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ORIGINAL ARTICLE

The differential association of conjugated equine estrogen and esterified estrogen with activated protein C resistance in postmenopausal women

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Summary. *Objectives:* Clinical trials have demonstrated that oral conjugated equine estrogen (CEE) therapy with or without medroxyprogesterone (MPA) increases venous thrombotic risk but this safety issue has not been investigated for other oral estrogens. Based on observational study findings that esterified estrogen (EE) was not associated with venous thrombotic risk whereas CEE was, we hypothesized that CEE users would be more resistant to activated protein C (APC), a prothrombotic phenotype, than EE users. *Methods:* We conducted an observational, cross-sectional study of postmenopausal women 30–89 years old who were controls in a case–control study of venous thrombosis. Use of CEE, EE, and MPA at the time of phlebotomy was determined using computerized pharmacy records. APC resistance was measured in plasma by the endogenous thrombin potential normalized APC sensitivity ratio. Adjusted mean APC resistance values were compared across estrogen type and CEE:EE ratios are presented. *Results:* There were 119 CEE and 92 EE users at the time of phlebotomy. Compared with EE users, CEE users had APC resistance measures that were 52% higher (1.52; 95% confidence intervals: 1.07–2.17) in adjusted analyses. Restricting to modal dose users (0.625 mg) and stratifying by MPA use did not materially change associations. *Conclusions:* CEE use was associated with higher levels of APC resistance when compared with EE use in postmenopausal women. These findings might provide an explanation for the higher risk of venous thromboembolism previously observed with CEE compared with EE use and, if replicated, may have safety implications for women when choosing an estrogen for symptom relief.

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Introduction

Clinical trials have repeatedly demonstrated that oral conjugated equine estrogen (CEE) therapy with or without medroxyprogesterone (MPA) increases venous thrombotic risk, often within the first year of treatment [1–3]. No large clinical trial has evaluated this safety issue for other oral estrogen or progestin compounds that continue to be used to treat menopause-related vasomotor symptoms in peri- and postmenopausal women.

In our population-based, case–control study, women who used oral CEE had an 80% higher risk of venous thrombosis than women who used oral esterified estrogen (EE) [4], a synthetic estrogen derived from yams and soybeans. We also observed that among estrogen users, MPA use compared with non-use increased risk by 60%. Based on these findings, we hypothesized that controls using oral CEE would have a more prothrombotic hemostatic profile than controls using oral EE and that among controls using either estrogen, MPA use would be associated with a more prothrombotic profile compared with non-use. Our primary assessment of a prothrombotic profile was increased resistance to activated protein C (APC), a global measure of anticoagulation that predicts venous thrombotic risk [5–10]. We also assessed five other parameters [factor (F) VII, D-dimer, antithrombin (AT), and total and free protein S] to characterize the hemostatic profile of hormone users [11–13].

Methods

Design and setting

This observational, cross-sectional study was conducted within Group Health Cooperative (GHC), a health maintenance organization in western Washington State, USA.

Subjects

Subjects were postmenopausal women 30–89 years of age who were identified and recruited from January 1, 1995 to December 31, 2001 as control subjects in an on-going case–control study of hormone therapy and risk of venous thrombosis [4]. All subjects provided signed informed consent.

Measures

All subjects had been assigned an index date: a computer-generated random date within the same year for which they had been sampled as controls. Medical history and clinical information through the index date, such as menopausal and hysterectomy status, height, and weight, were obtained from GHC ambulatory medical record; self-reported health status and race were collected by telephone interview. Controls who participated in the interview were also invited to donate a blood sample.

Phlebotomy Venous blood was drawn from the antecubital vein and the first 4.5 mL of blood was collected into a tube of 3.8% sodium citrate. Samples were transferred to a specimen-processing center where citrated samples were centrifuged at 4 °C for 10 min at $1300 \times g$ and stored at −70 °C. Samples that were processed and frozen outside a 4-h window were excluded from analyses.

