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Estrogenic activity and risk of invasive breast cancer among postmenopausal women in the Nurses' Health Study

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Abstract

Background: Estrogens increase breast cancer risk through estrogen receptor (ER) mediated pathway activation. It is unclear whether a broader assessment of plasma compounds that lead to ER activation would be more strongly related to risk than measurement of individual estrogens.

Methods: A prospective nested case-control study was conducted among postmenopausal women in the Nurses' Health Study, that included 371 cases with blood samples collected prior to breast cancer diagnosis and 731 matched controls. Total estrogen pathway activity (EA) was assessed via a luciferase reporter assay using plasma-treated T47D-Kbluc (ATCC, Manassas, VA) human breast cancer cells. We also assessed the contribution of EA to risk, independent of

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Authors' contributions:

EXH analyzed the data, interpreted the data, and wrote the original manuscript. SCH contributed to the analysis and interpretation of the data, and critically reviewed the manuscript. SSS performed the bioassays and critically reviewed the manuscript. AHE critically reviewed the manuscript. JQ provided statistical expertise and critically reviewed the manuscript. EBJ critically reviewed the manuscript. ZL critically reviewed the manuscript. SST critically reviewed the manuscript. MTS and SEH conceived of the study, secured funding, acquired the data, directed the analysis, contributed to the interpretation of the data, and critically reviewed the manuscript. All authors have read and approved the final manuscript.

circulating estrone, estradiol, and estrone sulfate concentrations. Multivariable odds ratios (OR) and 95% confidence intervals (CI) were calculated using conditional logistic regression adjusting for breast cancer risk factors.

Results: Women in the highest, vs. lowest EA quartile had an 86% increased risk of invasive breast cancer (OR_{Q4vsQ1} 1.86, 95%CI=1.16-2.97). After accounting for estradiol only, a weaker association was observed (OR_{Q4vsQ1} 1.27, 95%CI=0.75-2.17). No association was observed after accounting for all three estrogens (OR_{Q4vsQ1} 1.01, 95%CI=0.56-1.84).

Conclusions: A positive association between EA and breast cancer risk was observed. However, the association was substantially attenuated after accounting for levels of other estrogens.

Impact: Our study provides a first detailed assessment of a breast cancer cell line-based EA assay and postmenopausal breast cancer risk.

Introduction

Breast cancer is a leading cause of cancer incidence and mortality among women.(1) Estrogen, an essential steroid hormone, promotes breast carcinogenesis and high endogenous levels are a well-confirmed risk factor for postmenopausal breast cancer.(2) Prior epidemiologic studies have consistently linked the most common circulating estrogens, here termed classic estrogens (i.e., estradiol (E2), estrone (E1) and E1 sulfate (E1S)), to risk, with approximately a 2-fold increase in breast cancer risk comparing the top to bottom quartile of estrogen levels.(3-6)

The biologic effects of the classic estrogens are mediated through binding to the estrogen receptors (ERα and ERβ) leading to expression of multiple ER target genes.(2) Endogenous estrogen metabolites as well as environmental exposures, such as phytoestrogens (e.g., soy products) and xenoestrogens (e.g., phthalates and some pesticides), can also bind to ER and alter its function.(7-10) Given varying binding affinities, these compounds may activate (or inhibit) estrogen pathway signaling leading to, in the case of activation for example, increased cellular proliferation.(11) The influence of both classic estrogens and these other compounds on estrogen pathway activity should provide a cumulative, biologically relevant predictor of breast cancer risk, as well as offer insights into the contribution of these other compounds to risk. Several estrogen bioassays have been developed to assess the cumulative effect of estrogenic components, both endogenous and exogenous estrogens, on total estrogen activity (EA).(12-14)

Prior epidemiologic studies of EA and breast cancer were conducted using either a yeast-based assay(12,13) or a cervical cancer cell assay(14) and observed positive associations. However, when specifically evaluating risk of breast cancer these assays may not be as physiologically relevant as using a breast cancer cell line, leading to an underestimation of the association. For example, ER signaling in yeast cells is different than in humans due to variations in cell membrane permeability and ER co-regulator recruitment.(15,16) A bioassay, using T47D human breast cancer cells transformed by transfecting estrogen-responsive elements, has been shown to quantify total estrogenic (and antiestrogenic) activity that activates ER and induces gene transcription.(17)

We conducted a nested case-control study to evaluate the relationship between plasma EA as assessed in breast cancer cells and risk of invasive postmenopausal breast cancer in the Nurses' Health Study (NHS), a large ongoing prospective cohort. We further evaluated whether the association was independent of the classic estrogens which would suggest a potentially important association with other estrogenic compounds in driving breast cancer development.

