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Activated protein C resistance among postmenopausal women using transdermal estrogens: importance of the progestogen.

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Running title: progestogens and haemostasis

Abstract

Introduction. While the route of estrogen administration is known to be an important determinant of the thrombotic risk among postmenopausal women using hormone therapy, recent data have shown that norpregnane derivatives but not micronized progesterone would increase venous thromboembolism risk among transdermal estrogens users. However, differential effects of progesterone and norpregnanes on haemostasis have not yet been investigated.

Methods. We set up a cross-sectional study among healthy postmenopausal women aged 45 to 70 years. The impact of Activated Protein C (APC) on endogenous thrombin potential was investigated in plasma samples of 108 women who did not use any hormone therapy (n=40) or who were treated by transdermal estrogens combined with micronized progesterone (n=30) or norpregnane derivatives (n=38).

Results. After exclusion of women with factor V Leiden and/or G20210A prothrombin gene mutations, there was no significant change in APC sensitivity among women who used transdermal estrogens combined with micronized progesterone compared to non-users. Women using transdermal estrogens combined with norpregnanes were less sensitive to APC than were non-users (p=0.003) or users of transdermal estrogens combined with micronized progesterone (p=0.004). In addition, prothrombin fragment 1+2 concentration was higher in users of transdermal estrogens plus norpregnanes than in non-users (p=0.004). Other haemostatic parameters did not vary significantly across the different subgroups.

Conclusion. Transdermal estrogens combined with norpregnanes may induce an APC resistance and activate blood coagulation. These results provide a biological support to epidemiological data regarding the potential thrombogenic effects of norpregnanes. However, these findings need to be confirmed in a randomized trial.

Key words: Activated Protein C resistance, Haemostasis, postmenopausal hormone therapy, progestogens

Introduction

Venous thromboembolism (VTE), including deep vein thrombosis and pulmonary embolism, is one of the major harmful effects of hormone therapy use among postmenopausal women [1, 2]. Both observational studies and randomised clinical trials have shown that oral estrogens increased the risk of venous thromboembolism [3]. However, the ESTHER Study has recently suggested that transdermal estrogens might be safe with respect to thrombotic risk [4]. In addition, the type of progestogens might also be an important determinant of the thrombotic risk in women using combined estrogens [5]. In this case/control study, as well as in the E3N prospective cohort study, norpregnane derivatives, including nomegestrol acetate and promegestone could be thrombogenic. By contrast, micronized progesterone and pregnane derivatives were not associated with an increased thrombotic risk [5, 6]. Activated Protein C (APC) resistance, with or without associated with the presence of the factor V Leiden mutation, is a well established risk factor for venous thromboembolism [7, 8]. Randomized clinical trials have demonstrated that oral but not transdermal estrogens activated blood coagulation [9, 10] and induced an APC resistance state [10, 11], providing biological support to the differential association of oral and transdermal estrogens with VTE risk. However, whether or not the progestogen component of hormone therapy may play a role in haemostasis remains unclear. Therefore, we investigated the impact of micronised progesterone and norpregnane derivatives on haemostasis parameters in a cross sectional study among healthy postmenopausal women using transdermal estrogens.

Subjects and Methods

Study design

The SNAC (Study of NorpregnAnes on Coagulation) Study was a cross sectional study performed in France in a health care center (IPC, Paris) between 2006 and 2007 among healthy postmenopausal volunteers women aged 45 to 70 years who did not use any hormone therapy or who were treated by transdermal estrogens combined with either micronized progesterone or norpregnane derivatives. Menopause was defined by amenorrhea for more than 12 months, bilateral ovariectomy, or hysterectomy and age older than 52 years.

Exclusion criteria were anticoagulant treatment, personal history of thrombotic events (self-reported history of deep venous thrombosis or pulmonary embolism), arterial disease (self-reported history of myocardial infarction, coronary insufficiency, stroke, arterial occlusive disease) or cancer.

Overall, we screened 1652 women who came voluntarily in the Health Care Center during the recruitment period. We excluded women who were not menopausal (n=654), women who were younger than 45 years or older than 70 years (n=201), women who presented an exclusion criteria (n=147) and women who used a hormone therapy different than transdermal estrogens combined with progesterone or norpregnanes (n=110). On the 540 reminding postmenopausal women (470 non-users, 31 progesterone users and 39 norpregnanes users), 11 women, including 9 non-users, 1 progesterone user and 1 norpregnanes user, refused to participate to the study (rate of 2 % similar between subgroups). The final sample consisted of 529 subjects including women treated by transdermal estrogens combined with progesterone (n=30) or norpregnanes (n=38) and 461 non-users. Among the non-users, one subject was randomly included as a control when we included one user. The final control sample consisted of 41 women.

