A new automated functional screening assay (ProC Global) for the evaluation of the PC-system was tested to define its ability to identify patients with known inherited defects such as factor V (FV) Leiden mutation and PC and protein S (PS) deficiency. A total of 249 patients who were symptomatic or asymptomatic for previous venous thromboembolism (VTE) were evaluated, 50 of whom had FV Leiden mutation, 36 had PC deficiency, and 34 had PS deficiency. One hundred healthy subjects were also tested, as well as 40 blood donors of both sexes in whom coagulation abnormalities were not found. Results of ProC Global test were expressed as normalized ratio (NR) and values below an established cut-off level were consistent with a positive test. ProC Global was positive in all 50 patients with the FV Leiden mutation (mean NR = 0.59; range, 0.37 to 0.69). ProC Global correctly identified 32 of 36 (89%) PC defects (mean NR = 0.63; range, 0.34 to 1.21) and 25 of 34 (73.5%) PS defects (mean NR = 0.76; range, 0.5 to 1.23). Overall, 92.5% of hereditary defects of the PC system considered in this study were identified by ProC Global test. ProC Global exhibited NR above cut-off level in all 40 blood donors without coagulation defects. ProC Global is a new automated screening test with some diagnostic potential in identifying patients with defects of the PC system. However, ProC Global in its current form cannot substitute the assay of each single component of this inhibitory system in the daily screening for thrombophilia. **Key Words:** ProC Global—Protein C system—FV Leiden—Protein C—Protein S.

Deficiencies in the protein C (PC) system are the most frequent cause of hereditary thrombophilia (1,2). Protein C, once activated, is able to degrade the activated forms of factors V (FV) and VIII (FVIII) in the presence of its cofactor, protein S (PS). The prevalence of PC and PS deficiencies have been estimated to be around 3 to 8% among patients with objectively documented venous thromboembolism (VTE), depending on the selection criteria (2). In 1993, resistance to activated protein C (APC-R) has been identified as a new inherited condition predisposing to VTE (3). In about 90% of cases, APC-R is caused by a single point mutation (Arg506 to Gln) in FV gene (4), which is present in 20 to 50% of patients who have experienced VTE (5–7).

Currently, defects of the PC system are evaluated by means of specific and expensive assays. Because many laboratories cannot perform a complete screening for thrombophilia, a test that is able to screen the global function of the PC anticoagulant system, rather than each single component, may be of great interest and may facilitate the approach to laboratory diagnosis of patients with thrombosis.

Recently, new functional assays for the evaluation of PC system have been proposed as useful tools for first-line coagulation screening (8–11). One of these tests, named ProC Global, based on the activation of the endogenous PC present in plasma sample by Protac (snake venom from Agkistrodon Contortrix Contortrix), has been recently evaluated in several studies (11–15). In principle, this test differs substantially from those used for the evaluation of activated PC (APC) resistance in which the prolongation of the clotting time is assessed after the addition of exogenous APC to the assay mixture.

We tested by ProC Global plasma samples obtained from patients previously identified as carriers of FV Leiden mutation and those with PC and PS deficiency. Thrombotic patients with no identifiable thrombophilic defects and normal, healthy subjects were also studied

Address correspondence and reprint requests to Paolo Simioni, M.D., Ph.D., Department of Medical and Surgical Sciences, Via Ospedale Civile, 105, 35128 Padua, Italy; e-mail: simioni@ux1.unipd.it.

with a small set of patients taking oral anticoagulant treatments because of previous history of VTE.

PATIENTS AND METHODS

Patients

Plasma was obtained from probands and their family members with FV Leiden mutation (n = 50), PC deficiency (n = 36), and PS deficiency (n = 34). The laboratory diagnosis of the deficiency status had been previously performed by standard methods and criteria (16). Congenitally deficient patients were included with either PC antigen or free PS antigen levels below 70% and 60% of normal value, respectively. Eighty-two patients who had previously experienced VTE were also tested, in whom no known defects of the PC system were found. Additionally, 47 patients taking oral anticoagulant therapy for previous VTE (seven were also carriers of FV Leiden mutation) were tested with ProC Global. Finally, a total of 100 healthy subjects asymptomatic for previous VTE were included in this study with forty blood donors (20 men, 20 women; mean age, 37 years; range, 18 to 60 years) in whom both APC-R and PC/PS defects had been ruled out.

