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Associations between Markers of Inflammation and Physiological and Pharmacological Levels of Circulating Sex Hormones in Postmenopausal Women

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Abstract

Objective—Hormone therapy has been shown to reduce markers of vascular inflammation in postmenopausal women. C-reactive protein (CRP), a marker of generalized inflammation, is raised by oral estradiol therapy. It is not known how sex hormone concentrations relate to the markers of inflammation in postmenopausal women taking or not taking hormone therapy.

Methods—This observational study includes postmenopausal women participating in the Estrogen in the Prevention of Atherosclerosis Trial (EPAT). Multiple measures of serum sex hormone and sex hormone binding globulin (SHBG) levels from 107 postmenopausal women taking oral estradiol therapy (ET) and 109 taking placebo over 2 years were correlated with markers of inflammation over the same time period using generalized estimating equations.

Results—Levels of soluble intercellular adhesion molecule-1 (sICAM-1) were significantly inversely associated with estrone ($p = 0.05$), total and free estradiol ($p = 0.008$ and 0.02, respectively), and SHBG ($p = 0.03$) only among oral ET users. Serum homocysteine levels were also inversely associated with estrone ($p = 0.0001$), total and free estradiol ($p = 0.0006$ and 0.0009, respectively) in ET-treated women only. No such associations were observed among women

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taking placebo. C-reactive protein (CRP) was positively associated with estrogens and SHBG among women taking oral ET but inversely associated with SHBG among the placebo group.

Conclusions—The inverse associations of estrogens with sICAM-1, and homocysteine support an anti-inflammatory property of estrogen, which was only observed at pharmacologic levels in postmenopausal women. The positive associations between estrogens and CRP in the ET-treated women can be explained by the first-pass hepatic effect rather than a pro-inflammatory response.

Keywords

estrogen; androgen; CRP; sICAM; homocysteine

Introduction

Inflammation is a critical process in atherogenesis. Early stages of atherosclerosis involve an inflammatory process consisting of migration of leukocytes and monocytes into the subendothelium. Adhesion molecules such as soluble intercellular adhesion molecule-1 (sICAM), vascular cell adhesion molecule-1 (VCAM), E-selectin, and P-selectin mediate adhesion and migration of leukocytes into the arterial wall.[1] Circulating adhesion molecules are positively associated with levels of atherosclerosis 2 and risk for cardiovascular events.3

Coronary heart disease (CHD) is rare in women before menopause compared to men of the same age. The incidence of CHD in postmenopausal women is significantly higher than that of premenopausal women of the same age [4] suggesting a cardioprotective effect of endogenous sex hormones, particularly estrogen. In a cross-sectional study, circulating inflammatory markers including sICAM, VCAM, and E- and P-selectins were significantly higher in postmenopausal women not on hormone therapy (HT) compared with premenopausal women controlling for age and BMI.5

The impact of postmenopausal HT on markers of vascular inflammation such as adhesion molecules and cytokines have been studied by a number of investigators revealing an overall decrease in vascular inflammation among HT users.[6–8] In contrast, liver-derived C-reactive protein (CRP), a marker of generalized inflammation[9], increased with oral HT. 10

In postmenopausal women, endogenous or physiological levels of sex hormones differ markedly from the pharmacological levels induced by HT. It is unknown whether serum concentrations of estrogens, androgens, and sex hormone binding globulin (SHBG) correlate with markers of inflammation in postmenopausal women, or whether such associations differ at physiological and pharmacological levels of sex hormones.

We used data from the Estrogen in the Prevention of Atherosclerosis Trial (EPAT) to evaluate the association of estrogens, androgens and SHBG at physiological and pharmacological levels with markers of inflammation and pro-inflammatory factors in postmenopausal women. In EPAT, serum levels of sex hormones, CRP and sICAM-1, LDL-oxidation and homocysteine were measured longitudinally over 2 years. To our knowledge, this is the first study to relate both physiological and pharmacological sex hormone concentrations to inflammatory markers and pro-inflammatory factors longitudinally in the same cohort.

