Effect of Postmenopausal Hormones on Inflammation-Sensitive Proteins

The Postmenopausal Estrogen/Progestin Interventions (PEPI) Study

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Background—Observational studies in healthy women suggest postmenopausal hormone therapy reduces risk of coronary events. In contrast, in a recent clinical trial of women with coronary disease, a subgroup analysis demonstrated increased risk during the early months of therapy. Because higher levels of inflammation factors predict vascular disease outcomes, the effect of hormones on these factors is of interest.

Methods and Results—Four inflammation-sensitive factors, C-reactive protein, soluble E-selectin, von Willebrand factor antigen, and coagulation factor VIIIc were measured at baseline, 12, and 36 months in 365 participants of the Postmenopausal Estrogen/Progestin Interventions (PEPI) Trial, a randomized, placebo-controlled trial of the effects of 4 hormone preparations on cardiovascular disease risk factors. Compared with placebo, all 4 active preparations resulted in a large sustained increase in the concentration of C-reactive protein and a decrease in soluble E-selectin ($P=0.0001$). There were no effects of treatment on concentrations of von Willebrand factor or factor VIIIc. There were no differences in effects among treatment arms. Relative to placebo, when combining active treatment arms, final concentrations of C-reactive protein were 85% higher whereas E-selectin was 18% lower compared with baseline.

Conclusions—Postmenopausal hormones rapidly increased the concentration of the inflammation factor C-reactive protein. Such an effect may be related to adverse early effects of estrogen therapy. In contrast, hormones reduced the concentration of soluble E-selectin, and this might be considered an anti-inflammatory effect. Because PEPI was not designed to assess clinical endpoints, studies of the impact of hormone-mediated changes in inflammation on risk of subsequent coronary events are needed. (***Circulation*. 1999;100:717-722.**)

Key Words: risk factors ■ hormones ■ inflammation ■ coagulation ■ women

Most observational epidemiological evidence suggests that hormone replacement therapy reduces cardiovascular risk.[1] In contrast, a pooled analysis of short-term randomized clinical trials showed adverse effects, with a relative risk of 1.39 of cardiovascular events.[2] The recently published Heart and Estrogen/progestin Replacement Study (HERS) showed no reduction in rate of coronary events in women with coronary disease who were randomly assigned to estrogen plus medroxyprogesterone acetate (MPA) versus placebo.[3] Although these experimental findings cannot be generalized to all forms of estrogen, and other clinical trials of hormone therapy are ongoing, defining hormone therapy effects on newly described coronary risk markers, including inflammation factors, may provide useful data concerning mechanisms of therapy-mediated risk alteration.

Atherosclerosis, thrombosis, and inflammation are linked through several molecular pathways.[4,5] In recent studies, 4 inflammation-sensitive factors, C-reactive protein, von Willebrand factor (vWF), coagulation factor VIIIc, and soluble E-selectin, predicted increased risk of coronary heart disease in various populations,[6–11] some of which included women.[8–11] The latter 3 proteins may be involved as markers of endothelial damage reflecting preclinical atherosclerosis. Enhanced coagulation with estrogen use[12] may provide a partial mechanism for coronary risk modulation. However, experimental data on the effects of hormones on these 4 inflammation proteins is lacking.

In a cross-sectional study of long-term elderly users, hormone use was associated with over 50% higher levels of C-reactive protein compared with nonusers, and this was

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most apparent in women with higher body-mass index.[13] Factor VIIIc level was lower in users but this was not independent of other risk factors.[14] Hormone use was not associated with vWF concentration in another cross-sectional study,[15] however the only prospective report available was a small trial that showed reduced vWF concentration with hormone administration after surgical menopause.[16] To our knowledge, the effect of hormone therapy on soluble E-selectin has not been reported, nor are there randomized clinical trial data available for any of these factors.

