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Theme Issue Article

Evaluation of ProC Global assay in women with a history of venous thromboembolism on hormonal therapy

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Summary

The risk of thrombosis in women increases significantly during treatment with hormonal therapy (HT). The aim of this study was to evaluate ProC Global assay in women with a history of venous thromboembolism (VTE) while using HT. Protein C activation time normalized ratio (PCAT-NR) levels were significantly lower in 32 women with a history of VTE while using HT (0.72 ± 0.1) compared with 56 healthy controls without HT, matched by age at blood sampling (0.99 ± 0.2) and 40 healthy controls with HT, matched by age and HT at VTE event (0.94 ± 0.2) (P<0.001 for both). PCAT-NR lower than the cut-off level of 0.8 was found in 23/32 (72%) patients compared with 5/56 (9%) age-matched controls (OR=26, 95%CI: 7–106, P<0.001) and 9/40 (22.5%) of HT-matched controls (OR=9, 95%CI: 2.7–30, P<0.001). Any thrombophilic risk factor was found in 20/32 (62.5%) of patients compared with 12/56 (21.4%) of age-matched controls (OR=6, 95%CI: 2.1–10, P<0.001) and 12/40 (30%) of HT-matched controls (OR=4, 95%CI: 1.3–11.8, P=0.006). Out of the variables that are risk factors of VTE as age, HT or thrombophilic risk factor, ProC Global assay was found in the multivariate analysis – logistic regression, as the parameter that was the most associated with patient group [Exp(B)=15.8, 95% CI: 4.2–59.0, P<0.001]. In conclusion, abnormal PCAT-NR is associated with VTE in women using HT. ProC Global assay may potentially serve as a diagnostic tool for evaluating the risk of VTE in women prior to administration of HT.

Keywords

Thrombophilic risk factor, oral contraceptive, hormonal therapy, ProC Global assay

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Introduction

Over 100 million healthy women worldwide use hormonal therapy (HT), i.e. oral contraceptives (OC) or hormonal replacement therapy (HRT); however, the use of HT is associated with an increased risk of venous thromboembolism (VTE), probably due to the administration of exogenous estrogens (1, 2). The risk for a VTE is still present with current low-dose oral contraceptives (second and third generations; 20–30 mg of the estrogen ethinylestradiol). While the risk is low in absolute terms, oral contraceptives are the major cause of thrombotic events in young women (1), and HRT preparations, although they contain lower dosage of estrogen and progestin compared with OC, increase the risk of a VTE by two to three folds compared with the risk in women without hormonal therapies (3). The risk is higher during the first year of use (up to 1 per 1,000 per year), with the application of third generation OC (desogestrel or gestodene instead of levonorgestrel or norgestrel) and among women with thrombophilic risk factors (1). Women heterozygotes for factor V (FV) Leiden using OC have a 20- to 30-fold increased risk of a VTE compared with those without FV Leiden who do not use OC (4). Homozygosity for FV Leiden leads to a 50- to 100-fold increased risk of venous thrombosis (5). In a comparison of unselected relatives with various thrombophilic defects, the synergistic effect of OC appeared even higher for deficiencies of the natural anticoagulants protein C, protein S and antithrombin than for FV Leiden (6). The use of HT is associated with acquired resistance to activated protein C (APC) (7, 8), increased prothrombin and decreased plasma levels of total and free protein S (3, 9). Therefore, identification of women at high risk for VTE before starting administration of HT is important.

Since defects in the protein C anticoagulant pathway, including protein C and protein S deficiencies and APC-resistance with or without FV Leiden, are major risk factors of VTE (10), a screening assay for this pathway can be potentially useful in identification of women on HT with increased risk for VTE. ProC Global assay (Dade Behring, Marburg, Germany) devel-

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oped for evaluation of the protein C pathway, utilizes protein C activator of *Agkistrodon Contortrix Contortrix* venom, which activates the endogenous protein C in the detected plasma sample. The endogenous APC activity is measured by an activated partial thromboplastin time (APTT) base-clotting assay. The clotting time is measured with and without protein C activation, and the ratio between these times is calculated and expressed by the protein C activation time – normalized ratio (PCAT-NR) (11, 12).

