

Research Article

Circulating Steroid Hormone Levels and Risk of Breast Cancer for Postmenopausal Women

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Abstract

Epidemiologic studies have consistently reported that endogenous steroid hormone levels are associated with postmenopausal breast cancer risk, but little is known on the associations by tumor grade, hormone receptor status, or age at diagnosis.

We performed a case-cohort study of naturally postmenopausal women within the Melbourne Collaborative Cohort Study that included a random sample of 857 women and 197 breast cancer cases diagnosed during a mean of 9.2 years of follow-up. Concentrations of total estradiol, estrone sulfate, testosterone, DHEA sulfate, androstenedione, and sex hormone binding globulin were measured in plasma collected at baseline before diagnosis; free estradiol plasma concentration was calculated. Cox regression was used to estimate associations adjusted for known and potential confounders.

The HR for breast cancer comparing fourth and first quartiles was 1.44 [95% confidence interval (95% CI), 0.89-2.35] for total estradiol, 1.75 (95% CI, 1.06, 2.89) for free estradiol, 2.05 (95% CI, 1.24-3.37) for estrone sulfate, 1.25 (95% CI, 0.78-2.01) for testosterone, 1.41 (95% CI, 0.88-2.27) for DHEA sulfate, 1.49 (95% CI, 0.91-2.44) for androstenedione, and 0.33 (95% CI, 0.19-0.55) for sex hormone binding globulin. These associations did not differ by tumor grade and estrogen receptor/progesterone receptor status (all test for heterogeneity, $P > 0.05$). Risks associated with estrogen and androgen levels were stronger at older ages (test for interaction across age groups, $P = 0.59$ for total estradiol and $P = 0.01$ for testosterone).

Our prospective study confirms earlier findings and suggests that the associations of endogenous hormones with postmenopausal breast cancer risk are independent of tumor grade, and hormone receptor status and might increase in strength with age. *Cancer Epidemiol Biomarkers Prev*; 19(2); 492-502. ©2010 AACR.

Introduction

Prospective studies consistently report that higher levels of endogenous estrogens and androgens and lower levels of sex hormone binding globulin (SHBG) are associated with increased risk of postmenopausal breast cancer (1-6). Experimental and animal data, as well as clinical evidence, support a role for estrogens in breast carcinogenesis (7-9). The effect of androgens on the mammary gland is more complex. Androgens may increase breast cancer risk either directly, by stimulating breast cell proliferation, or indirectly, by providing the substrate for the synthesis of estrogens in peripheral or mammary

adipose tissue (4, 6). However, some evidence indicates that under certain circumstances, androgens may act as antiestrogens and exert an antiproliferative and apoptotic effect in the breast (10, 11).

Epidemiologic evidence suggests that breast cancers classified by estrogen receptor (ER) and progesterone receptor (PR) expression not only have different clinical, pathologic, and molecular features, but may also be etiologically distinct (12). Previous studies have suggested that hormone-related breast cancer risk factors, such as reproductive history and body mass index (BMI), might have a more pronounced effect on hormone receptor-positive tumors than on hormone receptor-negative tumors (12-15). In addition, postmenopausal exposure to exogenous hormones might increase the risk of ER-positive tumors more than ER-negative tumors (16-18). Few prospective studies have investigated the associations between endogenous sex hormone levels and breast cancer risk by tumor ER and PR status (5, 19, 20). The Nurses' Health Study reported that circulating levels of steroid hormones were most strongly associated with the risk of hormone receptor-positive cancers (5). Data from the New York University Women's Health Study suggested that the association of total and free estradiol and SHBG with breast cancer risk was independent of the ER status (20). A recent publication from the ORDET study

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reported stronger associations between testosterone and breast cancer risk for ER-positive tumors. However, the study did not find any statistical evidence of heterogeneity by ER and PR status of the association between estradiol, testosterone, or SHBG and breast cancer risk (19).

It has long been considered that established risk factors for breast cancer, such as early menarche, late menopause, alcohol consumption, postmenopausal obesity, hormone replacement therapy (HRT), can be thought of as measures of the “cumulative dose of estrogen that breast epithelium is exposed to over time” (7). There is evidence to support a role for sex hormones on breast cancer risk through the accumulation of genetic damage these hormones cause to DNA in breast cells (21). We therefore hypothesize that the associations between sex hormone levels and breast cancer risk depend on age.

In this study, we examined the role of circulating steroid hormone levels on postmenopausal breast cancer risk overall, and with respect to tumor grade and hormone receptor status, in the Melbourne Collaborative Cohort study. We also investigated whether these associations varied with age and with time since menopause.

Materials and Methods

The Melbourne Collaborative Cohort Study

The Melbourne Collaborative Cohort study is a prospective cohort study of 41,514 people (24,469 women) living in the Melbourne metropolitan area and with ages between 27 and 81 y at baseline (99.3% of whom were ages 40–69 y). Subjects were recruited through the electoral rolls (registration to vote is compulsory for adults in Australia), advertisements, and community announcements in local media (e.g., television, radio, newspapers). At baseline, extensive information was collected from face-to-face interviews that included diet, physical activity during leisure time, education, alcohol, smoking, and for women, use of HRT and oral contraceptives, and reproductive history. Physical measurements were taken including height, weight, and waist and hip circumference. Blood samples were drawn from all participants and plasma was stored.