All participants answered a standardized questionnaire concerning their demographic background, medical history, drug use and personal habits such as smoking and alcohol consumption.

Blood pressure was assessed three times on the right arm after 10-min rest. Height and weight of the subjects were systematically measured. Body mass index (BMI) was expressed as the ratio of the weight (kg) to the square of the height (m^2).

Women were classified into several groups according to hormone therapy use and its type. Women who did not use any hormone therapy during the last 3 months were included as non-users. Current users had to be treated by hormone therapy at the time of blood sampling or to have interrupted their treatment less than 3 months prior. Treated women were included as users of transdermal estrogens combined with either micronized progesterone or norpregnane derivatives, including nomegestrol acetate and promegestone. Estrogens were exclusively 17β-estradiol using at 50 µg daily dose. Women who were prescribed micronized progesterone took either 100 mg per day during the entire month or 200 mg per day 12 days a month. Nomegestrol acetate or promegestone were used either during the entire month at the daily dose of 2.5 mg and 250 µg respectively, or 12 days a month at an average dose of 5 mg and 500 µg per day respectively.

The study protocol was approved by an ethical committee. Written consent was obtained from all participants.

Blood samples

Venous blood was drawn between 08.00 h and 10.00 h after overnight fasting and 10 min rest. Cholesterol, triglycerides and glucose measurements were performed immediately following blood sample. For coagulation measurements, venous blood (nine volumes) was collected in 5 mL vacutainer tubes containing 0.105 mol/L trisodium citrate (one volume). Platelet-poor plasma (PPP) was obtained by two centrifugation steps at 2500 g for 15 min at 15 °C. Small aliquots (400 µl) were transferred into plastic tubes, quickly frozen and stored at - 40 °C. Venous blood was also collected in tubes containing 0.084 mL 15% EDTA for DNA extraction.

Haemostasis measurements

Thrombin generation in the presence or absence of APC was measured according to the method described by Hemker et al. [12] on a Fluoroscan Ascent fluorometer (Thermolabsystems OY, Helsinki, Finland), using PPP reagent (a mixture of phospholipids (PL) and tissue factor (TF)), thrombin calibrator and fluorogenic substrate FluCa (Thrombinoscope BV, Maastricht, The Netherlands), mainly following the manufacturer's instructions. Briefly, thrombin generation was triggered in 80µl citrated plasma by the addition of 20µl PPP reagent and 20µl of the fluorogenic substrate FluCa in the presence of 5µl APC 10µg/ml (Hyphen Biomed, Neuville

s/Oise, France) or 5µl NaCl 0.15M. Final concentration of PL and TF in the mixture was 3.9µM and 4.8pM respectively. Each thrombin generation measurement was calibrated against the fluorescence curve obtained under the same conditions except for PPP reagent which was replaced by a thrombin calibrator (Thrombinoscope BV). A pool of plasma samples from healthy subjects processed in the laboratory and stored frozen at -80°C was used to normalize the results. Inter-assay coefficients of variation calculated on the results obtained for the pool in 2 series of respectively 7 and 6 independent runs were less than 10% for the concentration-dependent parameters (Endogenous Thrombin Potential (ETP) and peak height).

For each plasma sample, a normalised APC sensitivity ratio ($APCsr_{ETP}$) was calculated by dividing the ratio of the ETP obtained in the presence of APC (ETP_{+APC}) and in its absence (ETP_{-APC}) by the ratio of the ETP_{+APC} and ETP_{-APC} determined in normal pooled plasma in the same experiment. A high $APCsr_{ETP}$ indicates an APC resistance.

Commercially available kits based on ELISA methods were used for measuring prothrombin fragment 1+2 (Enzygnost F1+2 micro, Behring), free Tissue Factor Pathway Inhibitor (TFPI) antigen (Asserachrom free TFPI, Stago) and Endothelial Protein C Receptor (EPCR) (Asserachrom EPCR, Stago). The other parameters (Factor VIII, fibrinogen, antithrombin (AT) activity, Protein S (PS) activity) were measured using reagents (FVIII deficient plasmas and Neoplastine, STA fibrinogen, STA Stachrom ATIII, STA Staclot PS) from Stago on a STAR analyser (Stago).

