Levels of Sex Steroid and Cardiovascular Disease Measures in Premenopausal and Hormone-Treated Women at Midlife

Implications for the "Timing Hypothesis"

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Background: The "timing hypothesis," in addressing findings from the Women's Health Initiative trial, suggests that hormone therapy (HT) should be initiated within 6 years of the menopause transition to extend a favorable estrogenic environment after menopause.

Methods: We compared sex steroid and cardiovascular profiles at the 5-year follow-up visit in a community-based, longitudinal study of the menopause transition (Study of Women's Health Across the Nation). Women aged 47 to 57 years were in 1 of 4 groups: premenopausal, women using conjugated equine estrogen with or without progestin, or postmenopausal (<5 years) without HT use. Cardiovascular assays included low-density lipoprotein cholesterol, oxidized low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglycerides, apolipoproteins A-I and B, F_{2a} -isoprostanes, C-reactive protein, and lipoprotein (a)-1. Sex steroid assays were performed for estradiol, estrogen receptor ligand load, 2-hydroxyestrone, 16α -hydroxyestrone, total testosterone, and sex hormone—binding globulin.

Results: Users of HT had 50% higher levels of sex hormone–binding globulin (P<.001 for both HT groups), which limits binding of sex steroids to their receptors, and higher excreted estrone metabolites (more than 60%; P<.001 for both HT groups) than premenopausal or postmenopausal women. These findings were, in turn, associated with higher levels of F_{2a} -isoprostanes, an oxidative stress measure, than in premenopausal women. The HT users had a more favorable ratio of high-density to low-density lipoprotein cholesterol than did premenopausal or postmenopausal women (P<.01), but higher triglyceride levels (P<.01).

Conclusion: Although HT users had some more favorable lipid profiles than premenopausal and postmenopausal women, there was evidence of adverse HT effects even in women free of atherosclerosis studied within the approximate 6-year period proposed with the timing hypothesis.

Arch Intern Med. 2008;168(19):2146-2153

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TUDIES TO UNDERSTAND UNderlying cardioprotection in women relative to men at midlife1 have focused on the importance of estrogens and their relation to lipids, especially highdensity lipoprotein cholesterol (HDL-C). Exogenous hormone therapy (HT) was the long-time paradigm thought to demonstrate that maintenance of estrogen levels after menopause contributed to heart health.^{2,3} However, when the Women's Health Initiative (WHI) and the Heart and Estrogen/Progestin Replacement Study demonstrated that widely prescribed exogenous hormone products were not cardioprotective, 4,5 as had been originally hypothesized, alternative explanations were proposed.

One proposal, the "timing hypothesis," is that a positive impact of HT on cardiovascular status is dependent on ex-

tending a favorable estrogenic environment after menopause without a substantial time discontinuity. A substantial interval between menopause and HT use could permit the development of atherogenic lesions and vascular compromise that would be less responsive to positive effects of HT use. In instances of HT initiation around menopause, it could also be hypothesized that HT use would confer sex steroid status that would help sustain favorable lipid or cardiovascular profiles.

To determine whether there were unappreciated changes in the endogenous sex steroid hormone environment among users of conjugated equine estrogen (with and without progestin) that might compromise lipids or other cardiovascular measures, we evaluated estradiol (E₂) levels and novel estrogen measures in 4 groups: (1) premenopausal women; (2) users of conjugated equine estrogen; (3) users of con-

jugated equine estrogen with progestin; and (4) women postmenopausal for less than 5 years without HT use. Novel measures included the amount of estrogen acting as a ligand to the estrogen receptor (estrogen receptor ligand load [ERLL]) and an estimate of E2 bioavailability (free estrogen index). Selected estrogen metabolites have been hypothesized to contribute biological activity, 7 so we assayed 2-hydroxyestrone (2-OHE₁) and 16 α hydroxyestrone (16α -OHE₁) on the premises that 2-OHE₁s may act as antioxidants⁸ and that 16α -OHE₁ activity may include covalent binding to the estrogen receptor.9 We characterized the potential antioxidant associations of estrogens by means of F2a-isoprostanes, a product of arachidonic acid oxidation and degradation. 10,11 We further characterized the endogenous sex steroid environment with measures of total testosterone and sex steroid bioavailability with sex hormone-binding globulin (SHBG).