Passive follow-up of the Melbourne Collaborative Cohort study by record linkage to cancer registries and death registries is conducted on a regular basis to identify cancer cases and deaths. Details of the study have been published elsewhere (22). The Cancer Council Victoria's Human Research Ethics Committee approved the study protocol. Subjects gave written consent to participate and for the investigators to obtain access to their medical records.

The Case-Cohort Study

Of the 24,469 women in the cohort, 10,573 (43%) were classified as naturally postmenopausal at baseline. Of these, 200 women were excluded because of a confirmed diagnosis of breast or ovarian cancer or unknown primary before baseline; 28 were excluded because they did not provide a blood sample; and another 1,312 were excluded

because they were taking HRT at baseline. This left 9,033 women eligible from which the study sample was selected. The study sample was composed of a random sample (hereafter called the subcohort) of 922 of the 9,033 eligible women together with all eligible women first diagnosed with breast cancer between baseline attendance and June 30, 2002.

Eligible cases were adenocarcinomas of the breast (International Classification of Disease 10th revision rubric C50.0–C50.9). Cases were ascertained by record linkage to the population-based Victorian Cancer Registry, which covers the state in which the cohort resides, and to the National Cancer Statistics Clearing House, which holds cancer incidence data from all Australian states.

Between baseline attendance and June 30, 2002, 9 eligible women left Australia and 456 died. A total of 218 women were newly diagnosed with invasive breast cancer over an average of 9.2 person-years of follow-up.

Hormone measurements were not made for 35 women, including 11 cases; 26 had insufficient plasma; one sample was contaminated; and 8 cases were identified after the measurements were completed. Therefore, measurements were made for 1,085 women: 898 members of the subcohort (97%) and 207 case subjects (95%); 20 of these cases were also members of the subcohort. There was virtually no difference in age at baseline, reproductive history (age at menarche, parity and age at first pregnancy, duration of lactation, HRT use) or demographic and life-style variables (country of birth, education, BMI, physical activity, energy from diet, alcohol intake, smoking) between women who had their levels of hormones measured and those who did not. For oral contraceptive use, there was a slightly higher proportion of never users among women with hormones measured than among those without hormones measured (58% versus 40%, $P = 0.04$). Women with missing values in potential confounders were excluded ($n = 25$), as were those who reported extreme values of total energy intake (<1st percentile or >99th percentile, $n = 25$).

The final sample included 1,035 women; 857 in the subcohort and 197 breast cancer cases (19 of whom were members of the subcohort).

Tumor Grade and ER and PR Status

The medical records of women with reported breast cancers were reviewed and their cancers were classified according to tumor grade and ER/PR status. Grade was used to categorize breast cancer into well-differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III) tumors. In addition to the ER and PR status obtained from histopathology report held at the Victorian Cancer Registry, we repeated the measure of ER and PR status using immunohistochemistry techniques for 149 cases with archival tissue available (76% of all cases). The original diagnostic tumor slides for these cases were retrieved from storage at pathology laboratories and were assessed by a single pathologist for ER and PR status. The archival material was sectioned at

4 μm and placed on superfrost plus slides. A routine de-waxing procedure was followed by heat-induced epitope retrieval with either citrate buffer (pH 6) or TRIS EDTA (pH 8) using a DAKO Pascal pressure chamber. The antibodies used were ER (Labvision rabbit monoclonal SP1) at 1/250 and PR (DAKO PGR636) at 1/1,200. Immunoreaction was performed using a Labvision autostainer using the Labvision horseradish peroxidase polymer detection system and DAKO DAB+. The agreements between the ER and PR status that were assessed by immunohistochemistry and the values held by the Victorian Cancer Registry were 96% and 78%, respectively (for ER: $\kappa = 0.88$, $P < 0.001$; for PR: $\kappa = 0.56$, $P < 0.001$). In this study, ER and PR status were assigned based on the results obtained by immunohistochemistry. Because of the high agreement between the ER and PR data, when archival tumor tissue was not available, ER and PR status was assigned according to the histopathology report held at the Victorian Cancer Registry.

Serum Analysis

Plasma samples were retrieved from liquid nitrogen, where they had been stored at baseline, aliquoted into 450- μL amounts, and shipped on dry ice in batches of an average of 25 samples each to the laboratory of one of us (HAM), where SHBG, estradiol, estrone sulfate, testosterone, DHEA sulfate (DHEAS), and androstenedione were measured. The median time in storage was 9.4 y (range, 6.3-13.3 y) for the subcohort and 9.3 y (range, 6.4-12.3 y) for the cases. To avoid the potential for differential measurement error due to batch-to-batch variability in hormone measurements, samples were randomly assigned to batches. The batches had similar proportions of samples from the cases and subcohort members: 80% of batches comprised between 12.5% and 37.5% of samples from cases and 50% comprised between 17% and 29% of samples from cases. Ten percent of the samples in each batch were aliquots from pooled plasma that had been stored with the samples from participants.

One scientist did all measurements blind to the status of the samples. Samples were thawed in a warm water bath, vortexed rapidly for a few seconds, and centrifuged at 2,000 rpm (210 \times g) for 10 min. Testosterone followed by estradiol was measured by electrochemiluminescence immunoassay (Elecys 2010 analyzer, Roche Diagnostics GmbH). Estrone sulfate was measured by RIA (DSL-5400). DHEAS was measured by competitive immunoassay (IMMULITE analyzer, DPC). Androstenedione was analyzed by RIA (DSL-4200). SHBG was measured by immunometric assay (IMMULITE analyzer, DPC). All hormones were measured between 6 and 13 y after blood collection (median, 9 y). Lower detection limits were 18 pmol/L for estradiol, 0.03 nmol/L for estrone sulfate, 0.1 nmol/L for testosterone, 0.2 $\mu\text{mol/L}$ for DHEAS, 0.02 nmol/L for androstenedione, and 2 nmol/L for SHBG.