Materials and Methods

Nurses' Health Study

The NHS is a U.S.-based cohort that began in 1976 with 121,700 female, registered nurses, ages 30 to 55 years. Participants are mailed biennial questionnaires to provide information on health and lifestyle factors (e.g., demographics, reproductive history, diet, disease diagnosis). Cohort details have been previously described(18), and all study protocols were approved by the Institutional Review Boards at Brigham and Women's Hospital and the Harvard T.H. Chan School of Public Health in Boston, MA, the University of Massachusetts Amherst in Amherst, MA, and those of participating registries as required. Heparin plasma blood samples were provided by 32,826 women (aged 43 to 69 years) from 1989 to 1990. Blood collection kits were sent to each woman, and after their blood was drawn, the samples were shipped with an ice pack to the laboratory for processing and storage in nitrogen freezers at −130 °C or colder.(19)

In our study, all women were postmenopausal and not currently using hormone therapy, with no prior cancer history, except non-melanoma skin cancer, at time of blood draw. Women were defined as postmenopausal if they reported having no menses in the past 12 months, a hysterectomy and/or a bilateral oophorectomy, or were at least 54 years of age for current smokers and 56 years of age for nonsmokers; these ages represent when 90% of the cohort had reported natural menopause.

Breast cancer confirmation and control selection

Women reported breast cancer diagnoses on the biennial questionnaires; permission to obtain medical records was requested from those reporting a new diagnosis. Medically trained cohort investigators confirmed diagnoses through medical record review. Over 99% of reported breast cancer diagnoses were confirmed after medical record review. In the NHS, tumor microarrays were constructed from paraffin blocks of breast cancer tumor tissue retrieved from the participant's hospital, and immunostained for ER and progesterone receptor (PR) using a Dako Autostainer (Dako Corporation, Carpinteria, CA). A breast pathologist reviewed the stains and determined positivity. Where tissue was not available, ER/PR status, histologic grade, and invasiveness were extracted from pathology reports.(20)

Cases in this study were diagnosed with invasive breast cancer from 1994 to 2004. Since plasma samples are particularly limited for cases diagnosed early in follow-up, we chose a period that avoided use of the earliest diagnosed cases and extended 10 years to provide sufficient statistical power for our study. Cases were matched to controls by date and time of blood draw, fasting status, and year of birth using incidence density sampling.(21) Two

controls per case were included for all hormone assays except for data from 2002 and 2004, when only one control per case was assayed for the measurement of E1. The total number of women with measured classic estrogens in our study were as follows: 1,000 (E1), 1,049 (E1S), and 1,060 (E2).

Assessment of estrogenic activity and classic estrogens

Total EA was assessed using a receptor-mediated Chemical-Activated LUciferase gene eXpression (CALUX) bioassay performed at the laboratory of Dr. Martyn Smith (UC Berkeley, Berkeley, CA). Details of the plasma assay methods were previously described.(17) Briefly, EA in human plasma was measured using transfected human breast cancer cells, T47D-Kbluc (ATCC, Manassas, VA), with a luciferase reporter. Each sample was run in triplicate. Reporter activity was measured by the fluorescent light emitted per well and results were averaged across three wells per sample; higher relative light units (RLUs) indicated greater EA. RLUs were converted to picomoles using a 17ß-estradiol concentration response standard curve (Tocris Bioscience, Bristol, UK), which was then converted to picograms per milliliter (pg/mL) for comparability with the measurement of the classic estrogens. For our project, plates with an average inter-well coefficient of variation (CV) ≥24% (n=14) or with a technical failure (n=1) were rerun once. In total, we had 76 plates and the average within plate CV was 9.5% after these plates were rerun once.

For all plasma analyses, matched case-control sample pairs were included together within each batch and lab personnel were blinded to case-control status. Replicate quality control samples (10% of samples) from three postmenopausal plasma pools were included among case and control samples and were indistinguishable from these samples to calculate CVs.

