Menopausal Hormone Therapy, Blood Thrombogenicity and Development of White Matter Hyperintensities in Women of the Kronos Early Estrogen Prevention Study

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Abstract

Objective: Development of white matter hyperintensities (WMH) in the brain is associated with blood thrombogenicity in recently menopausal women. This study examined the influence of menopausal hormone treatments (MHT) on this association.

Methods: Measures of blood thrombogenicity were examined in women of the Kronos Early Estrogen Prevention Study (n=95) who had brain magnetic resonance imaging prior to and during the 48 months of randomization to transdermal 17β-estradiol (n=30), oral conjugated equine estrogen (oCEE, n=29) both with progesterone for 12 days per month or placebo pills and patch (n=36). Principal components (PC) analysis was used to reduce the dimensionality of 14 markers of platelet activation and blood thrombogenicity. The first 5 PCs were assessed for association with treatment and changes in WMH. Within-person slopes were obtained to capture the extent of WMH change for each woman.

Results: WMH increased in all groups over the 48 months (P=0.044). The partial effect of PC_1, representing an average of 6 thrombogenicity variables (microvesicles derived from endothelium, leukocytes, and monocytes, and positive for tissue factor and adhesion molecules), on WMH was significant (P=0.003). PC_3, reflecting a contrast of platelet microaggregates and ATP secretion vs. total platelet count, differed across groups (P=0.006) with higher scores in the oCEE group. However, the global association between PCs and WMH increase did not differ significantly by MHT (P=0.207 for interaction between MHT and PC's).

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Conclusion: In recently menopausal women, the type of MHT did not significantly influence the association of markers of blood thrombogenicity with development of WMH in the brain.

Keywords

17β-estradiol; conjugated equine estrogen; KEEPS; menopause; postmenopausal; stroke

Introduction

White matter hyperintensities (WMH) on T2-weighted magnetic resonance imaging (MRI) are associated with ischemic small vessel disease (1, 2) and may precede exhibition of mild cognitive impairment (3–5). Conventional cardiovascular risk factors (i.e. age, hypertension, smoking, hyperlipidemia) are proposed to be risk factors for development of WMH, especially in elderly persons (5, 6). However, other factors not traditionally considered as cardiovascular risk factors, specifically, thrombogenic microvesicles, have been implicated in microvascular disease contributing to the formation of WMH (7–9). Thrombogenic microvesicles are blood-borne, cell-membrane derived vesicles that carry surface markers of the cell of origin, as well as phospholipids and proteins, associated with coagulation and inflammation (10). In recently menopausal women participating in the Kronos Early Estrogen Prevention Study (KEEPS) who were at low risk for cardio- and cerebrovascular disease as defined by a rigorous set of exclusion and inclusion criteria (11), WMH increased over the course of the four years of the study (12). An exploration of non-traditional risk factors that might affect cerebral blood flow and perhaps cerebral microvascular permeability suggested that the thrombogenicity and pro-inflammatory state of the blood, defined by activated circulating platelets and platelet-derived microvesicles at the time women enrolled in the study, that is prior to randomization to treatment, associated with development of WMH over the study course (12).

KEEPS participants were randomized to either oral conjugated equine estrogen (oCEE), transdermal 17β-estradiol (tE2) both with pulsed progesterone, or placebo pills and patch (PBO) over the four years of the study (11). These formulations of menopausal hormone treatments (MHT) were shown to affect platelet secretory products, reactivity, and aggregation (13–15). Therefore, the aim of this analysis was to determine if the type of MHT modified the association of WMH with thrombogenicity of the blood defined by a set of markers of platelet function and reactivity, intravascular cells, cell-derived microvesicles, endothelial activation, and inflammation.

Methods

Participants:

Women enrolled in an ancillary magnetic resonance imaging (MRI) study of the KEEPS (NCT000154180) at Mayo Clinic were eligible for this study. KEEPS was a double-blind, placebo controlled study to determine the effects of two different hormonal treatments on progression of atherosclerosis defined by increases in carotid intima-medial thickness in recently menopausal women (11). In brief, women were between 42–59 years old and within 6 months to 3 years past their last menses at the time of enrollment. Women were excluded

if they had a coronary artery calcium score of > 50 Agatston Units (AU), smoked over 10 cigarettes per day, had BMI >35 kg/m^2, had a history of cardiovascular disease, or had LDL cholesterol >190 mg/dl, triglycerides >400 mg/dl, diagnosis of diabetes, uncontrolled hypertension (systolic blood pressure > 150 mmHg and/or diastolic blood pressure >95 mm Hg) or current or recent (6 months) use of cholesterol lowering medications (statins, fibrate, or >500 mg/day niacin). Women were randomized to: oCEE (Premarin, 0.45 mg/day); transdermal tE2 (Climera, 50 μg/day; or PBO pills and patch. Micronized progesterone was given orally (Prometrium; 200 mg/day) for 12 consecutive days each month to both active menopausal hormone treatment groups (11). The study was approved by the Mayo Clinic Institutional Review Board and all participants gave written informed consent.