Methods

The EPAT study design has been described.[11] In brief, EPAT was a randomized, double-blind, placebo-controlled clinical trial designed to evaluate the impact of oral unopposed 17β-estradiol on subclinical atherosclerosis in postmenopausal women who had no clinically

evident cardiovascular disease. A total of 222 women were randomized to either placebo or active treatment of oral unopposed micronized 17β-estradiol (1mg/day). Women were eligible if estradiol level was <20pg/ml, LDL-cholesterol level ≥130mg/dl, fasting blood glucose level was <200mg/dl and were not smoking. Randomized participants were followed with clinic visits every month for the first six months, then every other month for the remainder of the 2-year trial period. Participants fasted for eight hours before sample collection. All study participants provided written informed consent and the study was approved by the Institutional Review Board of the University of Southern California.

Laboratory Measurements

Hormone Assays—Sex hormone concentrations were measured from fasting serum samples drawn at baseline and every 6 months during the trial period and stored at −70°C. Serum levels of androstenedione, dehydroepiandrosterone (DHEA), testosterone, estrone (E_1) and estradiol (E_2) were quantified by validated, previously described RIAs.[12] Prior to RIA, steroids were extracted from serum with hexane:ethyl acetate (3:2). Androstenedione (A4), DHEA and testosterone were then separated by Celite column partition chromatography using increasing concentrations of toluene in trimethylpentane. E_1 and E_2 were separated in a similar fashion by use of ethyl acetate in trimethylpentane. SHBG was quantified by direct immunoassay using the Immulite analyzer (Diagnostic Products Corporation, Inglewood, CA). Free testosterone was calculated using total testosterone and SHBG concentrations and an assumed constant for albumin in a validated algorithm.[13] Free E_2 was calculated in a similar manner.

All immunoassay methods were shown to be reliable. Specificity was achieved by using highly specific antisera and/or organic solvent extraction and chromatographic steps prior to quantification of the analytes. Assay accuracy was established by demonstrating parallelism between measured concentrations of a serially diluted analyte in serum with the corresponding standard curve. Intra- and inter-assay coefficients of variation (CV) ranged from 4 to 8% and from 8 to 13%, respectively. The sensitivities for the assays of androstenedione, DHEA, testosterone, estrone, estradiol and SHBG assay were 30 pg/ml, 0.05 ng/ml, 1.5 ng/dl, 5 pg/ml, 3 pg/ml and 1 nmol/L. The sensitivity of an RIA method was determined by the smallest amount of analyte that reduced the number of counts per minute of the radiolabeled analyte at zero mass by 2 standard deviations.

Inflammatory markers—Inflammatory markers were measured from fasting serum samples drawn at baseline and every 6 months during the trial period and stored at −70°C at the Laboratory for Clinical Biochemistry Research (University of Vermont, Burlington, Vermont). CRP was measured in fasting blood specimens by a validated high-sensitivity colorimetric competitive immunoassay (C-reactive protein antibodies and antigens from Calbiochem, La Jolla, California)[14]. The intra- and inter-assay CV was 3% and 6% respectively. sICAM-1 was measured using a commercially available ELISA assay (Parameter Human sICAM-1 Immunoassay; R&D Systems, Minneapolis, MN); intra- and inter-assay CV were 4.5% and 6.2% respectively.

***In vitro* oxidation of LDL—**The kinetics of LDL oxidation were analyzed by adding 10 μM $CuSO_4$ to 200 μg/mL LDL protein extracted from fresh serum collected in a fasting state. Formation of conjugated dienes was monitored continuously at 234 nm for up to sixteen hours using a Beckman DU-650 spectrophotometer equipped with a six position automated sample changer. Three measures of oxidation kinetics were analyzed: 1) the oxidation lag time which was defined as the interval between initiation of oxidation and the intercept of the tangent for the slope of the absorbance curve during the propagation phase, 2) the rate of oxidation during the lag time, defined as the initial oxidation rate before the

onset of the propagation phase, 3) the rate of oxidation during the propagation phase (the log rate) defined as the maximal rate of oxidation calculated from the slope of the absorbance curve during the propagation phase.

Homocysteine—Total fasting homocysteine concentrations were measured from stored frozen plasma samples by reverse phase HPLC with a C18 column on a Waters HPLC instrument equipped with a WISP automatic injector attached to a fluorimeter. Solutions containing 40 nmol/ml homocysteine and 200 nmol/ml cysteine were used as standards and for column calibration. For quality control, pooled plasma spiked with different quantities of cysteine and homocysteine were used. The CV for this assay was 7.8%.