In contrast to APC-resistance assay in which an exogenous APC is added, in ProC Global assay the endogenous protein C is activated and the whole protein C pathway function is detected. The ProC Global assay revealed a high sensitivity for detection of protein C deficiency, APC-resistance and FV Leiden mutation but controversial sensitivity for protein S deficiency (12–14). In a multicenter study, more than 40% of VTE patients without apparent thrombophilic risk factor were found to have an abnormal low result by the protein C Global assay (15), suggesting that ProC Global assay may potentially reveal yet undetermined abnormalities in the protein C pathway. Recently, the ProC Global assay was suggested as a potential predictor of recurrent VTE (16). Furthermore, abnormal ProC Global assay results were found to be more common (70%) in women with pregnancy loss, preeclampsia and HELLP syndrome compared with healthy women without these complications (17, 18).

In order to study the predictive value of the ProC Global assay for thrombotic events in women using HT, we have evaluated ProC Global assay in women with a history of VTE on HT.

Methods

Study group

The study group included 32 women who developed VTE while using OC or HRT: six women developed pulmonary embolism (PE), 23 had deep venous thrombosis (DVT), and three had both PE and DVT. DVT was diagnosed by compression ultrasonography, and PE was confirmed by lung perfusion scan or by CT angiogram.

In 23 patients the VTE event occurred while using OC (5 – second generation and 18 – third generation OC) and in nine patients while using various preparations of HRT. The most widely used HRT was composed of 0.625 conjugated equine estrogens and 5 mg medroxy progesterone acetate (MPA), Premaril Plus 0.625 (Dexxon, Israel). The average age of the nine patients on HRT at the time of VTE event was 59 ± 7.6 years.

ProC Global assay was performed at least six months after the VTE event. At the time of blood sampling, the patients did not receive any anticoagulant or hormonal therapies.

Control groups

The study included two control groups of healthy women without a history of VTE events. The first control group consisted of 56 women without HT, matched with patients by age at the time of blood sampling. The second control group included 40 healthy women matched by HT and patient's age while developing VTE.

In the second control group, seven women were on second generation OC, 26 on third generation OC and seven on various preparations of HRT. The most widely used HRT was the above-mentioned Premaril Plus 0.625. The average age of the seven controls on HRT was 58 ± 4.7 years.

The study was approved by the Ethics Committee of the Rambam Health Care Campus and the Israel Ministry of Health.

Coagulation assays

Upon obtaining informed consent of the study participants, blood samples were collected by venipuncture into 3.2% sodium citrate tubes and centrifuged at 2,000 g for 15 minutes (min). Plasma samples were frozen after second centrifugation at 2,000 g for 15 min. Supernatant plasma aliquots were frozen at –35°C for no more than three months. Before testing, plasma aliquots were thawed in 37 ± 0.5ºC water bath for 15 min.

Thrombophilic risk factors such as free protein S antigen, protein C activity, antithrombin activity, lupus anticoagulant, FV Leiden mutation, prothrombin G20210A and methylen-tetrahydrofolate reductase (MTHFR) were detected by methods that we have previously described (19).

Levels of factor VIII were determined by one-stage coagulation assay using factor VIII deficiency plasma (Instrumentation Laboratories, Barcelona, Spain) on the ACL 3000 analyzer (Instrumentation Laboratory, Milan, Italy).

ProC Global assay

ProC Global (Dade Behring, Marburg, Germany) is based on activation of endogenous protein C by Protac® (an extract from *Agkistrodon Contortrix* C*ontortrix* venom). The assay was performed on the STA-R coagulometer (Diagnostica Stago, Gennevilliers, France). Protein C activation time (PCAT) was measured either with Protac® or buffer (to determine PCAT-0). Results were expressed as PCAT normalized ratio (PCAT-NR) calculated by dividing the PCAT ratio (PCAT / PCAT-0) of sample's plasma by a PCAT ratio of lyophilized standard human plasma (ORKL; Dade Behring) and multiplying by a lot-specific sensitivity value (SV) defined by the manufacturer for each batch of standard plasma.