Brain imaging:

Women underwent MRI at baseline prior to randomization, and at 18, 36, and 48 months for measurement of WMH volumes on Fluid-attenuated inversion recovery sequences as previously described (16, 17).

Blood collection and analysis:

Women were asked to refrain from aspirin two weeks prior to blood collection. Fasting venous blood was collected into a syringe through a 19 gauge butterfly needle and dispensed into plastic tubes containing anticoagulants needed for each assay and maintained at 33°C until processed within 30 minutes (18). Platelet activation using this technique and as measured by surface expression of P-selectin and fibrinogen receptors is <5% (19). Collections occurred at baseline prior to randomization to study treatments and at each study time point (18). Total cholesterol, low-density lipoprotein cholesterol (LDL-C) and high-density lipoprotein cholesterol (HDL-C), triglycerides, blood glucose and 17β-estradiol were measured by Kronos Science Laboratories (Phoenix, AZ) and the Mayo Clinic Department of Laboratory Medicine and Pathology (Rochester, MN).

Platelet count was determined by Coulter counter, as previously described (20). Expression of activated platelet membrane P-selectin and glycoprotein IIb/IIIa complex binding to PAC-1 antibody (an indirect measure of the expression of membrane fibrinogen receptor) was measured by validated flow cytometry techniques (18–21).

Total numbers of thrombogenic (phosphatidylserine-positive defined by annexin V binding) microvesicles (MV) and other MV staining positive for selected cell-specific markers were measured using fluorophore conjugated recombinant proteins and antibodies by flow cytometry (18, 21, 22).

Statistical analysis:

Data reduction methods were used to achieve some degree of parsimony in these analyses so that the complexity could be reasonably supported by the sample size. Analysis of longitudinal WMH data was performed with a two-stage approach to first characterize the time-response profile and then assess differences in profiles across treatment. The goal of the first stage is to adequately summarize the repeat measurements into single WMH responses per person. For this, slope coefficients were computed using least squares

regression in fitting each woman's time-response data with a linear equation. To minimize the impact of skewed values, we took the logarithm of WMH, with a constant of 1 added to the raw value, before modeling. One-sample Wilcoxon signed-rank tests for zero slopes were used to test for a significant change in WMH over 48 months separately in each group. The slopes were then assessed for treatment difference using the proportional odds ordinal logistic model, which provides a generalization of the Kruskal-Wallis test for pairwise testing. The results of the two-stage approach were compared with generalized least squares (GLS) in which all the serial log-transformed data were analyzed jointly in a single model, and the correlation of the repeat measurements taken into account.

For each of the 14 cellular activation markers, repeat measurements on the same person were averaged across visits, and the resulting mean values were converted to normal scores based on their ranks. Scored dimensions of platelet reactivity and MVs were derived using principal component (PC) analysis on the 14 transformed variables. The multiple PCs were carried forward and analyzed for association with 3-level treatment in multi-nomial logistic regression model (an extension of the binary logistic model for >2 unordered outcome categories), and for association with WMH response in a proportional odd ordinal logistic regression model while adjusting for randomized treatment group. P-values are computed from the likelihood ratio χ^2 statistic for the model that is due to the individual or multiple variables of interest, with the exception of pairwise treatment comparisons which are based on approximate Wald χ^2 statistics.

Results

Baseline characteristics of the 95 participants, a subset of the 118 KEEPS participants at the Mayo Clinic site for whom WMH data were available (12) did not differ across treatment group assignments except for smoking status (Table 1). WMH increased in all 3 groups over the 48 months of treatment (P < 0.001 each; Table 2). The extent to which WMH increased, as summarized by within-person slopes, differed across treatments ($P = 0.044$), with pairwise comparisons indicating greater increases in oCEE than in PBO ($P = 0.011$). Results from repeated measures modeling (model-predicted WMH at 48 months are shown by treatment group in Supplemental Table 1) were in reasonable agreement with the summary measure analysis, supporting the validity of the simpler 2-stage approach, and with differences between oCEE and PBO reported in the original analysis (Tables1 and 3 of reference 17).

