Endogenous Steroid Hormone Concentrations and Risk of Breast Cancer Among Premenopausal Women

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Background: **Higher levels of endogenous sex steroid hormones are associated with increased risks of breast cancer in postmenopausal women. Data for premenopausal women are sparse, in part because of the complexity of measuring hormone levels that vary cyclically. We prospectively evaluated associations between plasma sex hormone levels and breast cancer risk among premenopausal women in a case–control study nested within the Nurses' Health Study II.** ***Methods:*** **From 1996 to 1999, blood samples were collected from 18 521 premenopausal women during the early follicular and midluteal phases of their menstrual cycles. A total of 197 cases of breast cancer were diagnosed among these women after blood collection and before June 1, 2003; these case subjects were matched to 394 control subjects. Logistic regression models, controlling for breast cancer risk factors, were used to calculate relative risks (RRs) and 95% confidence intervals (CIs). All statistical tests were two-sided.** ***Results:*** **Women in the highest (versus the lowest) quartiles of follicular total and free estradiol levels had statistically significantly increased risks of breast cancer (RR = 2.1 [95% CI = 1.1 to 4.1], P_{trend} = .08, and RR = 2.4 [95% CI = 1.3 to 4.5], P_{trend} = .01, respectively); the associations were stronger for invasive breast cancer and for estrogen and progesterone receptor–positive (ER+/PR+) tumors. Luteal estradiol levels were not associated with breast cancer risk. Higher levels of total and free testosterone and androstenedione in both menstrual cycle phases were associated with modest, non–statistically significant increases in overall risk of breast cancer and with stronger, statistically significant increases in risks of invasive and ER+/PR+ cancers (e.g., RR of invasive cancers for the top [versus bottom] quartile of luteal total testosterone levels = 2.0 [95% CI = 1.1 to 3.6], P_{trend} = .05, and RR of ER+/PR+ cancers = 2.9 [95% CI = 1.4 to 6.0], P_{trend} = .02). Levels of estrone, estrone sulfate, progesterone, and sex hormone–binding globulin were not associated with breast cancer risk. The absolute number of cases observed over 3 years were 30 among women in the lowest 25% of follicular total estradiol levels and 50 among women in the highest 25%.** ***Conclusions:*** **Levels of circulating estrogens and androgens may be important in the etiology of premenopausal breast cancer. [J Natl Cancer Inst 2006;98:1406–15]**

Hormones play a critical role in breast carcinogenesis *(1,2)*. Determining the associations between circulating sex steroid hormone levels and breast cancer risk may provide insight into the etiology of this disease and may help identify women who are at high risk and would therefore benefit from increased screening or chemoprevention. Although the relationships between circulating estrogen and androgen levels and breast cancer risk are well established among postmenopausal women *(3–6)*, most previous prospective studies among premenopausal women have been small and, for estrogens, have produced inconsistent results *(7–13)*. Three *(9–11)* of five *(7,9–12)* small studies (each with fewer than 80 cases) reported a non–statistically significant positive association between estradiol level and breast cancer risk; one large study [n = 285 cases; *(8)*] reported no association. Non–statistically significant positive associations between androgen levels and breast cancer risk were observed in two early studies *(7,11)*, whereas statistically significant positive associations were observed in two recent studies *(8,13)*. Investigations of the association between premenopausal estrogen levels and breast cancer risk are complicated by the cyclic variation of estrogen during the menstrual cycle; all published studies to date have included blood samples obtained without restriction to the phase or day of a woman's menstrual cycle and therefore had limited power to examine menstrual cycle phase–specific associations.

We conducted a prospective, nested case–control study within the Nurses' Health Study II cohort, using blood samples timed within the early follicular and midluteal phases of the participants' menstrual cycles, to examine separately the associations between levels of sex steroid hormones in each phase and breast cancer risk in premenopausal women.

Methods

Study Population and Blood Sample Collection

The Nurses' Health Study II was established in 1989, when 116 609 female registered nurses, aged 25–42 years, completed and returned a questionnaire. This cohort has been followed biennially by questionnaire to update exposures and ascertain newly diagnosed disease.

Between 1996 and 1999, 92 888 cohort members were invited to give a blood sample and 54 896 agreed and were eligible. Of these, 29 611 women who were cancer free and between the ages of 32 and 54 years provided blood samples. Participants were sent a short questionnaire and a blood collection kit that contained supplies necessary to have their blood samples drawn by a

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local laboratory or by a colleague. These women were similar to the overall cohort with respect to body mass index (BMI), parity, age at menarche, and past oral contraceptive use, but they differed slightly from the overall cohort in the prevalence of a family history of breast cancer (19% versus 15% in the overall cohort) *(14)*. Of the 29 611 women who provided blood samples, 18 521 were still having menstrual cycles (i.e., were premenopausal); each of these women provided two blood samples that were timed within their menstrual cycles. Premenopausal women who had used oral contraceptives, been pregnant, or had breastfed within 6 months of blood collection were excluded from the study. Participants were asked to provide two blood samples, one drawn during days 3–5 of their menstrual cycle (i.e., the follicular sample) and the other drawn 7–9 days before the anticipated start of their next menstrual period (i.e., the luteal sample). Participants were asked to refrigerate the follicular blood sample for 8–24 hours after blood collection. They then separated the plasma and stored it in their home freezer until their luteal blood sample was drawn. On the day of the luteal blood draw, both samples (i.e., follicular plasma and luteal whole blood) were shipped on ice via overnight courier to our laboratory, where the luteal blood sample was processed and separated into plasma, red blood cells, and white blood cells. Approximately 93% of the luteal blood samples were received within 1 day of collection; 96% were processed within 48 hours of collection. Sex steroids have previously been shown to be stable in whole blood for 24–48 hours *(15)*. All samples were stored in a liquid nitrogen freezer. Participants recorded, on the blood questionnaire, the first day of the menstrual cycle during which the blood samples were drawn. They also returned a postcard recording the first day of their next menstrual cycle so that the timing of their luteal-phase blood draw could be determined accurately. Follow-up of the 29 611 women who provided blood samples was 98% in 2003. This study was approved by the Committee on the Use of Human Subjects in Research at Harvard School of Public Health and Brigham and Women's Hospital. Informed consent was implied by receipt of completed questionnaires and blood samples.