Statistical analysis—Baseline demographic and clinical characteristics were compared between the treatment groups using student's t-test (for continuous variables) or chi-square test (for categorical variables). Mean on-trial levels of the sex hormones were compared between estradiol- and placebo-treated groups using t-tests for independent samples. Associations of sex hormone concentrations with inflammatory markers and pro-inflammatory factors were tested using linear regression with generalized estimating equations (GEE). The serial measures of inflammatory markers (CRP and sICAM-1) and proinflammatory factors (homocysteine and LDL oxidation) were the dependent variables and time-dependent measures of sex hormones/SHBG were the primary explanatory variables. Both the outcome and explanatory measures were modeled as continuous variables. Inflammatory markers/proinflammatory factors at a given follow-up time (trial visit) were regressed on the corresponding sex hormone measure measured from the same trial visit, accounting for the within-subject effect. Because age and/or BMI were associated with serum sex hormone and SHBG concentrations as well as sICAM and CRP levels, all models evaluating the association of serum sex hormones and SHBG with inflammatory markers were adjusted for age and BMI. Separate regression models were fitted for the estradiol- and placebo-treated groups. All analyses used SAS (version 9.1, Cary, NC); 2-sided p-values are reported.

Results

Data on markers of inflammation and sex hormones were available on 216 women from a total of 735 trial visits (median 4 (range 1–5) per subject). The average (SD) age of the EPAT participants was 61 (7) years and the majority of the women were non-Hispanic White (61%). The women were overweight with an average (SD) BMI of 29 (5) kg/M^2 and the mean (SD) LDL-cholesterol was 164 (28) mg/dl. The median (range) levels of inflammatory markers were: sICAM 282 (107–472) ng/mL, CRP 1.5 (0.37–13.4) mg/L and homocysteine 7.4 (4.1–40.2) nmol/ml. The baseline demographic and clinical characteristics were not statistically significantly different between the placebo and estradiol group (p-value for difference for all >0.30; Table 1).

Women treated with oral estradiol (1mg/day) had significantly higher estrone, total and free E2 and SHBG levels (all p-values <.0001; Table 2). In contrast, androgens including free testosterone (p = 0.0001), A4 (p<.0001), and DHEA (p<.0001) were significantly lower in estradiol-treated compared with placebo-treated women. Serum levels of total testosterone did not differ between the two treatment groups (p = 0.72).

Among women on ET, sICAM-1 was significantly inversely associated with estrone (p = 0.05), total E2 (p = 0.008), free E2 (p = 0.02) and SHBG (p = 0.03), adjusted for age and BMI (Table 3). A significant positive association was observed between sICAM-1 and free testosterone (p = 0.03). Total testosterone, A4 and DHEA were not associated with

sICAM-1. None of the sex hormones were statistically significantly associated with sICAM-1 among the placebo group (Table 4).

Women receiving ET had a significant positive association between CRP and estrone (p = 0.0003), total E2 (p<.0001), free E2 (p = 0.001) and SHBG (p = 0.002), adjusted for age and BMI (Table 4). A significant inverse association was observed between CRP and DHEA (p = 0.006); the remaining androgens were not associated with CRP. Among women receiving placebo, free E2 was positively (p = 0.05) associated with CRP whereas total testosterone and SHBG were inversely associated with CRP (p = 0.03 and 0.004 respectively).

Adjusted for age and BMI, homocysteine was significantly inversely associated with estrone (p = 0.001), total E2, (p = 0.0006) and free E2 (p = 0.0009) in estrogen users (Table 5). None of the androgens or SHBG was significantly associated with homocysteine among women taking ET. No hormone was significantly associated with homocysteine in the placebo group.

Lag time, lag rate and log rate, three parameters of LDL-oxidation kinetics, were not associated with any of the sex hormones in either treatment group (all p-values >0.10; data not shown). sICAM-1 was significantly positively associated with homocysteine (β estimate ±SE = 1.80±0.38; p<.0001) but not with LDL oxidation parameters. CRP was not associated with homocysteine or LDL oxidation lag time (data not shown).

Discussion

Few studies have examined the relationship between sex hormone concentrations and markers of inflammation in postmenopausal women. The majority of such studies included women not on HT, while a few others included HT users. Our population, half taking oral ET, the other half taking placebo, offers the unique opportunity to compare the associations of inflammatory markers with sex hormones at pharmacologic as well as physiologic levels. EPAT women using ET had significantly higher levels of estrogens and SHBG and lower levels of androgens including free T, DHEA and androstenedione.