The intra-assay coefficient of variation was determined for the PCAT-NR from the mean of two separate runs of 10 replicates each and was found to be 5.0%. The inter-assay coefficient of variation for the PCAT-NR was calculated from the mean of seven separate runs and was found to be 3.2%.

The cut-off level of 0.8 was found to have a sensitivity of 100% for FV Leiden mutation (13, 15). Therefore, PCAT-NR<0.8 were considered abnormal.

Statistical analysis

The data were evaluated by SPSS software, version 12 (SPSS Inc. Chicago, IL, USA). Fisher's exact test was used for detection of the differences in the prevalence of thrombophilic risk factors and PCAT<0.8 between the patient and control groups. ANOVA and t-test were applied to compare differences in age, factor VIII and PCAT-NR levels between study and control groups. Multivariate analysis by logistic regression was employed to study the parameters associated with VTE on HT (age, HT, any thrombophilic risk factor and ProC Global assay) and to analyze the parameters that could influence low levels of PCAT-NR (group, age, HT, any thrombophilic risk factor or abnormalities in the protein C pathway). Any known thrombophilic risk factor (FV Leiden mutation,

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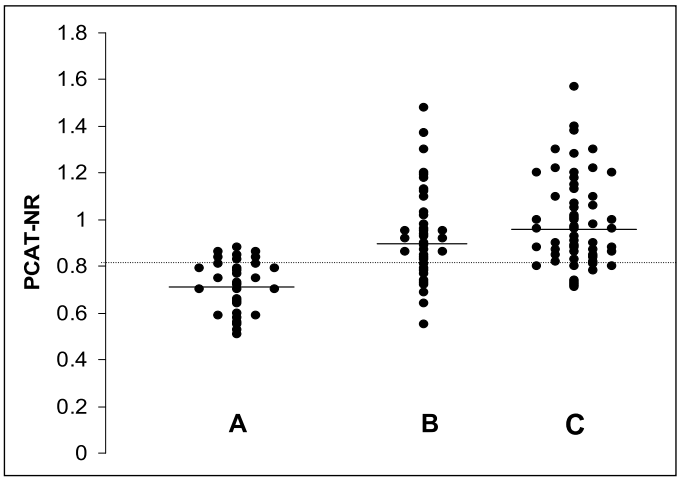

Figure 1: ProC Global assay in women with a history of VTE on HT and control groups. A – Women with a history of VTE on hormonal therapy (HT) (n=32). B – Controls on HT (n=40). C – Controls without HT (n=56). PCAT-NR: protein C activation time – normalized ratio (see *Methods*). Horizontal lines: mean values of the PCAT-NR in study and control groups. Dotted line: cut-off value (0.8).

free protein S antigen<60 u/dl, protein C activity<70 u/dl, factor VIII>150 u/dl, lupus anticoagulant, prothrombin G20210A and MTHFR) was included in the multivariate analysis as one variable. Patients were categorized as either with or without any of the known thrombophilic risk factors. The same categorization was performed for abnormalities in the protein C pathway (FV Leiden mutation, free protein S antigen<60 u/dl, protein C activity<70 u/dl, factor VIII>150 u/dl) which were also added to the multivariate analysis as one variable. Correction for multiple testing (age, HT, group, any thrombophilic risk factors, abnormalities in the protein C pathway and PCAT-NR levels) was performed by the general linear model (GLM) multivariate analysis with Bonferoni adjustment. P<0.05 was considered significant.

Results

In order to determine the diagnostic value of ProC Global assay in women receiving hormonal therapy, we studied 32 patients with a history of VTE while using OC or HRT compared with two healthy control groups. ProC Global assay was performed in the patient group, at least six months after the VTE event without anticoagulant or HT. Mean PCAT-NR levels were significantly lower in the patient group (0.72 ± 0.1) compared with controls without HT matched by age at the time of blood sampling (0.99 ± 0.2) and with controls receiving HT matched by type of HT and age at the time of the VTE event (0.94 ± 0.2) (P<0.001 for both) (Table 1 and Fig. 1).

PCAT-NR lower than the cut-off level of 0.8 was found in 23/32 (72%) patients compared with 5/56 (9%) controls matched

Table 1: ProC Global assay and thrombophilic risk factors in women with a history of VTE on hormonal therapy and control groups.

