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Research Article

Circulating Estrogen Metabolites and Risk of Breast Cancer in Postmenopausal Women

Alan A. Arslan[1,2,3,4], Karen L. Koenig[2,3], Per Lenner[5], Yelena Afanasyeva[2], Roy E. Shore[8], Yu Chen[2,3,4], Eva Lundin[6], Paolo Toniolo[1,4], Göran Hallmans[7], and Anne Zeleniuch-Jacquotte[2,3,4]

Abstract

Background: It has been hypothesized that predominance of the 2-hydroxylation estrogen metabolism pathway over the 16α-hydroxylation pathway may be inversely associated with breast cancer risk.

Methods: We examined the associations of invasive breast cancer risk with circulating 2-hydroxyestrone (2-OHE1), 16α-hydroxyestrone (16α-OHE1), and the 2-OHE1:16α-OHE1 ratio in a case–control study of postmenopausal women nested within two prospective cohorts: the New York University Women's Health Study (NYUWHS) and the Northern Sweden Mammary Screening Cohort (NSMSC), with adjustment for circulating levels of estrone, and additional analyses by tumor estrogen receptor (ER) status. Levels of 2-OHE1 and 16α-OHE1 were measured using ESTRAMET 2/16 assay in stored serum or plasma samples from 499 incident breast cancer cases and 499 controls, who were matched on cohort, age, and date of blood donation.

Results: Overall, no significant associations were observed between breast cancer risk and circulating levels of 2-OHE1, 16α-OHE1, or their ratio in either cohort and in combined analyses. For 2-OHE1, there was evidence of heterogeneity by ER status in models adjusting for estrone ($P \leq 0.03$). We observed a protective association of 2-OHE1 with ER+ breast cancer [multivariate-adjusted OR for a doubling of 2-OHE1, 0.67 (95% confidence interval [CI], 0.48–0.94; $P = 0.02$)].

Conclusions: In this study, higher levels of 2-OHE1 were associated with reduced risk of ER+ breast cancer in postmenopausal women after adjustment for circulating estrone.

Impact: These results suggest that taking into account the levels of parent estrogens and ER status is important in studies of estrogen metabolites and breast cancer. *Cancer Epidemiol Biomarkers Prev; 23(7); 1290–7.* ©*2014 AACR.*

Introduction

It is well recognized that higher levels of the endogenous estrogens estrone (E1) and estradiol (E2) are associated with breast cancer risk in postmenopausal women (1–5). Estrogens stimulate breast cell proliferation, increasing the likelihood of somatic DNA mutations and carcinogenesis (6–8). More recently, there has been increasing interest in the role of various estrogen metabolites that have been hypothesized to affect the risk of breast cancer. Among the most abundant estrogen metabolites, 2-hydroxyestrone (2-OHE1) does not seem to increase cell proliferation (9–11) and is rapidly eliminated from the circulation (12–14). The other major metabolite, 16α-hydroxyestrone (16α-OHE1), binds covalently to the estrogen receptor (ER) and induces cell proliferation (15, 16). On the basis of these observations, Bradlow and colleagues hypothesized that a metabolism favoring 2-OHE1 over 16α-OHE1, as indexed by the 2-OHE1:16α-OHE1 ratio, may be associated with a reduced risk of breast cancer (17)

Consistent with Bradlow's hypothesis, early case–control studies reported lower 2-OHE1:16α-OHE1 ratio levels among breast cancer cases compared with controls, particularly in premenopausal women (18–24). However, the interpretation of traditional case–control studies is limited because the presence of cancer may have affected estrogen metabolism among cases.

Several prospective studies of premenopausal (25–28) and postmenopausal women (25, 26, 29–31) measuring urine (25, 26, 30) or serum (29, 31) levels of estrogen metabolites did not find statistically significant associations between the 2-OHE1:16α-OHE1 ratio and breast cancer risk (32). In one study, a prospective case–control study nested in the Women's Health Initiative Hormone Trials (WHI-HT), higher levels of 2-OHE1 and the 2-

Authors' Affiliations: Departments of [1]Obstetrics and Gynecology, [2]Population Health, and [3]Environmental Medicine, New York University School of Medicine; [4]New York University Cancer Institute, New York, New York; Departments of [5]Radiation Sciences/Oncology, [6]Medical Biosciences/Pathology, and [7]Public Health and Clinical Medicine/Nutritional Research, Umeå University, Umeå, Sweden; and [8]Radiation Effects Research Foundation, Hiroshima, Japan

Note: Supplementary data for this article are available at Cancer Epidemiology, Biomarkers & Prevention Online (http://cebp.aacrjournals.org/).