Methods

ProC Global assay kit (Dade-Behring, Milan, Italy) was used to assess the function of the entire PC system. Briefly, an activated partial thromboplastin time (aPTT)-based assay was performed in which plasma test was incubated with Protac to activate the endogenous PC present in the sample. Pathromtin SL was used as the aPTT reagent, and after the addition of calcium chloride the clotting time was recorded. The clotting time obtained in the absence of Protac (PCAT/0) was used as the basal value to calculate a ratio.

In order to increase the specificity of tests for the diagnosis of FV Leiden mutation, ProC Global was also performed after mixing plasma samples 1+4 with FV-deficient plasma (FV-deficient, Dade-Behring). This modified test is referred to as ProC Global-FV in the text. ProC Global-FV was used also to test samples from patients taking oral anticoagulant treatment. Results of ProC Global test was expressed in ratio form, namely, ProC Global ratio = PCAT sample/PCAT/0 sample. ProC Global-FV ratios were expressed in the same way. All ratios were normalized by dividing for the ratio obtained in pooled normal plasma (from 50 healthy subjects; 25 men, 25 women; mean age, 32 years; range, 18 to 62 years) and were expressed as normalized Ratio (NR; NR = ratio sample/ratio pool). Samples with a PCAT/0 > 60 seconds were excluded from the evaluation because of the insufficient clotting capacity in the ProC Global assay. All assays were performed on an automated coagulometer (BCS, Dade-Behring, Milan, Italy).

Normal limits for ProC-Global were obtained as follows. Samples from 90 normal subjects (45 men, 45 women; mean age 32 years; range, 18 to 62 years) were tested for PCAT and PCAT/0. The ratio (PCAT/PCAT/0) was calculated and normalized according to the method described above. The same procedures were repeated in the same samples after 1+4 mixing with FV-deficient plasma. The 5th percentile of both series of samples were calculated, and values below the 5th percentile were considered abnormal and the test positive.

Linearity evaluation

To evaluate the response of ProC Global test to variable PC and PS concentrations, PC- and PS-deficient plasma was mixed to a standardized reference pooled normal plasma (Dade-Behring) with known concentrations of PC and PS. Thus, samples with defined concentrations of PC and PS (ranging from 20 to 80%) were obtained. Each sample was tested by ProC Global, and the results obtained at different concentrations of either PC or PS were plotted in a x–y linear diagram.

Additional assays

Tests and criteria used for the diagnosis of FV Leiden mutation, APC-R, and PC and PS deficiency have been described previously (16). These tests had already been performed in plasma samples used in this study.

Statistical analysis

Statistical analysis was performed using the BMDP, a statistical software package (1990, Berkeley, CA). Means and standard deviations of NR were calculated in each group.

RESULTS

The cut-off limits (5th percentiles) of NR were 0.80 and 0.70 for ProC Global and ProC Global-FV, respectively. Values below these limits were consistent with a positive test, suggesting an impairment of the PC system function.

The number of individuals investigated, the NRs obtained in different groups, and the number of individuals with a positive ProC Global test are shown in Table 1. NRs obtained with ProC Global in different groups are depicted in Figures 1 and 2.

Either ProC Global or ProC Global-FV tests performed in 50 patients with FV Leiden mutation (2 homozygous, 48 heterozygous) were positive in all cases (100%).

Thirty-two of 36 cases (88.8%) with PC deficiency and 25 of 34 cases (73.5%) with PS deficiency were identified as abnormal by ProC Global test.

ProC Global test was normal in all 40 blood donors (control group with no defects) (Figures 1 and 2).