Plasma E2, E1, and E1S assays were performed by the Nichols Institute at Quest Diagnostics (San Juan Capistrano, CA) and the Mayo Clinic (Rochester, MN). E2 and E1 were analyzed using radioimmunoassay (RIA) following organic extraction and celite column chromatography (1994-2000 follow-up cycles), and at the Mayo Clinic using liquid chromatography-tandem mass spectrometry (2002-2004 follow-up cycles). E1S (1994-2004 follow-up cycles) was extracted via an organic solvent and assayed by RIA after E1 extraction and enzymatically cleaving the E1S to release estrone by column chromatography.(22) The average (range) of within batch coefficients of variation reported by each laboratory are as follows: Quest Diagnostics: E2 8.9% (3.9% to 16.4%); E1 9.4% (8.7 to 12.0%); and E1S 10.3% (5.5% to 12.9%). The Mayo Clinic assays had an average (range) CV of 7.6% (4.0% to 9.5%) for E2 and E1 (2000-2004 follow-up cycles).

Classic estrogens were previously average batch recalibrated to account for batch and participant characteristics that may have varied by batch, (i.e., age and body mass index (BMI) at blood collection), as previously described.(4,23)

Covariate data

Information on breast cancer risk factors were measured via NHS biennial questionnaires and the questionnaire administered at the time of blood collection. Self-reported weight at age 18, height, and age at menarche were obtained from the baseline questionnaire administered in 1976. Family history of breast cancer was queried from 1976 to 1988.

History of benign breast disease confirmed by biopsy, age at natural menopause, parity/age at first birth, and alcohol intake were obtained from the biennial questionnaire administered in 1990. Date and time of blood draw, fasting status, age at blood draw, and current weight were recorded at the time of blood collection. Missing covariate information was substituted with the sample median value for continuous variables and the most frequent value for categorical variables.

Statistical Analysis

EA and hormone concentrations were natural log (ln) transformed to improve normality. One woman with an EA value below the limit of detection, we assigned a value of 0.5 pM, which is half the limit of detection for this bioassay. We performed the generalized extreme Studentized deviate test(24) to identify statistical outliers (n=7 for EA assay) which were removed from our analyses.

Our primary analysis examined the results of the EA assay without modification. To then assess the contribution of EA to risk, independent of the classic estrogens, we created two estrogen-adjusted residual variables (termed here E2-adjusted and 3-estrogen- [i.e., E1, E2, E1S] adjusted) by regressing EA on ln-transformed, continuous E2 only or E1, E2, and E1S simultaneously, taking the residual EA value, and then adding the EA mean to the residual value for each woman.(25) We also calculated an E2 subtraction variable by subtracting the plasma E2 value from the EA value, and adding the minimum estradiol value, rounded to the nearest whole number, as in prior research.(26) Quartiles for each EA variable were created based on the distribution among the controls. We calculated Pearson correlation coefficients with 95% confidence intervals (CI) between classic estrogens and EA concentrations and BMI at blood collection.

Participant characteristics between cases and controls were assessed using t-tests for continuous variables with reported means and standard deviations, and the Mantel-Haenszel tests for categorical variables with reported total number and frequency per category. For the primary analysis, conditional logistic regression was used to calculate odds ratios (OR) of incident breast cancer and 95% CI for each quartile of EA compared to the lowest quartile (referent). Tests for trend across quartiles were conducted by modeling the median of each EA quartile category as a continuous variable. Cubic splines were used to test for nonlinearity between EA concentrations and breast cancer risk. To assess differences by hormone receptor subtype, heterogeneity methods outlined by Wang et al.(27) for conditional logistic regression models were used, and the Wald test for heterogeneity was reported. We evaluated if associations varied across categories of family history of breast cancer (yes/no) and BMI (>25 vs. ≤25 kg/m^2) using unconditional logistic regression to maximize statistical power and determined statistical significance via likelihood ratio tests comparing models with and without multiplicative interaction terms. In multivariable models we adjusted for BMI at age 18 (continuous), weight change from age 18 to blood draw (continuous), family history of breast cancer (yes/no), history of benign breast disease (yes/no), age at menarche (continuous), parity/age at first birth (nulliparous, 1–2 children/<25 years, 1–2 children/≥25 years, ≥3 children/<25 years, ≥3 children/≥25 years), age at natural menopause (continuous), and alcohol intake (continuous) selected *a priori*;

matching factors were included in unconditional multivariable models. Controlling for BMI/weight change would account for potential confounding but could also result in overcontrol if these variables are on the same biologic pathway, thus we assessed models with and without these covariates, and results were essentially unchanged.

We observed substantial between batch variation in the bioassay (CV=27.3%). Therefore, for unconditional analyses, we created a categorical variable that grouped batches together based on the median bioassay value observed in each batch. These groupings resulted in a four-category batch variable to account for batch effects. Results from unconditional logistic regression models including this batch effect variable were similar to results of conditional logistic regression models.