METHODS

SAMPLING AND STUDY POPULATION

Data are from the Study of Women's Health Across the Nation (SWAN), a multicenter, multiethnic longitudinal study of the menopausal transition (see boxed copy on page 2151). Data and specimens were from the fifth annual follow-up examination, which took place in the year before the release of the WHI cardiovascular findings.⁴

At baseline, eligibility criteria for the SWAN study included age 42 to 52 years, presence of an intact uterus and at least 1 ovary, no use of exogenous hormones, menses in the 3 months before enrollment, and self-identification with a site's designated racial/ethnic group. Therefore, Caucasian and non-Caucasian samples were recruited including African-American women in Boston, Massachusetts; Chicago, Illinois; the Detroit, Michigan area; and Pittsburgh, Pennsylvania; and Japanese, Chinese, and Hispanic women in Los Angeles, California; Oakland, California; and Newark, New Jersey, respectively. 12

A total of 2606 women participated in the 5-year follow-up visit (78.9% of the 3302 baseline participants). However, data from 2 of the 7 sites (Chicago and Newark) were excluded from these analyses because their site protocols did not include urine collection for assay of estrogen metabolites. Also excluded from data analyses were women with diabetes mellitus, heart disease, nonestrogen hormone use, or surgical menopause without HT use. Women in the early (n=670) and late (n=196) perimenopause stages were excluded to allow a more clear delineation of hormone and cardiovascular measures in premenopausal women, postmenopausal women (without HT use), and women using 2 types of conjugated equine estrogen therapy, with and without a progestin. Institutional review board approval for the study protocol and repository storage was obtained at each study site.

MENOPAUSE STAGE AND HT USE

Premenopause was defined as the presence of menses within the previous 3-month period, with no decrease in predictability. Early perimenopause was the presence of menses within the previous 3 months and less predictable menstrual frequency; late perimenopause was defined as having 3 to 11 months of amenorrhea. ¹³⁻¹⁵ Twelve consecutive months of amenorrhea without alternative explanation indicated postmenopause. ¹⁵ Medication and HT use were self-reported with cor-

roborative visualization of the prescription container, when possible. Of 782 women available for this report, 98 (12.5%) were premenopausal without HT use, 53 (6.8%) used conjugated equine estrogen only, 243 (31.1%) used conjugated equine estrogen–progestin combinations, and 370 (47.3%) were postmenopausal without HT use. Eighteen women using transdermal formulations were excluded from analyses.

SPECIMENS AND ASSAYS

Annual morning blood and urine collections followed an overnight fast. Two attempts were made to obtain specimens in days 2 to 5 of a spontaneous menstrual cycle. If timed specimens could not be obtained, random fasting specimens were collected. Blood was refrigerated 1 to 2 hours after phlebotomy, centrifuged, aliquotted, frozen, and then sent on dry ice to laboratories for assay.

Specimens were analyzed for levels of hormones and SHBG at the University of Michigan (Clinical Laboratory Improvement Amendments certified) laboratory on an automated analyzer (ACS-180; Bayer Diagnostics, Tarrytown, New York). The E₂ was assayed in duplicate by means of a rabbit anti-E₂-6 immunoassay that was modified to reduce the lower limit of detection to 1.0 pg/mL (to convert to picomoles per liter, multiply by 3.671) and a coefficient of variation (CV) of 3% to 12%. The testosterone competitive immunoassay used testosterone labeled with dimethylacridinuimester, a polyclonal rabbit antitestosterone antibody, and a monoclonal mouse antirabbit antibody coupled to paramagnetic particles. The testosterone reporting range was 10 to 100 ng/dL (to convert to nanomoles per liter, multiply by 0.0347), and assay range was 2 to 478 ng/ dL. The testosterone assay was standardized analytically and confirmed by gas chromatography-mass spectrometry. Interassay and intra-assay CVs were 13.8% and 6.6%, respectively. The competitive SHBG assay was developed on-site with rabbit anti-SHBG antibodies, with a lower limit of detection of 1.95 nmol/L (to convert to micrograms per milliliter, divide by 8.896). Total testosterone level was indexed to SHBG to calculate the free androgen index ([100 × testosterone in nanograms per deciliter]/[28.84 \times SHBG in nanomoles per liter]). Total E₂ was indexed to SHBG as the free E2 index ([100×total E2]/ [272.11 \times SHBG]) to estimate nonbound E₂ bioavailability; $(10\times E_2)$ /testosterone was used to make the resulting ratio unit-

The 2-OHE₁ and 16α -OHE₁ were assayed by enzyme immunoassay (Estramet; Immuna Care Corp, Blue Bell, Pennsylvania) in triplicate. ¹⁶ Interassay and intra-assay CVs were less than 10% for each analyte. Because urinary 2-OHE₁ and 16α -OHE₁ are found as 3-glucuronides or 3,3,16-glucuronides, these sugars were removed to achieve recognition sites for the monoclonal antibodies by means of a mixture of β -glucuronidase and arylsulfatase enzyme isolated from *Helix pomatia*. The assay range was 0.6 to 40.0 ng/mL.