Case and Control Subjects

Breast cancer case subjects were identified by participants' responses to the biennial questionnaires; the National Death Index was searched for nonresponders. Case subjects were women who had no previously reported cancer diagnosis and who had been diagnosed with breast cancer after blood collection but before June 1, 2003. Overall, 197 cases of breast cancer (131 of which were invasive) were reported on biennial questionnaires and confirmed by medical record review (n = 183) or direct communication with the participant (n = 14); no additional cases were identified by the National Death Index search. Given that medical records confirmed 99% of cases, we also included the case subjects for whom there were no medical records but the diagnosis was confirmed by the participant herself. Information on breast cancer invasiveness and hormone receptor status was abstracted from the participants' medical record. The mean length of time from blood draw to diagnosis was 35 months (range = 1–87 months). Two control subjects (n = 394) were matched to each case subject on age at blood collection (±2 years); menopausal status at diagnosis; month and year of blood draw (±2 months); ethnicity (African American, Asian, Hispanic, Caucasian, or other); luteal day of menstrual cycle (defined as the date of the woman's next period minus the date of the luteal blood draw [±1 day]); and for each blood collection, time of the day (±2 hours) and the woman's fasting status (<2, 2–4, 5–7, 8–11, or ≥12 hours since previous meal). More than 90% of case–control matches were exact for each matching variable.

Reproducibility Study

Among the 18 521 women who provided timed blood samples, 236 collected two additional sets of timed blood samples over the next 2–3 years. The three sets of follicular and luteal samples from a random sample of 113 of these women were analyzed to assess the reproducibility of sex hormone levels over time, as previously described *(16)*. Intraclass correlation coefficients (ICCs) from the reproducibility study indicated that a single hormone measure provides a reasonable representation of hormone levels over at least 3 years, with ICCs for measured hormones ranging from 0.38 (follicular estradiol) to 0.83 (follicular and luteal sex hormone–binding globulin [SHBG]), with the exception of progesterone, which had an ICC of only 0.29.

Laboratory Assays

Hormone assays for estrogens and testosterone were performed by Quest Diagnostics (San Juan Capistrano, CA) as previously described *(17)*. In brief, plasma samples were assayed by radioimmunoassay following extraction and celite column chromatography. Estrone sulfate was assayed by radioimmunoassay of estrone after estrone extraction, enzyme hydrolysis, extraction, and column chromatography. The fractions of free estradiol and testosterone were calculated as described by Sodergard et al. *(18)*. Androstenedione was assayed by radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX) at the Royal Marsden Hospital, and SHBG and progesterone were measured using a chemiluminescence immunoassay and an Immulite autoanalyzer (Diagnostic Products, Gwynedd, U.K.).

Samples were assayed in two batches. The follicular and luteal samples from each woman were assayed together, as were samples from case–control sets. Samples were ordered randomly and labeled to mask case–control status. Masked replicates (10% of the samples) were included in each batch for quality control purposes. The interassay coefficients of variation for all hormones except progesterone ranged from 9% (testosterone) to 14% (estrone sulfate); progesterone had an intra-assay coefficient of variation of 3% and an interassay coefficient of variation of 17%.

Statistical Analysis

We performed separate analyses by menstrual cycle phase. Quartile cut points for hormone levels were based on distributions in the control subjects and were determined separately for follicular and luteal hormones. We used overall cut points for all hormones except androstenedione and progesterone because the results were similar whether we used batch-specific or overall cut points. For androstenedione and progesterone, we used batch-specific cut points. Although the correlation coefficients between batches from a subset of 12 samples run in both batches were ≥0.9, the mean hormone concentrations differed by batch, indicating some laboratory drift over time. In addition, for these two hormones, results of analyses that used overall cut points

differed somewhat from those that used batch-specific cut points (the latter approach being the most appropriate analytic approach in the setting of batch-to-batch variation such as we observed).

We identified statistical outliers using the generalized extreme studentized deviate many-outlier detection approach *(19)* and excluded subjects with extreme values from the analysis (i.e., those with follicular estrone levels >180 pg/mL [n = 2] or luteal free estradiol levels >10.6 pg/mL [n = 1]). Several subjects had missing hormone values related to technical difficulties or low sample volume; thus, the final sample size varied for each analysis by menstrual cycle phase and by hormone.

We used a mixed-effects regression model to test the paired differences in log-transformed hormone levels between case subjects and matched control subjects. We used conditional logistic regression models to estimate relative risks (RRs) and 95% confidence intervals (CIs). Multivariable models were adjusted for BMI at age 18 years, ages at menarche and first birth, parity, history of benign breast disease, and family history of breast cancer. Further adjustment for history of breast-feeding and past oral contraceptive use did not substantially alter the results, and therefore, the final models did not include the adjustments. Unless noted, results of multivariable-adjusted analyses are presented because they were essentially the same as results from the unadjusted models. In stratified analyses, we used unconditional logistic regression models with adjustment for matching factors because results from the multivariable unconditional and conditional logistic regression models were essentially identical. Only five case subjects were postmenopausal at diagnosis; therefore, we could not examine this group separately because of its small size. We performed separate analyses among case subjects with estrogen and progesterone receptor–positive (ER+/PR+) tumors (n = 89) but could not evaluate case subjects with other ER/PR subtypes because of their scarcity ($n \leq 25$ for each remaining subtype). Tests for trend were conducted by modeling the quartile median concentrations and calculating the Wald statistic. We used the Wald test to test for interactions between stratification variables and hormone levels by comparing the slope of the quartile median concentrations between strata. All *P* values were based on two-sided tests and were considered to be statistically significant if less than or equal to .05. In secondary analyses, we corrected point and interval estimates of the medians of the highest versus the lowest quartiles to evaluate the effect of laboratory measurement error and random within-person variation on our observed results *(20)*. The ICCs used to correct measurement error were calculated using the between-person variance from the case–control data and the within-person variance determined previously in the reproducibility study *(16)*. Spearman correlation coefficients were calculated for follicular and luteal hormone levels.