The Postmenopausal Estrogen/Progestin Interventions (PEPI) trial was a randomized, placebo-controlled trial designed to determine the effect of different preparations of postmenopausal hormones on cardiac risk factors.[17] Effect of treatment on lipoprotein(a) in a PEPI subgroup was recently reported.[18] To further define the biological effects of hormone therapy, we measured the levels of C-reactive protein, soluble E-selectin, vWF, and factor VIIIc in this PEPI subgroup.

Methods

Study

The PEPI trial was a 3-year randomized, double-blind, placebo-controlled clinical trial to compare 4 hormone therapy regimens and placebo for effects on cardiovascular risk factors. The design and main results have been published.[17] The estrogen regimens were: (1) placebo, (2) conjugated equine estrogens (CEE), 0.625 mg/d; (3) CEE, 0.625 mg/d plus MPA, 10 mg/d, days 1 through 12 each month; (4) CEE, 0.625 mg/d plus MPA, 2.5 mg/d; or (5) CEE, 0.625 mg/d plus micronized progesterone (MP), 200 mg/d, days 1 through 12 each calendar month. Randomization was stratified by study center and hysterectomy status.

Subjects

The study included ambulatory postmenopausal women who did not possess the following characteristics: (1) natural menopause before age 44 or <1 year or >10 years before enrollment, (2) hysterectomy within 2 months, (3) body-mass index $\geq$40 kg/m^2, and (4) medical history suggesting a possible contraindication to hormone use, or a factor which might limit follow-up. The study was approved by each center's institutional review committee, and participants provided written informed consent using approved guidelines. There were 875 women enrolled at 7 centers. The 383 women who formed the subset for this study were enrolled at the 3 centers where more detailed laboratory studies were done: George Washington University Medical Center, University of California at Los Angeles, and Stanford University.

At baseline and each yearly visit after randomization, demographic information, medical history, and lifestyle information were obtained in addition to measurements of weight, waist circumference, height, and seated blood pressure.[17]

Blood Collection and Analysis

Fasting morning blood samples were obtained from each participant at baseline, 12, and 36 months after randomization. Within 30 minutes, plasma was separated by centrifugation at 4°C and 30 000*g*, then stored at −70°C. All assays were run at the completion of the study with each participant's sequential samples run concurrently. Factor VIIIc was measured in citrated plasma, using factor VIII-deficient plasma and partial thromboplastin (Organon Teknika), with a coefficient of variation of 10%. An unassayed normal plasma pool (George King Biomedical Inc.) was used as the standard and calibrated with the World Health Organization reference plasma. One of the blood collection tubes contained 4.5 mmol/L EDTA plus protease inhibitors (0.15 KIU/L aprotonin, and 20 μmol/L D-Phe-Pro-Arg-chloromethyl ketone; Hematologic Technologies, Inc.) as the anticoagulant; this plasma was used for measurement of other inflammation factors. C-reactive protein was measured by immunoassay (antibodies and antigens from Calbiochem) with a coefficient of variation of 7.7%.[19] Soluble E-selectin and vWF antigen were measured by immunoassays (R&D Systems Inc. and Asserachrom vWF, American Bioproducts), with respective coefficients of variation of 10% and 3%. Concentrations of lipids, glucose, insulin, and fibrinogen were measured as previously reported.[17]

Inspection of the distributions of the inflammation factors (blind to treatment arm) indicated possible preanalytical influence on some values for factor VIIIc, with more than the expected number of low values. Most women with low values also had prothrombin fragment 1 to 2 (Behring Diagnostics, Inc., Westwood, Mass) concentrations >800 nmol·L^{-1}, consistent with preanalytical clotting artifact.[20] Therefore, for the analyses of factor VIIIc, 52 women with fragment 1 to 2 values of 800 nmol·L^{-1} were excluded.