At the University of California, Davis, archival serum specimens were analyzed for ERLL, a cell-based bioassay for measuring signal transduction activity of total circulating bioactive estrogens and detecting activation of estrogen-dependent gene expression. ^{17,18} Procedures are available from the authors

Specimens were assayed for cardiovascular measures at Medical Research Laboratory/Global Central Lab Services, which is certified by the National Heart, Lung, and Blood Institute, Centers for Disease Control Part III program. Lipid fractions were determined from plasma samples with EDTA. Total cholesterol and triglycerides concentrations were determined by enzymatic methods on an analyzer (Hitachi 747; Boehringer Mannheim Diagnostics, Indianapolis, Indiana). The HDL-C was

isolated by means of heparin-2M manganese chloride. 19,20 Lowdensity lipoprotein cholesterol (LDL-C) level was calculated by the Friedewald equation. 21 Lipoprotein (a)-1 (Lp[a]-1) was quantified by competitive enzyme-linked immunosorbent assay.²² Plasma oxidized LDL-C concentrations were measured by sandwich enzyme-linked immunosorbent assay. Apolipoproteins (apo) A-I and B were measured by immunonephelometry (BN1A-100; Behring Diagnostics, Westwood, Massachusetts) calibrated with a World Health Organization traceable standard.23 C-reactive protein (CRP) was quantified by an ultrasensitive rate immunonephelometric method (highsensitivity CRP on a BN100 nephelometer; Dade Behring, Marburg, Germany). The lower limit of detection of the CRP assay was 0.03 mg/L (to convert to nanomoles per liter, multiply by 9.524); the CV was 10% to 12% (0.05 mg/dL) and 5% to 7% (2.2 mg/dL). Plasma plasminogen activator inhibitor 1 (PAI-1) was measured with a sandwich procedure using a solid-phase monoclonal antibody and enzyme-labeled goat antiserum (IMU-BIND plasma PAI-1 enzyme-linked immunosorbent assay; American Diagnostica, Greenwich, Connecticut). Monthly interassay CVs were 5% to 9% and 4% to 9% at mean concentrations of 7 and 22.5 ng/dL, respectively (to convert to picomoles per liter, multiply by 1.923).

Samples analyzed for F_{2a} -isoprostanes were prepurified on affinity columns (Cayman Chemical, Ann Arbor, Michigan), washed, eluted with 95% ethanol, evaporated, dried, and then diluted 1:10 with 0.1M phosphate buffer, assayed by means of an F_{2a} -isoprostane enzyme immunoassay (Cayman Chemical), and read at 405 nm. The standard curve range was 3.9 to 500 pg/mL. Postextraction intra-assay and interassay CVs were 14.4% (51.2 pg/mL, n=83 pairs) and 17.5% (51.2 pg/mL, n=85), respectively; the intra-assay CV was 5.8% (n=1707 pairs).

PHYSICAL AND INTERVIEW-BASED MEASURES

Heights and weights were measured to estimate body mass index (calculated as weight in kilograms divided by height in meters squared). Waist circumference in centimeters was measured about 3 cm above the umbilicus after a relaxed expiration.

On the basis of self-report of race/ethnicity, the analytical sample was 17.0% African American (n=133), 61.0% Caucasian (n=477), 12.1% Japanese (n=94), and 10.1% Chinese (n=94). Exogenous hormone users in the analytical sample were 17.2% African American (n=51), 62.2% Caucasian (n=184), 12.2% Chinese (n=36), and 8.4% Japanese (n=25). Type of hormone delivery system did not vary according to race/ethnicity. Smoking and physical activity data were from self-administered questionnaires incorporating American Thoracic Society questions²⁴ and a modified Baecke instrument, ^{25,26} respectively.

STATISTICAL METHODS

On the basis of examination of distributions, log or square root transformations were applied to measures of lipids and hormones for use in statistical modeling and backtransformed (including variances with a Taylor series) for presentation. Primary analytical approaches included analysis of covariance, correlation analyses, and multivariable regression modeling, testing main effects and interactions (eg, the interaction between SHBG and group status or interactions between 5-year difference in cardiovascular disease [CVD] measures and group status). Analysis of covariance was used to evaluate whether there were significant mean differences in lipid or hormone levels according to menopause stage or HT group designation, after adjusting for site, age, body mass index, physical activity, race/ethnicity, and smoking status. Duration of HT use and difference in CVD measures between baseline and 5-year follow-up

were evaluated and did not change the reported relationships. Pearson and partial correlations ($r_{Y|X}$) were obtained before and after adjustment for covariates, respectively. Type III sums of squares were used to estimate the adjusted partial correlations. Data are shown with 95% confidence intervals (CIs) or P values (2-sided with α < .01). The statistical software used was SAS version 9.1 (SAS Institute Inc, Cary, North Carolina).