Results

Case subjects had slightly lower parity and were more likely to have a family history of breast cancer and a history of benign breast disease than control subjects (Table 1). Case subjects had statistically significantly higher levels of follicular total and free estradiol than control subjects (Table 2). Case subjects also had higher levels of follicular and luteal androgens than control subjects; however, these differences were not statistically significant.

Table 1. Characteristics of breast cancer case subjects and matched control subjects, Nurses' Health Study II*

Characteristic	Case subjects (n = 197)	Control subjects (n = 394)
Age at blood draw (y), mean (SD)	43.4 (3.8)	43.2 (3.8)
Menstrual cycle day at follicular blood draw, mean (SD)	3.8 (1.0)	3.9 (1.1)
Menstrual cycle day at luteal blood draw†, mean (SD)	7.7 (3.1)	7.6 (2.9)
Age at menarche (y), mean (SD)	12.5 (1.4)	12.5 (1.4)
Parity‡, mean (SD)	2.1 (0.8)	2.3 (1.0)
BMI (kg/m^2), mean (SD)		
At age 18	20.8 (3.1)	20.9 (2.6)
At blood draw	24.8 (5.2)	24.9 (5.1)
Family history of breast cancer, %	15.7	10.2
History of benign breast disease, %	20.8	15.2
Ever used oral contraceptives, %	84.3	84.0

*SD = standard deviation; BMI = body mass index.

†Days from luteal-phase blood draw to first day of the next menstrual cycle (i.e., backward dating method).

‡Among parous women only.

Women with high follicular total and free estradiol levels had a statistically significantly increased risk of breast cancer (RR for highest versus lowest quartile of follicular total estradiol = 2.1 [95% CI = 1.1 to 4.1], P_{trend} = .08; RR for highest versus lowest quartile of follicular free estradiol = 2.4 [95% CI = 1.3 to 4.5], P_{trend} = .01) (Table 3). These adjusted relative risks were slightly higher than those obtained from unadjusted models (unadjusted RR for follicular total estradiol = 1.9 [95% CI = 1.0 to 3.4]; unadjusted RR for follicular free estradiol = 2.1 [95% CI = 1.2 to 3.8]) primarily because of adjustment for BMI at age 18 years, parity, and age at first birth. The associations between follicular total and free estradiol and breast cancer risk were somewhat stronger for invasive breast cancer or ER+/PR+ tumors (Table 3). No clear associations were apparent for luteal estradiol levels except, possibly, among case subjects with ER+/PR+ tumors (e.g., RR for highest versus lowest quartile of luteal free estradiol = 2.0 [95% CI = 0.9 to 4.6]). Follicular estrone levels were not associated with overall breast cancer risk but showed a modest positive association with risk of ER+/PR+ tumors (RR = 1.9 [95% CI = 0.9 to 3.9]). Luteal estrone, follicular and luteal estrone sulfate, and luteal progesterone levels were not associated with risk of breast cancer.

Women who had high total testosterone levels during either the follicular or luteal phase of their menstrual cycle had a modest, non–statistically significant increased risk of breast cancer (RR for highest versus lowest quartile of follicular total testosterone = 1.3 [95% CI = 0.8 to 2.4], P_{trend} = .35; RR for highest versus lowest quartile of luteal total testosterone = 1.6 [95% CI = 0.9 to 2.8], P_{trend} = .10); however, the risk estimates—particularly those for follicular total testosterone—did not increase linearly across increasing quartiles (Table 4). The associations between total testosterone levels and breast cancer risk were stronger and, in some cases, statistically significant when the analysis was restricted to invasive breast cancer or ER+/PR+ tumors. For example, women in the top (versus bottom) quartile of luteal total testosterone levels had a twofold higher risk of invasive cancer (RR = 2.0 [95% CI = 1.1 to 3.6], P_{trend} = .05) and a nearly threefold higher risk of an ER+/PR+ tumor (RR = 2.9 [95% CI = 1.4 to 6.0], P_{trend} = .02). Our findings for free testosterone generally mirrored those for total testosterone. Women with high androstenedione levels had higher risks of breast

Table 2. Plasma sex steroid hormone and SHBG levels of case subjects and control subjects, Nurses' Health Study II*

Hormone	Case subjects		Control subjects		
	n	Median (range†)	n	Median (range†)	P‡
Estradiol (pg/mL)					
Follicular	185	48 (28–101)	368	44 (22–88)	.01
Luteal	175	125 (76–182)	349	120 (69–192)	.35
Free estradiol (pg/mL)					
Follicular	177	0.66 (0.38–1.20)	347	0.56 (0.30–1.06)	.002
Luteal	170	1.62 (0.98–2.53)	344	1.59 (0.90–2.49)	.11
Estrone (pg/mL)					
Follicular	193	40 (26–59)	381	39 (26–59)	.52
Luteal	193	74 (50–124)	392	79 (50–119)	.76
Estrone sulfate (pg/mL)					
Follicular	181	694 (379–1429)	361	667 (325–1414)	.47
Luteal	182	1364 (601–2933)	364	1521 (596–3141)	.18
Progesterone (ng/dL)					
Luteal	195	1572 (480–2491)	391	1447 (472–2514)	.80
Testosterone (ng/dL)					
Follicular	190	20 (13–33)	374	20 (12–31)	.11
Luteal	192	27 (17–41)	390	26 (16–39)	.09
Free testosterone (ng/dL)					
Follicular	189	0.17 (0.10–0.28)	372	0.16 (0.10–0.30)	.27
Luteal	191	0.23 (0.14–0.40)	388	0.21 (0.12–0.38)	.11
Androstenedione (ng/dL)					
Follicular	193	93 (55–152)	385	86 (54–149)	.26
Luteal	196	112 (74–169)	392	110 (69–171)	.60
SHBG (nmol/L)					
Follicular	193	61 (37–93)	386	59 (31–101)	.61
Luteal	196	61 (35–91)	392	59 (33–101)	.96

*Hormone levels calculated among all batches of case or control samples combined. SHBG = sex hormone–binding globulin.