Overall, ProC Global test correctly identified 92.5% of patients with hereditary defects of the PC system. Inter-

TABLE 1. *Mean normalized ratios (NRs) obtained in different groups of individuals investigated*

Groups of individuals	No. of individuals	NR, mean ± SD	No. of individuals with NR below the cut-off limits*
Healthy subjects	100	1.00 ± 0.17	5
DVT without defects	82	1.07 ± 0.24	8
FV Leiden mutation	50	0.59 ± 0.07	50
FV Leiden mutation taking OAT	7	0.53 ± 0.04†	7
Subjects taking OAT	40	0.81 ± 0.09†	6
Protein C deficiency	36	0.64 ± 0.16	32
Protein S deficiency	34	0.76 ± 0.15	25
Control group (blood donors)	40	0.99 ± 0.09	0

*0.80 and 0.70 for ProC Global and ProC Global-FV†, respectively.

DVT, deep venous thrombosis; FV, factor V; SD, standard deviation; OAT, oral anticoagulant treatment.

estingly, only one of 12 (8.3%) patients with inherited deficiency of PC or PS—a patient who was not identified by ProC Global—had a history of previous VTE, the remaining 11 patients being asymptomatic.

ProC Global was positive in 8 of 82 (9.7%) patients with VTE without known defects of the PC system (Table 1).

ProC Global-FV detected all seven patients with FV Leiden mutation taking anticoagulant treatment, while it was normal in 34 of 40 (85%) patients taking anticoagulant treatment. None of the six subjects taking oral anticoagulant treatment with a positive ProC Global test exhibited inherited defects of the PC system.

Figure 3 shows NRs obtained by ProC-Global test at different concentrations of PC and PS in the samples. A linear relationship was found for both PC and PS.

DISCUSSION

Protein C system is a major physiologic inhibitory mechanism regulating the coagulation cascade (1). Screenings for abnormalities in the PC system are often recommended in thrombotic patients and in their relatives to identify carriers of inherited deficiencies. This approach requires several expensive and time-consuming tests to be performed. In addition, many physiologic and pathologic conditions are maintained to cause a hypercoagulable state (17–20) due to interference with the PC system. For this reason, there is an increasing request for screening the function of PC system in individuals potentially exposed to thrombotic risks, such as women receiving oral contraceptive treatment or during pregnancy, or patients who undergo surgery or experience trauma and immobilization. Therefore, several tests have been proposed as new screening assays to evaluate the global anticoagulant function of the PC system in a single step (8–11).

In this study we assessed ProC Global, one commercially available global test, in a series of retrospectively collected plasma samples belonging to different groups of subjects with or without previous VTE or inherited thrombophilia. The data indicate that the ProC Global test can identify a large proportion of defects of the PC system. This is particularly true for the FV Leiden mu-