RESULTS

Mean ages of women in the premenopausal group, the conjugated equine estrogen group, the conjugated equine estrogen plus progestin group, and the postmenopausal group were 48.9, 52.1, 51.9, and 52.6 years, respectively; age was not different according to race/ethnicity. The numbers of women and duration of their use of conjugated equine estrogen alone were as follows: 5 women for 1 year, 11 for 2 years, 12 for 3 years, 17 for 4 years, and 8 for 5 years. The numbers and duration of conjugated equine estrogen plus progestin use were as follows: 40 women for 1 year, 55 for 2 years, 58 for 3 years, 50 for 4 years, and 40 for 5 years.

Mean E₂ values were similar in premenopausal women and the 2 HT groups and more than double the value in postmenopausal women (**Table 1**). There were no statistically significant mean differences by group in ERLL levels. Also, there were no differences in mean levels of total testosterone in the premenopausal women compared with the 2 HT groups, although testosterone levels were significantly higher in the postmenopausal women (Table 1).

There were pronounced estrone metabolite differences according to group (Table 1). Although mean 2-OHE $_1$ levels were significantly higher in the premenopausal group than in the postmenopausal group, values in HT users were more than double those in either premenopausal or postmenopausal women (Table 1). Furthermore, mean 16α -OHE $_1$ levels were more than 60% higher in HT users than in premenopausal or postmenopausal women.

Mean SHBG was almost 50% higher among the HT users than among the premenopausal or postmenopausal women (Table 1).

INTERMEDIATE CARDIOVASCULAR MARKERS

Mean oxidized LDL-C levels were similar in HT users and premenopausal women (**Table 2**). However, the LDL-C values in both premenopausal women and HT users were, on average, 11% to 12% lower than the mean value in postmenopausal women. The mean HDL-C values were approximately 10% lower in premenopausal women than in the other groups (Table 2). Collectively, there was a more favorable HDL-C:LDL-C ratio in women using HT (Table 2). Furthermore, the apo B to apo A-I ratio was more favorable in premenopausal and postmenopausal women than in HT users.

The triglyceride profile was less favorable in HT users than in either premenopausal or postmenopausal women (Table 2). Furthermore, mean Lp(a)-1 levels were approximately 10% higher in women using HT than in premenopausal and postmenopausal women.

Table 1. Age, BMI, and Hormones Values According to Menopause Status or Hormone Therapy Group, SWAN 5-Year Follow-upa

Variable	Conjugated Equine Estrogen HT				
	Premenopause (n = 98)	Without Progestin (n = 53)	With Progestin (n = 243)	Postmenopause (n = 370)	
Age, y	48.9 (0.3)	52.1 (0.3) ^b	51.9 (0.2) ^b	52.6 (0.2) ^{b,c,d}	
BMI	27.2 (0.6)	27.2 (0.8)	26.5 (0.4)	27.0 (0.3)	
Waist circumference, cm	83.8 (0.6)	83.9 (0.8)	84.8 (0.4)	84.9 (0.3)	
E ₂ , pg/mL	40.8 (4.3) ^e	34.5 (4.2) ^e	41.5 (2.5) ^e	14.8 (0.7)	
ERLL, pg/mL	37.2 (5.5)	48.0 (8.4)	33.6 (2.7)	38.6 (2.7)	
2-OHE ₁ , pg/mL	9.57 (1.09) ^e	23.0 (3.12) ^{b,e}	18.8 (1.25) ^{b,e}	5.64 (0.30)	
16α -OHE ₁ , pg/mL	6.3 (0.6) ^é	11.4 (1.3) ^{b,e}	10.0 (0.6) ^{b,e}	4.4 (0.20)	
Testosterone, ng/dL	29.8 (1.8)	26.0 (1.8)	29.8 (1.0)	34.6 (1.0) ^{ć,d}	
E ₂ to testosterone ratio	14.1 (1.6) ^e	13.8 (1.8) ^e	14.5 (0.9) ^e	4.7 (0.2)	
SHBG, nmol/L	42.8 (3.2)	61.4 (4.4) ^{b,e}	69.9 (2.4) ^{b,e}	42.0 (1.5)	
FEI (E ₂ /SHBG)	0.36 (0.04) ^{d,e}	0.24 (0.03) ^e	0.23 (0.02) ^e	0.14 (0.007)	
FAI (testosterone/SHBG)	2.55 (0.24) ^{c,d}	1.73 (0.19)	1.58 (0.09)	3.07 (0.13) ^{c,d}	
Current smoker, No. (%)	8 (8.2)	4 (7.5)	25 (10.3)	56 (15.1)	

Abbreviations: BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); E2, estradiol; ERLL, estrogen receptor ligand load; FAI, free androgen index; FEI, free E_2 index; HT, hormone therapy; 2-0HE₁, 2-hydroxyestrone; 16α -0HE₁, 16α -hydroxyestrone; SHBG, sex hormone-binding globulin; SWAN; Study of Women's Health Across the Nation.