Among all women who provided a blood sample in 1989-1990, 300 women were invited to participate in the Hormone Stability Sub-study by providing two additional blood samples over the following two- to three-years, as previously described.(28) The intraclass correlation coefficients (ICCs) from this substudy ranged from 0.68 (95% CI 0.59-0.80) to 0.75 (95% CI 0.67-0.84) for the classic estrogens. From this substudy, we selected up to 30 women with measured classical estrogens to assess EA reproducibility over time.

All statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC) for UNIX.

Results

A total of 374 cases and 735 controls with an EA value and at least one successful hormone (E2, E1, E1S) measurement were included in our study. After excluding outliers, the final sample size consisted of 371 cases and 731 controls.

At blood collection, women with breast cancer were more likely to have a family history of breast cancer, greater weight change from age 18 to blood draw, a slightly later age at menopause, and a higher concentration (pg/mL) of EA, E2, E1, and E1S compared to their matched controls (Table 1). Cases also were generally more likely to have a history of benign breast disease.

Among controls, levels of E1, E1S, and E2 were moderately correlated with EA (r=0.30 (0.23-0.37), r=0.34 (0.27-0.41), and r=0.41 (0.35-0.47), respectively) (Table 2). E2-adjusted, 3E-adjusted, and E2 subtraction EA were highly correlated with EA (0.91 (0.90-0.92), 0.89 (0.87-0.91), and 0.76 (0.73-0.79), respectively), though were minimally correlated, as expected, with the classic estrogens. Notably, EA was modestly correlated with BMI at blood collection (r= 0.23 (0.16-0.29), whereas there was no correlation observed once the variation due to E2, E1, and E1S was removed.

In analyses not adjusting for classic estrogens, women in the highest quartile had a 96% higher risk of invasive breast cancer (OR_{Q4vsQ1} 1.96, 95% CI 1.24-3.11, p_{trend}=0.003) compared to women in the lowest quartile. This association was modestly attenuated after accounting for covariates (OR_{Q4vsQ1} 1.86, 95% CI 1.16-2.97, p_{trend}=0.01). Additional attenuation was observed after accounting for the variation due to E2 in EA among women

with measured E2-adjusted EA (OR_{Q4vsQ1} 1.27, 95% CI 0.75-2.17, p_{trend}=0.51) and E2 subtracted EA (OR_{Q4vsQ1} 1.08, 95% CI 0.65-1.78, p_{trend}=0.42). The association was null after accounting for the cumulative contribution of the classic estrogens to EA (3E-adjusted EA OR_{Q4vsQ1} 1.01, 95% CI 0.56-1.84, p_{trend}=0.93) (Table 3). When modeled individually, and comparing the top vs. bottom quartile categories, the association of EA with breast cancer risk was 1.86 (1.16-2.97) and for E2 and breast cancer risk was 2.19 (1.45-3.31). When mutually adjusted for, these EA and E2 associations were both somewhat attenuated with OR_{Q4vsQ1}=1.42 (0.79-2.55) and OR_{Q4vsQ1}=1.89 (1.17-3.07), respectively.

In analyses considering hormone receptor status of the tumor, we observed a 2.5-fold increase in association between EA and invasive breast cancer risk among ER+/PR+ subtype (OR_{Q4vsQ1} 2.50, 95% CI 1.34-4.68), in analyses not adjusting for classic estrogens. Similar to our primary analyses, the association was attenuated for E2-adjusted, 3E-adjusted, and E2 subtraction EA (OR_{Q4vsQ1} 1.49, 95% CI 0.76-2.93; OR_{Q4vsQ1} 1.17, 95% CI 0.55-2.50; OR_{Q4vsQ1} 1.14, 95% CI 0.60-2.15, respectively) (Table 4). In the ER+/PR− and ER−/PR− subtypes, the associations were null with wide confidence intervals. Tests for heterogeneity between hormone receptor subtypes for each assessment of EA were not statistically significant (p-heterogeneity >0.34). When examining associations of EA and invasive breast cancer risk stratified by BMI at blood collection and family history of breast cancer, no statistically significant heterogeneity was observed (p-interaction >0.15).

When examining the reproducibility over 2-3 years of EA among a subgroup of our participants, the ICC was 0.87 (95% CI=0.76-0.94). For the estrogen-adjusted EA variables, ICCs were 0.80 (95% CI=0.62-0.90) for E2-adjusted EA and 0.57 (95% CI=0.31-0.79), among 26 women, for 3E-adjusted EA.