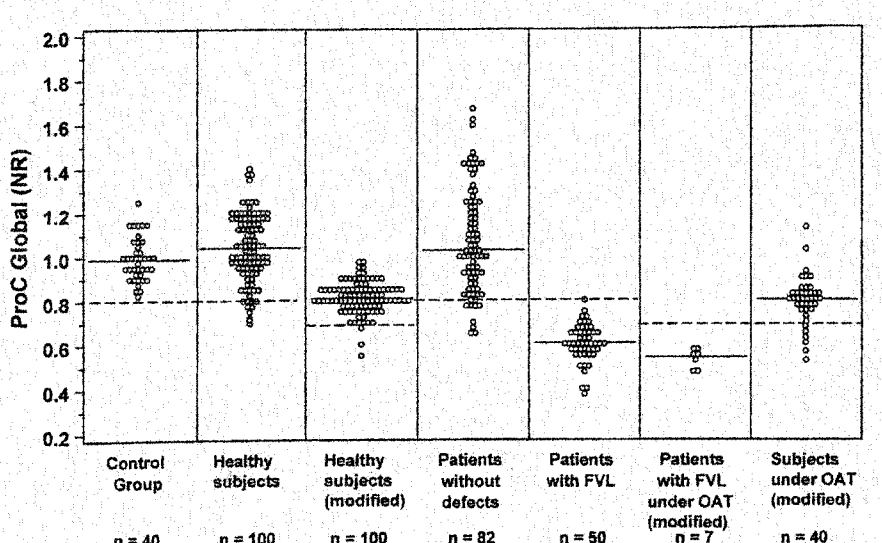

FIG. 1. Normalized ratios (NR) obtained by ProC Global test in the control group (blood donors), healthy subjects, and patients with and without defects of protein C system. Dashed lines indicate cut-off values according to the type of test used (standard or modified by 1:5 dilution of sample in factor V (FV)-deficient plasma). Solid lines indicate the mean values of NR in each group. "Modified" stands for ProC Global-FV test. OAT, oral anticoagulant treatment.

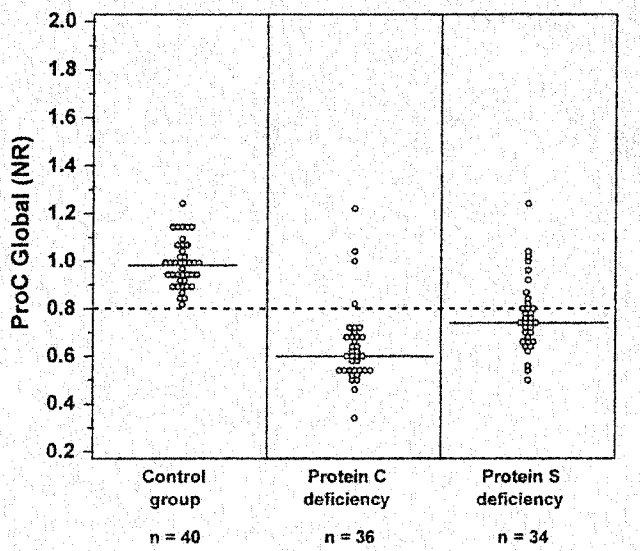

FIG. 2. Values of normalized ratios (NR) obtained by ProC Global test in the control group (blood donors) or protein C- and protein S–deficient patients. Data dealing with the control group are the same as in figure 1. Dashed line represents cut-off value. Solid line indicates the mean NR values in each group.

tation, which can be diagnosed by ProC Global with the same accuracy as the commonly used (modified) aPTT-based methods for APC-R. In this study, ProC Global-FV identified all carriers of FV Leiden mutation among subjects taking oral anticoagulant treatment. These data suggest that ProC Global-FV is able to identify the APC-R due to the FV Leiden mutation without having to discontinue the anticoagulant treatment.

The results obtained in PC- and PS-deficient samples indicate that ProC Global can detect correctly the majority of patients with these deficiencies (90% of PC- and 73% of PS-deficient subjects). A major limit of this global test is the failure to identify a large proportion of cases with PS defects. Recently, Toulon et al. (12), in a European multicenter evaluation, found a weak sensitivity (69%) of ProC Global test in detecting the PS defect. Conversely, recently Gemmati et al. (21) outlined that a modification of this test, using different concentrations of the PC activator and a previous dilution of samples in PC- or PS-deficient plasma, could increase its sensitivity to abnormal values of PC and PS. In another study, the use of the extrinsic pathway for the global test was associated with a better sensitivity for PS defects (9).

In this study, only one of 12 patients with inherited defects of PC or PS and normal ProC Global test results had a history of a previous thrombotic event. Whether subjects with defects in the PC system and a positive global test are at a higher risk for VTE than those with a negative global test is a matter of speculation and should be addressed in properly designed studies.