Statistical Analysis

SAS was used for statistical analysis. Analyses were limited to adherent women, defined as those who, on the basis of pill counts, took $\geq$80% of their assigned pills during the 6 months before the 12- and 36-month clinic visit. The distributions of C-reactive protein, E-selectin, and vWF were skewed; analyses were conducted on log-transformed data. For presentation, means and standard deviations were transformed back to their original units. Baseline characteristics, including values for the inflammation factors, were compared among randomized treatment groups using ANOVA or χ^2 test. Cross-sectional correlates of the inflammation factors with other vascular disease risk factors were determined by Pearson correlation coefficients or ANOVA. Changes in inflammation factors over time were compared among treatment groups using Wald tests from Laird-Ware models for repeated measures,[21] adjusting for the stratification factors (study center and hysterectomy status). For C-reactive protein and E-selectin, 10 pairwise comparisons of treatment arms were made, with Bonferroni adjustments to control the overall type I error. Pearson correlation coefficients were used to characterize associations between changes in inflammation factors and changes in other factors.

Results

Baseline characteristics, including baseline levels of the inflammation factors, were similar among groups defined by treatment assignment (Table 1). The mean age was 56 years, 87% were white, there were few current smokers (12%), and 26% reported prior hysterectomy. Prior estrogen use was reported by 55%. At baseline, 12, and 36 months there were 365, 316, and 264 women studied, respectively. Reasons for exclusion were either a missing blood sample (n=18, 18, and 27, respectively) or nonadherence to assigned treatment (by pill count or discontinuation; n=49 and 92, at 12 and 36 months). The main reason for exclusion was protocol-mandated discontinuation of unopposed estrogen due to endometrial hyperplasia.[17]

Combining all randomized arms, mean pretreatment concentrations of C-reactive protein and soluble E-selectin were higher in women with prior hysterectomy (1.28 versus 1.05 mg/L, P=0.02; and 38.2 versus 34.6 μg/L, P=0.05, respectively), whereas factor VIIIc was lower (102% versus 113%, P=0.03). Mean C-reactive protein and E-selectin concentrations were higher in the 49 nonwhite compared with the 316 white participants, and this difference was statistically significant for E-selectin (41.6 versus 34.6 μg/L, P=0.005). There were no differences in concentrations of any of the factors by smoking status, alcohol use, or prior use of hormones.

Baseline concentrations of C-reactive protein and E-selectin were correlated with each other (r=0.32,

TABLE 1. Baseline Characteristics by Treatment Assignment*

Characteristic	Treatment Assignment				
	Placebo (n=72)	CEE (n=74)	CEE+MPA cyc (n=73)	CEE+MPA cont (n=74)	CEE+MP cyc (n=75)
Age, y	55.7±4.4	56.3±4.2	55.7±4.3	56.1±4.0	55.7±4.1
Race, white	60 (83)	66 (89)	62 (85)	64 (87)	67 (89)
Hysterectomy, yes	20 (28)	20 (27)	18 (25)	18 (24)	20 (27)
Prior hormone use	36 (52)	41 (57)	41 (56)	37 (50)	45 (61)
Smoking					
Current	11 (15)	7 (10)	6 (8)	9 (12)	12 (16)
Former	32 (44)	28 (38)	30 (41)	31 (42)	22 (29)
Never	29 (40)	39 (53)	37 (51)	34 (46)	41 (55)
Body-mass index, kg/m^2	26.1±4.5	25.6±4.1	26.0±4.5	25.9±4.1	25.4±4.5
Fasting glucose, mmol/L	5.27±0.50	5.44±0.72	5.27±0.44	5.44±0.72	5.33±0.56
LDLc, mmol/L	3.70±0.80	3.68±0.65	3.47±0.75	3.70±0.67	3.50±0.67
HDLc, mmol/L	1.61±0.41	1.66±0.44	1.74±0.44	1.74±0.39	1.66±0.44
Triglyceride, mmol/L	1.12±0.50	1.09±0.54	1.09±0.48	1.14±0.60	1.09±0.61

Values are presented as mean±SD or frequency (%).