From the pooled plasma, the overall coefficients of variation were 10% (8% within batches and 6% between batches) for total estradiol at a concentration of 157

pmol/L; 15% (13% within batches and 8% between batches) for estrone sulfate at a concentration of 5.7 nmol/L; 7% (4% within batches and 5% between batches) for testosterone at a concentration of 4.3 nmol/L; 10% (9% within batches and 6% between batches) for DHEAS at a concentration of 4.0 $\mu\text{mol/L}$; 15% (11% within batches and 9% between batches) for androstenedione at a concentration of 2.6 nmol/L; and 7% (6% within batches and 4% between batches) for SHBG at a concentration of 45.0 nmol/L.

Concentration of protein-unbound estradiol (free estradiol) was calculated from the total concentration and from the concentration of SHBG using the law of mass action under the assumption of a fixed albumin concentration of 40 g/L (23, 24).

A reliability study was performed before the study commencement for estrone sulfate, testosterone, DHEAS, androstenedione, and SHBG. Plasma samples from 45 women who had given blood at baseline and again ~ 1 y later were divided into two aliquots and were measured in separate batches a week apart. To measure reliability, we calculated the intraclass correlation coefficient, which is the proportion of the total variance due to variation between persons, where the total variance included components due to between-persons, between-sampling occasions, and residual variance. From the reliability study, the intraclass correlation coefficient was 0.85 [95% confidence interval, (95% CI), 0.78-0.92] for estrone sulfate, 0.65 (95% CI, 0.52-0.77) for testosterone, 0.87 (95% CI, 0.81-0.93) for DHEAS, 0.61 (95% CI, 0.44-0.78) for androstenedione, and 0.90 (95% CI, 0.85-0.95) for SHBG. There were insufficient samples to perform an equivalent reliability study for estradiol.

Statistical Analysis

To remove the variation in circulating levels of hormones and SHBG between laboratory batches and by age, quartiles were assigned following a two-step procedure. First, using linear regression for the subcohort, log-transformed values of hormones were regressed on batch (as a categorical covariate) and age at blood collection; second, the predicted values of these regressions were calculated for all women and the residuals were categorized into quartiles according to the distribution of the values for the subcohort.

Cox proportional regression, with age as the time axis (25), was used to estimate hazard ratios (HR) and 95% CIs. Follow-up for a subcohort member began at baseline and ended at diagnosis of breast cancer or ovarian cancer or cancer of unknown primary site, death, the date last known to be in Australia, or June 30, 2002, whichever came first. We used the Prentice method to take the case-cohort sampling into account, and a robust method was used to calculate the variance-covariance matrix (26, 27).

We adjusted for country of birth, level of education, age at menarche, parity and age at first pregnancy (calculated from the live births and gestations with duration

>24 wk), duration of lactation, oral contraceptive use, past HRT use, physical activity, BMI, smoking, alcohol consumption, and energy from diet (see Table 1 for description of all the confounders).

Tests for linear trend were based on pseudocontinuous variables under the assumption that all subjects within each quartile had the same concentration equal to the within-quartile median. The pseudocontinuous variables were \log_2 transformed before inclusion in the models so that the HR would represent the HR associated with a doubling of the concentration.

We estimated the HRs for each hormone level overall, and then by grade (well differentiated versus moderately versus poorly differentiated) and hormone receptor status (ER- versus ER+; PR- versus PR+; ER+/PR+ versus ER+/PR- versus ER-/PR-) of the tumors. To test for heterogeneity in the HRs by tumor grade and ER and PR status, Cox proportional regression models were fitted using a data duplication method (28). The method is used in the presence of k competing risks and consists of duplicating data k times and treating failures for one competing event as censored observations for each of the other competing events. We also estimated separate HRs for three follow-up age groups that approximately correspond to tertiles of age at diagnosis of cases (<65, 65-69, and >69), and for two categories of time since blood collection (up to 2 and >2 y). For these analyses, we split the follow-up time into the specified groups and fitted models with the inclusion of a term for the interaction between hormones and groups.

Statistical analyses were done using Stata/SE 10.0 (Stata Corp.). Student's t test was used to test the hypothesis that hormone levels have the same distribution for cases and noncases. Because a robust method was used to calculate the variance-covariance matrix, the Wald test was used to test for trend and heterogeneity. All P values were two sided, and $P < 0.05$ was considered nominally statistically significant.

Results

Table 1 summarizes the baseline characteristics of the 1,035 women in the study sample, including the 197 breast cancer cases. Mean age at diagnosis was 67 years (range, 49-79 years); 13% of cases were diagnosed before age 60 years; 59% were diagnosed between 60 and 70 years of age; 28% were diagnosed at 70 years and older; and 25% were diagnosed within the 2 years of follow-up. Information on grade was available for 176 cases, including 39 (22%) well differentiated, 82 (47%) moderately differentiated, and 55 (31%) poorly differentiated. Information on ER and PR status was available for 177 and 176 cases, respectively (84% from immunohistochemistry and 16% from the Victorian Cancer Registry): 132 (75%) cases were ER+ and 45 (25%) ER-; 87 (49%) cases were PR+ and 89 (51%) PR-. Eighty-three cases were ER+/PR+, 48 ER+/PR- and 41 ER-/PR-. Only four cases were ER-/PR+, and this category was excluded from the

analyses by ER/PR status. The proportions of ER+, PR+, or ER+/PR+ cases increased with age at diagnosis: 71% of cases diagnosed before the age of 67 years were ER+ compared with 77% of those diagnosed at or after age 67 years; these proportions were 46% and 53% for PR+, and 45% and 49% for ER+/PR+. The baseline characteristics of cases and age at diagnosis by ER and PR status are summarized in Supplementary Table S1.