DNA isolation and genotyping

Genomic DNA was extracted from EDTA blood samples using a standard method. Screening for the FV Leiden mutation and the G20210A prothrombin mutation was performed as previously described [13].

Statistical analysis

Based on a previous study [10], we estimated that 40 subjects per group were needed to detect a difference between groups of about two third SD for the rPCA distribution with a 5% two-sided level and a 80% statistical power.

Distribution of each parameter was tested for normality. Mean levels are given in arithmetic form. Analysis of variance was used to compare the baseline

characteristics of the subjects. Multiple comparison procedures were used to assess the differences in haemostatic variables between users and non-users of hormone therapy. Bonferroni correction for multiple testing was applied and treatments were significantly different at the 0.05 level if there was a p value less then 0.016 within the subgroups. Stepwise multiple linear regression models were used to assess the relative contribution of the haemostatic variables to the prediction of APC sensitivity.

All statistical analyses were performed with the Statistical analysis System (SAS) software, version 9.1(SAS Institute, Inc., Cary, NC, USA).

Results

Table 1 shows the general characteristics of women according to hormone therapy use. Blood samples were obtained from 108 women, of whom 40 were non-users, 30 used transdermal estrogens combined with micronized progesterone and 38 were treated by transdermal estrogens combined with norpregnane derivatives. A large majority of HT users were treated by gel (n=65) while only 5 women used a patch. Among users of transdermal estrogens combined with norpregnane derivatives, 23 women were treated by nomegestrol acetate and 15 by promegestone.

Overall, 9.3% of women were heterozygous for the factor V Leiden mutation or the 20210 G>A prothrombin gene mutation and the repartition did not significantly differ between groups (p=0.38). Considering the known influence of these thrombogenic mutations on haemostasis, these women were excluded from the statistical analysis. Regarding the general characteristics of the subjects, no significant differences between the groups were found in terms of age, BMI, smoking status, blood pressure or lipidemia. Women who used transdermal estrogens combined with norpregnane derivatives were specifically prescribed these progestogens because of hyperestrogenic syndromes (breast tenderness (n=23) or uterus bleeding (n=12)).

Parameters of thrombin generation according to hormone therapy use are given in the table 2. Inter-group differences were observed for $APCsr_{ETP}$, ETP_{+APC} and Peak $height_{+APC}$. While there was no significant change in $APCsr_{ETP}$ between non-users and women using transdermal estrogens combined with micronized progesterone, women who used transdermal estrogens combined with norpregnane derivatives were significantly less sensitive to APC than both non-users and users of transdermal estrogens combined with micronized progesterone ($p<0.01$). In addition, the norpregnanes-treated group had borderline significant higher levels of ETP_{+APC} and Peak $height_{+APC}$ compared to the two other groups ($p<0.05$ for both ETP_{+APC} and Peak $height_{+APC}$). This pattern reflected the individual correlation of ETP_{+APC} and Peak $height_{+APC}$ with $APCsr_{ETP}$ ($r=0.77$, $p<0.0001$ for ETP_{APC} and $r=0.75$, $p<0.0001$ for peak $height+_{APC}$) and the strong correlation between peak $height+_{APC}$ and ETP_{APC} ($r=0.97$, $p<0.0001$). Other parameters of thrombin generation did not differ across the different groups.

The Table 3 shows the results for others haemostatic parameters measurements according to the hormone therapy use. Women treated by transdermal estrogens combined with norpregnane derivatives had an increased mean value of prothrombin activation peptide (F1+2) compared to non-users ($p<0.01$). Other haemostatic parameters, especially PS activity and free TFPI, did not vary significantly across the different subgroups.

Discussion

Our results showed the importance of the progestogen component in determining the APC resistance state among postmenopausal women using transdermal estrogens. While micronized progesterone did not affect haemostasis, norpregnane derivatives might induce APC resistance and might activate blood coagulation. These data provide a biological support to the recent findings regarding the increased risk of venous thrombosis among postmenopausal women using transdermal estrogens combined with norpregnane derivatives [5, 6].

