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ARTICLE

Sex Hormone Levels and Risks of Estrogen Receptor–Negative and Estrogen Receptor–Positive Breast Cancers

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Background Endogenous sex hormone levels are associated with risks of breast cancer overall and estrogen receptor (ER)–positive breast tumors; however, their associations with ER-negative tumors remain unclear.

Methods In a case–cohort study within the Women's Health Initiative Observational Study among postmenopausal women aged 50–79 years, we examined associations between endogenous testosterone and estradiol levels and the risks of ER-negative and ER-positive breast cancers. Serum levels of bioavailable testosterone and estradiol were assessed at the baseline visit in 317 invasive breast cancer case subjects and in a subcohort of 594 women. Bioavailable sex hormone levels were calculated using the total hormone level and the sex hormone–binding globulin concentration (measured by radioimmunoassays and a chemiluminescent immunoassay, respectively). Cox proportional hazards regression was used for statistical analysis. All statistical tests were two-sided.

Result The unadjusted absolute rates of ER-negative breast cancer for testosterone quartiles 1–4 were 0.34, 0.20, 0.23, and 0.21 per 10 000 person-years, respectively. Compared with women in the lowest quartile of testosterone level, those in quartile 2 had a 56% lower risk of ER-negative cancer (hazard ratio [HR] = 0.44, 95% confidence interval [CI] = 0.23 to 0.85), those in quartile 3 had a 45% lower risk (HR = 0.55, 95% CI = 0.30 to 1.01), and those in quartile 4 had a 49% lower risk (HR = 0.51, 95% CI = 0.28 to 0.94), independent of other risk factors. Estradiol level was not associated with ER-negative breast cancer. ER-positive breast cancer risk increased with higher testosterone levels (P_{trend} = .04), but this trend was not statistically significant after adjustment for estradiol (P_{trend} = .15). ER-positive cancer risk was approximately twofold higher in women with estradiol levels in quartiles 2–4 compared with women in quartile 1, independent of risk factors.

Conclusion Higher serum levels of bioavailable testosterone are associated with lower risks of ER-negative breast cancer in postmenopausal women.

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Estrogen receptor (ER)–positive and ER-negative breast cancers are emerging as distinct disease subtypes with different risk factors. Accumulating evidence (1–4) supports the need to evaluate breast cancer etiology and risk separately for each of these tumor subtypes.

The preponderance of observational epidemiological studies has reported strong associations between higher levels of estradiol and testosterone and an increased risk of postmenopausal breast cancer, especially ER-positive breast cancers (3,5–10). Preclinical data indicate that testosterone has dual effects on breast tumorigenesis: a proliferative effect mediated by the ER and an antiproliferative effect mediated by the androgen receptor (11,12). Therefore, testosterone has the potential to regulate the growth of both ER-positive and ER-negative tumors. Epidemiological studies that have investigated the effects of sex hormones have generally focused on associations with total breast cancer or have excluded ER-negative cancers from the analysis (3,5,9,10). The few reports that have evaluated the role of sex hormones separately in ER-negative breast cancer did not detect statistically significant associations; however, those studies included only a limited number of ER-negative cancers (7,8,13).

We used the Women's Health Initiative Observational Study (WHI-OS) to test the hypotheses that the risks of ER-negative as well as ER-positive breast cancer in postmenopausal women are associated with circulating levels of endogenous testosterone and estradiol.

Materials and Methods

Study Population

Participants were enrolled in the WHI-OS, a large multicenter prospective cohort study that was designed to examine the epidemiology of various disease outcomes in postmenopausal women. A description of the WHI-OS design and rationale has been reported elsewhere (14). The cohort included 93 676 postmenopausal women aged 50–79 years who were recruited between September 1993 and December 1998 at 40 clinical centers in the United States. Women were ineligible for the WHI-OS if they had a predicted survival time of less than 3 years, were known to have conditions inconsistent with study participation (ie, alcohol or drug dependency, mental illness, or dementia), or were participating in a clinical trial. All participants had a baseline clinic visit at which they underwent a physical examination and provided a blood specimen and risk factor information according to standardized procedures and protocols performed by trained staff. Subsequently, the women were followed up annually via mailed questionnaires and at a repeat visit 3 years after enrollment. The institutional review board at each clinical center approved the study protocol, and written informed consent was obtained from all participants.

By the end of follow-up through March 31, 2005, 2723 incident cases of invasive breast cancer had been diagnosed (2309 were ER positive and 414 were ER negative).

Selection of Case–Cohort Sample

This study used a case–cohort design and included 317 women with incident invasive breast cancer (111 were ER negative and 206 were ER positive) in addition to a randomly selected subcohort of 594 women.

Women in the original WHI-OS cohort (n = 93 676) were excluded from this case–cohort study based on the following criteria (applied in hierarchical order): 1) lacked sufficient baseline serum for sex hormone measurements (n = 4830); 2) reported a history of breast cancer (n = 5142); 3) were using hormone therapy at the baseline visit or within 2 years before the baseline visit (n = 44 742); 4) were using an androgen, dehydroepiandrosterone, or raloxifene at the baseline visit (n = 87); 5) had initiated hormone therapy or use of an androgen, dehydroepiandrosterone, or raloxifene during follow-up (n = 9375); 6) did not provide information about breast cancer status during follow-up (n = 309); or 6) did not indicate their race or ethnicity (n = 489).