Approximately 10% of patients with VTE without known defects of the PC system tested positive with the ProC Global assay. The false-positive tests represent another limiting factor for the validity of the test. This could be due to other coagulation defects, such as the presence of haplotype HR2, which can be variably detected even by aPTT-based methods for APC-R testing (22). Other acquired coagulation abnormalities due to the presence of antiphospholipid antibodies or related to hor-

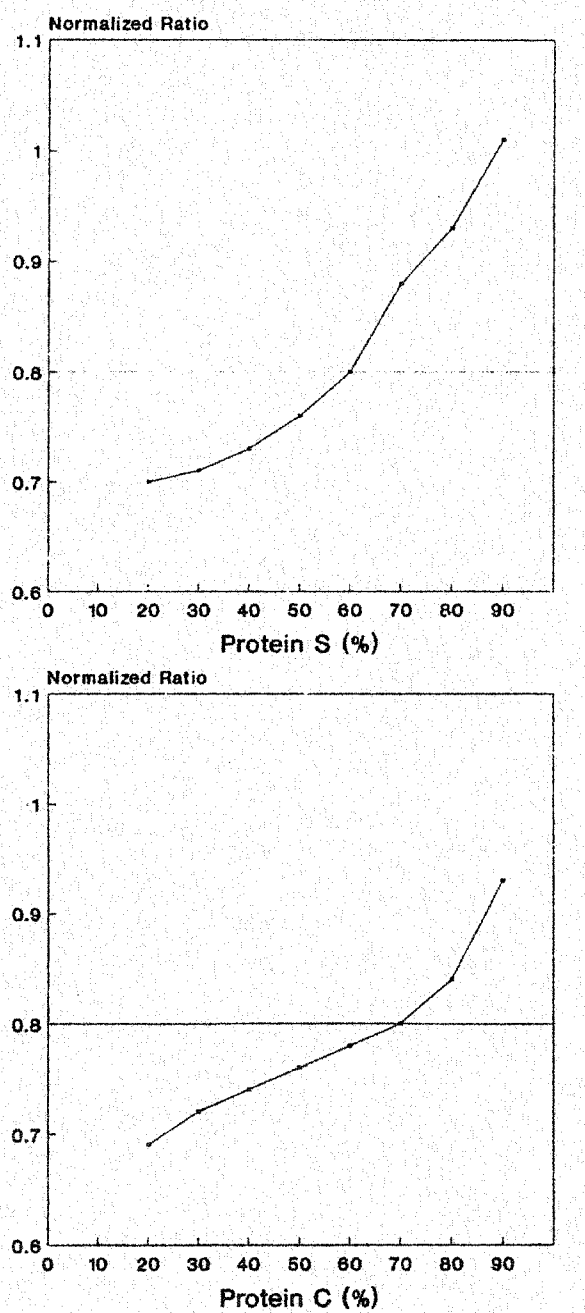

FIG. 3. Normalized ratios (NR) obtained by ProC Global test at different concentrations of protein S (PS) (upper panel) or protein C (PC) (lower panel) in samples obtained by mixing PC- or PS-deficient plasma with a standardized reference pooled normal plasma (Dade-Behring, Milan, Italy) containing known concentrations of PC and PS. A linear relationship was found between NR values and PS or PC concentrations.

monal therapy might interfere with the PC Global test, especially when dilution of plasma sample in FV-deficient plasma is not performed. Interestingly, none of these conditions was present in the 10% of patients with VTE who had a positive ProC Global test. It has been suggested that a positive ProC Global test in itself might be associated with an increased risk for VTE, independent of the presence of a known defect of the PC system (12,14).

In conclusion, the ProC Global test appears to be have some potential in the identification of defects affecting the PC system. The test, however, fails to identify some PC- and many PS-deficient subjects. Thus, although the ProC Global test was designed for large-scale use in daily clinical practice for thrombophilia screening (23), in its current form it appears not to be sensitive and global enough. The assay of each single component of the PC system still remains the most tedious but effective way for the diagnostic workup of thrombophilic conditions. There may be room, however, to improve the sensitivity of the ProC Global test by possible modifications of reagent concentrations. Owing to the large request for coagulation screenings, efforts should be devoted to finding and improving global tests for thrombophilia diagnosis, and to assess their diagnostic accuracy in proper prospective studies examining large number of individuals with thrombosis.

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