The average of each of the 14 intravascular cellular activation markers (6 platelet reactivity variables and 8 MV variables) over treatment follow-up is shown by treatment group in Table 3. Due to the multiplicity of variables, and to differences in directional change (increases or decreases compared to placebo) for each of the variables, we used PC analysis to reduce these dimensions to their most important components. The first five PCs were retained as they could explain most (62%) of the variability in the 14 standardized variables (Supplemental Table 2).

A global test of association with treatment for these five PCs approached significance (P=0.059), with partial tests revealing an overall group difference for PC_3 (P=0.006). PC_3

represents a contrast of platelet microaggregates, ATP secretion, basal expression of P-selectin, and fibrinogen receptor complex (PAC-1 binding) vs. total platelet count and numbers of leukocyte-derived MV. The composite scores in oCEE were marginally to significantly higher compared to other groups (P=0.003 for oCEE vs. tE2, and P=0.063 for oCEE vs. PBO; Supplemental Table 3).

Using multivariable regression to test the joint influence of the PCs and treatment on the slope measure for WMH, the global contribution of all 5 PCs did not reach statistical significance (P=0.104). However, of the individual components PC_1 reflecting MV positive for expression of tissue factor, ICAM-1 and VCAM-1, and microvesicles derived from leukocytes and monocytes showed the most prominent effect (P=0.003). This finding indicates that, after controlling for treatment, the higher the composite score for PC_1 the greater the rate of increase in WMH. Also from this model, the association between treatment group and WMH increase persisted after adjustment for PC variables (P=0.009). Based on the global test of interaction on 10 degrees of freedom, there was no evidence (p=0.204) that the overall association between PCs and WMH increase differed by MHT (Table 4).

Discussion

The results of this study support previous observations that blood thrombogenicity and pro-inflammatory status associate with WMH (7–9, 12), and extend those observations that this association may be influenced by factors others than the type and dose of menopausal hormones used for the treatment in Kronos Early Estrogen Prevention Study.

Factors influencing generalized inflammation and, indirectly, blood thrombogenicity, include conventional cardiovascular risk factors such as age, blood pressure, hyperlipidemia, insulin resistance, and life-style choices such as diet, activity and smoking. However, in KEEPS, the conventional cardiovascular risk factors such as body mass index, blood pressure, triglycerides, high and low density lipoprotein, glucose and smoking status did not associate with WMH at 48 months, which is consistent with other studies (9, 12, 23, 24). Other potential sources for inflammation in KEEPS participants are unclear. Only 5% of participants were current smokers (12), but other behavior factors such as diet and activity were not analyzed, nor were potential sources of commensal or low-grade infectious or inflammatory conditions such as periodontal disease, asthma or prior histories of hypertensive pregnancy disorders (25, 26). Each of these conventional and non-conventional risk factors may individually be insufficient to initiate an inflammatory response of the cells within the vascular compartment. However, their collective effects of the endothelium, platelets and monocytes may reach a threshold to alter changes in the macro-vasculature (carotid artery intima-media thickness) (18, 27) and cerebral microvasculature affecting development of WMH.

In the present study, the overall association between treatment and the 5 PCs describing a number of cellular activity measures did not reach statistical significance at the P<0.05 level. However, the PC_3 that represented a contrast of platelet reactivity measures differed significantly in the oCEE compared to the tE2 or PBO groups. This result is consistent with

previous findings that of significant differences in platelet functions between tE2 and oCEE groups (13, 14, 28). Effects of various genetic variants on the responses to treatment might also have masked potential treatment effects as genetic variants associated with metabolism and uptake of estrogen, with innate immunity, and with *APOE* ε4 was observed for MHT effects measured by differences in chronological age for onset of menopause, in carotid artery intima-medial thickness, and deposition of β-amyloid in the brain (9, 29–33). In spite of these effects, after controlling for treatment, the overall association of the 5 PCs with increase in WMH reflected the strong positive correlation between PC_1 score and WMH increase. Taken together these results suggest that both MHT and the composite of the MV measurements explaining PC_1 show an independent effect on development of WMH.

There are several limitations of this study that should be considered. First, the results may not be applicable to the general population as the KEEPS enrolled recently menopausal women within a relatively narrow age range. In addition, these women were predominantly white, healthy, educated, and most were non-smokers. However, the advantage of this homogenous population is that the findings may reflect general physiological processes that are not confounded by manageable cardiovascular risk factors. Second, the influences of the MHT used in KEEPS on development of the WMH may not apply to other doses or formulations of MHT used in other studies. Third, the overall association between PCs and WMH increase did not apparently differ by MHT. However, the relatively small sample in our study may have limited the power to detect such a difference.