Discussion

In this large prospective nested case-control study, we observed a two-fold higher risk of invasive breast cancer among those in the top vs. bottom quartile of ER activated estrogen pathway activity as measured by total plasma EA concentrations. However, this association was substantially attenuated after accounting for plasma levels of E2 and no association was observed after accounting for the three classic estrogens together.

A number of estrogen bioassays have been previously developed.(12-14) The assays are intended to measure the cumulative impact on ER activity by both classic estrogens and a range of endogenous (e.g., estrogen metabolites) and exogenous compounds (e.g., phytoestrogens and certain pesticides) not typically measured in epidemiologic studies. Although ER binding affinities for these other compounds are generally lower than for E2, the compounds have similar molecular structure to E2, and thus can compete with classic estrogens for ER binding and activation (or inhibition).(29) Further, because E2 levels are relatively low among postmenopausal women, these other compounds could play a particularly important role by influencing the ER.(2,29)

ERα and ERβ are nuclear receptors that regulate the expression of ER target genes involved in various biological processes, e.g., cell proliferation, and are responsible for

binding as dimers to estrogen-response-elements for transcription.(2,30) Both ERs have high affinity for E2, and although similar homology is observed in their ligand-binding domains (53%), the ERs respond differently to various ligands.(31) For example, ERβ has a greater affinity for phytoestrogens, and ERα has both antagonist and agonist effects in the presence of selective ER modulator drugs (e.g., tamoxifen).(32,33) Notably, ERα and ERβ have opposing effects in the promoter region of various proliferation genes, and therefore, understanding the ratio and fluctuation of both ERs in target tissue may be an important component in assessing risk.(2,31)

To date, three prior epidemiologic studies have utilized EA reporter bioassays to evaluate the relationship between EA and breast cancer in postmenopausal women(12-14). All three studies separately evaluated ERα and ERβ activity whereas our study reported cumulative ERα and ERβ activity. Each observed significant positive associations of similar magnitude to our findings, in at least a subset of the data. Two studies utilized the same yeast reporter bioassay(12,13) and the third used a cervical cancer HELA cell assay(14). In a retrospective assessment(12) with 182 cases, women in the highest (vs lowest) category were at a significant 2.5-fold higher risk for both ERα and ERβ activity, assessed separately or together, in the same model. In the prospective nested case-control study using a yeast-based assay(13) (n=200 ER+ cases), no significant association was observed overall, although significant positive associations of a similar magnitude to associations seen in our study, for both ERα and ERβ activation, were observed in cases diagnosed >2 years since blood collection (95 cases). In the prospective nested case-control study using the HELA cell assay(14) (169 cases), positive associations for ERα were suggestively stronger than for ERβ (comparable comparison, OR_{Q4vsQ1}=2.80 [95% CI 1.58-4.97] for ERα and 1.74 [95% CI 0.89-3.41] for ERβ). Overall, these studies provide further support that activation of estrogen receptors is an important etiologic pathway in breast tumorigenesis.

Prior studies also evaluated if associations of EA and breast cancer were attenuated after accounting for E1 and/or E2, although differences in the estrogen assays used limit comparisons. In two studies(12,13), direct assays that often have lower sensitivity and specificity in detecting estradiol (or estrone) specifically were used, which would lead to misclassification in postmenopausal women.(34,35) Perhaps because of this, E2 concentrations were not associated with breast cancer risk in those studies, limiting the ability to evaluate how E2 adjustment influenced associations for the EA assay results. Further, the yeast-based assay used was not correlated with serum E2 ($r \leq 0.08$), possibly due to the use of a non-human cell type for the EA assay or measurement error in the EA or E2 assay. In contrast, we observed a correlation of 0.41 (95% CI=0.35-0.47) between plasma E2 and the EA bioassay. In Fourkala et al.(13), E1 was significantly positively associated with breast cancer. However, in the smaller subgroup with 95 cases where EA associations were observed, E1 and E2 were only weakly related to risk and they did not substantially alter the EA associations. In Lim et al.(14), where more sensitive and specific liquid chromatography-mass spectroscopy assays were used, E1 and E2 were non-significantly positively associated with risk. E1, E2, and ERα bioassay values were included in the same model, and only the ERα bioassay remained significantly associated with risk. Our use of sensitive and specific estrogen assays, additional consideration of E1S, and use of a regression method that may better remove variation due to the classic estrogens, may

account, at least in part, for the greater attenuation we observed in the EA/breast cancer association. Given the limited data to date, further assessment of this issue is warranted.