†From the median of the bottom quartile (12.5 percentile) to the median of the top quartile (87.5 percentile).

‡From mixed-effects regression models that compared paired differences in log hormone levels between case subjects and matched control subjects; two-sided.

cancer than women with low levels, although the risk estimates over increasing quartiles did not increase in a linear fashion and were statistically significant only for the risk of ER+/PR+ tumors (RR for highest versus lowest quartile of luteal androstenedione = 2.7 [95% CI = 1.2 to 6.2], P_{trend} = .17). SHBG level was not clearly associated with breast cancer risk; although several point estimates for breast cancer risk association with follicular SHBG were elevated, adjustment for estradiol attenuated these estimates (data not shown).

When estradiol and testosterone were modeled together, the associations for each were essentially unchanged. No statistically significant associations were observed when luteal estradiol and progesterone levels were evaluated together or when low levels of both were compared with high levels of both (data not shown).

All associations were essentially unchanged when we excluded women with anovulatory menstrual cycles (i.e., those with progesterone levels <400 ng/mL; 11 case subjects, 28 control subjects), women who had blood drawn either fewer than 3 days or more than 21 days before the start of their next menstrual cycle (six case subjects, five control subjects), or women with anovulatory menstrual cycles who had blood drawn fewer than 3 days or more than 21 days before the start of their next menstrual cycle (10 case subjects, 17 control subjects) (data not shown). Analyses restricted to the 77% of women who reported having regular menstrual cycles between the ages of 18 and 22 years yielded stronger associations for both follicular total estradiol (RR = 3.5 [95% CI = 1.7 to 7.2], P_{trend} = .01) and luteal total testosterone (RR = 2.1 [95% CI = 1.1 to 3.8], P_{trend} = .03). Stratification by time since blood collection, age at blood draw, BMI at blood draw, past oral contraceptive use, family history of breast cancer, or history of benign breast disease did not substantially change the results for any of the hormones.

We further examined the characteristics of women in the first quartile because a number of the risk estimates did not increase in a linear fashion over increasing quartiles. Women in the lowest quartile of follicular estradiol level were similar to the rest of the women with respect to age at blood draw and menstrual cycle characteristics but had a higher mean BMI at blood draw (quartile 1 versus quartiles 2–4: 26.4 versus 24.5 kg/m^2) and a higher prevalence of anovulatory cycles (quartile 1 versus quartiles 2–4: 21% versus 7%). However, as noted above, the relative risks were similar when the analysis was stratified by BMI at blood draw or when women with anovulatory cycles were excluded. Therefore, these characteristics do not explain the differences in risk between quartile 1 and quartiles 2–4. Women in the lowest quartile of follicular testosterone level were similar to the rest of the women with respect to age at blood draw, menstrual cycle characteristics, BMI at blood draw, and prevalence of anovulatory cycles.

We next corrected for random within-person variability and laboratory measurement error; in these analyses, relative risks of breast cancer were calculated by comparing the median plasma hormone level of women in the highest quartile with that of women in the lowest quartile. Measurement error correction using an ICC of 0.50 increased the relative risk of breast cancer associated with follicular estradiol level from 1.8 (95% CI = 1.1 to 3.0) to 3.3 (95% CI = 1.2 to 9.3); results for invasive cases were identical. For follicular testosterone (ICC = 0.59), the relative risk for total breast cancer increased from 1.4 (95% CI = 0.9 to 2.2) to 1.8 (95% CI = 0.8 to 4.0) and for invasive breast cancer from 1.6 (95% CI = 1.0 to 2.6) to 2.2 (95% CI = 0.9 to 5.2).

Table 3. Relative risks (95% confidence intervals) of breast cancer by quartile of prediagnostic plasma estrogen and progesterone levels, Nurses' Health Study II*