SI conversion factors: To convert E, to picomoles per liter, multiply by 3.671; SHBG to micrograms per milliliter, divide by 8.896; testosterone to nanomoles per liter, multiply by 0.0347.

Table 2. Cardiovascular Intermediate Markers According to Menopause Status or Hormone Therapy Group^a

Marker		Conjugated Equine Estrogen HT		
	Premenopause (n = 98)	Without Progestin (n = 53)	With Progestin (n = 243)	Postmenopause (n = 370)
Oxidized LDL-C, mg/dL	39.1 (1.7)	38.0 (2.1)	39.3 (1.2)	38.6 (1.0)
LDL-C, mg/dL	108.8 (3.8)	105.1 (4.4)	106.7 (2.2)	121.8 (2.0) b,c,d
HDL-C, mg/dL	55.6 (1.5)	64.3 (2.1) ^b	62.6 (1.0) ^b	60.7 (0.8) ^b
HDL-C:LDL-C ratio	0.51 (0.02)	0.62 (0.03) ^e	0.59 (0.02) ^{b,e}	0.50 (0.01)
Apo B, mg/dL	103.8 (3.2)	110.2 (4.0)	108.4 (1.9)	112.0 (1.6)
Apo A-I, mg/dL	157 (3.1)	181 (4.3) ^{b,e}	180 (2.1) ^{b,e}	167 (1.6) ^b
Apo B:apo A-I ratio	0.70 (0.03)	0.64 (0.03)	0.63 (0.02)	0.70 (0.01) ^d
Triglycerides, mg/dL	98 (5.2)	122 (7.6) ^{b̂,e}	122 (3.8) ^{b,e}	102 (2.5)
Lp(a)-1, mg/dL	50.0 (1.7)	59.4 (2.4) ^{b,e}	55.5 (1.1) ^{b,e}	51.1 (0.8)
F _{2a} -isoprostanes, pg/mL	423 (43)	403 (49)	499 (30)	434 (21)
C-reactive protein, mg/L	2.0 (0.24) ^e	2.3 (0.33) ^e	2.7 (0.19) ^e	1.3 (0.07)
PAI-1, ng/dL	16.6 (1.8) ^{ć,d}	10.0 (1.3)	9.6 (0.6)	14.4 (0.7) ^{c,d}

Abbreviations: apo A-I, apolipoprotein A-I; apo B, apolipoprotein B; HDL-C, high-density lipoprotein cholesterol; HT, hormone therapy; LDL-C, low-density lipoprotein cholesterol; Lp(a)-1, lipoprotein (a)-1; PAI-1, plasminogen activator inhibitor 1.

Mean CRP level was significantly higher in women using the conjugated equine estrogen preparations than in postmenopausal women (Table 2). Mean level of $F_{2\alpha}$ isoprostanes, a measure of oxidative stress, was about 10% higher in women using the conjugated equine estrogen plus progestin preparation than in the other 3 groups, a

difference significant at the P < .05 level. In contrast, the mean level of PAI-1, an inhibitor of fibrinolysis, was almost 50% lower in HT users than mean values in premenopausal and postmenopausal women (Table 2). Adjusting for duration of conjugated equine estrogen product use did not alter these associations.

^aData are adjusted for age, site, smoking behavior, body size, and race/ethnicity and backtransformed. Values are given as mean (SE) unless otherwise indicated. Statistically significant data are given in boldface type.

^b Groupwise comparison, $P \le .01$ vs premenopause.

^cGroupwise comparison, $P \le .01$ vs HT without progestin.

d Groupwise comparison, $P \le .01$ vs HT with progestin. e Groupwise comparison, $P \le .01$ vs postmenopause.

SI conversion factors: To convert apolipoprotein to grams per liter, multiply by 0.01; C-reactive protein to nanomoles per liter, multiply by 9.524; HDL-C and LDL-C to millimoles per liter, multiply by 0.0259; Lp(a)-1 to micromoles per liter, multiply by 0.0357; PAI-1 to picomoles per liter, multiply by 1.923; triglycerides to millimoles per liter, multiply by 0.0113.

a Data are adjusted for age, site, smoking behavior, body size, and race/ethnicity and backtransformed. Values are given as mean (SE) unless otherwise indicated. Statistically significant data are given in boldface type.