Our study has both strengths and limitations. It is the first to use a breast cancer cell line-based bioassay, which may be more biologically relevant than previously used bioassays. Our bioassay did not, however, distinguish ERα from ERβ activation and the T47D-KBluc cell line expresses somewhat higher levels of ERβ than ERα.(17) Our exposure was assessed using a single blood sample, and we noted laboratory variation in our cell-based assay, which would attenuate the observed associations. However, we saw very good reproducibility of the bioassay over a two-year period both overall and after removing the variation due to E2. Although after accounting for all three classic estrogens, the ICC appeared somewhat attenuated. We also assessed the contribution of classic estrogens to EA, using state-of-the-art and precise measurements of E2, E1, and E1S. Our sample size was sufficient to evaluate the primary association between EA and total breast cancer risk, however it was small for assessing potential interactions by BMI and family history of breast cancer, and when considering less common tumor hormone receptor subtypes. Our population was largely non-Latina white, and given the distribution of EA levels varies across racial/ethnic groups (26), additional studies are warranted.

Our findings suggest a broader assessment of plasma compounds contributing to estrogen receptor activation, as measured specifically using the T47D-KBluc cell line bioassay, does not provide additional information on breast cancer risk beyond measuring classic estrogens alone. Further large prospective assessments that can distinguish ERα and ERβ pathway activation using a breast cancer relevant assay, and that can also account for classic estrogen levels, would provide additional insight.

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Disclosure of Potential Conflicts of Interest:

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Table 1.

Characteristics of breast cancer cases and matched controls at blood collection (1990) in the Nurses' Health Study

Characteristics mean ± SD or % (n)	**Cases (*n* = 371)**	**Controls (*n* = 731)**	***P-value***
Age, y	61.3 ± 4.9	61.3 ± 4.9	*a*
Age at first birth, y	23.3 ± 8.4	24.2 ± 6.5	0.08
Age at menarche, y	12.5 ± 1.9	12.6 ± 1.7	0.26
Age at natural menopause, y	50.2 ± 3.4	49.7 ± 4.4	**0.03**
BMI, kg/m^2, at age 18, y	21.4 ± 3.0	21.6 ± 3.0	0.30
BMI at blood collection, kg/m^2	26.8 ± 4.9	26.0 ± 4.6	**0.01**
Weight change, kg, since age 18, y	36.2 ± 40.6	29.8 ± 33.8	**0.01**
Parity	3.1 ± 1.8	3.3 ± 1.9	0.20
History of benign breast disease, %(n)	35.9 (133)	30.2 (221)	0.06
Family history of breast cancer, %(n)	19.1 (71)	14.1 (103)	**0.03**
Alcohol intake (g/day)	4.8 ± 9.0	5.0 ± 9.3	0.70
Plasma Concentrations			
- Estrogenic activity (pg/mL)	23.2 ± 58.7	16.7 ± 39.0	**0.03**
- Estradiol (pg/mL)	9.3 ± 8.7	7.4 ± 5.3	**<.0001**
- Estrone sulfate (pg/mL)	337 ± 251	286 ± 249	**0.002**
- Estrone (pg/mL)	32.7 ± 17.6	29.4 ± 16.6	**0.003**

[a]Matching factor

Abbreviations: SD, standard deviation; BMI, body mass index

Bolding indicates statistical significance (p<0.05)

Table 2.

Pearson correlation coefficients (95% confidence intervals) between estrogenic activity and classic estrogens and body mass index at blood collection among control participants (N=731)

	EA	E2-adjusted[1] EA	3E-adjusted[2] EA	Estradiol subtraction[3] EA	Estradiol	Estrone	Estrone sulfate	BMI at blood collection
EA	1.00	0.91 (0.90-0.92)	0.89 (0.87-0.91)	0.76 (0.73-0.79)	0.41 (0.35-0.47)	0.30 (0.23-0.37)	0.34 (0.27-0.41)	0.23 (0.16-0.29)
E2-adjusted[a] EA	0.91 (0.90-0.92)	1.00	0.98 (0.979-0.983)	0.81 (0.78-0.83)	0.0002 (−0.07-0.07)	0.03 (−0.05-0.11)	0.13 (0.05-0.20)	0.04 (−0.03-0.12)
3E-adjusted[b] EA	0.89 (0.87-0.91)	0.98 (0.976-0.983)	1.00	0.80 (0.77-0.82)	−0.01 (−0.09-0.07)	−0.01 (−0.09-0.07)	−0.03 (−0.11-0.05)	0.02 (−0.06-0.10)
E2 subtraction[c] EA	0.76 (0.73-0.79)	0.81 (0.78-0.83)	0.80 (0.77-0.82)	1.00	0.06 (−0.01-0.13)	0.03 (−0.05-0.11)	0.13 (0.05-0.20)	0.06 (−0.01-0.13)

Abbreviations: EA, estrogen pathway activity; BMI, body mass index; E2, estradiol.