Corresponding Author: Anne Zeleniuch-Jacquotte, Department of Population Health, New York University School of Medicine, 650 First Avenue, New York, NY 10016. Phone: 212-263-6512; Fax: 212-263-8570; E-mail: Anne.Jacquotte@nyumc.org

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OHE1:16αOHE1 ratio were associated with modestly increased breast cancer risk; higher 16α-OHE1 levels were associated with increased risk only of tumors that were both ER- and progesterone receptor (PR)–positive (33). However, the three most recent prospective studies in postmenopausal women, nested case–control studies from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial (PLCO; ref. 34), the Columbia Missouri Serum Bank (35), and the Breast and Bone Follow-up to the Fracture Intervention Trial (B∼FIT; ref. 36) reported inverse associations between the 2:16 pathway ratio and breast cancer risk after adjustment for parent estrogens. These three recent studies measured serum concentrations of estrogens and estrogen metabolites using a new liquid chromatography/mass spectrometry (LC/MS) technique (34–36)

Most of the previous studies on the relationship between estrogen metabolites and breast cancer risk, except one report (33), did not take into account tumor receptor status. The objective of this study was to examine the association between circulating estrogen metabolites (2-OHE1, 16α-OHE1, and their ratio) and risk of invasive breast cancer with additional analyses considering tumor receptor status and circulating levels of parent estrone.

Materials and Methods

Description of cohorts

Descriptions of the New York University Women's Health Study (NYUWHS) and the Northern Sweden Mammary Screening Cohort (NSMSC), have been provided previously (3, 37). Briefly, the NYUWHS enrolled 14,274 healthy women ages 34 to 65 years at a breast cancer screening center in New York City between 1985 and 1991. The NSMSC enrolled approximately 28,800 women ages 40 to 69 between 1995 and 2006 within a county breast screening program in northern Sweden. Each cohort collected information about medical and reproductive history, family history of cancer, medication use, smoking history, and diet during enrollment and/or follow-up. Blood was collected at enrollment as serum (NYUWHS) or plasma (NSMSC, in tubes containing EDTA) and stored at −80°C. Participants who reported using exogenous hormones within 6 months of enrollment were not eligible for enrollment in the NYUWHS cohort or for case–control selection in the NSMSC. Women were classified as postmenopausal if they reported absence of menstrual cycles in the previous 6 months, a total bilateral oophorectomy, or a hysterectomy without total oophorectomy if their age was 52 years or older.

Case ascertainment and control selection

All incident invasive breast cancer cases diagnosed before July 1, 2003 for the NYUWHS or before January 1, 2007 for the NSMSC, who were postmenopausal at the time of blood donation, were eligible for inclusion in the current study. A total of 400 eligible cases were identified in the NYUWHS and 170 in the NSMSC. Of those, 2 NYUWHS cases were excluded because of insufficient amount of serum, 6 cases (2 in the NYUWHS and 4 in the NSMSC) because estrogen metabolites were not detectable and 63 cases (38 in the NYUWHS and 25 in the NSMSC) because estrone data were not available. As a result, 499 incident cases of breast cancer (358 from the NYUWHS and 141 from the NSMSC) were included in this study.

For each case, one control was selected at random from women who were alive and free of cancer at the time of diagnosis of the case. We used incidence-density sampling to select a control who matched the case on cohort, menopausal status and age at entry (±6 months), and date of blood donation (±90 days).

The Institutional Review Board of the New York University School of Medicine and the Regional Ethical Committee of the University of Umeå, Sweden reviewed and approved this study annually.