Hormone use Use of hormones at the time of phlebotomy was determined using the GHC computerized pharmacy database that contains information on drug name, prescription fill date, medication strength and quantity prescribed, and dosing instructions or the days supply of medication. More than 95% of GHC members in this age group fill all or almost all their prescriptions through GHC pharmacies [14].

A woman was considered a current user of a hormone if she received enough medication with her last prescription to last until the phlebotomy date and if she also had accumulated at least 90 days of use immediately prior to the phlebotomy date. The type of estrogen a woman was using at phlebotomy was determined primarily by when blood was drawn. Women who had draws prior to October 1999 were primarily EE users as EE was the standard postmenopausal estrogen therapy dispensed at GHC pharmacies in this time period. GHC pharmacies switched the standard estrogen therapy for current and new users from EE to CEE in October 1999 so women with blood draws after the switch date were primarily CEE users. Formulary switches such as these occur in health maintenance organization settings when medications are thought to be therapeutically interchangeable [15–17]. Progestin use was primarily determined by hysterectomy status.

Oral estrogens were classified into two groups: CEE, such as Premarin® (Wyeth Pharmaceuticals Inc., Philadelphia, PA, USA); and EE, such as Estratab® (Solvay Pharmaceuticals, Marietta, GA, USA) and Menest® (Monarch Pharmaceuticals, Inc., Bristol, TN, USA). The progestin prescribed was almost exclusively MPA and was dispensed as a separate pill from estrogen in virtually all subjects. Women who used non-oral estrogens and oral hormones other than CEE, EE, and MPA at the time of the blood draw were excluded from the study. Any subject who received a prescription for anticoagulant or antiplatelet medications or vitamin K in the 180 days before phlebotomy was excluded.

Laboratory assays Citrated plasma was shipped at −70 °C from Seattle, WA, USA, to Leiden, the Netherlands. Plasma was thawed and centrifuged at $14\,000 \times g$ for 5 min and half the content was immediately used to measure APC resistance using the endogenous thrombin potential (ETP) normalized APC sensitivity ratio (nAPCsr) assay [6,18]. The unused content was refrozen at −70 °C for the remaining five assays. The normalized APC sensitivity ratio is the ratio of the amount of thrombin generated in the absence of APC and the quantity generated in the presence of APC in plasma free of fibrinogen and then normalized to the same ratio derived from the plasma of normals [7]. All laboratory normals had APC measured in 3.2% citrated plasma (new laboratory standard) whereas all subjects had APC measured in 3.8% concentration so APC sensitivity ratios are less than 1. Coagulation FVII activity was determined by clotting time (STA Factor VII; Diagnostica Stago, Asnières, France); D-dimer was measured by enzyme immunoassay (Asserachrom D-Dimer; Diagnostica Stago); AT activity was measured by thrombin neutralization (STA Antithrombin III; Diagnostica Stago); and total and free protein S antigen levels were measured separately (Liatest® Protein S and Liatest® Free Protein S; Diagnostica Stago). All tests were done in duplicates. Lab technicians were blinded to the type of hormone therapy used by the subjects. The inter-assay coefficient of variation for ETP-nAPCsr, FVII, D-dimer, AT, and protein S total and free were 6.4%, 7.0%, 4.7%, 3.2%, 3.6%, and 5.0%, respectively. High values for ETP-nACPsr, FVII and D-dimer and low values for AT and total and free protein S indicated a more prothrombotic profile. Hemostatic factor levels were measured in 109 control women who were not using estrogen and who are not included in these analyses; median levels were 0.22 for nAPCsr, 132% for FVII, 578 ng mL^{-1} for D-dimer, 111% for AT, 96% for free protein S and 123% for total protein S.

Carriers of the FV 1691A (Leiden) variant were identified using standard methods [19].