*There were no significant differences among treatment arms for any variables shown. Cyc indicates cyclical; cont, continuous.

$P<0.001$). C-reactive protein, but not E-selectin, was associated with higher fibrinogen ($r=0.37$, $P<0.001$). Higher concentrations of both of these factors were associated with higher body-mass index and waist-hip ratio, higher LDLc, glucose and insulin concentrations, and lower HDLc (data not shown). There was a positive correlation of vWF with factor VIIIc ($r=0.49$, $P<0.001$), and both of these were negatively associated with soluble E-selectin ($r=-0.15$ and -0.22, $P<0.01$, respectively). Unlike C-reactive protein and E-selectin, concentrations of these 2 proteins were not correlated with other risk factors, but both increased with age.

The effect of treatment on each inflammation factor over 36 months is shown in Figure 1 and Table 2. C-reactive protein and soluble E-selectin did not change over time in the placebo group (mean estimated changes: −0.05 mg/L and +0.90 μg/L over 36 months, respectively). In contrast, the concentration of C-reactive protein rose in each active treatment group while the concentration of soluble E-selectin decreased. All pairwise comparisons between the active arms and placebo were significant ($P=0.001$), although there were no significant differences among active treatment arms. Most of the effect of therapy on C-reactive protein and E-selectin occurred in the first 12 months of therapy; these effects were sustained over 3 years. The treatment effect on C-reactive protein was substantial. At 3 years, the mean estimated increase in C-reactive protein in all active treatment groups relative to placebo was 1.06 mg/L (85% increase). The mean estimated decrease of E-selectin relative to placebo was 5.8 μg/L (18% decrease). The effect of treatment on fibrinogen was similar to that previously reported in the entire PEPI cohort.[17] Baseline fibrinogen differed by treatment assignment, and power limited interpretation of the findings; the only significant pairwise difference over time was between CEE and placebo (mean change −0.02 versus +0.06 g/L, $P<0.05$). Relative to placebo, there was no significant increase in fibrinogen in any of the active treatments, as was observed for C-reactive protein.

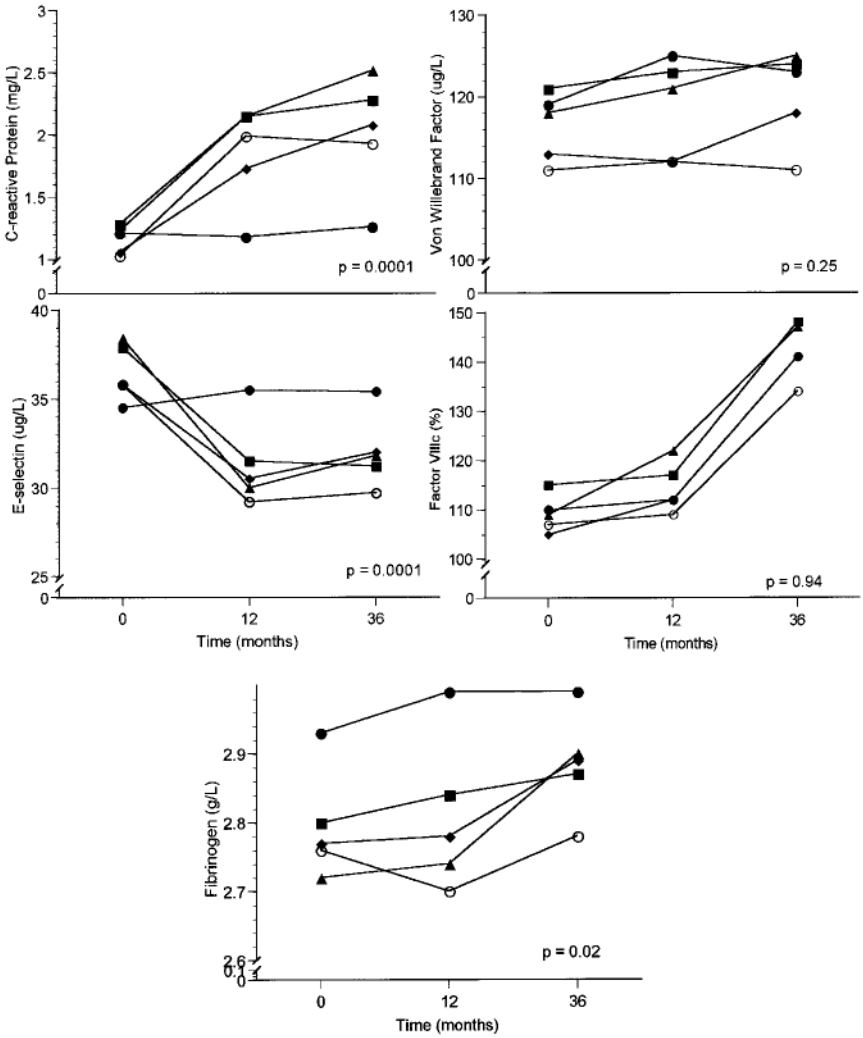
In comparison to placebo, there were no effects of treatment on the concentrations of factor VIIIc or vWF antigen over time (Figure 1, Table 2). Combining the placebo and active treatment arms, factor VIIIc increased over time, from a mean baseline concentration of 109% to 143% 36 months later. The vWF antigen concentration did not change over time.