Conclusions

The findings of the present study are consistent with those of other investigations that implicate thrombogenicity of the blood and inflammation as contributors to development of WMH (9, 12, 34). Activation of blood platelets, endothelium and monocytes associated with development of WMH are most likely multifactorial including synergistic effects of conventional risk factors such as age, blood pressure and components of metabolic syndrome. In addition, other potential sources of platelet and cellular activation such as effects of natural menopausal aging processes, adverse pregnancy histories, commensal infections and co-morbid inflammatory conditions and behaviors could have additive effects. Specific mechanisms by which these activated cells and MV affect cerebral microvascular function leading to formation of WMH remain to be determined.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Sources of funding:

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Table 1.

Baseline clinical characteristics of participants by treatment assignment

Variable	N	No. (%) Missing	PL (n=36)	tE2 (n=30)	oCEE (n=29)	P-value
Age at randomization (years)^	95	0	52.7 (51.8, 54.3)	53.3 (51.7, 54.3)	53.8 (52.1, 54.8)	0.561
Smoking	80	15 (15.8%)				0.031
Never			25 (73.5%)	13 (56.5%)	16 (69.6%)	
Past			9 (26.5%)	6 (26.1%)	7 (30.4%)	
Current			0 (0.0%)	4 (17.4%)	0 (0.0%)	
Menopausal age (months, at randomization)^	95	0	12.7 (10.0, 21.2)	18.5 (14.1, 25.8)	21.4 (10.1, 27.8)	0.108
Body mass index (kg/m2)^	95	0	25.7 (24.7, 30.6)	26.8 (22.2, 30.2)	28.4 (25.0, 32.2)	0.330
Waist Circumference^	94	1 (1.1%)	84.8 (76.0, 91.5)	82.5 (72.0, 89.0)	85.0 (77.8, 92.5)	0.564
MSBP^	95	0	121.8 (114.5, 128.0)	116.5 (110.5, 129.5)	124.0 (112.5, 130.0)	0.538
MDBP^	95	0	76.0 (70.0, 81.3)	73.8 (66.5, 79.5)	78.5 (71.5, 82.0)	0.282
TC^	95	0	218.5 (199.5, 232.5)	222.5 (209.0, 248.0)	209.0 (193.0, 237.0)	0.177
HDL^	95	0	59.5 (50.0, 66.0)	61.0 (53.0, 71.0)	58.0 (49.0, 63.0)	0.371
LDL^	95	0	136.5 (117.5, 152.3)	150.6 (123.8, 168.6)	133.4 (120.4, 153.4)	0.373
Trig^	95	0	83.5 (65.0, 107.0)	88.0 (67.0, 118.0)	94.0 (59.0, 117.0)	0.775
hs-CRP^	92	3 (3.2%)	1.2 (0.5, 2.2)	1.3 (0.4, 2.0)	1.5 (0.8, 3.8)	0.404
Fasting Glucose^	95	0	91.0 (87.5, 96.5)	94.0 (87.0, 98.0)	89.0 (82.0, 99.0)	0.404

^
Median (25th, 75th percentiles); Kruskal-Wallis test

Baseline characteristics of these 95 participants, a subset of the 118 KEEPS participants at the Mayo Clinic site for whom WMH data were available, were published previously (12). Treatment groups differed with regard to smoking status (P=0.031; four [17.4%] women in the tE2 were smokers at time of baseline, compared with none in the other groups), but were otherwise similar.

Table 2.

White matter hyperintensities prior to (baseline) and 18, 36, and 48 months after randomization to placebo or menopausal hormone treatments

White Matter Hyperintensity Volume	N	Placebo pills or patch (n=36)	Transdermal 17β-estradiol (n=30)	Oral conjugated equine estrogen (n=29)	
Baseline	95	1.34 (1.00, 2.02)	1.99 (1.57, 2.85)	2.13 (1.64, 3.66)	
Month 18 visit	92	1.42 (1.01, 2.13)	1.98 (1.64, 2.90)	2.31 (1.67, 3.55)	
Month 36 visit	85	1.52 (1.09, 2.17)	2.13 (1.77, 3.08)	2.59 (1.76, 4.07)	
Month 48 visit	79	1.41 (1.09, 2.35)	2.33 (1.79, 3.38)	2.55 (1.93, 4.63)	
Within-Woman Slope	*95*	*0.016 (−0.001, 0.028)*	*0.019 (0.013, 0.035)*	*0.025 (0.010, 0.051)*	*P*=0.044 [*]

Results reported as median (25th, 75th percentile).