Analyses We compared levels of hemostatic measures across hormone regimens using multivariate linear regression with adjustment for age, race (white vs. other), body mass index [weight (kg) height $(m)^{-2}$], self-reported health (good, very

good, or excellent vs. poor or fair), hysterectomy status, MPA use, estrogen dose [indicator variables for low dose (< 0.625 mg day^{-1}) and high dose (> 0.625 mg day^{-1})], and recency of starting current therapy (less than 2 years vs. 2 or more years). All measures were log transformed and differences in hemostatic measure levels are reported as a CEE : EE ratio or a MPA : no-MPA ratio of the adjusted medians with 95% confidence intervals (CI). In secondary analyses, we examined associations restricted to users of the modal estrogen dose (0.625 mg day^{-1}) and among subgroups defined by MPA use, FV Leiden (FVL) carriership, and recency of starting hormone therapy.

Results

We identified 71 women using CEE alone, 45 using CEE + MPA, 45 using EE alone, and 47 using EE + MPA at the time of phlebotomy and who met all eligibility criteria. Table 1 presents characteristics of the 208 subjects in the study and hormone duration and dose information. Table 2 presents unadjusted median values and interquartile range values for the six hemostatic measures according to estrogen type and MPA use. In descriptive analyses, median values of nAPCsr and FVII activity appeared higher and median values of AT activity, total and free protein S lower in CEE users than in EE users. In general, median values did not appear to vary by MPA use.

Table 3 presents unadjusted median values for the six hemostatic measures according to estrogen type. Compared with EE users, CEE users had APC resistance measures that were 52% higher after adjusting for age, race, and body mass index (CEE:EE ratio: 1.52; 95% CI: 1.07–2.17; $P = 0.020$). Further adjustment for hysterectomy status, MPA use, dose, and recency of starting did not materially change estimates (ratio: 1.49; 95% CI: 1.00–2.20; $P = 0.049$). For the remaining five parameters, the hemostatic profile was more prothrombotic among CEE users than EE users (higher levels of FVII and D-dimer, and lower levels of AT, and free and total protein S) but not all differences reached statistical significance.

We further investigated the CEE and EE comparison by investigating subgroups. The FVL mutation was strongly associated with increased resistance to APC: mean APC resistance measures were 0.77 and 2.91 among those without and with the mutation, respectively. Among women without the mutation, CEE users remained more resistant to APC than EE users (ratio: 1.54; 95% CI: 1.07–2.23; $P = 0.021$); among women with the mutation, the association was similar (ratio: 1.30; 95% CI: 0.70, 2.41; $P = 0.362$). There was no statistical evidence of an interaction between estrogen type and the

Table 1 Subject characteristics by hormone use

	Hormone therapy			
Characteristic	CEE alone ($n = 71$)	CEE + MPA ($n = 45$)	EE alone ($n = 45$)	EE + MPA ($n = 47$)
Mean age, years (SD)	68.3 (10.0)	65.4 (10.2)	66.6 (9.7)	63.3 (9.4)
White (%)	92	89	96	96
Good/excellent health (%)	82	89	86	91
Body mass index (SD)	29.1 (6.3)	26.9 (5.4)	27.5 (6.3)	30.4 (9.3)
Hysterectomy (%)	92	2	87	0
Perimenopausal (%)	1	9	0	13
Factor V Leiden carrier (%)	8	9	9	9
Starting estrogen use within 2 years (%)	61	58	24	34
Estrogen daily dose (%)				
< 0.625 mg	13	4	13	15
0.625 mg	69	89	82	85
> 0.625 mg	18	7	4	0

EE, esterified estrogen; CEE, conjugated equine estrogen; MPA, medroxyprogesterone; SD, standard deviation.