The inverse associations between sICAM-1 and estrogens among ET users indicate a beneficial anti-inflammatory effect of estrogens at pharmacologic levels. However, lack of such associations in the placebo group indicate that the beneficial anti-inflammatory impact of estrogens can only be expected at higher levels of estrogens as in ET users but not at the endogenous physiologic levels of postmenopausal women not using ET. According to a cross-sectional study, postmenopausal women (n = 74) not using HT had 24% higher mean levels of circulating ICAM-1 compared to premenopausal women (n = 60) (p<.001).[5] Our results support the evidence that inflammatory marker levels rise after menopause due to a sharp decline of estrogen. Our findings also support the results from multiple studies showing favorable effects of HT on soluble markers of vascular inflammation.[6–8, 15] In EPAT, ET also significantly reduced plasma sICAM-1 levels.[16] In-vivo studies have also demonstrated an inhibitory effect of estradiol on cytokine-induced intercellular adhesion molecule.[17]

We did not find any significant associations between endogenous physiological levels of estrogens and sICAM-1 among the non-ET using postmenopausal women. Consistent with our finding, a cross-sectional study among a subset of participants (n=623) from the Postmenopausal Estrogen Progestin Intervention (PEPI) Trial [18] showed no association of endogenous serum levels of total estradiol measured at baseline (prior to HT initiation) with IL-6 and sICAM-1. These results indicate that physiological levels of estrogen in postmenopausal women are too low to have any beneficial impact on this inflammatory marker.

We also found that higher SHBG levels were associated with lower sICAM-1 concentrations in ET users. No such association was observed at the physiological concentration of SHBG. Lower serum SHBG has consistently been associated with increased risk of atherosclerosis and CVD in postmenopausal women.[19–21] We recently reported a significant beneficial impact of SHBG on atherosclerosis progression in the EPAT population.[22] However, the mechanisms involved in the cardioprotective effect of SHBG are largely unknown. Several hypotheses have been proposed including maintenance of the estrogen to androgen ratio and a direct effect of SHBG on endothelial cell-mediated cyclic AMP.[19] Our analyses showed that a higher SHBG concentration was significantly associated with lower sICAM-1 levels, which may partly explain the cardioprotective effect of SHBG. The molecular mechanism of such an effect remains to be explored.

Among women with physiologic sex hormone levels (placebo group), there was a significant inverse relationship between SHBG and CRP, which is consistent with previous reports.[18, 23–25] On the other hand, pharmacologic levels of estrogen (oral ET group) were associated with an increase in CRP. It is noteworthy that despite a reduction in the levels of other markers of inflammation such as E-selectin, VCAM, sICAM-1, serum amyloid A, and IL-6 as reported in many studies, oral ET has been consistently associated with elevation of CRP levels and has been attributed to the hepatic first-pass metabolism of estradiol taken orally.[6, 7, 10, 15, 26] Like CRP, SHBG is also produced in the liver and oral ET has been consistently shown to increase the levels of SHBG, a finding also supported by our study (Table 2). Therefore, the strong positive associations we found between estrogens/SHBG and CRP among oral ET users could be explained by a first-pass hepatic effect rather than a generalized pro-inflammatory response. In support of this explanation, CRP was not associated with atherosclerosis progression in EPAT participants.[16] However, our finding of an inverse association of total testosterone and CRP at physiologic concentrations among ET non-users is novel and requires further understanding.

Homocysteine, an intermediate metabolite of methionine can cause endothelial dysfunction and has been suggested to be a potent inducer of inflammation.[27, 28] Elevated levels of homocysteine have been associated with increased IL-6 production from monocytes, and can enhance monocyte adhesion to the vascular endothelium by upregulating VCAMs.[29, 30] Several HT trials have shown that oral estrogen use, alone or combined with progestin, lowered serum homocysteine levels in postmenopausal women.[31–34] Our data support another potential anti-inflammatory property of estrogen showing a significant inverse association between estrogens and homocysteine concentrations. Of note, this beneficial effect of estrogen was only observed at pharmacologic levels in women using ET.

