Circulating 2-Hydroxy- and 16α-Hydroxy Estrone Levels and Risk of Breast Cancer among Postmenopausal Women

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Abstract

Circulating estrogens are associated with breast cancer risk in postmenopausal women. Given that estrogen metabolites are potentially both mitogenic and genotoxic, it is possible that plasma levels of estrogen metabolites are related to breast cancer risk. We conducted a prospective, nested case-control study within the Nurses' Health Study. Blood samples, collected in 1989 to 1990, were assayed for 2-OH and 16α-OH estrone among 340 cases and 677 matched controls not taking postmenopausal hormones. Multivariate relative risks (RR) and 95% confidence intervals (95% CI) were calculated by conditional logistic regression, adjusting for breast cancer risk factors. Neither 2-OH nor 16α-OH estrone concentrations were significantly associated with breast cancer risk overall (top versus bottom quartile: RR, 1.19; 95% CI, 0.80-1.79; P_{trend} = 0.40 for 2-OH estrone and RR, 1.04; 95% CI, 0.71-1.53; P_{trend} = 0.81 for 16α-OH estrone). The ratio between the two metabolites (2-OH:16α-OH estrone) **was similarly unrelated to risk overall (1.30; 95% CI, 0.87-1.95; P_{trend} = 0.35). Although no associations were detected among women with estrogen receptor (ER)–positive/progesterone receptor (PR)–positive tumors, significant positive associations were observed for 2-OH estrone and the 2-OH:16α-OH estrone ratio among women with ER-negative/PR-negative tumors (RR, 3.65; 95% CI, 1.23-10.81; P_{trend} = 0.01; $P_{heterogeneity}$ = 0.02 for 2-OH estrone; RR, 3.70; 95% CI, 1.24-11.09; P_{trend} = 0.004; $P_{heterogeneity}$ = 0.005 for 2-OH:16α-OH estrone). These data do not support the hypothesized inverse associations with 2-OH estrone and the 2-OH:16α-OH estrone ratio or the hypothesized positive association with 16α-OH estrone. The significant positive associations with 2-OH estrone and the 2-OH:16-OH estrone ratio among women with ER-negative/PR-negative tumors needs to be replicated in future studies.** (Cancer Epidemiol Biomarkers Prev 2008;17(8):2029–35)

Introduction

Circulating estrogens, including estradiol, estrone, and estrone sulfate, are positively associated with breast cancer risk in postmenopausal women (1-4). The metabolism of these estrogens yields products that are potentially both estrogenic and genotoxic (5-10). It is possible that circulating levels of estrogen metabolites are related to breast cancer risk.

Oxidation of estrogens occurs at the C-2 and C-4 positions to yield 2-hydroxy (2-OH) and 4-hydroxy (4-OH) estrogens and at the C-16 position to yield 16α-OH estrone (5, 11). The estrogenic and genotoxic potential varies by metabolite. Although 2-OH estrogens bind to the estrogen receptor (ER) with affinity equivalent to or greater than estradiol (12, 13), they may act as only weak mitogens (14, 15) or as inhibitors of

proliferation (16, 17). Although 16α-OH estrone binds to the ER with lower affinity than estradiol, it binds covalently (18-20) and, once bound, fails to down-regulate the receptor (21). Thus, 16α-OH estrone stimulates cell proliferation in a manner comparable with estradiol in ER+ breast cancer cell lines (6, 22, 23) and may have stronger estrogenic properties than 2-OH estrone. Animal and *in vitro* studies have shown that hydroxy estrogens can induce DNA damage either directly, through the formation of quinones and DNA adducts, or indirectly, through redox cycling and the generation of reactive oxygen species (11). Although 2-OH estrogens are capable of redox cycling, the semi-quinones and quinones (the oxidized forms) form stable DNA adducts that are reversible without DNA destruction (24-26). 16α-OH estrone increases unscheduled DNA synthesis in mouse mammary cells (27) and hence also may be genotoxic. Given the different potential for estrogenic and genotoxic activity by these metabolites, it has been hypothesized that metabolism favoring the 2-OH over the 16α-OH pathway may be inversely associated with breast cancer risk (28).