Of the 28 702 eligible women, 111 had incident ER-negative breast cancer and 571 had incident ER-positive cancer. We included all 111 ER-negative case subjects and a randomly chosen sample of 206 ER-positive case subjects, after excluding five women who had an ER-positive cancer that was diagnosed after August 31, 2004, the date of diagnosis of the last ER-negative cancer that occurred in the cohort. The comparison subcohort constituted a random sample of 594 women who were selected from the entire baseline eligible population of 28 702 women to achieve an approximate 2:1 ratio for the number of women in the subcohort to the number of breast cancer case subjects. The subcohort included eight women who developed breast cancer during follow-up (four ER-negative case subjects and four ER-positive case subjects).

CONTEXT AND CAVEATS

Prior knowledge

Observational epidemiological studies have reported strong associations between higher levels of estradiol and testosterone and an increased risk of postmenopausal breast cancer, especially estrogen receptor (ER)–positive breast cancers. The few studies that have examined the role of sex hormones separately in ER-negative breast cancer detected no statistically significant associations; however, those studies included few ER-negative cancers.

Study design

A prospective case–cohort study within the Women's Health Initiative Observational Study among postmenopausal women aged 50–79 years who were not taking exogenous hormones examining associations between serum levels of bioavailable (ie, free plus albumin bound) testosterone and estradiol and the risks of ER-negative and ER-positive breast cancers.

Contribution

Higher serum levels of testosterone were associated with a lower risk of ER-negative breast cancer, independent of putative breast cancer risk factors and serum estradiol level. Higher serum level of estradiol was associated with an increased risk of ER-positive breast cancer.

Implications

These results shed new light on the etiology of ER-negative breast cancer and further reinforce the need to assess risk separately for ER-positive and ER-negative breast cancers.

Limitations

Sex hormone concentrations were measured once. Women who were diagnosed with breast cancer during follow-up may have had subclinical disease at baseline. The results may not be generalizable to postmenopausal women who use hormone therapy, racial groups other than non-Hispanic whites, and premenopausal women.

From the Editors

Follow-up time was defined as the time from the date of enrollment in WHI-OS to the date of breast cancer diagnosis, the last contact with the participant, or August 31, 2004 (date of diagnosis of the last ER-negative cancer that occurred in the cohort), whichever occurred first.

Ascertainment and Validation of Breast Cancer Cases

Breast cancer outcomes were initially ascertained through participants' responses on the annual self-administered questionnaires. Events were subsequently adjudicated through a centralized review of medical records and pathology reports. Clinical and pathological characteristics of the breast tumors, including ER status (ER positive vs ER negative) and progesterone receptor (PR) status (PR positive vs PR negative), were obtained from the pathology reports and coded according to the National Cancer Institute's Surveillance, Epidemiology, and End Results program guidelines (15,16). Of the 111 ER-negative case subjects included in this study, 102 (92%) were also PR negative. Of the 206 ER-positive case subjects, 165 (80%) were also PR positive.

Blood Samples and Measurements of Endogenous Sex Hormones

Endogenous sex hormone levels were measured using blood samples that were collected at the baseline visit from women who had fasted for at least 12 hours. The blood samples were centrifuged at 1300*g* for 10 minutes, and the separated sera were stored in aliquots at −70°C within 2 hours of blood collection (17). For this study, serum aliquots were shipped on dry ice to the Reproductive Endocrine Research Laboratory (University of Southern California, Los Angeles, CA), where sex hormones and sex hormone-binding globulin (SHBG) levels were measured. Laboratory personnel were blinded to case status, and the samples were analyzed in random order.

Total estradiol and testosterone concentrations were quantified using sensitive and specific radioimmunoassays following organic solvent extraction and Celite column partition chromatography, as previously described (18). For the estradiol radioimmunoassay, the intra-assay coefficient of variation (CV) was 7.9% at 34 pg/mL (124 pmol/L), and the interassay CVs were 8.0% at 16 pg/mL (58.7 pmol/L) and 12.0% at 27 pg/mL (99.1 pmol/L). For the testosterone radioimmunoassay, the intra-assay CV was 6% at 14.3 ng/dL (0.50 nmol/L), and the interassay CVs were 12.0% at 4.9 ng/dL (0.17 nmol/L), 11.0% at 14.3 ng/dL (0.5 nmol/L), and 10.0% at 47.9 ng/dL (1.66 nmol/L). The sensitivity of the estradiol assay was 2 pg/mL (11.0 pmol/L) and the sensitivity of the testosterone assay was 1.5 ng/dL (0.052 pmol/L).