The median hormone levels (and interquartile ranges) are summarized in Table 1. The distributions of total and free estradiol, testosterone, DHEAS, and androstenedione were similar for cases and noncases (all $P > 0.05$). Estrone sulfate was significantly higher for cases than for noncases ($P = 0.04$). SHBG was significantly lower for cases than for noncases ($P < 0.001$).

Associations Between Hormone Concentrations and Breast Cancer Risk

Associations between breast cancer risk and quartiles of steroid hormone levels are shown in Table 2. For all estrogens and androgens, the HRs were higher for women with higher concentrations. The test for trend was statistically significant only for free estradiol (HR for doubling hormone concentration, 1.61; 95% CI, 1.12-2.33; $P_{\text{trend}} = 0.01$) and estrone sulfate (HR, 1.52; 95% CI, 1.11-2.07; $P < 0.01$). SHBG was inversely associated with breast cancer risk with a dose-response relationship; the HR for doubling the plasma concentration was 0.41 (95% CI, 0.29-0.60; $P < 0.01$).

Only minor differences were observed in HRs for breast cancer when excluding tumors diagnosed during the first 2 years of follow-up, whereas no association was found for any hormone and breast cancer risk during the first 2 years of follow-up; only SHBG was inversely associated with breast cancer risk in both periods. The HRs associated with a doubling of hormone level for durations of follow-up of up to 2 years and of >2 years were as follows: 0.94 (95% CI, 0.40-2.22) and 1.65 (95% CI, 1.00-2.71) for total estradiol ($P_{\text{heterogeneity}} = 0.23$), 1.24 (95% CI, 0.64-2.42) and 1.74 (95% CI, 1.18-2.56) for free estradiol ($P = 0.35$), 1.08 (95% CI, 0.63-1.84) and 1.68 (95% CI, 1.19-2.38) for estrone sulfate ($P = 0.14$), 1.10 (95% CI, 0.75-1.63) and 1.10 (95% CI, 0.85-1.43) for testosterone ($P = 0.99$), 1.03 (95% CI, 0.73-1.47) and 1.19 (95% CI, 0.95-1.51) for DHEAS ($P = 0.47$), 0.96 (95% CI, 0.66-1.38) and 1.34 (95% CI, 1.02-1.76) for androstenedione ($P = 0.12$), and 0.41 (95% CI, 0.23-0.73) and 0.42 (95% CI, 0.28-0.63) for SHBG ($P = 0.92$).

For all hormones, the HRs for the breast cancer associated with a doubling of hormone concentration were higher for women who had never used HRT than for past HRT users. The HRs for never and past HRT users, respectively, were as follows: 1.54 (95% CI, 0.93-2.53) and 0.93 (95% CI, 0.33-2.63) for total estradiol ($P_{\text{heterogeneity}} = 0.38$), 1.68 (95% CI, 1.14-2.47) and 1.16 (95% CI, 0.46-2.94) for free estradiol ($P = 0.47$), 1.67 (95% CI, 1.21-2.29) and 0.66 (95% CI, 0.24-1.86) for estrone sulfate ($P = 0.09$), 1.22 (95% CI, 0.95-1.56) and 0.55 (95% CI, 0.28-1.07) for

Table 1. Characteristics and hormone levels of subjects (cases and subcohort) at baseline