To date, several epidemiologic studies have examined the association between the 2-OH and 16α-OH estrogen metabolites and breast cancer risk with inconclusive

results. Five prospective studies of either urinary (29-31) or serum (32, 33) estrogen metabolites among postmenopausal women have been published to date. No significant associations have been observed between 2-OH estrone, 16α-OH estrone, and the 2-OH:16α-OH estrone ratio and breast cancer risk and the direction of the estimates is not consistent across studies.

We investigated the associations of the 2-OH and 16α-OH estrone metabolites and the 2-OH:16α-OH estrone ratio with breast cancer risk in a nested case-control study among postmenopausal women within the Nurses' Health Study (NHS). A total of 340 cases and 677 controls were included, which is a subset of our previous breast cancer case-control study of estradiol and estrone sulfate (4).

Materials and Methods

Study Population. In 1976, 121,700 female, married, registered nurses, ages 30 to 55 years, were enrolled in the NHS. Biennially, participants have completed mailed questionnaires that collect information on various exposures, including many breast cancer risk factors, and new disease diagnoses.

During 1989 to 1990, blood samples were collected from 32,826 cohort members ages 43 to 69 years. Details regarding the blood collection methods have been published previously (34, 35). Briefly, each woman arranged to have her blood drawn and shipped, via overnight courier and with an icepack, to our laboratory, where it was processed; 97% of samples arrived at our laboratory within 26 h of being drawn. The stability of estrogens in whole blood for 24 to 48 h has been documented previously (36). Samples have been stored in continuously monitored liquid nitrogen freezers since collection. As of 2000, the follow-up rate among the blood cohort was 99%. The study was approved by the Committee on the Use of Human Subjects in Research at the Brigham and Women's Hospital.

Cases and controls are women who, at blood collection, were postmenopausal [defined as having a natural menopause (no periods in previous 12 months) or bilateral oophorectomy or a hysterectomy with at least one ovary remaining if they were at least 56 years old (if a nonsmoker) or 54 years old (if a current smoker), ages at which natural menopause had occurred in 90% of these groups in the overall cohort] and had not used postmenopausal hormones (PMH) for at least 3 months. Cases had no reported cancer diagnosis (other than nonmelanoma skin cancer) before blood collection and were diagnosed with breast cancer between June 1, 1992 and May 30, 2000. Overall, 340 cases of breast cancer were reported ($n = 277$ invasive) and confirmed by medical record review ($n = 334$) or by verbal confirmation of the diagnosis by the nurse ($n = 6$). Median time from blood collection to diagnosis was 80 months. Two controls (total $n = 677$) were matched per case by age (± 2 years), and month (± 1 month), time of day (± 2 h), and fasting status at blood collection (≥ 10 h since a meal, <10 h since a meal, or unknown). Ten controls from earlier follow-up cycles became cases in later follow-up cycles; these women serve as both cases and controls in this analysis, as is appropriate in incidence density sampling.

Laboratory Analyses. Estrogen metabolites were measured by a monoclonal antibody–based enzyme assay (ESTRAMET 2/16, Immuna Care). The assays for 2-OH estrone and 16α-OH estrone in serum/plasma were developed from reagents and methods for measuring the metabolites in urine (37-39). The assays for urinary estrogen metabolites have been validated against gas chromatography-mass spectroscopy methods (37, 40). Serum assays were then validated against urine assays by adding known amounts of urinary metabolites to serum samples and then performing the serum assay. Assays for estrone were conducted in three batches [for cases diagnosed through June 1998 ($n = 249$) and their matched controls] and estrone sulfate and estradiol were conducted in four batches (all cases and controls) at Quest Diagnostic's Nichols Institute. Assay methods have been described previously in detail (2, 34). In brief, samples were assayed by RIA following extraction and celite chromatography (41-45). After extraction of estrone, estrone sulfate was assayed by RIA of estrone after enzyme hydrolysis, extraction, and column chromatography (46).