We calculated the concentrations of bioavailable (ie, free plus albumin bound) estradiol and testosterone with a validated algorithm that used the measured total concentrations of estradiol or testosterone, SHBG level, an assumed constant representing the normal albumin concentration, and the association constants for the binding of estradiol and testosterone to SHBG and albumin (19,20). Calculated values for bioavailable estradiol and testosterone have been previously shown to be highly correlated ($r \geq .85$) with direct measures of free hormone levels (21). SHBG was quantified with the use of an Immulite Analyzer (Siemens Medical Solutions, Malvern, PA) that performs a solid-phase two-site chemiluminescent immunoassay for SHBG. The solid phase consisted of polystyrene beads coupled to a mouse monoclonal antibody specific for SHBG (Siemens Medical Solutions). The intra-assay CVs for the SHBG assay were 2.5% at 21 nmol/L, 2.7% at 63 nmol/L, and 5.3% at 80 nmol/L. The interassay CVs were 5.2% at 21 nmol/L, 5.2% at 63 nmol/L, and 6.6% at 80 nmol/L. The SHGB assay had a sensitivity of 0.2 nmol/L.

We used the calculated bioavailable levels of testosterone and estradiol in our analyses rather than the total or free levels because the bioavailable levels of sex hormones reflect the unbound fraction of the hormone as well as the fraction that is loosely bound to albumin and thus are more indicative of the bioactive hormone concentrations.

Breast Cancer Risk Factors

All breast cancer risk factors used in this analysis were collected at the baseline WHI-OS study visit. Self-administered questionnaires were used to collect information on demographic characteristics (age, race and ethnicity, educational level, and marital status), menstrual and reproductive histories (age at menarche and menopause, age at birth of first child birth, number of pregnancies, and breastfeeding practices), family history of breast cancer in a first-degree relative, number of breast biopsies, medical history (including hysterectomy and bilateral oophorectomy), and lifestyle and dietary habits (current smoking status, alcohol intake, and physical activity level). Total energy expenditure from physical activity (expressed in metabolic equivalents as kilocalorie hours per week per kilogram) was calculated based on responses to questions about the frequency and duration of a range of physical activities in the previous week as previously described (22).

Current use of medications, vitamins, and supplements was assessed through direct review of participants' medication bottles at the baseline visit. Information regarding past use of hormone therapy was assessed by use of an interviewer-administered questionnaire.

Weight was measured to the nearest 0.1 kg with the use of a balance beam scale while the participant was wearing indoor clothing. Height was measured to the nearest 0.1 cm with the use of a fixed stadiometer. Body mass index (BMI) was calculated as weight (in kg) divided by height squared (in m).

The Gail model (23) was used to estimate the 5-year absolute risk of breast cancer (Gail risk score) based on age, ethnicity, age, age at menarche, age at birth of first child, number of breast biopsies, and number of first-degree relatives with breast cancer. Information on the presence of atypical hyperplasia was not available, and this variable was coded as "unknown" in the Gail risk calculation. Gail risk scores were dichotomized as greater than vs less than or equal to 1.7%, the risk score that represents the average 5-year breast cancer risk of a 60-year-old woman and the cut point used for inclusion in the National Surgical Adjuvant Breast and Bowel Project P-1 tamoxifen prevention trial (24).

Statistical Analyses

All analyses were performed separately for ER-negative and ER-positive breast cancers. Differences in baseline characteristics and hormone levels between breast cancer case subjects and noncase subjects were compared using a *t* test or the Wilcoxon rank sum test for continuous data and the Pearson χ^2 test for categorical data. To evaluate associations between endogenous circulating sex hormones and incident ER-negative and ER-positive breast cancers, we used a modified Cox proportional hazards model that accounted for the case–cohort sampling design. The modification to the model entailed setting the entry time for case subjects who were not part of the random subcohort to be approximately equal to, but slightly less than, their time of diagnosis. For case–cohort modeling, we used the Prentice method (25), which uses an "unweighted" approach for pseudo-likelihood estimation. Hazard ratios (HRs) and 95% confidence intervals (CIs) were calculated by quartiles of sex hormone levels, using the lowest quartile (Q1) as the referent group. Quartile cutoff points were established based on the distribution of hormone levels in the subcohort. Robust standard errors were used to derive unbiased 95% confidence intervals. The proportional hazards assumption was confirmed by testing the statistical significance of interaction terms for covariates with time. Unadjusted and risk factor–adjusted analyses were performed. Variables were selected for entry into the regression

model if they were associated with either ER-negative or ER-positive breast cancer in univariate analyses (at the .10 level of statistical significance), with the exception of BMI, which was forced into the model. Thus, we adjusted for age, race, age at menopause, BMI, physical activity, prior needle aspiration of a breast lump, lifetime use of estrogen plus progestin therapy, and time since ending hormone therapy. Although the Gail risk score was associated with the risk of ER-positive breast cancer in univariate analysis, it was not included in the final models because some of the component variables (such as age and race) were adjusted for individually. Models for ER-negative and ER-positive breast cancer were adjusted for the same set of covariates to ensure that the two models would be comparable. To assess whether the associations of the two sex hormones with breast cancer were independent of each other, the individual estradiol and testosterone models were further adjusted for the other sex hormone. We tested for linear trend across quartiles of hormone level to determine if their associations with breast cancer followed a linear dose–response pattern. These tests were performed by including a four-level variable for sex hormone quartiles as a continuous predictor in the Cox regression model and assessing the statistical significance of this variable by using a Wald test.