Table 3 shows the correlation of longitudinal changes in C-reactive protein and E-selectin with changes in selected risk factors, by treatment assignment. In active treatment arms, the increase in C-reactive protein and decrease in E-selectin were associated with respective increase and decrease in body-mass index. These relationships were similar for the placebo group, suggesting the weight change associations were not mediated by treatment assignment. The increase in C-reactive protein was weakly associated with an increase in fibrinogen concentration, regardless of treatment assignment. Lowering of E-selectin by active treatment was correlated with a decrease in LDLc, which was not observed in the placebo group. Among women on active treatment, change in C-reactive protein or E-selectin was not associated with change in HDLc, waist-hip ratio, or fasting insulin or glucose levels.

The main analyses were repeated after stratification by baseline levels of cardiovascular risk factors. The effect of active treatment on levels of C-reactive protein or E-selectin did not differ by baseline level of any factors studied, including baseline levels of C-reactive protein, E-selectin, fibrinogen, lipids, glucose, body-mass index, or waist-hip ratio. There was also no difference by race, prior estrogen use, or smoking status.

Discussion

The main findings of this study were that compared with placebo, estrogen alone or in combination with MP or MPA increased the concentration of C-reactive protein and de-



Estimated mean level of each inflammation factor over time by randomized treatment assignment. ● indicates placebo; ○, CEE; ▲, CEE + MPA cyc; ■, CEE + MPA con; ♦, CEE + MP. Probability value from log-transformed data, adjusted for clinical center and hysterectomy status, and for 10 pairwise comparisons. For C-reactive protein and E-selectin, the significant differences were between the placebo group and each active treatment arm. For fibrinogen, analyses were adjusted for the baseline value, and the significant difference was between CEE and placebo.

creased the concentration of soluble E-selectin over 12 and 36 months of follow-up, in women who adhered to their treatment. There were no effects of therapy on concentrations of coagulation factor VIIIc or vWF. Administration of MPA or MP, compared with no progestin, did not influence the magnitude of the changes. The lack of effect of progestin confirmed, in part, findings of cross-sectional studies[13,15] that examined fibrinogen, factor VIII, and vWF. Effects of active treatment on E-selectin and LDLc were correlated, suggesting common regulation by treatment. A longitudinal increase in factor VIIIc was observed in all study arms, and this increase was larger than an expected 14% absolute age-related increase.[22]