Each case and her two matched controls were assayed together in the same batch; samples were ordered randomly and labeled so that laboratories were masked to case-control status. The interassay coefficient of variation from masked replicate plasma samples in each batch was 6% (16α-OH estrone) and 15% (2-OH estrone); coefficients of variation for the other estrogens were within this range. When plasma hormone values were reported as less than the detection limit (2-OH estrone, 20 pg/mL; 16α-OH estrone, 10 pg/mL; estradiol, 2 pg/mL; estrone, 10 pg/mL; estrone sulfate, 40 pg/mL), we set the value to half this limit [16α-OHE1 ($n = 9$), estradiol ($n = 2$), estrone ($n = 21$), and estrone sulfate ($n = 6$)].

Reproducibility Study. A subset of 186 postmenopausal NHS participants who gave blood samples during the 1989 to 1990 collection also provided two additional samples during the following 2 years. These women had not used PMH for at least 3 months and had no previous diagnosis of cancer (except nonmelanoma skin cancer) at the time of each blood collection. Blood samples from 70 of these women, chosen randomly, were assayed for estrogen metabolites at the same laboratory to assess hormone reproducibility over time as has been published previously for other hormones (47).

Covariate Data. We obtained breast cancer risk factor information from the biennial NHS questionnaires. Age at menarche and height were queried in 1976. Age at first birth and parity were assessed in 1976 and updated until 1984. Family history of breast cancer was queried in 1976, 1982, 1988, 1992, 1996, and 2000. Weight at age 18 years was queried in 1980; current weight was queried at blood collection. Menopausal status, type of and age at menopause, PMH use, and history of benign breast disease were assessed biennially. Alcohol consumption was assessed with a semiquantitative food frequency questionnaire in 1990.

Statistical Analysis. Using the log-transformed hormone values, we estimated between-person and within-person variances from the three sets of metabolite measurements by random-effects models. Reproducibility

Table 1. Spearman correlation coefficients between estrogens and estrone metabolites among controls

	Estradiol	Estrone	Estrone sulfate	2-OH estrone	16α-OH estrone	2-OH:16α-OH estrone ratio	BMI
2-OH estrone							
r	0.06	0.11	0.12	1.00	0.26	0.71	0.01
P	0.12	0.02	0.002		<0.001	<0.001	0.81
16α-OH estrone							
r	0.14	0.20	0.08	0.26	1.00	-0.43	0.05
P	<0.001	<0.001	0.05	<0.001		<0.001	0.19
2-OH:16α-OH estrone ratio							
r	-0.03	-0.03	0.05	0.71	-0.43	1.00	0.06
P	0.48	0.57	0.17	<0.001	<0.001		0.15

NOTE: n ranges from 460 (estrone) to 675 (2-OH, 16α-OH, and 2-OH:16α-OH ratio).

of estrone metabolites over time was assessed by calculating intraclass correlation coefficients by dividing the between-person variance by the sum of the within- and between-person variances.

Plasma hormone levels were categorized into quartiles, with cut points based on the control distribution. For estrone, estrone sulfate, and estradiol, the control distribution varied across batches such that quartiles based on all controls combined resulted in uneven batch-specific distributions. Because the mean value of quality-control replicates in each batch varied similarly, much (if not all) of this difference was due to laboratory drift over time rather than true differences between batches. Thus, we combined batches that had similar cut points but otherwise used batch-specific cut points (4).

We removed one matched set from the analysis because the case's estrogen values were in the premenopausal range. We used the Studentized deviate many-outlier procedure (48) to identify and exclude statistical outliers (two 2-OH estrone values ≥626 pg/mL and three estradiol values ≥76 pg/mL).

We used a mixed-effects regression model to test the paired differences in log-transformed hormone levels between cases and their matched controls. To estimate relative risks (RR) and 95% confidence intervals (95% CI), we used conditional logistic regression, controlling for breast cancer risk factors (see Table 3 footnote). Estimates from age-adjusted regression models were similar to those from multivariate models; therefore, only multivariate results are presented. We calculated tests for trend by modeling the medians of the quartiles as a continuous variable and calculating a Wald statistic. Interactions, on the multiplicative scale, between hormone levels and breast cancer risk factors were evaluated by adding an interaction term (log hormone quartile medians × presence or absence of risk factor) to the logistic models and calculating a Wald statistic. In stratified analyses, we used unconditional logistic regression, adjusting for matching factors, because multivariate unconditional and conditional logistic regression models were essentially identical. To test whether associations differed by ER and PR status of the tumor, we used polychotomous logistic regression (49) with three endpoints (ER+/PR+, ER-/PR-, and no breast cancer). We used a likelihood ratio test to compare a model with separate metabolite slopes in each case group with a model with a common slope. All analyses were conducted using SAS software version 9 (SAS Institute).