The effect of progestogens on VTE risk remains largely unknown among postmenopausal women using hormone therapy. However, randomised clinical trials, as well as observational studies, provided some evidence for a more thrombogenic tendency of estrogens associated with progestogens compared to estrogens alone [3, 14-16]. In the Women's Health Initiative (WHI) Clinical trials as well as in the Women's International Study of long Duration Oestrogen after Menopause (WISDOM) trial, there was an increased VTE risk among women receiving opposed estrogens compared with the users of estrogen only. Data regarding the differential association of VTE risk with type of progestogens remain scarce and only two observational studies have so far assessed the thrombotic risk associated with the different pharmacological subgroups of progestogens [5, 6]. Taking into account the difficulties to obtain new clinical data for studying the effect of progestogens on thrombotic risk, assessing their impact on surrogate markers of VTE risk may therefore be useful. In addition, APC resistance has been shown to be a valid marker of VTE risk [17]. Since transdermal estrogens have no or little effect on haemostasis [11, 18-21], assessment of progestogens on blood coagulation appears to be more relevant among postmenopausal women using transdermal estrogen therapy. Previous observational studies and randomised controlled trials have investigated the effect of transdermal estrogens in combination with micronized progesterone [9, 10], medroxyprogesterone acetate [22-24] or nortestosterone derivatives [25-27]. In the randomised controlled trials, transdermal estrogens combined with micronized progesterone did not have an effect either on APC resistance or on prothrombin fragment 1+2 concentration [9, 10] and the present findings are consistent with this previous results. One randomised study showed an increase in prothrombin fragment

1+2 concentration among users of transdermal estrogens combined with medroxyprogesterone [22]. In another trial, nortestosterone derivatives used in transdermal estrogen therapy had little or no effect on blood coagulation activation and haemostasis [25-27]. No data are currently available on the effects of norpregnane derivatives on haemostasis among transdermal estrogens users. However, some studies have investigated their impact in association with oral estrogen therapy among postmenopausal women. While studies have thus far failed to show any changes in levels of haemostatic parameters between norpregnane derivatives and dydrogesterone users [28-30], recent data suggested that trimegestone, a norpregnane derivative, could have a stronger impact on fibrinolysis compared to dydrogesterone [31].

The mechanisms underlying the effects of transdermal estrogens combined with norpregnane derivatives on haemostasis remain unclear. It has been reported that changes in free PS and TFPI might have important roles in determining the sensitivity of plasma to APC in women receiving oral estrogens [10, 11, 32]. We have previously described no influence of transdermal estrogens combined with micronized progesterone on these parameters [10, 33]. However, whether or not transdermal estrogens plus norpregnane derivatives have a significant influence on these factors remains unknown. In the present study, the reduced APC sensitivity observed in women using transdermal estrogens combined with norpregnane derivatives was not mediated by PS or free TFPI, the levels of which were not significantly different from those observed among non-users and among users of transdermal estrogens combined with micronized progesterone. We have also measured plasma levels of the soluble form of EPCR which was previously reported to inhibit APC anticoagulant activity [34] and might therefore be a candidate to explain differences in APC resistance level. However, no significant inter-group differences have been observed. Future research will therefore be necessary to explain the influence of norpregnane derivatives on APC resistance.

The SNAC Study is an observational study and we cannot exclude the possibility that these findings may be due in part to bias, especially selection bias. Norpregnane derivatives, either nomegestrol acetate or promegestone, are potent progestogens which have both high antiestrogenic and antigonadotrophic actions. Thus, these

progestogens may oppose the estrogen proliferation effect. Norpregnane derivatives are therefore preferentially prescribed to women with hyperestrogenic symptoms (endometrial susceptibility, benign breast diseases, …) [35-37]. Since endogenous estrogens exposure has been positively related to VTE among postmenopausal women [38], women with stronger estrogenic climate would be more likely to be at high thrombotic risk. As a consequence, we cannot exclude the possibility that APC resistance - which is observed among women using norpregnane derivatives - might also be related to a hypercoagulability state due to the presence of a hyperestrogenic status rather than exclusively to the deleterious effect of these progestogens on haemostasis. Therefore, this bias could in part explain the increased level of blood coagulation activation and APC resistance among users of norpregnane derivatives. Another selection bias could also occur among users of progesterone which is preferentially used by obese women because of its neutral impact on cardiovascular risk factors. However, in our study, women using progesterone had a similar average BMI than control women and means of haemostatic parameters were adjusted for BMI. Finally, a limitation of our study relates to the extrapolation of results. Our data on norpregnanes were restricted to nomegestrol acetate and promegestone and the results cannot be extrapolated to others norpregnane derivatives, including trimegestone.