		Breast cancer cases* n = 197	Subcohort n = 857
Age, y; mean, SD (range)		62, 5 (48-70)	61, 6 (46-70)
Country of birth, n (%)	Australia/New Zealand	144 (73.1)	569 (66.4)
	United Kingdom	9 (4.6)	47 (5.5)
	Italy	26 (13.2)	134 (15.6)
	Greece	18 (9.1)	107 (12.5)
Age at menarche, n (%)	Less than 12 y	27 (13.7)	109 (12.7)
	12 y	35 (17.8)	160 (18.7)
	13 y	56 (28.4)	217 (25.3)
	14 y or more	79 (40.1)	371 (43.3)
Parity and age at first pregnancy (live birth or gestation >24 wk), n (%)	Nulliparous	30 (15.2)	100 (11.7)
	1 and <25	5 (2.5)	15 (1.8)
	>1 and <25	75 (38.1)	358 (41.8)
	1 and ≥25	11 (5.6)	52 (6.1)
	>1 and ≥25	76 (38.6)	332 (38.7)
Lactation, n (%)	Never	63 (32.0)	214 (25.0)
	Up to 6 mo	35 (17.8)	184 (21.5)
	7-12 mo	32 (16.2)	147 (17.2)
	13-24 mo	43 (21.8)	173 (20.2)
	>24 mo	24 (12.2)	139 (16.2)
Oral contraceptive use, n (%)	Never user	123 (62.4)	492 (57.4)
	Past user	74 (37.6)	365 (42.6)
HRT use, n(%)	Never user	174 (88.3)	760 (88.7)
	Past user	23 (11.7)	97 (11.3)
Physical activity, n (%)	None	38 (19.3)	182 (21.2)
	Low	45 (22.8)	176 (20.5)
	Medium	78 (39.6)	341 (39.8)
	High	36 (18.3)	158 (18.4)
Alcohol, n (%)	Abstainers	89 (45.2)	403 (47.0)
	Exdrinkers	6 (3.0)	31 (3.6)
	1-19 g/d	82 (41.6)	339 (39.6)
	20-39 g/d	14 (7.1)	69 (8.1)
	40 g/d or more	6 (3.0)	15 (1.8)
Smoking, n (%)	Never	150 (76.1)	612 (71.4)
	Current	11 (5.6)	74 (8.6)
	Past	36 (18.3)	171 (20.0)
Education, n (%)	Primary school	46 (23.4)	247 (28.8)
	Some high school	98 (49.7)	378 (44.1)
	Completed high school	33 (16.8)	130 (15.2)
	Degree/diploma	20 (10.2)	102 (11.9)
BMI (kg/m ²); mean, SD		27.7, 4.7	27.3, 4.7
Energy from diet (MJ/d); mean, SD		8.3, 2.7	8.5, 2.9
Hormones and SHBG, median (interquartile range)[†]			
Total estradiol (pmol/L)		57.00 (46.00-74.00)	57.00 (45.35-71.00)
Free estradiol (pmol/L)		0.87 (0.63-1.13)	0.80 (0.60-1.05)
Estrone sulfate (nmol/L)		3.22 (2.29-4.41)	2.95 (2.15-4.03)
Testosterone (nmol/L)		0.70 (0.50-1.10)	0.70 (0.50-1.10)
DHEAS (μmol/L)		1.50 (1.00-2.30)	1.50 (0.90-2.40)
Androstenedione (nmol/L)		2.19 (1.60-3.17)	2.16 (1.41-3.06)
SHBG (nmol/L)		43.10 (32.90-57.30)	51.10 (38.55-67.80)

*Nineteen breast cancer cases were also in the subcohort.

[†]The number of missing measures among cases and in the subcohort were as follows: 2 and 15 for total estradiol, 2 and 16 for free estradiol, 5 and 16 for estrone sulfate, 1 and 4 for testosterone, 0 and 3 for DHEAS, 1 and 3 for androstenedione, 0 and 1 for SHBG.

testosterone ($P = 0.03$), 1.23 (95% CI, 0.99-1.53) and 0.70 (95% CI, 0.38-1.29) for DHEAS ($P = 0.08$), 1.38 (95% CI, 1.07-1.77) and 0.58 (95% CI, 0.30-1.12) for androstenedione ($P = 0.01$), and 0.42 (95% CI, 0.28-0.62) and 0.39 (95% CI, 0.14-1.09) for SHBG ($P = 0.91$).

Associations by Estrogen and Progesterone Receptor Status and Tumor Grade

The HRs in relation to hormone levels did not differ greatly between ER, PR, and ER/PR subtypes (Table 3; all tests for heterogeneity, $P > 0.05$). The analyses by ER, PR, and ER/PR status as measured by immunohistochemistry were similar to those obtained using the values from the Victorian Cancer Registry (data not shown).

No significant differences were observed in the HRs between tumors of different grades of differentiation (results not shown).

Similar results by hormone receptor status and grade were obtained when past HRT users were excluded from the analysis (data not shown).

Associations by Attained Age During Follow-up

Table 4 shows the associations between breast cancer risk and the levels of circulating hormones by attained age during follow-up. For both estrogens and androgens, the strongest associations with risk were observed at older ages, although the test for heterogeneity across age groups was statistically significant only for androgens: the breast cancer HRs associated with a doubling of hormone concentration for women of ages <65 years, between 65 and 69 years, and >69 years were 0.92 (95% CI, 0.66-1.29), 0.83 (95% CI, 0.55-1.25), and 1.70 (95% CI, 1.16-2.48) for testosterone (test for heterogeneity across age groups: $P = 0.01$); 1.05 (95% CI, 0.76-1.45), 0.79 (95% CI, 0.55-1.13), and 1.74 (95% CI, 1.26-2.42) for DHEAS ($P < 0.01$); and 1.03 (95% CI, 0.74-1.45), 0.92 (95%

Table 2. HRs and 95% CIs of breast cancer by quartiles of steroid hormone levels

	Quartiles*				Doubling hormone concentration [†]	$P_{\text{trend}}^{\ddagger}$
	Q1	Q2	Q3	Q4		
Total estradiol						
Cases/PY [§]	39/1,950	47/1,946	49/1,917	60/1,904		
HR (95% CI)	Reference	1.09 (0.66-1.79)	1.21 (0.74-1.99)	1.44 (0.89-2.35)	1.45 (0.91-2.30)	0.12
Free estradiol						
Cases/PY [§]	37/1,934	39/1,948	53/1,904	66/1,923		
HR (95% CI)	Reference	1.05 (0.62-1.77)	1.49 (0.90-2.44)	1.75 (1.06-2.89)	1.61 (1.12-2.33)	0.01
Estrone sulfate						
Cases/PY [§]	35/1,954	48/1,914	43/1,939	66/1,898		
HR (95% CI)	Reference	1.53 (0.93-2.52)	1.20 (0.71-2.03)	2.05 (1.24-3.37)	1.52 (1.11-2.07)	<0.01
Testosterone						
Cases/PY [§]	45/2,007	47/1,934	48/1,930	56/1,946		
HR (95% CI)	Reference	1.09 (0.68-1.74)	1.10 (0.68-1.78)	1.25 (0.78-2.01)	1.11 (0.88-1.40)	0.37
DHEAS						
Cases/PY [§]	45/1,984	47/1,940	44/1,957	61/1,948		
HR (95% CI)	Reference	1.06 (0.65-1.74)	1.06 (0.64-1.73)	1.41 (0.88-2.27)	1.15 (0.94-1.42)	0.17
Androstenedione						
Cases/PY [§]	36/1,958	49/1,944	56/1,950	55/1,970		
HR (95% CI)	Reference	1.39 (0.85-2.28)	1.66 (1.03-2.69)	1.49 (0.91-2.44)	1.24 (0.97-1.57)	0.08
SHBG						
Cases/PY [§]	79/1,985	51/1,960	36/1,940	31/1,955		
HR (95% CI)	Reference	0.57 (0.37-0.88)	0.38 (0.24-0.61)	0.33 (0.19-0.55)	0.41 (0.29-0.60)	<0.01