Results

Reproducibility of the estrone metabolites over 3 years was comparable with other steroid hormones in this population (47), with intraclass correlation coefficients of 0.63 for 2-OH estrone, 0.80 for 16α-OH estrone, and 0.73 for the 2-OH:16α-OH estrone ratio. Among controls, neither metabolite was strongly correlated with circulating estrogen levels [e.g., the strongest correlation was $r = 0.20$ ($P < 0.001$) between estrone and 16α-OH estrone; Table 1]. In addition, the two metabolites were only modestly correlated with one another ($r = 0.26$; $P < 0.001$).

Cases were slightly heavier than controls [body mass index (BMI) = 27.1 versus 26.1 kg/m^2], were more likely to be nulliparous (9.4% versus 5.3%), and had a higher prevalence of both benign breast disease (46.2% versus 37.4%) and family history of breast cancer (19.7% versus 14.0%; Table 2). There were no significant differences in estrogen metabolite concentrations or the 2-OH:16α-OH estrone ratio between cases and controls, but cases had significantly higher levels of estradiol, estrone, and estrone sulfate compared with controls ($P < 0.001$ for each) as published previously (Table 3; refs. 2, 4).

2-OH estrone was not significantly associated with breast cancer risk overall (top versus bottom quartile: RR, 1.19; 95% CI, 0.80-1.79; $P_{\text{trend}} = 0.40$; Table 4). No association was observed when cases were restricted to ER+/PR+ ($n = 164$; top versus bottom quartile: RR, 1.00; 95% CI, 0.60-1.67; $P_{\text{trend}} = 0.95$), but a significant positive association was observed among ER-/PR- cases [$n = 41$; comparable RR, 3.65; 95% CI, 1.23-10.81; $P_{\text{trend}} = 0.01$;

Table 2. Characteristics [mean (SD) or %] of breast cancer cases and matched controls, NHS

Characteristics	Cases ($n = 340$)	Controls ($n = 677$)
Age, y	61.5 (4.7)	61.5 (4.7)
BMI, kg/m^2	27.1 (5.3)	26.1 (4.6)
Age at menarche, y	12.6 (1.4)	12.7 (1.5)
Parity, children	3.2 (1.8)	3.3 (1.8)
Age at first birth*, y	25.8 (3.5)	25.7 (3.5)
Age at menopause[†], y	49.9 (4.0)	49.6 (4.6)
Nulliparous, %	9.4	5.3
History of benign breast disease, %	46.2	37.4
Family history of breast cancer, %	19.7	14.0

*Among parous women only.
[†]Among women with natural menopause or bilateral oophorectomy only.

Table 3. Median (range) of hormones among breast cancer cases and matched controls

	Cases ($n = 340$)	Controls ($n = 677$)	P
2-OH estrone (pg/mL)	129 (86-209)	125 (82-195)	0.20
16α-OH estrone (pg/mL)	344 (262-473)	349 (254-466)	0.80
2-OH:16α-OH estrone ratio	0.37 (0.24-0.60)	0.37 (0.23-0.59)	0.54
Estradiol (pg/mL)	8 (4-16)	6 (4-12)	<0.001
Estrone (pg/mL)	26 (15-42)	22 (13-37)	<0.001
Estrone sulfate (pg/mL)	272 (121-669)	207 (93-516)	<0.001

NOTE: Range is median of the bottom quartile (12.5 percentile) to the median of the top quartile (87.5 percentile).

$P_{heterogeneity}$ = 0.02]. No significant associations were observed for ER+/PR- cases ($n = 33$; data not shown); there were too few cases of ER-/PR+ ($n = 6$) to evaluate separately. When invasive ($n = 277$) and in situ ($n = 57$) cases were evaluated separately, results were similar to the overall results with both case types combined (data not shown).