[*] P-value is based on the likelihood ratio test from a proportional odds ordinal logistic model, which is a generalization of the Kruskal-Wallis test with pairwise testing fully embedded in the overall model. The significant 2 d.f. overall test (likelihood ratio $\chi^2 = 6.2$, $P = 0.044$) indicates differences in WMH rate of increase by treatment group. Pairwise comparisons showed this difference was driven by the oral conjugated equine estrogen versus placebo contrast (Wald $\chi^2 = 6.4$, $P = 0.011$), with the other two contrasts nonsignificant (Wald $\chi^2 = 1.2$, $P = 0.274$ for tE2 versus placebo, and Wald $\chi^2 = 2.1$, $P = 0.150$ for oral conjugated equine estrogen versus transdermal 17β-estradiol).

For comparison, a GLS model was fit on log-transformed WMH data, with repeated measurements at 18, 36, and 48 month visits modeled as the response and baseline measurement as a covariate. Results were fairly consistent with the summary measure analysis, based on the interaction between 3-level treatment and (linear) time trending toward significance (Wald $\chi^2 = 4.8$, $P = 0.089$

Table 3:

Platelet reactivity and blood-borne microvesicles (MV) averaged across baseline, 18, 36, and 48 month visits

Measurements (Averaged over treatment period)	Placebo pills or patch (n=36)	Transdermal 17β-estradiol (n=30)	Oral conjugated equine estrogen (n=29)
Platelet reactivity			
Platelet count (×10⁹/μL)	235.60 (221.70, 260.10)	246.75 (219.40, 287.60)	239.60 (212.60, 264.20)
Platelet microaggregates (% difference)	1.90 (−0.30, 5.04)	2.79 (−0.14, 4.72)	3.58 (0.64, 7.72)
ATP secretion(attomoles/platelet)	25.78 (22.68, 29.46)	23.96 (20.28, 28.10)	28.90 (23.10, 32.36)
PGE1 sensitivity of ATP secretion (% suppression)	24.92 (15.16, 31.96)	22.05 (16.00, 29.74)	20.30 (14.10, 27.94)
Basal expression of membrane P-Selectin (%)	1.61 (1.38, 2.02)	1.40 (1.09, 1.67)	1.72 (1.30, 1.99)
Basal expression of membrane fibrinogen receptor (PAC-1, %)	0.72 (0.64, 0.89)	0.76 (0.61, 0.87)	0.81 (0.64, 0.92)
Microvesicles (MV)/μL plasma			
Phosphatidylserine positive MV	208.84 (170.60, 294.23)	226.94 (141.74, 354.53)	252.54 (178.44, 381.23)
Tissue factor positive MV	22.96 (14.24, 28.62)	23.67 (18.06, 39.35)	17.05 (14.32, 24.97)
Leukocyte (CD45) -derived MV	5.72 (3.48, 10.10)	6.71 (4.83, 8.58)	4.74 (3.73, 6.85)
Monocyte (CD14) -derived MV	16.75 (11.30, 25.42)	16.92 (11.42, 26.96)	17.54 (10.16, 19.07)
Platelet (CD42a) -derived MV	170.95 (132.38, 257.48)	214.58 (135.23, 314.32)	194.14 (141.75, 313.66)
Endothelium (CD62-E) -derived MV	11.17 (8.13, 17.32)	12.96 (8.38, 20.55)	11.96 (9.44, 17.50)
ICAM-1 positive MV	11.16 (7.57, 18.75)	10.45 (5.90, 16.67)	8.98 (5.99, 16.79)
VCAM-1 positive MV	1.18 (0.84, 3.08)	2.02 (0.89, 3.40)	0.91 (0.59, 1.58)

Results reported as median (25th, 75th percentile).

Table 4.

Modeling rate of change in white matter hyperintensities as a function of PCs and treatment

Model Outcome: WMH Slope	Model Terms	LR χ^2 test P value (df)[*]
Model 1: Additive model	**PC$_{1-5}$**	Global, $P = 0.104$ (5 df)
	PC$_1$	Partial, $P = 0.003$ (1 df)^
	PC$_2$	$P = 0.953$ (1 df)
	PC$_3$	$P = 0.961$ (1 df)
	PC$_4$	$P = 0.634$ (1 df)
	PC$_5$	$P = 0.853$ (1 df)
	Treatment	Overall, $P = 0.006$ (2 df)
Model 2: Non-additive model	Treatment × PC$_{1-5}$ Interaction	Global, $P = 0.207$ (10 df)

[*] Degrees of freedom, df