NOTE: HRs from the Cox model were adjusted for country of birth, age at menarche, parity and age at first pregnancy, duration of lactation, oral contraceptive use, HRT use, alcohol consumption, energy from diet, smoking, BMI, level of education, and level of physical activity. Estimates were based on the following number of women (cases): total estradiol, 1,019 (195); free estradiol, 1,018 (195); estrone sulfate, 1,014 (192); testosterone, 1,031 (196); DHEAS, 1,032 (197); androstenedione, 1,031 (196); SHBG, 1,034 (197).

*Quartiles were adjusted for variations between batches and by age at time of blood collection, according to the procedure described in the method section.

[†]Estimates from the model including the pseudocontinuous variable \log_2 transformed.

[‡]Test for linear trend using the pseudocontinuous variable \log_2 transformed.

[§]Breast cancer cases and person-years (PY).

Table 3. HRs (95% CIs in parentheses) of breast cancer associated with a doubling of hormone levels in relation to tumor characteristics

ER status	HR (95% CI)*	P†	PR status	HR (95% CI)*	P†	ER/PR status	HR (95% CI)*	P†
Total estradiol								
ER+	1.46 (0.86-2.48)	0.79	PR+	1.52 (0.82-2.81)	1.00	ER+/PR+	1.68 (0.90-3.14)	0.69
ER-	1.67 (0.70-3.99)		PR-	1.52 (0.80-2.89)		ER+/PR-	1.23 (0.54-2.82)	
						ER-/PR-	2.02 (0.82-4.96)	
Free estradiol								
ER+	1.69 (1.11-2.56)	1.00	PR+	1.99 (1.21-3.28)	0.34	ER+/PR+	2.29 (1.38-3.79)	0.16
ER-	1.69 (0.87-3.25)		PR-	1.46 (0.90-2.39)		ER+/PR-	1.13 (0.60-2.13)	
						ER-/PR-	2.12 (1.07-4.19)	
Estrone sulfate								
ER+	1.38 (0.96-2.00)	0.50	PR+	1.54 (0.99-2.41)	0.74	ER+/PR+	1.55 (0.99-2.43)	0.59
ER-	1.70 (0.99-2.91)		PR-	1.41 (0.93-2.11)		ER+/PR-	1.20 (0.69-2.07)	
						ER-/PR-	1.74 (1.00-3.00)	
Testosterone								
ER+	1.14 (0.86-1.50)	0.53	PR+	1.09 (0.78-1.53)	0.95	ER+/PR+	1.14 (0.81-1.60)	0.92
ER-	0.99 (0.66-1.47)		PR-	1.11 (0.82-1.49)		ER+/PR-	1.17 (0.79-1.73)	
						ER-/PR-	1.05 (0.70-1.58)	
DHEAS								
ER+	1.09 (0.86-1.39)	0.86	PR+	1.22 (0.93-1.60)	0.27	ER+/PR+	1.22 (0.92-1.60)	0.44
ER-	1.13 (0.77-1.67)		PR-	1.00 (0.74-1.34)		ER+/PR-	0.91 (0.62-1.34)	
						ER-/PR-	1.12 (0.74-1.69)	
Androstenedione								
ER+	1.27 (0.96-1.68)	0.64	PR+	1.27 (0.91-1.76)	0.75	ER+/PR+	1.29 (0.92-1.80)	0.92
ER-	1.14 (0.73-1.76)		PR-	1.18 (0.85-1.64)		ER+/PR-	1.23 (0.80-1.87)	
						ER-/PR-	1.15 (0.73-1.82)	
SHBG								
ER+	0.41 (0.27-0.63)	0.85	PR+	0.33 (0.19-0.56)	0.18	ER+/PR+	0.30 (0.17-0.51)	0.11
ER-	0.44 (0.23-0.83)		PR-	0.51 (0.32-0.81)		ER+/PR-	0.64 (0.35-1.14)	
						ER-/PR-	0.37 (0.19-0.71)	

NOTE: Estimates from the Cox's model including the pseudocontinuous variable \log_2 transformed and fitted using a data duplication method. All estimates were adjusted for country of birth, age at menarche, parity and age at first pregnancy, duration of lactation, oral contraceptive use, HRT use, alcohol consumption, energy from diet, smoking, BMI, level of education, and physical activity.

*Estimates from the model including the pseudocontinuous variable \log_2 transformed.

†Test for heterogeneity in HRs between groups.

CI, 0.61-1.39), and 1.91 (95% CI, 1.26-2.88) for androstenedione ($P = 0.02$).