No significant associations were observed between 16α-OH estrone and breast cancer risk either overall (top versus bottom quartile: RR, 1.04; 95% CI, 0.71-1.53; P_{trend} = 0.81) or by hormone receptor status [comparable RR, 1.05; 95% CI, 0.63-1.73; P_{trend} = 0.85 for ER+/PR+ tumors ($n = 164$) and RR, 1.39; 95% CI, 0.47-4.06; P_{trend} = 0.78 for ER-/PR- tumors ($n = 41$), $P_{heterogeneity}$ = 0.81; Table 4]. Results were similar among invasive and in situ cases (data not shown).

Similar to the association with 2-OH estrone, levels of 2-OH:16α-OH estrone were not associated with breast cancer risk overall (top versus bottom quartile: RR, 1.30; 95% CI, 0.87-1.95; P_{trend} = 0.35) or with ER+/PR+ tumors ($n = 164$; comparable RR, 0.88; 95% CI, 0.52-1.48; P_{trend} = 0.51). However, we observed a statistically significant positive association with ER-/PR- tumors ($n = 41$; comparable RR, 3.70; 95% CI, 1.24-11.09; P_{trend} = 0.004) and the difference in the associations by tumor receptor status was statistically significant ($P_{heterogeneity}$ = 0.005; Table 4). Results did not differ from overall when stratified by invasive and in situ tumors (data not shown).

When we adjusted for estrone, estrone sulfate, or estradiol, results were not substantially different for either metabolite or their ratio (data not shown). In addition, results were unchanged when stratified by estrogen levels (top two versus bottom two quartiles). Stratification by BMI (<25 versus ≥25 kg/m²) resulted in similar observations for 2-OH estrone. Among leaner

Table 4. Multivariate RR (95% CI) of breast cancer according to quartiles of estrogen metabolites

	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}
2-OH estrone, pg/mL	<98	98-<125	125-<164	≥164	
Cases/controls	73/165	84/171	95/168	88/171	
RR (95% CI)	1.00 (reference)	1.13 (0.75-1.69)	1.30 (0.88-1.93)	1.19 (0.80-1.79)	0.40
ER+/PR+*					
Cases/controls	40/165	43/171	40/168	41/171	
RR (95% CI)	1.00 (reference)	1.07 (0.65-1.77)	1.04 (0.62-1.74)	1.00 (0.60-1.67)	0.95
ER-/PR-*					
Cases/controls	5/165	7/171	13/168	16/171	
RR (95% CI)	1.00 (reference)	1.62 (0.47-5.54)	3.03 (1.00-9.15)	3.65 (1.23-10.81)	0.01
16α-OH estrone, pg/mL	<292	292-<349	349-<413	≥413	
Cases/controls	78/168	102/166	76/173	84/170	
RR (95% CI)	1.00 (reference)	1.38 (0.95-2.02)	1.01 (0.68-1.50)	1.04 (0.71-1.53)	0.81
ER+/PR+*					
Cases/controls	40/168	45/166	37/173	42/170	
RR (95% CI)	1.00 (reference)	1.27 (0.77-2.09)	0.89 (0.53-1.50)	1.05 (0.63-1.73)	0.85
ER-/PR-*					
Cases/controls	7/168	18/166	7/173	9/170	
RR (95% CI)	1.00 (reference)	3.05 (1.16-8.03)	0.88 (0.28-2.73)	1.39 (0.47-4.06)	0.78
2-OH:16α-OH estrone ratio	<0.28	0.28-<0.37	0.37-<0.48	≥0.48	
Cases/controls	74/169	89/168	87/170	90/168	
RR (95% CI)	1.00 (reference)	1.33 (0.89-1.99)	1.24 (0.83-1.85)	1.30 (0.87-1.95)	0.35
ER+/PR+*					
Cases/controls	41/169	44/168	43/170	36/168	
RR (95% CI)	1.00 (reference)	1.16 (0.70-1.90)	1.12 (0.68-1.85)	0.88 (0.52-1.48)	0.51
ER-/PR-*					
Cases/controls	5/169	5/168	15/170	16/168	
RR (95% CI)	1.00 (reference)	0.95 (0.25-3.57)	2.89 (0.97-8.66)	3.70 (1.24-11.09)	0.004