The presence of a threshold effect in the sex hormones–breast cancer associations was investigated by using linear spline models that were fitted using a single knot at the potential threshold value.

We also assessed the joint association between estradiol and testosterone levels and the risk of ER-positive breast cancer. The joint categories of estradiol and testosterone were defined a posteriori and based on examination of the individual associations between these hormones and the risk of ER-positive breast cancer. For estradiol, given the similar effect sizes of the associations between quartiles 2, 3, and 4 (Q2, Q3, and Q4) and the risk of breast cancer, these quartiles were combined and labeled "high estradiol," whereas Q1 was labeled "low estradiol." For testosterone, based on the observed associations across quartiles, Q3 was combined with Q4 (and designated the "high testosterone" group), and Q1 was combined with Q2 (designated the "low testosterone" group). The resulting joint categories of estradiol and testosterone were defined as follows: low estradiol and low testosterone (being in Q1 for estradiol and Q1 or Q2 for testosterone), low estradiol and high testosterone (being in Q1 for estradiol and Q3 or Q4 for testosterone), high estradiol and low testosterone (being in Q2, Q3, or Q4 for estradiol and Q1 or Q2 for testosterone), and high estradiol and high testosterone (being in Q2, Q3, or Q4 for estradiol and Q3 or Q4 for testosterone). We did not assess the joint association between testosterone and estradiol levels and the risk of ER-negative breast cancer because we did not observe that these hormones were independently associated with risk of this cancer.

All estradiol models excluded women with missing values for the hormone (n = 30; three ER-negative case subjects, seven ER-positive case subjects, and 20 noncase subjects) and women with extreme total estradiol levels (values >40 pg/mL, n = 17; one ER-negative case subject, seven ER-positive case subject, and nine noncase subjects). The testosterone models excluded women with missing hormone values (n = 3; all noncase subjects) and women with extreme total testosterone concentrations (values >150 ng/dL, n = 5; three ER-positive case subjects and two noncase subjects). The cut points for extreme hormone levels indicate values beyond which hormone measurements are considered to be biological outliers, as defined by the laboratory.

Statistical significance was defined as P less than .05. All tests of statistical significance were two-sided. All analyses were done using SAS software (version 9.1; SAS Institute, Inc, Cary, NC). Case–cohort models were performed using the PHREG procedure in SAS.

Results

Population Characteristics

The average follow-up time was 6.0 years (SD = 2.5 years) for the whole study sample, 3.9 years (SD = 2.4 years) for ER-negative case subjects, 3.5 years (SD = 2.2 years) for ER-positive case subjects, and 7.1 years (SD = 1.7 years) for women in the subcohort.

Compared with noncase subjects, women diagnosed with ER-negative breast cancer were younger (P = .04), more physically active (P = .04), and more likely to have had a needle aspiration of a breast lump (P < .001). ER-negative case subjects and noncase subjects were similar with regard to race, reproductive factors, Gail risk score, BMI, and family history of breast cancer (Table 1).

ER-positive case subjects were slightly older than noncase subjects (P = .05) and more likely to be white (P = .002). In addition, compared with noncase subjects, ER-positive case subjects were older at menopause (P = .04) and more likely to have a Gail risk score greater than 1.7% (P = .009). Reproductive history (including age at menarche and age at birth of first child), BMI, lifestyle factors, and family history of breast cancer did not differ statistically significantly between ER-positive case subjects and noncase subjects (Table 1).

Bioavailable Sex Hormone Levels

Endogenous levels of bioavailable testosterone and estradiol were statistically significantly correlated with each other (Spearman correlation coefficient = 0.48, P < .001). The median level of bioavailable testosterone was 10.4 ng/dL in ER-negative case subjects, 12.8 ng/dL in ER-positive case subjects, and 11.0 ng/dL in noncase subjects. The median level of bioavailable estradiol was 6.5 pg/mL in ER-negative case subjects, 7.1 pg/mL in ER-positive case subjects, and 6.3 pg/mL in noncase subjects. ER-positive case subjects had statistically significantly higher median levels of testosterone (P = .004) and estradiol (P = .01) compared with noncase subjects. However, there was no statistically significant difference in the median level of either sex hormone between ER-negative case subjects and noncase subjects (Table 1).

Bioavailable Sex Hormone Levels and ER-Negative Breast Cancer

The unadjusted absolute rates of ER-negative breast cancer by testosterone quartiles were 0.34 per 10 000 person-years in Q1, 0.20 per 10 000 person-years in Q2, 0.23 per 10 000 person-years in Q3, and 0.21 per 10 000 person-years in Q4. The corresponding absolute rates by estradiol quartiles 1–4 were 0.31, 0.18, 0.21, and 0.28 per 10 000 person-years, respectively.