Since ET significantly reduced serum concentrations of all the androgens except for total testosterone in EPAT, we expected that androgens might not show significant associations with markers of inflammation among ET users. However, we did see a significant positive association between free testosterone and sICAM-1 and an inverse association between DHEA and CRP among the ET users. Unlike estrogens, the effect of androgens on markers of inflammation in postmenopausal women has not been frequently studied. A cross-sectional report from PEPI demonstrated a borderline inverse association between endogenous levels of bioavailable testosterone and sICAM-1 among women not treated with HT.[18] Our results showed no associations between sICAM-1 and any of the androgens at physiologic concentrations, but a significant positive association between free testosterone and sICAM-1 among ET users. The PEPI substudy also reported a significant positive association between bioavailable testosterone and CRP among postmenopausal women not using HT, a finding supported by the longitudinal study of women of mid-life in the Study of Women Across the Nation (SWAN).[25] In contrast, we found a significant inverse association between CRP and free testosterone at physiologic concentrations. A possible

explanation for such differences between our study and the PEPI and SWAN sub-studies could be the cross-sectional nature of those latter studies, whereas in the current study, we had multiple assessments of the hormones over 2 years. Although there are reports showing a detrimental effect of testosterone on human vascular endothelial cells 35 and an anti-inflammatory effect of DHEA 36, we could not explain why we observed the pro- and anti-inflammatory association of testosterone and DHEA, respectively, only at pharmacologic levels. Further evaluation of the role of testosterone in vascular inflammation and risk of cardiovascular disease in postmenopausal women is warranted.

The primary results of EPAT showed a significant reduction in the progression carotid atherosclerosis in women taking oral ET compared to the placebo group [11] . We have also shown in EPAT that serum levels of estrogens including E1, total and free E2, and SHBG were significant inverse correlates of carotid atherosclerosis progression, and these associations were partly mediated by the beneficial effect of estrogens and SHBG on lipids. [22] Results reported in the current manuscript suggest an anti-inflammatory impact of estrogen and SHBG at pharmacological levels. Although oral ET-induced changes in the inflammatory markers was not associated with subclinical atherosclerosis progression in this group of women, as reported in a previous publication[16], results of the current study may have implications for cardiovascular events including ischemic heart disease and cardiovascular mortality. Evidence shows soluble adhesion molecules, including sICAM, can predict future death among patients with coronary artery disease.[37] Homocysteine has been linked with ischemic heart disease and stroke.[38]

Conclusion

In conclusion, our results support the anti-inflammatory effect of estrogens in postmenopausal women as these hormones were inversely associated with sICAM-1 and homocysteine. These results are suggestive of a potential protective role of estrogen on cardiovascular disease. We also report that these anti-inflammatory effects are only achieved at ET-induced pharmacologic levels. The positive associations of estrogens and SHBG with CRP at pharmacologic levels may not be a pro-inflammatory effect but rather might be explained by a hepatic first-pass mechanism. However, the underlying mechanism for the inverse association between SHBG and CRP levels at physiologic levels needs further attention. Future studies are warranted to understand the impact of androgens on markers of inflammation.

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Table 1

Demographic and baseline clinical characteristics of EPAT women (n=216)

Treatment group	Placebo (n=109)	Estradiol (n=107)
Age (years)	62 (7)	61 (7)
Race		
White	64 (59%)	58 (54%)
African-American	10 (9%)	14(13%)
Hispanic	24 (22%)	22(21%)
Others	11 (10%)	13(12%)
BMI (kg/M^2)	29 (5)	29 (6)
Smoking		
Never	58 (53%)	49 (46%)
Former smokers	51 (47%)	58(54%)
Blood pressure (mmHg)		
Systolic	129 (14)	127 (15)
Diastolic	78 (7)	78 (8)
Total cholesterol (mg/dl)	216 (35)	218 (34)
HDL-cholesterol (mg/dl)	54 (12)	53(12)
LDL-cholesterol (mg/dl)	162 (26)	165(29)
Glucose (mg/dl)	94 (25)	90(15)
sICAM-1 (ng/ml)	284 (61)	285(59)
CRP (μg/ml)	2 (2)	2(2)
Homocysteine (nmol/ml)	8 (2)	8(3)

Mean (SD) or n(%)

P-value for difference for all variables > 0.30

Table 2

Mean serum levels of sex hormones and SHBG by treatment group (n = 180)[§]