[a] E2-adjusted: EA regressed on E2, then EA mean added to the EA residual value for each woman.

[b] 3E-adjusted: EA regressed on E1, E2, and E1S, then EA mean added to the residual value for each woman.

[c] E2 subtraction: E2 plasma value subtracted from the EA value, added the minimum EA value.

Table 3.

Odds ratios and 95% confidence intervals of invasive breast cancer by quartile of total estrogenic activity (pg/mL) among postmenopausal women with no recent use of hormone therapy in the Nurses' Health Study

		Estrogenic activity concentrations (pg/mL) OR (95%CI)					
		Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**		
	case/control	**<5.19**	**5.19-9.00**	**9.01-14.65**	**>14.65**	***P* for trend**[a]	**OR (95% CI) per 1 SD increase**[b]
Simple conditional model	371/731	*1.0 referent*	1.27 (0.84-1.94)	1.19 (0.75-1.90)	1.96 (1.24-3.11)	**0.003** [d]	
Multivariable conditional model[c]	371/731	*1.0 referent*	1.29 (0.84-1.97)	1.17 (0.73-1.88)	1.86 (1.16-2.97)	**0.01** [d]	1.29 (1.08-1.54)
		Estradiol-adjusted estrogenic activity concentrations[e] **(pg/mL) OR (95%CI)**					
	case/control	**<6.00**	**6.01-9.77**	**9.78-14.72**	**>14.73**	***P* for trend**[a]	
Simple conditional model	359/701	*1.0 referent*	1.12 (0.72-1.74)	0.92 (0.57-1.49)	1.36 (0.81-2.28)	0.36	
Multivariable conditional model[c]	359/701	*1.0 referent*	1.07 (0.68-1.68)	0.86 (0.52-1.41)	1.27 (0.75-2.17)	0.51	1.13 (0.93-1.38)
		3 Estrogen-adjusted estrogenic activity concentrations[f] **(pg/mL) OR (95%CI)**					
	case/control	**<5.92**	**5.93-9.87**	**9.88-15.20**	**>15.21**	***P* for trend**[a]	
Simple conditional model	345/607	*1.0 referent*	1.20 (0.75-1.94)	1.07 (0.64-1.80)	1.05 (0.59-1.87)	0.97	
Multivariable conditional model[c]	345/607	*1.0 referent*	1.16 (0.71-1.89)	1.00 (0.59-1.71)	1.01 (0.56-1.84)	0.93	1.03 (0.83-1.28)
		Estradiol subtraction estrogenic activity concentrations[g] **(pg/mL) OR (95%CI)**					
	case/control	**<−0.72**	**−0.73-2.81**	**2.82-7.30**	**>7.31**	***P* for trend**[a]	
Simple conditional model	359/701	*1.0 referent*	0.79 (0.52-1.20)	0.64 (0.39-1.04)	1.12 (0.69-1.83)	0.30	
Multivariable conditional model[c]	359/701	*1.0 referent*	0.79 (0.52-1.22)	0.63 (0.38-1.03)	1.08 (0.65-1.78)	0.42	1.04 (0.88-1.22)

Abbreviations: OR, odds ratio; CI, confidence interval; PMH, postmenopausal hormone therapy use.

[a] Quartile medians, modeled continuously.

[b] OR (95% CI) for a 1 standard deviation increase in the natural log transformed estrogen pathway activity bioassay measure.

[c] Adjusted for BMI at age 18, weight change from age 18 to blood draw, family history of breast cancer, history of benign breast disease, age at menarche, parity/age at first birth (nulliparous, 1–2 children/<25 years, 1–2 children/≥25 years, ≥3 children/<25 years, ≥3 children/≥25 years), age at natural menopause, alcohol intake, and matching factors (age at blood draw, date and time of blood draw, and fasting status).

[d] Bolding indicates statistical significance ($p<0.05$).

[e] E2-adjusted: EA regressed on E2, then EA mean added to the EA residual value for each woman.

[f] 3E-adjusted: EA regressed on E1, E2, and E1S, then EA mean added to the residual value for each woman.

[g] E2 subtraction: E2 plasma value subtracted from the EA value, added the minimum EA value.