Higher bioavailable testosterone levels were associated with a lower risk of ER-negative breast cancer in both unadjusted and risk

Table 1. Baseline characteristics and sex hormone levels of estrogen receptor (ER)–negative and ER-positive breast cancer case subjects and noncase subjects*

Characteristic	Noncase subjects† (n = 586)	ER-negative case subjects (n = 111)	*P*‡	ER-positive case subjects (n = 206)	*P*§
Mean age at screening, y (SD)	65.9 (7.3)	64.3 (7.3)	.04	67.1 (7.2)	.05
White, No. (%)	475 (81.1)	90 (81.1)	.88	186 (90.3)	.002
Education level, No. (%)					
Less than high school diploma	41 (7.1)	7 (6.5)	.47	7 (3.5)	.14
High school diploma	95 (16.4)	23 (21.5)		39 (19.3)	
Some college or associate degree	221 (38.2)	43 (40.2)		69 (34.2)	
College degree or higher	222 (38.3)	34 (31.8)		87 (43.1)	
Smoking status, No. (%)					
Never	295 (51.3)	63 (56.8)	.54	98 (48.3)	.58
Past	246 (42.8)	43 (38.7)		95 (46.8)	
Current	34 (5.9)	5 (4.5)		10 (4.9)	
Alcohol consumption, No. (%)					
Never	93 (16.0)	15 (13.6)	.21	24 (11.7)	.15
Past	121 (20.8)	36 (28.2)		42 (20.5)	
Current					
1–7 drinks/wk	299 (51.3)	56 (50.9)		103 (50.2)	
>7 drinks/wk	70 (12.0)	8 (7.3)		36 (17.6)	
Mean BMI, kg/m^2 (SD)	28.5 (7.1)	28.8 (7.3)	.56	28.6 (5.8)	.85
Median total energy expenditure from physical activity, MET hr/wk (IQR)§	7.7 (2.5–17.5)	10.5 (5.0–21.0)	.04	8.0 (1.9–16.9)	.58
Mean age at menopause, y (SD)	48.5 (6.1)	49.6 (5.1)	.36	49.6 (6.1)	.04
Mean age at menarche, y (SD)	12.6 (1.5)	12.5 (1.4)	.38	12.6 (1.5)	.53
Age at birth of first child, No. (%)					
<20 y	60 (14.1)	12 (14.5)	.69	15 (9.9)	.54
20–24 y	197 (46.4)	39 (47.0)		71 (47.0)	
25–29 y	125 (29.4)	27 (32.5)		46 (30.5)	
≥30 y	43 (10.1)	5 (6.0)		19 (12.6)	
Family history of breast cancer in a first-degree relative, No. (%)	133 (24.7)	27 (25.0)	.94	57 (29.2)	.21
Prior breast biopsy, No. (%)	122 (22.2)	30 (27.5)	.15	52 (26.0)	.16
Prior needle aspiration of a breast lump, No. (%)	63 (11.0)	27 (24.8)	<.001	26 (13.1)	.43
History of hysterectomy, No. (%)	169 (28.8)	34 (30.9)	.66	49 (23.8)	.16
History of bilateral oophorectomy, No. (%)	71 (12.5)	14 (12.6)	.98	22 (11.0)	.56
Gail risk score > 1.7%, No. (%)	271 (46.2)	50 (45.0)	.82	117 (56.8)	.009
Median serum level of bioavailable estradiol, pg/mL (IQR)‖	6.3 (4.0–9.7)	6.5 (3.6–10.4)	.85	7.1 (4.7–10.9)	.01
Median serum level of bioavailable testosterone, ng/dL (IQR)‖	11.0 (7.9–16.5)	10.4 (7.9–16.5)	.16	12.8 (9.2–17.6)	.004

* All statistical tests were two-sided. BMI = body mass index; MET = metabolic equivalents; IQR = interquartile range.

† The comparison group included only the noncase subjects of the random subcohort (N = 586) and excluded the eight breast cancer case subjects that were part of the random subcohort (N = 594).

‡ *P* value for comparison of ER-negative case subjects with noncase subjects obtained from *t* tests (for normally distributed continuous data), Wilcoxon rank sum tests (for nonnormally distributed continuous data), or χ^2 tests (for categorical data).

§ *P* value for comparison of ER-positive case subjects with noncase subjects obtained from *t* tests (for normally distributed continuous data), Wilcoxon rank sum tests (for nonnormally distributed continuous data), or χ^2 tests (for categorical data).

‖ Median (IQR) and Wilcoxon rank sum test *P* value are reported due to the skewed distribution of this variable.

factor–adjusted models. In a model that adjusted for putative breast cancer risk factors, compared with women whose testosterone level was in the lowest quartile (Q1), those in Q2 had a 56% lower risk of ER-negative cancer (HR = 0.44, 95% CI = 0.23 to 0.85, *P* = .01), those in Q3 had a 45% lower risk (HR = 0.55, 95% CI = 0.30 to 1.01, *P* = .05), and those in Q4 had a 49% lower risk (HR = 0.51, 95% CI = 0.28 to 0.94, *P* = .03) (model 2; Table 2). However, the level of bioavailable estradiol was not associated with the risk of ER-negative breast cancer, and adjusting for bioavailable estradiol level did not materially change the magnitude or statistical significance of associations between testosterone and ER-negative breast cancer (Table 2).