Conclusion

Our results emphasize the importance of the progestogen components in hormone therapy on plasma sensitivity to APC resistance and other haemostatic variables. They may serve to provide a biological support to the differences in thrombotic risk by different progestogens variety. Taken together, both clinical [4, 5] and biological data [9, 10] suggest that transdermal estrogens combined with micronized progesterone might be safe with respect to thrombotic risk. However, these findings need to be confirmed in randomized controlled trials.

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Disclosure of conflict of interest

None

Tables

Table 1: General characteristics of women according to hormone therapy

	No hormone therapy (n=40)	Transdermal estrogens + progesterone (n=30)	Transdermal estrogens + norpregnanes# (n=38)
Age (years)	59.5 (5.8)	58.4 (6.1)	58.7 (5.8)
Body-mass index (kg/m²)	24.3 (3.9)	23.3 (2.2)	23.4 (4.1)
Waist/Hip ratio	0.8 (0.1)	0.8 (0.1)	0.8 (0.1)
Natural menopause	39 (98)	30 (100)	37 (97)
Smoking status			
Never users	24 (60.0)	12 (40.0)	22 (58.0)
Past users	11 (28.0)	12 (40.0)	13 (34.0)
Current users	5 (12.0)	6 (20.0)	3 (8.0)
Systolic pressure (mmHg)	125.7 (20.4)	120.6 (16.4)	132.2 (24.2)
Diastolic pressure (mmHg)	74.2 (11.2)	71.2 (9.3)	74.6 (12.5)
Cholesterol (mg/dl)	230.1 (30.8)	223.7 (31.6)	217.9 (31.3)
HDL (mg/dl)	68.7 (15.4)	69.7 (15.7)	71.9 (15.3)
Triglyceride (mg/dl)	80.5 (28.1)	80.0 (36.9)	74.7 (39.8)
FV Leiden mutation	2 (5.0)	1 (3.5)	2 (5.4)
FII 20210 G>A mutation	2 (5.0)	0 (0)	3 (8.1)

Values are mean (SD) or number (percentages)

* Women received either nomegestrol acetate (n=23) or promegestone (n=15)

Table 2: Parameters of thrombin generation according to hormone therapy

	No hormone therapy (n=36)	Transdermal estrogens + progesterone (n=29)	Transdermal estrogens + norpregnanes (n=33)[#]
Lag Time$_{-APC}$ (min)	2.02 (0.38)	1.92 (0.59)	1.79 (0.41)
Lag Time$_{+APC}$ (min)	2.44 (0.77)	2.64 (0.65)	2.82 (0.63)
Peak height$_{-APC}$ (nM IIa)	280.42 (22.71)	281.21 (28.59)	280.61 (27.38)
Peak height$_{+APC}$ (nM IIa)	16.15 (14.47)	15.03 (13.63)	23.79 (18.84) * [£]
ETP$_{-APC}$ (nM/min)	1639 (158)	1668 (196)	1693 (188)
ETP$_{+APC}$ (nM/min)	134 (102)	133 (104)	197 (132) * [£]
APCsr$_{ETP}$	1.00 (0.63)	0.98 (0.72)	1.64 (1.17) ** [££]

Mean values (SD) adjusted for age and body-mass index

[#] Women received either nomegestrol acetate (n=19) or promegestone (n=14)

Comparison to non-user group: * $p<0.05$; ** $p<0.01$

Comparison to the group treated by transdermal estrogens combined with micronized progesterone: [£] $p<0.05$; [££] $p<0.01$

Table 3: Haemostatic characteristics according to hormone therapy

	No hormone therapy (n=36)	Transdermal estrogens + progesterone (n=29)	Transdermal estrogens + norpregnanes[#] (n=33)
Antithrombin (%)	103.4 (7.7)	100.0 (6.1)	104.4 (7.6)
Protein S (%)	103.9 (16.8)	105.9 (16.9)	104.7 (23.6)
Fibrinogen (g/L)	3.3 (0.6)	3.4 (0.6)	3.1 (0.6)
TFPI (ng/mL)	10.1 (5.0)	8.8 (3.2)	8.8 (4.5)
F1+2 (pmol/L)	253.9 (88.8)	284.9 (93.7)	330.0 (129.3) *
Factor VIII (%)	108.3 (38.3)	91.4 (32.3)	99.0 (25.4)
sEPCR (ng/ml)	126.44 (96.70)	130.66 (83.22)	101.48 (54.62)

Mean values (SD) adjusted for age and body-mass index

[#] Women received either nomegestrol acetate (n=19) or promegestone (n=14)

Comparison to non-user group: * $p<0.01$