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Table 4.

Multivariable odds ratios and 95% confidence intervals of invasive breast cancer by tumor hormone receptor status, by quartile of total estrogenic activity (pg/mL) among postmenopausal women (no recent PMH) in the Nurses' Health Study

		Estrogenic activity concentrations OR (95%CI)				
		Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	
	case/control	**<5.19**	**5.19-9.00**	**9.01-14.65**	**>14.65**	***P* for heterogeneity**[a]
ER+,PR+[b]	237/463	*1.0 referent*	1.34 (0.78-2.31)	1.49 (0.80-2.80)	2.50 (1.34-4.68)	0.34
ER+,PR−[b]	43/83	*1.0 referent*	1.18 (0.30-4.64)	0.38 (0.07-2.12)	1.51 (0.42-5.36)	
ER−,PR−[b]	57/113	*1.0 referent*	0.82 (0.23-2.88)	0.31 (0.07-1.37)	0.61 (0.14-2.63)	
		Estradiol-adjusted estrogenic activity concentrations[c] **OR (95%CI)**				
	case/control	**<6.00**	**6.01-9.77**	**9.78-14.72**	**>14.73**	***P* for heterogeneity**[a]
ER+,PR+[b]	230/437	*1.0 referent*	1.01 (0.57-1.79)	0.74 (0.39-1.41)	1.49 (0.76-2.93)	0.92
ER+,PR−[b]	40/79	*1.0 referent*	0.52 (0.13-2.09)	0.33 (0.06-1.73)	1.15 (0.24-5.57)	
ER−,PR−[b]	55/106	*1.0 referent*	0.89 (0.20-4.05)	0.72 (0.15-3.39)	0.83 (0.16-4.33)	
		3 estrogen-adjusted estrogenic activity concentrations[d] **OR (95%CI)**				
	case/control	**<5.92**	**5.93-9.87**	**9.88-15.20**	**>15.21**	***P* for heterogeneity**[a]
ER+,PR+[b]	210/370	*1.0 referent*	1.08 (0.57-2.05)	0.83 (0.41-1.67)	1.17 (0.55-2.50)	0.55
ER+,PR−[b]	38/71	*1.0 referent*	1.65 (0.37-7.37)	1.62 (0.29-9.08)	1.43 (0.24-8.62)	
ER−,PR−[b]	52/88	*1.0 referent*	0.33 (0.08-1.40)	0.50 (0.13-12.31)	0.22 (0.03-1.50)	
		Estradiol subtraction estrogenic activity concentrations[e] **OR (95%CI)**				
	case/control	**<−0.72**	**−0.73-2.81**	**2.82-7.30**	**>7.31**	***P* for heterogeneity**[a]
ER+,PR+[b]	230/437	*1.0 referent*	0.72 (0.43-1.21)	0.54 (0.28-1.02)	1.14 (0.60-2.15)	0.56
ER+,PR−[b]	40/79	*1.0 referent*	0.47 (0.12-1.78)	0.35 (0.08-1.45)	1.61 (0.38-6.80)	

		Estrogenic activity concentrations OR (95%CI)				
		Quartile 1	**Quartile 2**	**Quartile 3**	**Quartile 4**	
	case/control	**<5.19**	**5.19-9.00**	**9.01-14.65**	**>14.65**	***P* for heterogeneity**[a]
ER−,PR−[b]	55/106	*1.0 referent*	3.17 (0.48-20.98)	1.39 (0.21-9.33)	1.55 (0.23-10.21)	

Abbreviations: OR, odds ratio; CI, confidence interval; PMH, postmenopausal hormone therapy use; ER, estrogen receptor; PR, progesterone receptor.

[a] *p* for heterogeneity compares differences between hormonal receptor subtype among quartiles of respective estrogen pathway activity variable.

[b] Adjusted for BMI at age 18, weight change from age 18 to blood draw, family history of breast cancer, history of benign breast disease, age at menarche, parity/age at first birth (nulliparous, 1–2 children/<25 years, 1–2 children/≥25 years, ≥3 children/<25 years, ≥3 children/≥25 years), age at natural menopause, alcohol intake, and matching factors (age at blood draw, date and time of blood draw, and fasting status).

[c] E2-adjusted: EA regressed on E2, added the EA mean to the residual value for each woman.

[d] 3E-adjusted: EA regressed on E1, E2, and E1S, added the EA mean to the residual value for each woman.

[e] E2 subtraction: E2 plasma value subtracted from the EA value, added the minimum EA value.