In a sensitivity analysis, we excluded women who were diagnosed with breast cancer in the first year after their enrollment in the study. This exclusion did not materially affect the strength or statistical significance of the observed associations of sex hormones with ER-negative breast cancer (data not shown).

We tested for the presence of a threshold effect in the association between testosterone level and ER-negative breast cancer at the lower boundary of testosterone Q2 (7.90 ng/dL). We observed a threshold effect of borderline statistical significance at this level: Below the 7.90 ng/dL threshold, the risk of ER-negative breast cancer decreased linearly with higher testosterone concentrations ($\beta = -0.13$, *P* = .04), and then the slope of the decrease leveled off

for testosterone concentrations greater than 7.90 ng/dL (β = 0.01, P = .49). This finding suggests that breast cancer risk decreased linearly with higher testosterone concentrations up to the concentration of 7.9 ng/dL, but above this concentration the decrease in breast cancer risk became rather constant.

Bioavailable Sex Hormone Levels and ER-Positive Breast Cancer

The unadjusted absolute rates of ER-positive breast cancer by testosterone quartiles were 0.31 per 10 000 person-years in Q1, 0.34 per 10 000 person-years in Q2, 0.52 per 10 000 person-years in Q3, and 0.48 per 10 000 person-years in Q4. The corresponding absolute rates by estradiol quartiles 1–4 were 0.26, 0.47, 0.47, and 0.44 per 10 000 person-years, respectively.

We observed a statistically significant trend of increasing risk of ER-positive cancer with increasing testosterone level in unadjusted (P_{trend} = .005) and risk factor–adjusted (P_{trend} = .04) models (Table 3). In unadjusted analyses, women in Q3 and Q4 of testosterone level had a 1.89-fold (95% CI = 1.19- to 2.99-fold, P = .007) and a 1.68-fold (95% CI = 1.04- to 2.70-fold, P = .03) greater risk of ER-positive cancer, respectively, compared with women in Q1 of testosterone level. Adjustment for risk factors attenuated these risk estimates (Q3: HR = 1.67, 95% CI = 1.01 to 2.78, P = .047; Q4: HR = 1.55, 95% CI = 0.92 to 2.61, P = .10). Adding estradiol to the model further diminished the strength, statistical significance, and trend (P_{trend} = .15) of the association between testosterone level and ER-positive breast cancer (model 3; Table 3).

Higher estradiol levels were statistically significantly associated with increased risk of ER-positive breast cancer in unadjusted and risk factor–adjusted models. In the risk factor–adjusted model, compared with women in Q1 of estradiol level, women in Q2 had a 2.14-fold higher risk of breast cancer (95% CI = 1.22- to 3.78-fold, P = .008), women in Q3 had a 1.90-fold higher risk (95% CI = 1.08- to 3.36-fold, P = .02), and women in Q4 had a 1.86-fold higher risk (95% CI = 1.00- to 3.45-fold, P = .05) (model 2; Table 3). After further adjustment for testosterone level, higher estradiol level continued to be associated with an increased risk of ER-positive breast cancer, an association that was statistically significant for estradiol Q2 (HR = 2.03, 95% CI = 1.11 to 3.71, P = .02), but not for Q3 (HR = 1.68, 95% CI = 0.88 to 3.20, P = .12) or Q4 (HR = 1.53, 95% CI = 0.74 to 3.17, P = .25) (model 3; Table 3).

Excluding women who were diagnosed with breast cancer in the first year after their enrollment in the study did not materially affect the strength or statistical significance of the observed associations of sex hormones with ER-positive breast cancer (data not shown).

In post hoc analyses, we assessed the joint association between estradiol and testosterone levels and the risk of ER-positive breast cancer risk by using sex hormone level categories that were defined based on the observed associations across quartiles of each sex hormone (as described in "Statistical Analyses"). Women with high estradiol and low testosterone, low estradiol and high testosterone, and high estradiol and high testosterone all had a 2.6- to 3.1-fold increased risk of ER-positive breast cancer compared with women with low estradiol and low testosterone (HR for high estradiol and low testosterone = 2.64, 95% CI = 1.30 to 5.37; HR for low estradiol and high testosterone = 2.82, 95% CI = 1.10 to 7.26;

Table 2. Risk of estrogen receptor–negative breast cancer by baseline bioavailable level of testosterone or estradiol*