^b Groupwise comparison, $P \le .01$ vs premenopause.

^cGroupwise comparison, $P \le .01$ vs HT without progestin.

^dGroupwise comparison, $P \le .01$ vs HT with progestin.

^eGroupwise comparison, $P \leq .01$ vs postmenopause.

Table 3. Partial Correlations of Cardiovascular Measures With Serum Sex Hormone-Binding Globulin^a

Measure		r _{Y X} (95% CI)				
		Conjugated Equine Estrogen HT				
	Premenopause	Without Progestin	With Progestin	Postmenopause		
HDL-C, mg/dL	0.19 (-0.04 to 0.41)	0.36 (0.04 to 0.61)	0.10 (-0.03 to 0.24)	0.24 (0.13 to 0.34)		
logLDL-C, mg/dL	-0.33 (-0.53 to -0.11)	-0.17 (-0.47 to 0.16)	-0.15 (-0.29 to -0.01)	-0.07 (-0.18 to 0.04)		
Oxidized LDL-C, mg/dL	-0.38 (-0.58 to -0.14)	-0.18 (-0.50 to 0.17)	-0.02 (-0.16 to 0.13)	-0.10 (-0.21 to 0.02)		
logApo A-I, mg/dL	0.13 (-0.11 to 0.36)	0.37 (0.05 to 0.62)	0.18 (0.04 to 0.31)	0.18 (0.07 to 0.29)		
logApo B, mg/dL	-0.36 (-0.55 to -0.13)	-0.03 (-0.35 to 0.30)	-0.06 (-0.20 to 0.08)	-0.12 (-0.23 to -0.01		
log Triglycerides, mg/dL	-0.30 (-0.50 to -0.07)	-0.07 (-0.39 to 0.25)	0.01 (-0.13 to 0.15)	-0.24 (-0.34 to -0.13		
log C-reactive protein, mg/L	-0.26 (-0.47 to -0.01)	0.24 (-0.08 to 0.53)	0.17 (0.03 to 0.31)	-0.21 (-0.32 to -0.10		
logPAI-1, mg/dL	-0.44 (-0.61 to -0.22)	-0.52 (-0.73 to -0.22)	-0.48 (-0.58 to -0.36)	-0.49 (-0.57 to -0.40		

Abbreviations: Apo A-I, apolipoprotein A-I; Apo B, apolipoprotein B; CI, confidence interval; HDL-C, high-density lipoprotein cholesterol; HT, hormone therapy; LDL-C, low-density lipoprotein cholesterol; PAI-1, plasminogen activator inhibitor 1; $\kappa_{Y|X}$, partial correlation coefficient.

RELATING SEX STEROID MEASURES TO CVD MARKERS

In exogenous hormone users, the partial correlations between E_2 and HDL-C and apo A-I were 0.17 (95% CI, 0.05-0.29) and 0.19 (0.06-0.31), respectively. There were no other notable associations of CVD measures with E_2 , free estrogen index, or ERLL.

In premenopausal women, estrone metabolites were positively associated with LDL-C levels and with apo B. Partial correlations between LDL-C and the 2-OHE $_{\rm l}$ and 16α -OHE $_{\rm l}$ metabolites were 0.25 (95% CI, 0.02-0.47) and 0.33 (0.09-0.54), respectively. Partial correlations between apo B and 2-OHE $_{\rm l}$ and 16α -OHE $_{\rm l}$ metabolites were 0.20 (95% CI, 0.05-0.43) and 0.32 (0.08-0.53), respectively. However, in HT users, estrone metabolites and lipid measures were not statistically associated although mean metabolite levels were almost 50% higher in HT users.

Higher estrone metabolite levels were associated with higher levels of F_{2a} -isoprostanes in all groups, indicating that estrone metabolites were associated with greater oxidative stress. In premenopausal women, the partial correlations between the $_{\rm log}F_{2a}$ -isoprostanes and $_{\rm log}2$ -OHE $_{\rm l}$ and $_{\rm log}16\alpha$ -OHE $_{\rm l}$ metabolites were 0.57 (95% CI, 0.38-0.72) and 0.63 (0.46-0.76), respectively. In users of conjugated equine estrogen only, $r_{Y/X}$ values between $_{\rm log}F_{2a}$ -isoprostanes and $_{\rm log}2$ -OHE $_{\rm l}$ and $_{\rm log}16\alpha$ -OHE $_{\rm l}$ metabolites were 0.30 (95% CI, 0.05-0.58) and 0.43 (0.10-0.68), respectively. In users of conjugated equine estrogen plus progestin, $r_{\rm Y/X}$ values between $_{\rm log}F_{2a}$ -isoprostanes and $_{\rm log}2$ -OHE $_{\rm l}$ and $_{\rm log}16a$ -OHE $_{\rm l}$ metabolites were 0.44 (95% CI, 0.31-0.55) and 0.49 (0.37-0.59), respectively.