Plasma hormone	Quartile categories 1	2	3	4	P_{trend}†
Estradiol					
Follicular					
Cut points (pg/mL)	≤29	>29–44	>44–66	>66	
No. of case subjects/No. of control subjects	30/94	55/91	50/94	50/89	
All breast cancer‡	1.0 (referent)	2.0 (1.1 to 3.6)	1.7 (1.0 to 3.2)	2.1 (1.1 to 4.1)	.08
Invasive breast cancer§	1.0 (referent)	2.9 (1.5 to 5.7)	2.0 (1.0 to 4.0)	2.7 (1.3 to 5.4)	.07
ER+/PR+ breast cancer§	1.0 (referent)	2.5 (1.2 to 5.6)	1.8 (0.8 to 4.1)	2.7 (1.2 to 6.0)	.07
Luteal					
Cut points (pg/mL)	≤90	>90–120	>120–159	>159	
No. of case subjects/No. of control subjects	36/90	46/86	60/88	33/85	
All breast cancer‡	1.0 (referent)	1.2 (0.7 to 2.3)	1.8 (1.0 to 3.3)	1.0 (0.5 to 1.9)	>.99
Invasive breast cancer§	1.0 (referent)	1.3 (0.7 to 2.6)	2.0 (1.1 to 3.8)	0.9 (0.4 to 1.9)	.91
ER+/PR+ breast cancer§	1.0 (referent)	2.0 (0.9 to 4.7)	3.0 (1.3 to 6.8)	1.5 (0.6 to 3.6)	.56
Free estradiol					
Follicular					
Cut points (pg/mL)	≤0.40	>0.40–0.56	>0.56–0.80	>0.80	
No. of case subjects/No. of control subjects	28/86	40/86	52/86	57/89	
All breast cancer‡	1.0 (referent)	1.6 (0.9 to 2.9)	2.0 (1.1 to 3.6)	2.4 (1.3 to 4.5)	.01
Invasive breast cancer§	1.0 (referent)	1.8 (0.9 to 3.6)	2.1 (1.0 to 4.1)	2.7 (1.4 to 5.3)	.01
ER+/PR+ breast cancer§	1.0 (referent)	1.4 (0.6 to 3.4)	2.4 (1.1 to 5.3)	2.8 (1.3 to 6.2)	.01
Luteal					
Cut points (pg/mL)	≤1.18	>1.18–1.59	>1.59–2.07	>2.07	
No. of case subjects/No. of control subjects	36/87	45/86	43/85	46/86	
All breast cancer‡	1.0 (referent)	1.5 (0.8 to 2.8)	1.4 (0.8 to 2.6)	1.5 (0.8 to 2.8)	.30
Invasive breast cancer§	1.0 (referent)	1.4 (0.7 to 2.8)	1.3 (0.7 to 2.6)	1.3 (0.7 to 2.7)	.56
ER+/PR+ breast cancer§	1.0 (referent)	1.7 (0.7 to 3.8)	1.4 (0.6 to 3.3)	2.0 (0.9 to 4.6)	.15
Estrone					
Follicular					
Cut points (pg/mL)	≤31	>31–39	>39–49	>49	
No. of case subjects/No. of control subjects	48/104	46/95	51/87	48/95	
All breast cancer‡	1.0 (referent)	1.1 (0.6 to 1.8)	1.3 (0.8 to 2.1)	1.2 (0.7 to 1.9)	.48
Invasive breast cancer§	1.0 (referent)	1.3 (0.7 to 2.3)	1.2 (0.7 to 2.2)	1.4 (0.8 to 2.6)	.25
ER+/PR+ breast cancer§	1.0 (referent)	1.8 (0.9 to 3.7)	1.6 (0.7 to 3.3)	1.9 (0.9 to 3.9)	.11
Luteal					
Cut points (pg/mL)	≤61	>61–79	>79–99	>99	
No. of case subjects/No. of control subjects	67/102	40/101	41/95	45/94	
All breast cancer‡	1.0 (referent)	0.5 (0.3 to 0.9)	0.5 (0.3 to 0.9)	0.6 (0.3 to 1.0)	.13
Invasive breast cancer§	1.0 (referent)	0.6 (0.3 to 1.1)	0.5 (0.3 to 1.0)	0.7 (0.4 to 1.3)	.30
ER+/PR+ breast cancer§	1.0 (referent)	0.8 (0.4 to 1.5)	0.8 (0.4 to 1.6)	0.8 (0.4 to 1.6)	.58
Estrone sulfate					
Follicular					
Cut points (pg/mL)	≤459	>459–667	>667–998	>998	
No. of case subjects/No. of control subjects	42/91	46/90	43/90	50/90	
All breast cancer‡	1.0 (referent)	1.0 (0.6 to 1.7)	1.1 (0.6 to 1.9)	1.1 (0.7 to 1.9)	.63
Invasive breast cancer§	1.0 (referent)	1.0 (0.5 to 1.8)	1.0 (0.5 to 1.9)	1.1 (0.6 to 2.0)	.66
ER+/PR+ breast cancer§	1.0 (referent)	0.9 (0.5 to 1.9)	1.1 (0.6 to 2.3)	1.1 (0.5 to 2.3)	.69
Luteal					
Cut points (pg/mL)	≤863	>863–1521	>1521–2303	>2303	
No. of case subjects/No. of control subjects	44/91	58/91	44/91	36/91	
All breast cancer‡	1.0 (referent)	1.2 (0.7 to 2.1)	0.8 (0.4 to 1.5)	0.6 (0.3 to 1.2)	.06
Invasive breast cancer§	1.0 (referent)	1.3 (0.7 to 2.4)	0.7 (0.4 to 1.3)	0.8 (0.4 to 1.6)	.23
ER+/PR+ breast cancer§	1.0 (referent)	1.5 (0.8 to 3.0)	0.8 (0.4 to 1.7)	0.9 (0.4 to 2.0)	.46
Progesterone					
Luteal					
Cut points‖ (ng/dL)					
No. of case subjects/No. of control subjects	53/101	33/98	61/94	48/98	
All breast cancer‡	1.0 (referent)	0.6 (0.3 to 1.1)	1.2 (0.7 to 2.0)	0.9 (0.5 to 1.7)	.42
Invasive breast cancer§	1.0 (referent)	0.7 (0.4 to 1.3)	1.3 (0.7 to 2.4)	0.9 (0.5 to 1.7)	.74
ER+/PR+ breast cancer§	1.0 (referent)	0.6 (0.3 to 1.3)	1.2 (0.6 to 2.5)	0.9 (0.4 to 1.8)	.78

*Cut points are based on the distribution in control subjects using values from combined batches except where otherwise indicated.

†The medians of the quartiles were entered into the model as a continuous variable; two-sided.

‡Conditional logistic regression models controlling for body mass index at age of 18 years (<21, 21 to <23, ≥23 kg/m^2), family history of breast cancer (yes, no), age at menarche (<12, 12, 13, ≥14 years), history of benign breast disease (yes, no), and parity/age at first birth (nulliparous, age at first birth <25 years/1–2 children, age at first birth 25–29 years/1–2 children, age at first birth ≥30 years/1–2 children, age at first birth <25 years/≥3 children, age at first birth ≥25 years/≥3 children).

§Unconditional logistic regression models controlling for covariates above and matching factors: days from luteal draw to the next menstrual cycle (0–5, 6–7, 8–9, 10–28 days), age at blood collection (continuous), date of blood collection (continuous), fasting at blood collection (yes, no), time of blood collection (1 AM–4 AM, 5 AM–6 AM, 7 AM–12 PM, continuous), and race/ethnicity (Caucasian, other). ER+/PR+ = estrogen and progesterone receptor positive.

‖Cut points for progesterone are batch specific—batch 1: ≤877, >877–1418, >1418–2170, >2170 ng/dL; batch 2: ≤1132, >1132–1478, >1478–1903, >1903.