Table 2 Unadjusted median and interquartile range values of hemostatic factors by estrogen type and MPA use

	Hormone therapy			
Hemostatic parameter	CEE alone ($n = 71$)	CEE + MPA ($n = 45$)	EE alone ($n = 45$)	EE + MPA ($n = 47$)
ETP-nAPCsr	0.86 (0.26–1.44)	0.81 (0.51–1.65)	0.49 (0.27–1.18)	0.36 (0.21–0.83)
FVII activity (%)	140 (127–167)	125 (102–148)	126 (118–151)	122 (110–136)
D-dimer (ng mL^{-1})	474 (362–664)	524 (367–788)	509 (390–724)	414 (289–679)
AT activity (%)	104 (99–110)	103 (98–108)	107 (99–115)	108 (100–115)
Free protein S (%)	89 (76–99)	88 (74–102)	90 (77–102)	98 (77–116)
Total protein S (%)	110 (100–120)	100 (93–117)	112 (102–120)	117 (102–128)

AT, antithrombin; CEE, conjugated equine estrogen; EE, esterified estrogen; ETP-nAPCsr, endogenous thrombin potential normalized activated protein C sensitivity ratio; FVIII, factor VIII; MPA, medroxyprogesterone.

Table 3 Unadjusted median hemostatic factor values by estrogen type and adjusted ratios

Hemostatic parameter	Estrogen type comparisons		
	CEE median (IQ range) ($n = 116$)	EE median (IQ range) ($n = 92$)	Adjusted* CEE:EE ratio (95% CI), *P*-value
ETP-nAPCsr	0.85 (0.37–1.54)	0.46 (0.21–1.07)	1.52 (1.07–2.17), 0.020
FVII activity (%)	137 (116–160)	124 (116–146)	1.07 (0.99–1.14), 0.073
D-dimer (ng mL^{-1})	490 (362–683)	473 (346–698)	1.01 (0.89–1.14), 0.856
AT activity (%)	103 (98–110)	107 (100–115)	0.97 (0.94–1.00), 0.027
Free protein S (%)	89 (74–101)	91 (77–109)	0.95 (0.90–1.01), 0.115
Total protein S (%)	107 (97–118)	113 (102–125)	0.95 (0.91–0.99), 0.008

AT, antithrombin; CEE, conjugated equine estrogen; CI, confidence interval; EE, esterified estrogen; ETP-nAPCsr, endogenous thrombin potential normalized activated protein C sensitivity ratio; FVIII, factor VIII; IQ, interquartile; MPA, medroxyprogesterone. *Adjusted for age, race, and body mass index.

mutation ($P = 0.652$). Among the 175 women without the FVL mutation, median nAPCsr measures for low, modal, and high CEE doses were 0.69, 0.76, 0.74, respectively, and were 0.42, 0.42, 1.06 for EE doses, respectively. Eighty per cent of women were using a modal dose (Table 1). Among the 151 women without the FVL mutation and using the modal dose of estrogen (0.625 mg), we again found that CEE users were more resistant to APC than EE users (ratio: 1.57; 95% CI: 1.04–2.36; $P = 0.030$). When data were stratified by MPA use among women without the FVL mutation, CEE users remained more resistant to APC than EE users both among women not using MPA (ratio: 1.22; 95% CI: 0.69–2.26; $P = 0.495$) and among women using MPA (ratio: 2.15; 95% CI: 1.35–3.42; $P = 0.002$). Although the point estimate was larger among MPA users, there was no statistical evidence of an interaction between estrogen type and MPA use ($P = 0.212$). Recency of starting either EE or CEE (< 2 year vs. > 2 years) was not associated with APC resistance and there was no suggestion of an interaction between estrogen type and recency ($P = 0.908$).

The median daily dose of prescribed MPA was 2.5 mg: 4% of women were using lower doses and 25% were using higher doses. The use of MPA was not associated with increased resistance to APC among CEE users (MPA:no-MPA ratio: 1.36; 95% CI: 0.83–2.23; $P = 0.220$) nor among EE users (ratio: 0.90; 95% CI: 0.52–1.55; $P = 0.689$) after adjusting for age, body mass index, and race. The use of MPA was associated with lower FVII levels in both CEE (ratio: 0.91; 95% CI: 0.82–1.00; $P = 0.047$) and EE (ratio: 0.85; 95% CI: 0.85–0.93; $P = 0.001$) users. For the remaining four hemostatic measures, no clear or consistent differences were found.