In premenopausal women, higher SHBG levels were associated with lower LDL-C, apo B, triglyceride, CRP, and PAI-1 levels (**Table 3**), suggesting a potential protective effect for SHBG. However, these relationships were not replicated in HT users, whose SHBG levels were remarkably higher than levels in premenopausal women. In women using conjugated equine estrogen only, SHBG levels were positively correlated with levels of HDL-C, apo A-I, and CRP; in women using conjugated equine estrogen plus proges-

tin, associations were mixed (negative associations with LDL-C and PAI-1 levels, but positive associations with CRP level). The SHBG level was negatively associated with CRP levels in premenopausal women but positively associated with CRP levels in HT users.

COMMENT

The WHI and the Heart and Estrogen/Progestin Replacement Study demonstrated that widely prescribed conjugated equine estrogen-based products were not identified as cardioprotective^{4,5} in women remote from their final menstrual period, thereby generating debate about explanatory mechanisms. This debate is currently centered about the timing hypothesis,6 which speculates that HT use should be initiated within 6 years of the menopause transition (on the basis of data extrapolated from monkeys subjected to ovariectomy)²⁷ so that optimal sex steroid and CVD profiles observed in the premenopause stage can be sustained before CVD progresses to an irreversible state. We related a panel of usual and more novel estrogen and androgen measures with cardiovascular markers in HT users, premenopausal women, and postmenopausal women under the theory that cardiovascular values observed in premenopausal women were an expression of the desirable CVD environment during HT use.

Traditionally, studies of HT use have focused on the HDL-C levels, citing the greater cardioprotective aspects with this lipid fraction. ^{2,3} We too found that HDL-C:LDL-C ratios and their apolipoprotein ratios were favorable in both conjugated equine estrogen groups. However, triglyceride levels were much less favorable in the HT users than in premenopausal women. This previously reported finding motivated the recommendation for using HT agents that do not require "first-pass" hepatic metabolism. ²⁸ The markedly higher SHBG levels, which affect sex steroid binding, may be a reflection of altered hepatic metabolism that indirectly affect cardiovascular measures. There were higher Lp(a)-1 and PAI-1 levels among HT users than in the other 2 groups.

^a Adjusted for age, site, smoking behavior, body size, and race/ethnicity. Sex hormone-binding globulin values were square root-transformed in nanomoles per liter. Statistically significant data are given in boldface type.

The Study of Women's Health Across the Nation

Clinical Centers

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Lipoprotein(a) is thought to recruit inflammatory cells through interaction with Mac-1 integrin.²⁹ The Lp(a) structure is similar to plasminogen and tissue plasminogen activator and it competes with plasminogen for its binding site, leading to reduction in fibrinolysis.

Although the effect of HT use on some, but not all, lipids appeared favorable, users had a less favorable oxidative environment and more pronounced inflammatory response. Premarin or the Premarin-progestin combination Prempro (Wyeth Pharmaceuticals Inc, Madison, New Jersey), which compose the preponderance of formulations used by SWAN (and all WHI) enrollees, include conjugated estrogens of which more than 50% are estimated to be estrone. Consistent with this, we identified much greater estrone metabolite excretion in HT users. Recent work suggests that higher estrone metabolite levels were highly associated with higher F_{2a}-isoprostane levels, 30 a product of the free radical-induced peroxidation of arachidonic acid. 31,32 F_{2a}-isoprostanes have been characterized as a superior marker of the in vivo oxidative stress response. $^{10,33-35}$ The mean F_{2a} -isoprostane level was highest in women using the conjugated equine estrogen plus progestin combination. Notably, this treatment arm of the WHI was terminated early because of excess cardiovascular risk.4

There were higher levels of CRP, an inflammation marker, in women using conjugated equine estrogen formulations than in postmenopausal women. Elevated CRP levels in hormone users have been reported previously by our group.³⁶ In the Postmenopausal Estrogen/Progestin Interventions Study, hormone use was associated with 50% higher levels of CRP than in nonusers.³⁷ The CRP level was higher in each treatment group, including estrogen alone or in combination with micron-

ized progesterone or medroxyprogesterone acetate.³⁷ The investigators in that study suggested that a mechanism for adverse cardiovascular events with HT use is through increasing inflammatory response, with the potential for accelerated atherosclerosis, plaque destabilization, or thrombosis.