	P	95% CI	OR	Controls without HT	Patients	Controls with HT	OR	95% CI	P
Number	-	-	-	56	32	40	-	-	-
Age (Y) at patients' VTE event *	-	-	-	-	36.4 ± 17	36.2 ± 13	N.S	N.S	N.S
Age (Y) at blood sampling	N.S	N.S	N.S	42.0 ± 8	42.6 ± 16	-	-	-	-
Thrombophilic risk factor									
FV-Leiden	<0.001	2.5–479	25	1 (1.7%)	9 (28%)	1 (2.5%)	15	1.8–342	<0.001
Free PS antigen <60 u/dl	N.S	N.S	N.S	2 (3.5%)	5 (16%)	7 (17.5%)	N.S	N.S	N.S
PC activity<70 u/dl	-	-	-	-	2 (6%)	-	-	-	-
Factor VIII u/dl mean ± SD	<0.001	-	-	76 ± 28	134 ± 60	83.5 ± 27	-	-	0.001
Lupus anticoagulant	0.02	1.06–242	10	1 (1.7%)	5 (16%)	2 (5%)	N.S	N.S	N.S
Prothrombin G20210A	N.S	N.S	N.S	3 (5.3%)	4 (12.5%)	2 (5%)	N.S	N.S	N.S
MTHFR	N.S	N.S	N.S	6 (10.7%)	4 (12.5%)	4 (10%)	N.S	N.S	N.S
Any thrombophilia	<0.001	2.1–10	6	12 (21.4%)	20 (62.5%)	12 (30%)	4	1.3–12	0.001
Combined thrombophilia	0.001	2.1–413	18	1 (1.7%)	8 (25%)	3 (7.5%)	4	0.9–22	0.05
ProC Global assay									
PCAT-NR mean ± SD	<0.001	-	-	0.99 ± 0.2	0.72 ± 0.1	0.94 ± 0.2	-	-	<0.001
PCAT-NR < 0.8	<0.001	7.0–106	26	5 (9%)	23 (72%)	9 (22.5)	9	2.7–30	<0.001
PCAT-NR < 0.8 without thrombophilia	-	-	-	-	8 (25%)	4 (10%)	N.S	N.S	N.S

* Age at patients' VTE event vs. age at blood sampling of controls with HT. N.S – Not significant, PS – Protein S, PC – Protein C, MTHFR – Methylen –tetrahydrofolate reductase, HT- hormonal therapy. OR, 95%CI and P values of patients vs. controls without HT are located in the left side of the table and values of patients vs. controls with HT values are on the right side of the table.

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by age at the time of blood sampling and 9/40 (22.5%) controls matched by HT and age at the time of a VTE event (P<0.001 for both) (Table 1).

At least one thrombophilic risk factor was revealed in 62.5% of women with a history of VTE while using HT, compared with 21.4% of controls without HT and 30% of controls with HT (Table 1). The high prevalence of thrombophilic risk factors in the controls on HT is probably due to low protein S levels (17.5%) caused by the HT (Table 1). Among the detected thrombophilic risk factors, FV Leiden (28%) was found to be associated with the patient group (Table 1). Lupus anticoagulant was more common in the patient group (16%) compared with controls, but this reached statistical significance only when compared to the controls without HT (P=0.02) (Table 1). Protein C deficiency was detected in two patients but not in controls.

Significantly higher levels of factor VIII were documented in the patient group (134 ± 60 u/dl) compared to the controls with HT (83.5 ± 27 u/dl) or without HT (76 ± 28 u/dl) (P=0.001 or P<0.001, respectively) (Table 1). Elevated factor VIII levels (>150 u/ml) were detected in five patients but not in controls.

No differences were found in the prevalence of either homozygosity to MTHFR or carriers of the prothrombin G20210A mutation between patient and control groups (Table 1).

Combined thrombophilic risk factors were revealed in 25% of the patients compared with 1.7% of controls without HT (P=0.001) and 7.5% of controls with HT (P=0.05) (Table 1).