Discussion

Previously, we reported that CEE use in postmenopausal women was associated with an increased risk of venous thrombosis compared with EE use [4]. To support this finding, we have investigated the hemostatic profile in healthy users of both types of hormones and found that CEE users were more resistant to APC and had a more prothrombotic hemostatic profile than EE users. Restricting analyses to women using only modal doses of CEE and EE did not change the interpretation nor did stratification by MPA use. We also found that CEE users generally had lower AT and total and free protein S levels and higher FVII levels than EE users and these findings are similar to published observations of APC resistance phenotype [10]. We also had reported that MPA use was associated with an increased risk of venous thrombosis but we did not find that MPA use was associated with a more prothrombotic hemostatic profile than non-use of MPA.

Several limitations should be considered. The use of hormone therapy was not randomly assigned. Women and their physicians chose whether to use hormones according to clinical indication, which could induce confounding. The type of estrogen received, however, was dictated primarily by changes over time in the GHC formulary and not by patient or physician choice. Although we adjusted for patient characteristics that may be associated with hemostatic parameter levels and hormone use, residual confounding by these or other unmeasured characteristics may be present.

Equivalence of estrogen exposure relied exclusively on daily dosing and not on a standard of bioequivalence of the estrogenic compounds in CEE and EE. Conjugated equine estrogens contain 10 known biologically active estrogen compounds as well as others that are not characterized [20,21]. The primary estrogen compounds in CEE are estrone sulfate, constituting ~53% of the estrogens, and equilin sulfate, constituting about ~25% of the product [21,22]. Esterified estrogens contain ~80% estrone sulfate and ~11% equilin sulfate [22]. There is a large literature demonstrating prothrombotic characteristics of oral CEE and estradiol estrogen use [11,12,23–30], but a comparable literature for EE use does not exist. The mechanism by which some estrogens increase thrombotic risk is not known. Our data do not suggest a strong role for protein S, AT, or FVII as estrogen-type differences were generally not strong, although some were statistically significant. It has been hypothesized that tissue factor pathway inhibitor and its influence on activated FX levels may underlie estrogen's role in modulating nAPCsr levels [31]. Estrogen has been shown to decrease levels of glucosylceramide, which serves as a co-factor for APC [32].

The prothrombotic characteristics of MPA are not known. The lack of an association between MPA use and APC resistance suggests that if MPA is associated with increased thrombotic risk, as was observed in clinical trials [2,3], it may

operate through a different biologic pathway. The MPA comparison, however, is nearly completely confounded by hysterectomy status, which limits the interpretation of these findings. Comparison of MPA use with other progestin types among women with an intact uterus would be a clinically more informative investigation.

Menopausal women continue to use hormones to treat vasomotor symptoms, and venous thrombosis remains the most serious side effect associated with short-term use. Our data demonstrate that CEE use was associated with higher levels of APC resistance when compared with EE use in postmenopausal women. These findings need to be replicated in a clinical trial of women seeking medicinal relief from vasomotor symptoms related to menopause to understand better the relative safety and effectiveness of these drugs. If replicated, they may have health-related implications for women and their physicians when choosing an estrogen for symptom relief.

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Addendum

Drs Smith, Heckbert, Doggen, Lemaitre, Lumley, Psaty, and Rosendaal were involved in the planning of the study. Drs Smith, Heckbert, Lemaitre, Lumley and Psaty were responsible for data collection and Drs Smith, Doggen, Meijers, and Rosendaal were responsible for laboratory analyses. Statistical analyses were the primary responsibility of Drs Smith and Lumley and all authors were involved with data interpretation. Dr Smith drafted the manuscript and all eight co-authors provided substantial scientific contribution to revisions.

Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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