Markedly higher SHBG levels were observed in women using HT. Sex hormone-binding globulin is synthesized in the liver with concentrations being regulated by the androgen-estrogen balance, thyroid hormones, and insulin. Other publications have identified that SHBG levels were significantly correlated with intermediate markers of CVD risk, 36,38 including positive associations with HDL-C concentrations and negative associations with LDL-C, oxidized LDL-C, triglyceride, and apo B levels during the menopausal transition.³⁹ Traditionally, descriptions of SHBG activity have focused on its binding capacity with testosterone and E₂ in determining bioavailability so that less than 2% to 3% of these sex steroids are available for hormone receptor-mediated action. 40 Expanded actions of SHBG are also being identified. 40 It remains to be determined whether the elevated 2-OHE₁ and 16α -OHE₁ metabolites bind with SHBG and disrupt the favorable role of SHBG seen in premenopausal women. It also remains to be determined whether alterations in liver metabolism alter the impact of sex steroids on CVD metabolism via the increased SHBG production.

We observed no significant associations of the ERLL assay. Potentially, estrogen receptor binding is a minor mechanism in the lipid synthesis and degradation system relative to other relevant receptors such as the apo B receptor.

Strengths of this report include infrequently characterized lipid measures (oxidized LDL-C and the

apo B:apo A-I ratio) for a more complete depiction of how hormone measures relate to CVD measures in HT users and nonusers. An expanded battery of sex steroid markers, especially the estrone metabolites, was also associated with CVD markers. Women were all premenopausal or in early perimenopause at study inception, so that transitions to postmenopause occurred within the 5 years of study observation. All study enrollees were free of hormone use at study inception, so that hormone therapy was initiated early in the menopausal transition. Women with heart disease at baseline were excluded from analysis. Data also reflect relationships observed before the release of WHI findings. However, there are limitations. Although we evaluated 2 specific formulations (conjugated equine estrogen with and without progestin) and duration of use, we did not specifically address dose. This cross-sectional evaluation precludes experimentally assigned HT use. Furthermore, SWAN includes measures of cardiovascular intermediates but not hard end points.

Collectively, our findings demonstrate positive and negative aspects of conjugated equine estrogen use relative to cardiovascular intermediate end points observed in women during the menopausal transition. The evidence suggests that conjugated equine estrogen use may provide positive effects via some, but not all, lipids; however, there were less favorable aspects of the use of conjugated equine estrogen, including higher estrone metabolite and SHBG levels and increased propensity for greater oxidative stress, thrombotic activity, and inflammation even in middle-aged women free of heart disease. From a research perspective, this study indicates that there should be a much closer examination of liver metabolism of SHBG and the estrone metabolites during conjugated equine estrogen use in relation to potential CVD risk. From a practice perspective, these results suggest very thoughtful consideration in the clinical use of HT because there was evidence of adverse HT effects even in women free of atherosclerosis who were studied in a time frame that was well within the approximate 6-year period proposed by the timing hypothesis.

Accepted for Publication: May 1, 2008.

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Author Contributions: All authors had full access to the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: Sowers. Acquisition of data: Sowers and McConnell. Analysis and interpretation of data: Sowers, Randolph, Jannausch, Lasley, and Jackson. Drafting of the manuscript: Sowers and Jannausch. Critical revision of the manuscript for important intellectual content: Sowers, Randolph, Lasley, Jackson, and McConnell. Statistical analysis: Sowers and Jannausch. Obtained funding: Sowers. Administrative, technical, and material support: Sowers, Randolph, and McConnell. Study supervision: Lasley and Jackson.

Financial Disclosure: None reported.

Funding/Support: The SWAN and the SWAN Reposi-

tory have grant support from the National Institutes of Health, Department of Health and Human Services, through the National Institute on Aging, the National Institute of Nursing Research, and the National Institutes of Health Office of Research on Women's Health (grants AG17104, AG017719, NR004061, AG012505, AG012535, AG012531, AG012539, AG012546, AG012553, AG012554, AG012495, and AG17104). The oxidized LDL-C, ERLL, estrogen metabolites, and isoprostane data are from an estrogen metabolism grant using specimens from the SWAN Repository (AG17104).

Additional Information: This report is based on samples from the SWAN Core Repository. If scientists are interested in developing studies based on this resource, a description of the SWAN Core and DNA Repositories and how to obtain access to the resources can be found at http://www.swanrepository.org.

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