ProC Global assay successfully detected most abnormalities in the protein C pathway in the patient group; two women with low levels of protein C, five with low free protein S antigen levels, five with elevated factor VIII levels and eight heterozygotes for FV Leiden mutation. One heterozygote for FV-Leiden had normal PCAT-NR, probably because of high protein C levels (244 u/ml) (Fig. 2).

In relation to the control groups, while ProC Global assay successfully identified two heterozygotes for FV Leiden mutation, it failed to reveal low levels of free protein S. Normal PCAT-NR levels were detected in six controls on HT and one without HT, all of them having low levels of free protein S. Abnormal levels of PCAT-NR (lower than the cut-off level of 0.8) associated with low levels of free protein S were found only in one control on HRT and one without HT that was also found to be a heterozygote for prothrombin 20210 mutation. Of interest, all six controls with low free protein S and normal PCAT-NR levels were on third generation OC.

Low PCAT-NR levels were found in eight out of 12 patients without a known thrombophilic risk factor (67%) (Fig. 2), in four controls on HT and in none of the controls without HT (Table 1).

Applying logistic regression multivariate analysis for the variables that are risk factors for VTE, revealed that the ProC Global assay was the parameter mostly associated with the patient group [Exp(B)=15.8, 95% CI: 4.2–59.0, P<0.001]. In this statistical model, age, HT and any thrombophilic risk factor were not found to be associated with the patient group (P=0.2).

Furthermore, studying the parameters that could influence PCAT-NR levels by the same type of multivariate analysis, revealed that the VTE event was the parameter most associated with low levels of PCAT-NR [Exp(B)=13.2, 95% CI: 4.0–48.0, P<0.001]. Abnormalities in the protein C pathway were also found to be associated with low levels of PCAT-NR [Exp(B)=4.8, 95% CI: 1.5–15.2, P=0.007]. In this statistical model, age and HT were found not to influence low levels of PCAT-NR (P=0.2 and P=0.1, respectively).

In addition, correction for multiple testing by the GLM multivariate analysis with Bonferroni adjustment for age, HT, group, any thrombophilic risk factor and any abnormality in the protein C pathway revealed that the patient group was the only parameter significantly associated with PCAT-NR levels (P=0.006).

Discussion

The aim of the current study was to evaluate the ProC Global assay in women with a history of VTE on OC or HRT.

Abnormal ProC Global assay results were found by the multivariate analysis to be the variable mostly associated to women with a history of VTE while on HT, suggesting that ProC Global assay can potentially serve as a diagnostic tool for evaluating the risk for VTE in women prior to HT administration. Moreover, within this group of patients with a history of VTE on HT, abnormal PCAT-NR levels were documented with the same proportions in patients with thrombophilia (75%) and those without a known thrombophilic risk factor (67%), suggesting that abnormal results of the ProC Global assay may be considered as a new risk factor in women with or without thrombophilia who are at risk of developing VTE on HT.

Previous studies also demonstrated the association between abnormal ProC Global assay results and the first episode of VTE (15, 20), recurrent VTE (16) and vascular pregnancy complications (17, 18). Furthermore, these trials also showed that 40–50% of patients with abnormal PCAT-NR levels had no known thrombophilic risk factor. This observation could be the