The clinical meaning of increased C-reactive protein with postmenopausal hormones remains to be clarified, but the finding is in accord with a cross-sectional study of older women using hormones.[13] Increased C-reactive protein with hormone treatment provides a possible mechanistic correlate to results of a pooled analysis of short-term trials[2] and the recent subgroup finding of the HERS trial, which demonstrated an early increase in coronary risk with hormone treatment in women with established coronary disease.[3] Individuals with existing coronary disease,[23] those with noninvasively measured subclinical disease (M. Cushman, MD, unpublished data, 1998), and those at greatest risk of future cardiovascular events[7,8] have higher levels of C-reactive protein, presumably reflecting upregulation of at least some aspects of inflammation involved with atherosclerosis and/or thrombosis. Taken together, the findings suggest that one mechanism for a putative adverse cardiovascular effect of postmenopausal hormones is through increasing inflammation, possibly related to accelerated atherosclerosis, plaque destabilization, or thrombosis, which might be enhanced in women with existing disease. There were too few women in PEPI with prevalent or incident coronary disease to test this hypothesis.

In this study, hormone therapy had no effect on vWF or its associated protein, factor VIIIc. To address these null findings, post hoc power calculations indicated 80% power with alpha of 0.05 to detect a 24% difference between treatments in longitudinal change of factor VIII and a 1 μg/L difference in vWF. Both effect sizes are reasonable.

The correlation of change in E-selectin with change in LDL-c in women assigned to hormones, but not placebo, suggests a hypothesis that part of the influence of hormones on E-selectin is mediated by lipid effects. Regardless, a decrease of soluble E-selectin with hormone use may be thought of as a benefit of treatment, under the assumption that soluble E-selectin levels contribute to atherosclerosis or

TABLE 2. Baseline and Final Visit Estimated Means of Inflammation Factors by Treatment Assignment*

Variable	Time (Months)	Treatment Assignment					
		Placebo	CEE	CEE+MPA cyc	CEE+MPA cont	CEE+MP cyc	*P*
C-reactive protein, mg/L	0	1.21 (0.11)	1.03 (0.09)	1.23 (0.11)	1.28 (0.11)	1.06 (0.09)	0.0001†
	36	1.26 (0.12)	1.93 (0.20)	2.52 (0.24)	2.28 (0.21)	2.08 (0.19)	
E-selectin, μg/L	0	34.5 (1.8)	35.8 (1.9)	38.4 (2.0)	37.9 (2.0)	35.8 (1.8)	0.0001†
	36	35.4 (1.9)	29.7 (1.6)	31.8 (1.7)	31.2 (1.6)	32.0 (1.7)	
Factor VIII, %	0	110 (5)	107 (5)	109 (5)	115 (5)	105 (5)	0.94
	36	141 (6)	134 (7)	147 (6)	148 (6)	141 (6)	
von Willebrand factor, μg/L	0	119 (4)	111 (4)	118 (4)	121 (4)	113 (4)	0.25
	36	123 (4)	111 (4)	125 (4)	124 (4)	118 (4)	

Values in parentheses represent SE.

*Adjusted for study center and hysterectomy status. *P* values determined using log-transformed data. †Intergroup differences were significant between the placebo arm and each treatment group ($P=0.001$).

reflect regulation of endothelial cell E-selectin by cytokine stimulation. Higher concentrations of adhesion molecules have been associated with risk of first myocardial infarction in 2 studies, where observed effects were independent of lipid levels.[9,24] Apart from reflecting cytokine stimulation of endothelial cells by interleukin-1 and tumor necrosis factor, an active role for circulating E-selectin itself has not been clarified.[25] Possible roles include anti-inflammatory, pro-inflammatory, and procoagulant effects.[25,26] In one study, estradiol-treated cultured endothelial cells exhibited an increase in E-selectin mRNA expression, and leukocyte binding.[27] The relationship of this to our findings is not clear.