Table 4. Relative risks (95% confidence intervals) of breast cancer by quartile of prediagnostic plasma androgen and sex hormone–binding globulin (SHBG) levels, Nurses' Health Study II*

Plasma hormone or SHBG	Quartile categories 1	2	3	4	P_{trend}†
Testosterone					
Follicular					
Cut points (ng/dL)	≤15	>15–20	>20–26	>26	
No. of case subjects/No. of control subjects	42/105	55/98	45/84	48/87	
All breast cancer‡	1.0 (referent)	1.3 (0.8 to 2.2)	1.4 (0.8 to 2.3)	1.3 (0.8 to 2.4)	.35
Invasive breast cancer§	1.0 (referent)	1.9 (1.0 to 3.4)	1.6 (0.8 to 3.0)	1.8 (0.9 to 3.4)	.17
ER+/PR+ breast cancer§	1.0 (referent)	2.2 (1.0 to 4.4)	2.1 (1.0 to 4.6)	2.0 (0.9 to 4.3)	.17
Luteal					
Cut points (ng/dL)	≤20	>20–26	>26–32	>32	
No. of case subjects/No. of control subjects	43/114	42/81	49/99	58/96	
All breast cancer‡	1.0 (referent)	1.3 (0.8 to 2.3)	1.4 (0.8 to 2.3)	1.6 (0.9 to 2.8)	.10
Invasive breast cancer§	1.0 (referent)	1.6 (0.9 to 3.1)	1.3 (0.7 to 2.4)	2.0 (1.1 to 3.6)	.05
ER+/PR+ breast cancer§	1.0 (referent)	2.3 (1.1 to 5.1)	1.4 (0.6 to 3.0)	2.9 (1.4 to 6.0)	.02
Free testosterone					
Follicular					
Cut points (ng/dL)	≤0.12	>0.12–0.16	>0.16–0.23	>0.23	
No. of case subjects/No. of control subjects	38/98	45/84	57/99	49/91	
All breast cancer‡	1.0 (referent)	1.5 (0.8 to 2.6)	1.5 (0.9 to 2.6)	1.6 (0.9 to 2.8)	.17
Invasive breast cancer§	1.0 (referent)	1.3 (0.7 to 2.4)	1.4 (0.8 to 2.6)	1.5 (0.8 to 2.8)	.25
ER+/PR+ breast cancer§	1.0 (referent)	1.2 (0.6 to 2.6)	1.4 (0.7 to 2.9)	1.8 (0.9 to 3.7)	.09
Luteal					
Cut points (ng/dL)	≤0.15	>0.15–0.21	>0.21–0.29	>0.29	
No. of case subjects/No. of control subjects	37/90	45/106	54/94	55/98	
All breast cancer‡	1.0 (referent)	0.9 (0.5 to 1.5)	1.3 (0.7 to 2.2)	1.4 (0.8 to 2.5)	.14
Invasive breast cancer§	1.0 (referent)	1.5 (0.8 to 3.0)	2.0 (1.1 to 4.0)	1.9 (1.0 to 3.8)	.08
ER+/PR+ breast cancer§	1.0 (referent)	2.0 (0.9 to 4.7)	2.6 (1.1 to 6.1)	2.9 (1.2 to 6.6)	.03
Androstenedione					
Follicular					
Cut points§ (ng/dL)					
No. of case subjects/No. of control subjects	39/102	52/91	53/99	49/93	
All breast cancer‡	1.0 (referent)	1.7 (1.0 to 3.0)	1.5 (0.9 to 2.7)	1.7 (0.9 to 3.1)	.28
Invasive breast cancer§	1.0 (referent)	1.4 (0.7 to 2.5)	1.7 (1.0 to 3.2)	1.5 (0.8 to 2.9)	.23
ER+/PR+ breast cancer§	1.0 (referent)	1.1 (0.5 to 2.4)	1.9 (0.9 to 3.8)	1.8 (0.9 to 3.7)	.08
Luteal					
Cut points‖ (ng/dL)					
No. of case subjects/No. of control subjects	40/99	60/97	46/100	50/96	
All breast cancer‡	1.0 (referent)	1.4 (0.9 to 2.4)	1.1 (0.6 to 1.9)	1.3 (0.7 to 2.3)	.77
Invasive breast cancer§	1.0 (referent)	2.4 (1.3 to 4.5)	2.0 (1.0 to 3.9)	1.7 (0.8 to 3.4)	.52
ER+/PR+ breast cancer§	1.0 (referent)	2.9 (1.3 to 6.3)	1.9 (0.8 to 4.4)	2.7 (1.2 to 6.2)	.17
SHBG					
Follicular					
Cut points (nmol/L)	≤42	>42–59	>59–80	>80	
No. of case subjects/No. of control subjects	41/97	46/96	61/96	45/97	
All breast cancer‡	1.0 (referent)	1.2 (0.7 to 2.0)	1.5 (0.9 to 2.4)	1.1 (0.6 to 1.9)	.77
Invasive breast cancer§	1.0 (referent)	1.1 (0.6 to 2.0)	1.6 (0.9 to 3.0)	1.3 (0.7 to 2.3)	.37
ER+/PR+ breast cancer§	1.0 (referent)	0.8 (0.4 to 1.6)	1.5 (0.8 to 3.0)	1.2 (0.6 to 2.4)	.30
Luteal					
Cut points (nmol/L)	≤42	>42–59	>59–79	>79	
No. of case subjects/No. of control subjects	42/98	52/98	57/98	45/98	
All breast cancer‡	1.0 (referent)	1.2 (0.7 to 2.1)	1.2 (0.7 to 2.1)	1.0 (0.6 to 1.8)	.95
Invasive breast cancer§	1.0 (referent)	1.4 (0.8 to 2.5)	1.4 (0.8 to 2.5)	1.1 (0.6 to 2.1)	.85
ER+/PR+ breast cancer§	1.0 (referent)	1.0 (0.5 to 2.1)	1.4 (0.7 to 2.8)	1.1 (0.5 to 2.2)	.67

*Cut points are based on the distribution in control subjects using values from combined batches except where otherwise indicated.

†The medians of the quartiles were entered into the model as a continuous variable; two-sided.