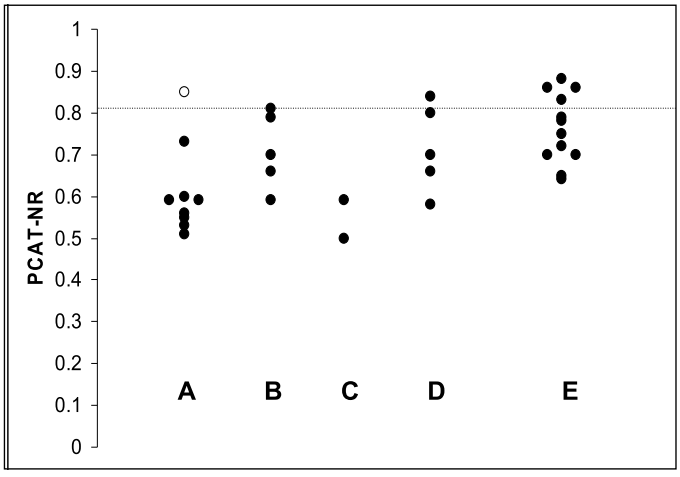

Figure 2: ProC Global assay in women with a history of VTE on HT. A – Women with FV Leiden mutation (n=9). B – Women with free protein S antigen<60 u/dl (n=5). C – Women with protein C activity<70 u/dl (n=2). D – Women with lupus anticoagulant (n=5). E – Women without thrombophilic risk factor (n=12). Open circle – Woman Heterozygote for FV Leiden and protein C activity level of 244 u/dl. PCAT-NR: protein C activation time – normalized ratio (see *Methods*). Dotted line: cut-off value (0.8).

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result of an as yet undetermined abnormality in the protein C pathway or the net effect of the interaction of borderline levels of known protein C pathway components, but this needs to be further investigated.

The current study also supports the association between thrombophilic risk factors and VTE in women on HT, particularly with regard to FV Leiden, protein C deficiency, elevated levels of factor VIII and lupus anticoagulant.

A recent meta-analysis found that the reported OR for VTE among women with FV Leiden who were HT users, ranged from 6 to 35 (21), which is in close proximity to our results (Table 1). In contrast, in the current study, protein S deficiency was not found to be more common in patients compared with controls on HT.

ProC Global assay successfully detected all the known protein C pathway abnormalities in the patient group, including FV Leiden, low protein C and protein S levels, and elevated factor VIII levels. However, normal PCAT-NR was detected in one heterozygote for FV Leiden with high protein C level, which probably masked the PCAT-NR results.

In the controls, while ProC Global assay successfully detected two heterozygotes for FV Leiden, it failed to reveal low levels of free protein S in women on third generation OC.

The present study demonstrates that abnormal PCAT-NR levels are associated with low levels of free protein S in patients, but not in healthy women on third generation OC. This observation is in agreement with a previous publication that demonstrated a reduced ProC Global assay sensitivity to low levels of protein S in asymptomatic subjects (55%) compared with higher sensitivity in those who had a thrombotic event (92%) (22).

No differences were observed in the mean PCAT-NR values between controls with and without HT, leading to the assumption that ProC Global assay results in the current study were not affected by intake of HT, differing from the increased resistance to APC, which was demonstrated by the global thrombin generation assay in healthy women under OC administration (23). Despite the observed insensitivity of the ProC Global assay to HT, abnormal results of this assay were found to be associated to VTE on HT. The current study was designed to investigate ProC Global assay in patients with a history of VTE while using HT and not to study the effect of HT on ProC Global assay. This issue can be further investigated by crossover studies.

A potential limitation of the current study is the size of the patient group, especially when considering the subgroups of patients on different types of OC or HRT. However, the significant differences in PCAT-NR levels between patients and the two healthy control groups, one matched by age at the time of blood sampling and the other paired by the type of HT and age at the time of patients' VTE event, strengthen the validity of our study.

An additional potential limitation is the multiple tests of all the known thrombophilic risk factors and the ProC Global assay that were performed in this study. However, in the critical multivariate analysis, all the known thrombophilic risk factors were combined into a single variable – "any thrombophilic risk factor". Therefore, the statistical analysis included actually only five independent variables and one dependent variable, and correction for multiple testing (beyond the Bonferroni adjustment) was deemed unnecessary.

Identification of women at risk of thrombosis before exposure to HT, i.e. OC or HRT, is important for prevention of VTE events. Although the screening of women for thrombophilia prior to prescribing HT still remains debatable (21, 24), application of global assays is expected to have the potential to improve cost-effectiveness in this setting. ProC Global assay is a global coagulation test that evaluates the protein C anticoagulant pathway and has emerged as an important test in the evaluation of the thrombophilic risk for VTE (15, 16, 20), and according to the current results may serve as an important test before prescription of HT.

In conclusion, abnormal PCAT-NR levels are associated with patients with a history of VTE while using HT. ProC Global assay may potentially serve as a diagnostic tool for evaluating the risk for VTE in women prior to administration of HT. Further studies comparing the ProC Global assay with established diagnostic markers are required before this assay can be recommended as a diagnostic tool in this setting.

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