Similar trends by attained age during follow-up were obtained when the analysis was restricted to ER+ tumors, to PR+ tumors, or to ER+/PR+ tumors or when past HRT users were excluded from the analysis (data not shown).

Associations with Androgen Levels with and without Adjusting for Estrogen Levels

Table 5 presents the HRs for doubling androgen concentrations with and without adjustment for estrogen concentrations. All HRs decreased slightly after adjusting for total estradiol and substantially after adjusting for estrone sulfate.

Similar results were obtained when past HRT users were excluded from the analysis (data not shown).

Discussion

In this cohort of naturally postmenopausal women, we found that breast cancer risk was associated with higher levels of estrogens and lower levels of SHBG. Although the associations between androgens and breast cancer risk were higher for women with higher levels, overall, we did not find any significant association between circulating androgens and breast cancer risk. Our results suggest that the association between circulating estrogens and androgens and breast cancer risk varies with age, being stronger at older ages. We did not find any evidence

of heterogeneity of the association between sex hormones and breast cancer risk by tumor ER and PR status and grade.

The main strengths of our study include its prospective design, duration and completeness of follow-up, and the availability of accurate information on potential confoun-

ders. Consistent with the reanalysis of nine prospective studies (1), we found that the increased risk was greater for cases diagnosed >2 years after baseline than for cases diagnosed within the first 2 years of follow-up. This suggests that the presence of incipient breast cancers at time of blood collection did not increase the strength of the associations, and that hormonal profile is a good predictor of the long-term risk of breast cancer. Also consistent with previous reports (1, 5, 29), we found that the association between circulating sex hormones and breast cancer risk was higher for women who never used HRT than for past users, although the small proportion of past HRT users in our sample (11%) make the estimates for past users quite imprecise and the tests for heterogeneity were not significant.

For SHBG and all hormones, the quality of our measurements was good, as evidenced by high intraclass correlations and low coefficient of variation for the measurements made in the pooled plasma samples. Nevertheless, it is possible that weaker associations seen with the androgens were due to greater within person variability as evidenced by the lower intraclass correlation coefficients for these hormones.

After menopause, estradiol plasma levels decrease by 90% (9) and the main estrogen is estrone, resulting from the aromatization of androgens in adipose tissue (30). Others have previously reported that estradiol level is not a reliable risk predictor for postmenopausal women, perhaps because of the low levels detected, preferring estrone levels instead (31). Our results for estradiol are consistent with those reported in the literature and with those found for estrone sulfate, the other estrogen we measured.

Estrogens are known to directly stimulate the proliferation of breast cells, whereas the effect of androgens on breast tissue is more complex and still unclear. It has been suggested that the conversion of androgens into estrogens may be a possible mechanism by which androgens stimulate proliferation of the breast cells (10, 32). The aromatase enzyme is responsible for the conversion of androgens into estrogens and it may control the local production of estrogens through an autocrine loop (10, 32). Data from *in vitro* and animal studies suggest that androgens may also exert an antiproliferative and apoptotic effect (10, 32). Overall, we did not observe any statistically significant association between androgens and breast cancer risk, but the HRs that we found for women with the highest concentrations were similar to those reported from other studies (1-6). We observed strong positive associations between androgens and breast cancer risk at older ages, and in agreement with the results of other prospective studies (1, 3-5), we also found that the associations between androgen levels and breast cancer risk decreased after adjusting for estrogen levels. Both these findings are consistent with the argument that the contribution of androgens to breast cancer might be largely through their role as estrogen precursors, and that at older

Table 4. HRs and 95% CIs of breast cancer for a doubling of hormone concentration by attained age during follow-up

	HR (95% CI)*	$P_{\text{trend}}^{\dagger}$	P^{\ddagger}
Total estradiol			
<65 y	1.14 (0.55-2.37)	0.72	0.59
65-69 y	1.40 (0.64-3.04)	0.40	
>69 y	1.93 (0.93-4.01)	0.08	
Free estradiol			
<65 y	1.27 (0.73-2.21)	0.40	0.48
65-69 y	2.00 (1.10-3.66)	0.02	
>69 y	1.75 (0.98-3.15)	0.06	
Estrone sulfate			
<65 y	1.34 (0.84-2.14)	0.22	0.20
65-69 y	1.16 (0.68-1.96)	0.58	
>69 y	2.10 (1.28-3.44)	<0.01	
Testosterone			
<65 y	0.92 (0.66-1.29)	0.65	0.01
65-69 y	0.83 (0.55-1.25)	0.37	
>69 y	1.70 (1.16-2.48)	<0.01	
DHEAS			
<65 y	1.05 (0.76-1.45)	0.75	<0.01
65-69 y	0.79 (0.55-1.13)	0.20	
>69 y	1.74 (1.26-2.42)	<0.01	
Androstenedione			
<65 y	1.03 (0.74-1.45)	0.85	0.02
65-69 y	0.92 (0.61-1.39)	0.70	
>69 y	1.91 (1.26-2.88)	<0.01	
SHBG			
<65 y	0.59 (0.35-0.98)	0.04	0.07
65-69 y	0.25 (0.13-0.46)	<0.01	
>69 y	0.43 (0.24-0.76)	<0.01	

NOTE: HRs from the Cox model were obtained by splitting the data into the specified age bands and fitting an interaction of hormones with age band. All estimates were adjusted for country of birth, age at menarche, parity and age at first pregnancy, duration of lactation, oral contraceptive use, HRT use, alcohol consumption, energy from diet, smoking, BMI, level of education, and physical activity.