Sex hormones	Placebo (n=89)	Estradiol (n=91)	
	Mean (SD)[†]	Mean (SD)[†]	p-value[*]
Estrone (pg/ml)	48.1(42.0)	310.3(167.2)	<.0001
Estradiol (pg/ml)	14.7(6.3)	68.2(16.2)	<.0001
Free estradiol (pg/ml)	0.6(0.2)	1.6(0.6)	<.0001
Total testosterone (ng/dl)	22.3(9.0)	22.8(10.8)	0.72
Free testosterone (pg/ml)	4.1(1.8)	3.1(1.6)	0.0001
Androstenedione (pg/ml)	522(189)	492 (180)	<.0001
DHEA (ng/ml)	2.14(1.34)	1.77(0.78)	<.0001
SHBG (nmol/L)	36.5(18.2)	58.2(23.6)	<.0001

[§]Of the total 216, 36 women had baseline but no ontrial hormone assessment, either due to drop-out or lack of sample.

[*]P-values were from t-test for independent samples.

[†]Mean (SD) of all follow-up measures.

Table 3

Association between sex hormone/SHBG levels and sICAM-1 (ng/ml) by treatment group

Hormones	Placebo		Estradiol	
	β (SE) estimate*	p-value	β (SE) estimate*	p-value
Estrone (pg/ml)	−.06(0.11)	0.56	−.02(0.008)	0.05
Estradiol (pg/ml)	0.03(0.15)	0.84	−.12(0.04)	0.008
Free estradiol (pg/ml)	2.7(4.5)	0.55	−4.3(1.8)	0.02
Total testosterone (ng/dl)	0.35(0.31)	0.25	−.34(0.25)	0.16
Free testosterone (pg/ml)	0.90(1.9)	0.63	2.4(1.2)	0.04
Androstenedione (pg/ml)	0.006(0.091)	0.50	0.02(0.01)	0.19
DHEA (ng/ml)	−1.2(1.5)	0.40	5.0(2.6)	0.06
SHBG (nmol/L)	−.10(0.27)	0.72	−.17(0.07)	0.03

*β estimates are from generalized estimating equations models, adjusted for age and BMI.

Table 4

Association between sex hormone/SHBG levels and CRP (μg/ml) by treatment group

Hormones	Placebo		Estradiol	
	β (SE) estimate*	p-value	β (SE) estimate*	p-value
Estrone (pg/ml)	0.01(0.01)	0.32	0.002(0.0005)	0.0003
Estradiol (pg/ml)	0.02(0.01)	0.09	0.007(0.002)	<.0001
Free estradiol (pg/ml)	0.93(0.47)	0.05	0.41(0.12)	0.001
Total testosterone (ng/dl)	−.02(0.01)	0.03	0.02(0.01)	0.27
Free testosterone (pg/ml)	−.02(0.05)	0.67	−.09(0.06)	0.12
Androstenedione (pg/ml)	−.0001(0.0004)	0.90	−.0002(0.0005)	0.67
DHEA (ng/ml)	−.10(0.06)	0.10	−.22(0.08)	0.006
SHBG (nmol/L)	−.02(0.006)	0.004	0.01(0.004)	0.002

*β estimates are from generalized estimating equations models, adjusted for age and BMI.

Table 5

Association between sex hormone/SHBG levels and homocysteine (mg/ml) by treatment group

Hormones	Placebo		Estradiol	
	β (SE) estimate*	p-value	β (SE) estimate*	p-value
Estrone (pg/ml)	−.002(0.002)	0.35	−.002(0.0005)	0.001
Estradiol (pg/ml)	0.0001(0.007)	0.98	−.01(0.003)	0.0006
Free estradiol (pg/ml)	−.01(0.31)	0.96	−.48(0.15)	0.0009
Total testosterone (ng/dl)	−.02(0.01)	0.09	−.01(0.01)	0.38
Free testosterone (pg/ml)	−.09(0.06)	0.09	0.11(0.08)	0.18
Androstenedione (pg/ml)	0.0005(0.005)	0.36	−.0002(0.001)	0.71
DHEA (ng/ml)	0.01(0.08)	0.90	0.10(0.10)	0.31
SHBG (nmol/L)	0.003(0.63)	0.63	−.008(0.006)	0.14

*β estimates are from generalized estimating equations models, adjusted for age, and BMI.