	Bioavailable testosterone					Bioavailable estradiol				
Quartile	Quartile range, ng/dL	No. of events	Model 1,†HR (95% CI)	Model 2,‡HR (95% CI)	Model 3,§HR (95% CI)	Quartile range, pg/mL	No. of events	Model 1,†HR (95% CI)	Model 2,‡HR (95% CI)	Model 3,§HR (95% CI)
1	<7.89	39	1.00 (referent)	1.00 (referent)	1.00 (referent)	<3.99	32	1.00 (referent)	1.00 (referent)	1.00 (referent)
2	7.90–10.89	21	0.56 (0.32 to 0.99)	0.44 (0.23-0.85)	0.45 (0.22 to 0.92)	4.00–6.19	20	0.58 (0.32 to 1.05)	0.61 (0.32 to 1.18)	0.76 (0.37 to 1.53)
3	10.90–16.29	28	0.65 (0.38 to 1.11)	0.55 (0.30 to 1.01)	0.50 (0.24 to 1.06)	6.20–9.39	24	0.69 (0.39 to 1.23)	0.72 (0.38 to 1.35)	1.04 (0.49 to 2.19)
4	16.30–50.80	23	0.58 (0.33 to 1.02)	0.51 (0.28 to 0.94)	0.43 (0.19 to 0.99)	9.40–31.4	31	0.90 (0.52 to 1.55)	0.83 (0.43 to 1.61)	1.38 (0.57 to 3.33)
P_{trend}			.08	.05	.08			.86	.60	.56

* Statistical tests were two-sided. HR = hazard ratio; CI = confidence interval.

† Model 1: unadjusted.

‡ Model 2: adjusted for age, race, age at menopause, body mass index, physical activity, prior needle aspiration of a breast lump, lifetime use of estrogen plus progestin therapy, and time since stopping hormone therapy.

§ Model 3: adjusted for all variables in Model 2 plus estradiol or testosterone level.

Table 3. Risk of estrogen receptor–positive breast cancer by baseline bioavailable level of testosterone or estradiol*

Quartile	Bioavailable testosterone					Bioavailable estradiol				
	Quartile range (pg/mL)	No. of events	Model 1,†HR (95% CI)	Model 2,‡HR (95% CI)	Model 3,§HR (95% CI)	Quartile range (pg/mL)	No. of events	Model 1,†HR (95% CI)	Model 2,‡HR (95% CI)	Model 3,§HR (95% CI)
1	<7.89	35	1.00 (referent)	1.00 (referent)	1.00 (referent)	<3.99	26	1.00 (referent)	1.00 (referent)	1.00 (referent)
2	7.90–10.89	36	1.07 (0.64 to 1.80)	1.09 (0.62 to 1.92)	0.95 (0.50 to 1.80)	4.00–6.19	56	2.01 (1.19 to 3.37)	2.14 (1.22 to 3.78)	2.03 (1.11 to 3.71)
3	10.90–16.29	73	1.89 (1.19 to 2.99)	1.67 (1.01 to 2.78)	1.36 (0.74 to 2.52)	6.20–9.39	57	1.99 (1.19 to 3.34)	1.90 (1.08 to 3.36)	1.68 (0.88 to 3.20)
4	16.30–50.80	59	1.68 (1.04 to 2.70)	1.55 (0.92 to 2.61)	1.39 (0.73 to 2.65)	9.40–31.4	53	1.90 (1.13 to 3.21)	1.86 (1.00 to 3.45)	1.53 (0.74 to 3.17)
P_{trend}			.005	.04	.15			.03	.08	.44

* Statistical tests were two-sided. HR = hazard ratio; CI = confidence interval.

† Model 1: unadjusted.

‡ Model 2: adjusted for age, race, age at menopause, body mass index, physical activity, prior needle aspiration of a breast lump, lifetime use of estrogen plus progestin therapy, and time since stopping hormone therapy.

§ Model 3: adjusted for all variables in Model 2 plus estradiol or testosterone level.

HR for high estradiol and high testosterone = 3.10, 95% CI = 1.59 to 6.04).

Discussion

In this prospective study of postmenopausal women who were not taking hormone therapy, higher serum levels of testosterone were associated with a lower risk of ER-negative breast cancer, independent of putative breast cancer risk factors and serum estradiol level. In addition, a higher level of both testosterone and estradiol was associated with an increased risk of ER-positive breast cancer, but the association between testosterone level and ER-positive breast cancer was partially explained by estradiol level.

To our knowledge, this is the first prospective study to report a statistically significant inverse association between serum testosterone level and the risk of ER-negative breast cancer. Indeed, only a few reports (7,8,13) have evaluated associations between sex hormones and ER-negative breast cancer separately from ER-positive breast cancer. The Nurses' Health Study (8) reported that women in the higher quartiles of testosterone level had a lower risk ER- and PR-negative breast tumors compared with those in the lowest quartile (relative risk [RR] for Q2 vs Q1 = 0.4, 95% CI = 0.2 to 1.1; RR for Q3 vs Q1 = 0.6, 95% CI = 0.3 to 1.6; RR for Q4 vs Q1 = 0.7, 95% CI = 0.3 to 1.6); however, the associations were not statistically significant, perhaps because of the small sample size of ER-negative tumors (n = 38). The two other studies, which included 30 (7) and 23 case subjects (13), also observed no statistically significant associations between testosterone (7) or estradiol levels (7,13) and ER-negative breast cancer.