NOTE: Multivariate models controlled for BMI at age 18 y (<21, 21-22.9, 23-24.9, ≥25 kg/m²), family history of breast cancer (yes, no), age at menarche (<12, 12, 13, ≥14 y), age at first birth and parity (nulliparous; 1-4 children, first birth <25 y; 1-4 children, first birth 25-29 y; 1-4 children, first birth ≥30 y; ≥5 children, first birth <25 y; ≥5 children, first birth ≥25 y), age at menopause (<46, 46-50, 51-55, ≥56 y), and duration of past PMH use (continuous); unconditional multivariate models (for ER/PR analyses) additionally controlled for matching factors [age (<55, 55-59, 60-64, ≥65 y), date of blood draw [6-mo groups], time of blood draw [1:00-8:00 a.m., 9:00 a.m.-12:00 p.m., 1:00 p.m.-12:00 a.m.], and fasting status (<10 versus ≥10 h)].
*$P_{heterogeneity}$ values between ER+/PR+ and ER-/PR- were 0.02 for 2-OH estrone, 0.81 for 16α-OH estrone, and 0.005 for 2:16α OH estrone ratio.

women (BMI < 25 kg/m^2), levels of 16α-OH estrone were suggestively inversely associated with breast cancer risk (top versus bottom quartile: RR, 0.56; 95% CI, 0.28-1.11; P_{trend} = 0.06), whereas levels of the 2-OH:16α-OH estrone ratio were significantly positively associated with risk [quartiles 2-4: RR (95% CI), 2.11 (1.02-4.35), 2.43 (1.18-5.02), and 2.68 (1.27-5.66); P_{trend} = 0.02]. However, the interactions between BMI and either 16α-OH estrone levels or the 2-OH:16α-OH estrone ratio were not statistically significant (P = 0.12 and 0.08, respectively). Associations with both metabolites and the ratio were unchanged when stratified by time since blood collection (<6 versus 6-10 years) or age at blood collection (<62 versus ≥62 years). When analyses were restricted to women with no family history of breast cancer or women who had never used PMH, results were similar to those of the overall analysis (data not shown).

Discussion

In this large prospective study of 2-OH and 16α-OH estrone metabolites and breast cancer risk, we did not observe any significant associations overall with either individual metabolite or with the ratio of the two metabolites. Although we observed significant positive associations of both 2-OH estrone and the 2-OH:16α-OH estrone ratio with ER-/PR- tumors, these results should be interpreted with caution given the small number of ER-/PR- tumors and that we are the first, to our knowledge, to report such an association. In addition, although we observed a significant positive association between the 2-OH:16α-OH estrone ratio and breast cancer risk among lean women, the differences observed by BMI were not statistically significant.

The reproducibility of these estrogen metabolites is comparable with or better than other biomarkers with well-established relationships to disease outcomes in epidemiologic studies, such as cholesterol (intraclass correlation coefficient = 0.65; ref. 50) and blood pressure (intraclass correlation coefficient = 0.60-0.64; ref. 51) as well as estradiol (0.68), estrone (0.74), and estrone sulfate (0.75; ref. 47), which have been consistently associated with breast cancer risk in this and other populations (1-4). Thus, the lack of observed associations likely is not a result of poor reproducibility of a single measure of these metabolites.

To date, several epidemiologic studies have examined the association between 2-OH and 16α-OH estrone and breast cancer risk. Several retrospective case-control studies have produced conflicting results, although the analysis of hormone levels after diagnosis, which may reflect tumor-driven activity, is a limitation of these studies (52-59). Five prospective studies of either urinary (29-31) or serum (32, 33) estrogen metabolites among postmenopausal women have been published to date, with case numbers ranging from 42 (29) to 272 (32) among women who were not using PMH. No significant associations have been observed between 2-OH estrone and breast cancer risk, with RR ranging from 0.80 (33) to 1.61 (30) for the top versus bottom quartile or quintile or a doubling of 2-OH estrone concentration (comparable with our top versus bottom quartile comparison). Three (30, 32, 33) of four (30-33) studies observed RR above 1 for the association between 16α-OH estrone and breast

cancer risk (range of RR, 1.23-2.47); none of the point estimates was statistically significant, although one trend was suggestive (top versus bottom quartile: RR, 2.47; 95% CI, 0.90-6.80; P = 0.06; ref. 33). No significant associations have been observed with the 2-OH:16α-OH estrone ratio, with two studies reporting point estimates below 1 and two reporting estimates above 1 (range of RR, 0.71-1.31; refs. 29-32). Thus, similar to our overall findings, previous prospective studies have not observed any significant associations with either 2-OH or 16α-OH estrone or the ratio of the two metabolites and breast cancer risk overall.