‡Conditional logistic regression models controlling for body mass index at the age of 18 years (<21, 21 to <23, ≥23 kg/m^2), family history of breast cancer (yes, no), age at menarche (<12, 12, 13, ≥14 years), history of benign breast disease (yes, no), and parity/age at first birth (nulliparous, age at first birth <25 years/1–2 children, age at first birth 25–29 years/1–2 children, age at first birth ≥30 years/1–2 children, age at first birth <25 years/≥3 children, age at first birth ≥25 years/≥3 children).

§Unconditional logistic regression models controlling for covariates above and matching factors: days from luteal draw to the next menstrual cycle (0–5, 6–7, 8–9, 10–28 days), age at blood collection (continuous), date of blood collection (continuous), fasting at blood collection (yes, no), time of blood collection (1 AM–4 AM, 5 AM–6 AM, 7 AM–12 PM, continuous), and race/ethnicity (Caucasian, other). ER+/PR+ = estrogen and progesterone receptor positive.

‖Cut points for androstenedione are batch specific—follicular batch 1: ≤68, >68–93, >93–123, >123; follicular batch 2: ≤57, >57–77, >77–115, >115; luteal batch 1: ≤89, >89–117, >117–152, >152; luteal batch 2: ≤72, >72–99, >99–126, >126.

To further explore the different results we observed for the estrogens by menstrual cycle phase, we examined correlations between follicular and luteal levels. Low correlations were observed between follicular and luteal phases for both total (r = .13) and free (r = −.02) estradiol. Estrone sulfate levels were more highly correlated between the follicular and luteal phases (r = .54), and estrone levels were weakly correlated between phases (r = .22).

In terms of absolute case numbers, we observed 30 cases of breast cancer in the 25% of women (~4630 women) with the lowest plasma follicular total estradiol levels over 3 years of follow-up and 50 cases in the 25% of women with the highest plasma follicular total estradiol levels. For follicular free estradiol, 28 cases were observed among women in the lowest 25% and 57 cases were observed in the highest 25%. We observed 43 cases in the 25% of women with the lowest plasma luteal total testosterone levels and 58 cases in the 25% of women with the highest plasma luteal total testosterone levels.

Discussion

In this prospective nested case–control study of premenopausal women, higher plasma levels of total and free estradiol in the early follicular phase and total and free testosterone in both menstrual cycle phases were associated with an increased risk of breast cancer. These associations appeared to be stronger among women with invasive breast cancer or ER+/PR+ tumors and were independent of other known breast cancer risk factors.

To our knowledge, this is the first prospective study to observe a statistically significant association between premenopausal estradiol levels and the risk of breast cancer. No association between estradiol and breast cancer risk was observed in the European Prospective Investigation into Cancer and Nutrition (EPIC), a large prospective study with 285 cases *(8)*. The EPIC study was similar to our study in that control subjects were matched to case subjects on the menstrual cycle day of blood collection; however, blood samples in the EPIC study were collected on any day of the menstrual cycle, whereas samples in our study were collected during specific times within the follicular and luteal phases. The EPIC study also reported no association between estradiol levels in either the follicular or luteal phase and breast cancer risk. However, estradiol levels change dramatically during the follicular phase, and the EPIC study had limited power to restrict the analyses to specific segments of the menstrual cycle (e.g., the early follicular phase, when estrogen levels are low). Retrospective case–control studies have, in general, reported that case subjects had higher estradiol levels than control subjects in the follicular *(21)* or early follicular *(22)* phase but not in the luteal phase. However, few of the reported associations were statistically significant, and the postdiagnostic hormone assessment used in those studies is susceptible to bias.

Our finding of a positive association between follicular estradiol level and breast cancer risk but no association with luteal estradiol level might reflect the fact that luteal estradiol levels derive primarily from ovarian production *(23)*, whereas a greater proportion of early follicular estradiol levels derives from nonovarian sources (e.g., adipose tissue) *(24,25)* and thus may better reflect the estrogen levels in breast tissue. Evidence supporting this hypothesis includes the fact that ovarian expression of aromatase (an enzyme that catalyzes the conversion of androgens to estrogens) varies across the menstrual cycle and aromatase activity is low in the early follicular phase *(23)*. While aromatase expression is driven by follicle-stimulating hormone in the ovaries, in adipose tissue and normal breast tissue it is regulated by a different promoter that is activated by factors such as interleukin 6, interleukin 11, and tumor necrosis factor-α *(23,26,27)*. In fact, adipose tissue is the primary source of estrogen in postmenopausal women, in whom the positive association between circulating estradiol levels and breast cancer risk is well established *(3,4,28)*. The low correlations we observed between follicular and luteal total and free estradiol levels also support the hypothesis that follicular and luteal estradiol levels reflect different sources of circulating estrogens. By contrast, estrone sulfate levels, which were not associated with breast cancer risk, may more consistently reflect ovarian estrogen production across the menstrual cycle because of the higher correlation between follicular and luteal estrone sulfate levels. However, although the correlation between follicular and luteal estrone levels (r = .22) was more similar to that for estradiol (r = .13) and we expected the associations with breast cancer risk to be similar, we did not observe an association between estrone levels and breast cancer risk.

Another possible explanation for our findings is that hormone exposure in the follicular phase may be more relevant than in the luteal phase, given that women who are closer to menopause, such as those included in this study, have menstrual cycles with longer follicular and slightly shorter luteal phases *(29,30)*. Finally, estrogen activity may differ between the low- and high-progesterone environments of the follicular and luteal phases. ER expression in the breast tissue is higher in the follicular phase than in the luteal phase *(31–34)*, due, in part, to the decreased expression of ER caused by luteal progesterone *(35,36)*. In addition, breast tissue concentrates estradiol to a greater degree when circulating levels of estradiol are low, as they are in the follicular phase *(37,38)*. Thus, despite the lower circulating estrogen levels in the follicular phase, follicular estrogen may have a greater impact than luteal estrogen on breast tissue. In addition, estradiol increases expression of antiapoptotic proteins, whereas progesterone decreases antiapoptotic protein expression *(39–41)*, so that apoptosis in lobuloalveolar cells is higher in the luteal phase than in the follicular phase *(42)*. Thus, the proliferative effects of high estrogen levels in the luteal phase may be offset by the effects of apoptosis. Given this range of potential hypotheses and considering that this is the first study to observe an association between premenopausal estrogen levels and breast cancer risk, further examination of this relationship is warranted.