*Estimates from the model including the pseudocontinuous variable \log_2 transformed.

† Test for linear trend using the pseudocontinuous variable \log_2 transformed.

‡ Test for heterogeneity in HRs for hormone concentration (pseudocontinuous \log_2 transformed) in the three age groups.

Table 5. HRs and 95% CIs (in parentheses) for breast cancer associated with a doubling in androgen level with and without adjustment for estrogens

	Unadjusted	Adjusted for	
		Total estradiol	Estrone sulfate
Testosterone			
All	1.11 (0.88-1.40)	1.04 (0.81-1.35)	0.92 (0.68-1.23)
<65 y	0.92 (0.66-1.29)	0.89 (0.59-1.33)	0.75 (0.45-1.23)
65-69 y	0.83 (0.55-1.25)	0.73 (0.47-1.14)	0.71 (0.44-1.16)
>69 y	1.70 (1.16-2.48)	1.63 (1.09-2.43)	1.36 (0.88-2.11)
DHEAS			
All	1.15 (0.94-1.42)	1.12 (0.91-1.39)	0.98 (0.76-1.27)
<65 y	1.05 (0.76-1.45)	1.04 (0.74-1.44)	0.92 (0.61-1.38)
65-69 y	0.79 (0.55-1.13)	0.75 (0.52-1.08)	0.65 (0.43-0.99)
>69 y	1.74 (1.26-2.42)	1.68 (1.20-2.34)	1.50 (0.99-2.27)
Androstenedione			
All	1.24 (0.97-1.57)	1.17 (0.91-1.50)	1.08 (0.81-1.44)
<65 y	1.03 (0.74-1.45)	1.01 (0.69-1.46)	0.90 (0.57-1.43)
65-69 y	0.92 (0.61-1.39)	0.83 (0.55-1.28)	0.85 (0.54-1.34)
>69 y	1.91 (1.26-2.88)	1.80 (1.19-2.73)	1.60 (1.00-2.54)

NOTE: All estimates were from the Cox's regression model including the pseudocontinuous variable \log_2 transformed and adjusted for country of birth, age at menarche, parity and age at first pregnancy, duration of lactation, oral contraceptive use, HRT use, alcohol consumption, energy from diet, smoking, BMI, level of education, and physical activity.

ages, aromatase activity increases to maintain high concentrations of estrogens in the breast tissues (10).

Our observation that the associations between hormone levels and breast cancer risk are highest for women ages >69 years agrees with the age-dependent association between peptide hormones and breast cancer risk that we described in the same cohort of women (33), and could be explained by the cumulative damage that these hormones might produce in breast tissue (21). In a pooled reanalysis of nine prospective studies, no statistically significant difference in the association of any hormone levels with breast cancer risk was reported when women were categorized according to age at diagnosis (1). In subsequent reports from the Nurses' Health Study, age at diagnosis did not modify the associations between endogenous hormone levels and breast cancer risk for nonusers of postmenopausal hormones (5), but both estradiol and free estradiol levels were positively associated with breast cancer risk primarily for women ages >60 years at time of blood collection (but not for younger women) who were postmenopausal hormone users (29). Only a small proportion of the women in our sample reported the use of postmenopausal hormones in the past, and the age-dependent trend of the association remained after the exclusion of past HRT users. Recently, it has been reported that higher levels of circulating estrogens and androgens might be important not only for the etiology of postmenopausal breast cancer but also for premenopausal breast cancer (34). Longitudinal studies are necessary to help clarify the role of sex hormones with

regard to breast cancer risk before or during the menopausal transition. Because the heterogeneity by age persisted when the analysis was restricted to tumors expressing hormone receptors, we can rule out the possibility that the higher association at older ages was due to the higher proportion of breast tumors expressing hormone receptors diagnosed in older women, for which tumor subtypes, the association with steroid hormones levels could be higher (5, 19).

Our HRs varied little in relation to ER and PR status. The literature contains only a few and conflicting reports on the association between endogenous hormone levels and breast cancer risk by tumor hormone receptor status (5, 19, 20, 31). The classic mechanism of estrogen carcinogenicity is its binding to its specific nuclear receptor α (ER- α) to form an ER complex that binds to specific DNA sites and exerts a stimulus on breast cell proliferation by enhancing the production of growth factors (9). We should, therefore, expect that the increased risk associated with higher levels of endogenous hormones would be limited to ER-positive tumors. However, results from *in vitro* experiments show that estrogens may cause breast cancer through a genotoxic, non-ER- α -mediated mechanism (9) and other receptors such as ER- β or other mechanisms could also play a role in the estrogen-induced transformation of human breast epithelial cells. Because ER- β is not currently considered as a clinically useful breast tumor marker, testing for this receptor is not part of routine clinical practice (35). Our study, where the

antibody used to determine ER status in the majority of the breast cancer cases recognizes ER- α protein, provides evidence that estrogens can induce breast cancer independently of the tumor ER status. It is likely that once hormone receptor-positive breast tumors are initiated, they are more susceptible to stimulation by estrogens than hormone receptor-negative tumors. Studies investigating the effect of circulating levels of sex hormones on survival by ER/PR status need to be conducted to test this hypothesis.

In conclusion, our prospective study confirms earlier findings that, for postmenopausal women, exposure to higher levels of endogenous hormones is associated with an increased risk of breast cancer, and also suggests that the associations might be stronger for breast cancer diagnosed at older ages.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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