Including our study, there have been a few reports of significant associations among subgroups of women. However, the specific subgroup is not consistent across studies, nor do the subgroups follow a predicted pattern. For example, we observed a suggestive inverse association with 16α-OH estrone and a significant positive association with the 2-OH:16α-OH estrone ratio among lean women, suggesting possible associations in a low-estrogen environment. However, significant associations with both metabolites have been observed in two other studies in environments suggestive of higher estrogen levels (high BMI and among women on PMH). Specifically, Modugno et al. (33) observed a combined effect of high BMI and high 16α-OH estrone (RR, 3.51; 95% CI, 1.34-9.16) for women in the top tertile of BMI and top half of 16α-OH estrone compared with lean women with lower 16α-OH estrone and Wellejus et al. (31) observed significant positive associations among PMH users with 2-OH estrone (RR for doubling, 1.28; 95% CI, 1.04-1.56) and 2-OH:16α-OH estrone ratio (RR for doubling, 1.25; 95% CI, 1.02-1.53).

To our knowledge, the study by Wellejus et al. (31) is the only other prospective study to examine these associations by hormone receptor status, although their results were not consistent with ours. In our population of PMH nonusers, we observed no associations with ER+/PR+ tumors but significant positive associations with 2-OH estrone and the 2-OH:16α-OH estrone ratio among women with ER-/PR- tumors. In the Danish study, no associations were observed with either ER+ or ER- tumors among PMH nonusers but significant positive associations with 2-OH estrone and the 2-OH:16α-OH estrone ratio were observed among PMH users with ER+ tumors but not ER- tumors. In a retrospective case-control study, Kabat et al. (59) observed a stronger inverse association of the 2-OH:16α-OH estrone ratio with ER- tumors than with ER+ tumors among postmenopausal women. Because circulating estrogen levels have been associated more strongly with ER+/PR+ tumors than with ER-/PR- tumors (2), it seems contrary that estrogen metabolites may be associated with ER-/PR- tumors. In addition, based on animal studies, 2-OH estrone and the 2-OH:16α-OH estrone ratio have been hypothesized to be inversely associated with breast cancer risk (28) rather than positively associated as we observed. Given that there are two different, although not necessarily mutually exclusive, hypotheses of the mechanism by which estrogen metabolites may affect breast cancer risk, it is possible that the genotoxicity of 2-OH estrone plays a role in hormone receptor–negative tumors (60).

This study has several strengths, including that it is the largest study to date among postmenopausal women not

using PMH. Blood samples and risk factor information were collected before diagnosis, minimizing the possibility of reverse causality or recall bias. Only one blood sample per woman is a potential limitation, although our reproducibility data suggest that one sample is an adequate representation of these metabolites over at least a few years. Another limitation is the selectivity of estrogen metabolites, with no data on other potentially important metabolites including 4-OH estrone. 4-OH estrogens have a greater estrogenic potential than 2-OH estrogens, given the lower dissociation rate from ERs compared with estradiol (61), and are potentially more genotoxic because the quinones form unstable adducts, leading to depurination and mutation *in vitro* and *in vivo* (10, 25, 62-64). Furthermore, the balance between the catechol (2-OH and 4-OH) and methoxy (2-Me and 4-Me) estrogens may affect risk. Thus, the investigation of just 2-OH and 16α-OH estrone may be inadequate to rule out the importance of estrogen metabolites on breast cancer risk.

In conclusion, our results do not support the hypothesis that metabolism favoring the 2-OH estrone pathway is more beneficial to breast cancer risk than that favoring the 16α-OH estrone pathway. Although we observed positive associations with 2-OH estrone and the 2-OH:16α-OH estrone ratio among women with lower BMI and women with ER-/PR- tumors, these results were unexpected and require replication. Future studies should include a broader panel of metabolites to investigate the estrogen metabolism pathway and its possible role in breast cancer risk more thoroughly.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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