It has been hypothesized that progesterone may either decrease breast cancer risk, by mitigating the estrogen-induced proliferation of breast epithelial cells *(43,44)*, or increase risk because of the higher breast cell proliferation in the luteal phase *(45)* and the increased risk associated with estrogen-plus-progesterone hormone replacement therapy *(46–48)*. Although we observed no association between endogenous progesterone levels and breast cancer risk, others have observed suggestive *(11)* or statistically significant *(8,13)* inverse, although not linear, associations between luteal progesterone levels and breast cancer risk. The method of dating within the luteal phase (forward dating from the last menses versus backward dating from the next menses) could contribute to these discrepant results because the follicular phase is more variable in length than the luteal phase, which makes backward dating the more accurate approach *(49,50)*. In this context, it is interesting that the inverse association between luteal-phase progesterone levels and breast cancer risk reported in EPIC *(8)* was apparent only among case–control sets that were matched by forward dating. Similarly, the inverse association observed in the Hormones and Diet in the Etiology of Breast Tumors (ORDET) study *(13)* may have occurred, at least

in part, because luteal blood samples were timed by forward dating (i.e., they were collected on days 20–24 of the menstrual cycle) and the case subjects had shorter menstrual cycles than control subjects. Thus, the case subjects' blood samples may have been collected closer to the end of the luteal phase, when levels of luteal progesterone were decreasing, whereas the control subjects probably had levels closer to the midluteal peak, possibly resulting in a spurious inverse association with breast cancer risk. Alternatively, it is possible that the level of progesterone is an important determinant of breast cancer risk but that we were not able to detect an association because of the fluctuations in progesterone levels in the luteal phase and the relatively low within-woman ICC over 3 years (0.29) *(16)*. However, we would not expect the ICC to vary substantially between studies. Given the inconsistent results among studies, the association with progesterone level needs further evaluation, with particular attention to the timing of samples in the luteal phase. Our finding of a lack of association between SHBG levels and breast cancer risk is consistent with previous reports *(8,9,11,13)*.

The results to date from the small number of prospective studies that have evaluated associations between endogenous plasma androgen levels and breast cancer risk in premenopausal women have shown consistently an increased risk in women with higher androgen levels. For example, suggestive positive associations between testosterone *(11)* and androstenedione *(7)* levels and breast cancer risk were observed in two small studies. Although our overall results for total testosterone revealed a weak association with breast cancer risk, when we restricted our analyses to invasive cases the magnitude of the association we observed was similar to those reported in the EPIC and ORDET studies, which included only invasive cases [RR for highest versus lowest category of testosterone = 1.7 (95% CI = 1.2 to 2.6), *(8)*; RR = 2.2 (95% CI = 0.6 to 7.6), *(13)*]. Although we observed a slightly stronger and more linear association between luteal testosterone levels and breast cancer risk than between follicular testosterone levels and risk, the small numbers limited our ability to address this difference in detail. Our findings for androstenedione are similar to those reported in the EPIC study *(8)* but are in contrast with the null association reported in the smaller ORDET study, with 65 cases *(13)*. The possible mechanism underlying the association between androgens and breast cancer risk is not well understood. Androgens may act directly, promoting growth via binding to the androgen receptor or the ER *(51)*, or indirectly, via conversion to estrogens, either peripherally or in breast tissue *(28)*. Our results suggest that the androgen association may be at least partly independent of estrogen because adjustment for estradiol did not eliminate the association between testosterone levels and breast cancer risk.

The stronger associations we observed between estradiol and testosterone levels and breast cancer risk among women who reported that they had regular menstrual cycles between the ages of 18 and 22 years may have arisen if in these women, a single blood sample better reflected their long-term hormone exposure. In addition, some of the women excluded because their cycles were irregular may have had polycystic ovarian syndrome, a condition that is characterized by irregular and frequently anovulatory menstrual cycles. Although breast cancer risk among women with polycystic ovarian syndrome is unclear *(52)*, the hormonal profile of these women (i.e., high androgen levels and low estrogen and progesterone levels) is clearly different from that of women with normal cycles *(23)*, and therefore, their risk may differ.

To our knowledge, no other studies have reported associations between premenopausal hormone levels and breast cancer risk with respect to tumor hormone receptor status. The stronger associations we observed among case subjects who had ER+/PR+ tumors are similar to our findings in postmenopausal women *(4)*. The higher risk is biologically plausible because ER+ tumors can be stimulated by estrogen *(53)*. Among these tumors, PR expression indicates an intact ER signaling pathway *(54)*, and such tumors are more likely to respond to endocrine treatments that block estrogen signaling *(55)*.

Our study has several important strengths, of which the most important is the carefully timed, prospective collection of samples in both the follicular and luteal phases of the menstrual cycle. This feature and our large sample size allowed us to perform phase-specific analyses and to examine specific tumor types. Our study also has limitations. A single blood sample (i.e., follicular or luteal) may provide an imprecise measure of long-term average hormone levels. However, results of our reproducibility study suggest that, with the exception of progesterone, levels of most hormones measured at specific points in the menstrual phase are fairly stable over time *(16)*. Quantification and correction for this within-woman variation suggested that the associations between estrogen and androgen levels and breast cancer risk are probably stronger than what we were able to observe. Despite the relatively large number of case subjects in the phase-specific analyses, additional follow-up is necessary to further evaluate the shape of the dose–response curve because several of the associations appeared nonlinear. In addition, more follow-up is needed to further assess the associations by tumor characteristics, such as the ER/PR status, or menopausal status of the case subjects at diagnosis.

Overall, our data suggest that circulating levels of sex steroid hormones are important in the etiology of premenopausal breast cancer. Further research is necessary to confirm the associations observed with estradiol levels and by the hormone receptor status of the tumor. In addition, assessments of the determinants of premenopausal hormone levels are needed. Finally, the inclusion of premenopausal circulating hormone levels may improve breast cancer risk prediction models for premenopausal women.

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NOTES

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