The opposite effects of postmenopausal hormones on C-reactive protein and E-selectin may relate to the early increase and subsequent decline in myocardial infarction risk with hormones observed in the HERS trial.[3] It is possible that an increase in C-reactive protein represents changes associated with plaque destabilization and rupture, events that are more likely in women with existing coronary disease. This is supported by findings that the risk of myocardial infarction related to higher C-reactive protein concentration in older women is greatest for events occurring within 1 year of measurement.[8] On the other hand, the reduction of E-selectin with treatment may be associated with physiological changes that require a longer time to translate to cardioprotection, in a manner similar to that proposed for lipid effects of hormone use. This idea is suggested because the effects of therapy on E-selectin and LDLc were correlated. Moreover, high levels of soluble intercellular adhesion molecule-1 tend to predict later rather than earlier risk of myocardial infarction.[24]

Our findings illustrate the complexity of regulation of the inflammation factors studied. Cross-sectional correlates of C-reactive protein and E-selectin were similar, but effects of hormone therapy on these proteins were opposite, suggesting (1) different mechanisms for these effects, and (2) that effects of treatment on both proteins are not mediated by these other risk factors. Similarly, there were discordant effects of hormone therapy on C-reactive protein and fibrinogen, 2 proteins whose levels are also correlated with each other. The longitudinal correlations of fibrinogen and C-reactive protein concentrations did not differ by treatment assignment (Table 3), suggesting unrelated mechanisms for treatment effects on these 2 proteins. Our study cannot determine the reasons for these findings; further basic research is required.

The major strengths of this study are the prospective placebo-controlled design and well-characterized population, both of which allowed for detailed analysis of observed effects. Randomization reduced the possibility of confounding for unknown covariates, and women in the 5 study arms did not differ by known potential confounders. Limiting the study to adherers improved confidence that the true effects of the hormones were measured.

TABLE 3. Correlation Coefficients Between Percent Change in C-reactive Protein and E-selectin With Percent Change in Other Variables at 12 and 36 Months

Variable	C-reactive Protein (mg/L)				E-Selectin (μg/L)			
	Placebo		Active		Placebo		Active	
	12 mo	36 mo	12 mo	36 mo	12 mo	36 mo	12 mo	36 mo
Body-mass index, kg/m^2	0.27	0.31	0.21†	0.19*	0.42†	0.49†	0.28†	0.24†
LDLc, mmol/L	−0.33*	−0.20	0.01	0.01	−0.04	−0.001	0.21†	0.21*
HDLc, mmol/L	−0.11	−0.11	0.03	−0.03	0.08	−0.06	0.004	−0.07
Triglyceride, mmol/L	0.20	0.06	0.04	0.19*	0.24	0.15	0.02	0.05
Fibrinogen, mmol/L	0.19	0.15	0.15*	0.13	0.10	0.18	0.04	0.09

*$P\leq0.01$; †$P\leq0.001$.

The primary limitation of this study was the inability to assess the effects of changes in inflammation on risk of coronary events; PEPI was not designed to detect relationships of risk factor changes with clinical endpoints. High intraindividual variability of the factors, especially factor VIIIc, may have limited the ability to detect relatively small influences of therapy. Multiple secondary statistical analyses were performed, introducing the possibility of false-positive findings. For example, cautious interpretation for the correlation of change in soluble E-selectin with change in LDLc on treatment is necessary, because a mechanism is not known at this time. For the main analyses of effect of treatment, however, stringent statistical criteria were used to account for multiple comparisons.

In conclusion, in this 3-year randomized, placebo-controlled trial, each of 4 hormone regimens resulted in an early and sustained increase in C-reactive protein and decrease in soluble E-selectin concentrations, with no effect of treatment on concentrations of vWF and factor VIIIc. In the context of adverse early cardiovascular effects of hormones, results suggest that alterations of inflammation regulation may be in the causal pathway. In the context of cardioprotection from postmenopausal hormones, results suggest that this protection is not mediated by C-reactive protein (or the metabolic pathways it represents). To address the clinical sequelae of these findings, studies linking these biochemical changes to subsequent clinical events are needed.

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