Our results regarding the associations between testosterone and estradiol levels and ER-positive breast cancer confirm previous findings. For example, the 2002 meta-analysis of data from nine prospective cohort studies by Key et al. (5) reported strong associations between higher levels of estradiol and testosterone and the overall risk of breast cancer. Of the several studies published subsequently (3,6–10,26), all but one (26) observed statistically significant associations between higher sex hormone levels and higher risk of breast cancer, particularly for ER-positive tumors. In an updated report from the Nurses' Health Study (8), women whose testosterone and estradiol levels were in the highest quartiles had a twofold to threefold higher risk of ER- and PR-positive breast cancer compared with women in the lowest quartile. In the Study of Osteoporotic Fractures (3), testosterone was more strongly associated with the risk of ER-positive breast cancer than estradiol. Having a testosterone concentration in the highest two quintiles conferred a fourfold higher risk of ER-positive cancer compared with having a testosterone concentration in the lowest quintile, independent of estradiol level. However, estradiol level was not statistically significantly associated with the risk of ER-positive breast cancer after adjustment for testosterone level. By contrast, results of our study indicate that estradiol level is more strongly associated with ER-positive breast cancer risk than testosterone level, and further suggest that the effect of testosterone is partially explained by estradiol.

Our finding that endogenous testosterone level has opposite associations with ER-negative and ER-positive breast cancer risks is biologically plausible. The role of testosterone in the development

of breast cancer has been largely attributed to its conversion to estrogen by aromatase and the resultant stimulation of mammary cell proliferation via ER activation. However, preclinical evidence indicates that testosterone also has an antiproliferative effect on mammary cell growth that is regulated by the androgen receptor (11,12,27–30). For example, in vitro, testosterone inhibits the proliferation of breast cancer cell lines in a dose-dependent manner and is a more potent inhibitor of proliferation in ER-negative cell lines compared with ER-positive cell lines (27). Furthermore, adding physiological doses of testosterone to estrogen therapy markedly inhibited estrogen-induced mammary cell proliferation in ovariectomized rhesus monkeys and rats (28–30). Given this dual role of testosterone as both a stimulator and an inhibitor of mammary cell growth, the net effect of testosterone on the development of breast cancer could depend on the ER status of the tumor. In ER-positive breast tumors, the androgen receptor–mediated inhibitory effect of testosterone on proliferation may be countered by the ER-mediated proliferative effect. However, in ER-negative tumors, testosterone could have an overall antiproliferative effect.

Whereas preclinical studies have suggested that testosterone supplementation may have a protective effect against breast cancer, the clinical evidence for such an effect is limited. Combined estrogen and testosterone use was associated with an increased risk of breast cancer in the Nurse's Health Study (HR for current vs never users = 2.5, 95% CI = 1.53 to 4.04) (31) and in the WHI-OS (HR for current users vs nonusers = 1.42, 95% CI = 0.95 to 2.11) (32), but in only the former study was the association statistically significant. A clinical trial investigating the effect of testosterone patch use without estrogen on sexual dysfunction in postmenopausal women produced inconclusive results regarding the effects on breast cancer risk (33). By contrast, a retrospective analysis of 631 postmenopausal women who received testosterone in addition to conventional hormone therapy observed a breast cancer incidence rate close to that expected in never-users of hormone therapy, a finding that based on only 12 breast cancer cases (34). Although none of these studies evaluated associations by tumor ER status, it is likely that the majority of breast cancers that occurred in those studies were ER positive, given that the study populations consisted of postmenopausal women.

The strengths of this study include the rigorous centralized adjudication of breast cancer cases in a large and well-characterized cohort of postmenopausal women, and the large number of incident ER-negative case subjects with endogenous testosterone and estradiol measurements. The study limitations include the assessment of sex hormone concentrations at a single point in time and the fact that circulating levels of testosterone and estradiol may not reflect the local hormonal milieu of the breast tissue. In addition, it is possible that women who were diagnosed with breast cancer during follow-up may have had subclinical disease at baseline. However, excluding women who were diagnosed with breast cancer in the first year after their enrollment in the study did not affect the strength or statistical significance of the observed associations. Moreover, we did not conduct analyses by joint categories of tumor ER and PR status because only a small percentage of cancers were positive for one receptor but negative for the other. Finally, our results may not be generalizable to postmenopausal women who use hormone therapy, racial groups other than non-Hispanic whites, and premenopausal women.

In conclusion, this study provides evidence that higher levels of bioavailable testosterone are associated with lower risks of ER-negative breast cancer in postmenopausal women. These results shed new light on the etiology of ER-negative breast cancer and further reinforce the need to assess risk separately for ER-positive and ER-negative breast cancers. In addition, we observed that higher levels of bioavailable testosterone and estradiol are associated with an increased risk of ER-positive breast cancer. These findings confirm previous reports and further suggest that the effect of testosterone on ER-positive breast cancer is partially explained by that of estradiol.

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