Laboratory analyses

Estrogen metabolites 2-OHE1 and 16α-OHE1 were measured using a monoclonal antibody-based enzyme assay (ESTRAMET 2/16; Immuna Care Inc.). The enzyme immunoassays (EIA) for estrogen metabolites 2-OHE1 and 16α-OHE1 in serum were developed from reagents and buffers previously designed for the measurement of these metabolites in urine (38–41). Each case and her matched control were analyzed in the same laboratory batch. Samples within each batch were placed in random order and labeled so that laboratory personnel were blinded to case–control status. For serum, the intra-batch coefficients of variation (CV) from masked duplicate samples were 3.9% (2-OHE1) and 3.1% (16α-OHE1); the inter-batch CVs were 8.9% (2-OHE1) and 4.7% (16α-OHE1). For plasma, the intra-batch CVs from masked duplicate samples were 4.8% (2-OHE1) and 5.0% (16α-OHE1); the inter-batch CVs were 6.5% (2-OHE1) and 7.4% (16α-OHE1). Total estrone concentrations were measured as part of a previous study by a radioimmunoassay with a double-antibody system and a hydrolysis step for the separation of unconjugated and conjugated estrone (Diagnostics Systems Laboratories, Inc.) as previously described (42).

Statistical analysis

Conditional logistic regression analysis was used to assess the association between established risk factors, estrogen metabolites, and the metabolite ratio with breast cancer risk. Because of differences in levels of estrogen metabolites observed in serum versus plasma, odds ratios (OR) were computed for quartiles with cohort-specific cutpoints defined using the frequency distribution in the respective controls. Tests for trend in breast cancer risk across quartiles of estrogen metabolites were carried out using ordered categorical variables.

We conducted analyses adjusting for circulating level of estrone, the parent estrogen. We also present ORs and 95% confidence intervals (CI) from multivariate models that included, in addition to estrone level, the following known breast cancer risk factors: log-transformed body

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mass index (BMI, kg/m^2), height (continuous), age at menarche (continuous), age at menopause (continuous), family history of breast cancer (negative, positive), parity/age at first birth (≤20 years, 21–25, 26–30, >30, or nulliparous), oral contraceptive use (never, ever), hormone replacement therapy (never, ever), and complete bilateral oophorectomy (no, yes). Sensitivity analyses were performed excluding cases diagnosed within 5 years of enrollment ($n = 128$) to control for potential effects of early undiagnosed disease.

To allow all subjects to remain in the multivariate analyses, multiple imputation using fully conditional specification was performed for the variables with missing data: age at menarche, parity, age at first full-term pregnancy, BMI (all with <3% missing data); age at menopause, oral contraceptive use, and hormone replacement therapy (HRT) use (all with <10% missing data). The analyses including all subjects and imputed data generated results similar to complete-case analyses in which subjects with missing data were excluded.

Subgroup analyses were conducted by breast cancer ER status using $\log_2$-transformed continuous variables for 2-OHE1, 16α-OHE1, and the 2-OHE1:16α-OHE1 ratio. ER status was available for 324 (65%) of eligible cases. Tests for heterogeneity were conducted to assess consistency of the results in the two cohorts and in the different subgroups. All P values are two sided. Analyses were performed using SAS 9.3 (SAS Institute).

Results

The characteristics of the study participants are provided in Table 1. The average age at blood sampling was 60 years. Breast cancer cases had higher median weight than controls (67 vs. 64 kg, respectively; $P = 0.007$), higher median height (163.0 vs. 162.0 cm; $P = 0.006$), and a higher median BMI (25.5 vs. 24.8 kg/m^2, respectively; $P = 0.06$). Cases also had higher levels of circulating estrone compared with controls (median, 28.1 and 26.5 pg/mL, respectively; $P < 0.0001$). No significant differences were observed for other risk factors (Table 1).

Table 2 reports median levels of 2-OHE1, 16α-OHE1, and their ratio in cases and controls by cohort. We observed higher estrogen metabolite levels in the EDTA plasma samples, as compared with serum samples: among controls, median levels of 2-OHE1 and 16α-OHE1 were 92% and 41% higher in plasma than in serum, and the 2-OHE1:16α-OHE1 ratio was 40% higher. The median levels of 2-OHE1, 16α-OHE1, and their ratio, however, were not significantly different between breast cancer cases and controls in either the NYUWHS or the NSMSC cohort. There was also no evidence of heterogeneity between the NYUWHS and the NSMSC results in conditional logistic regression models ($P = 0.70$ for 2-OHE1, $P = 0.26$ for 16α-OHE1 and $P = 0.50$ for the 2-OHE1:16α-OHE1 ratio).

Table 3 presents Spearman correlations (r_s) between estrogen metabolite measures and estrone. 2-OHE1 and 16α-OHE1 were positively correlated ($r_s = 0.44$; $P <$

Table 1. Characteristics of postmenopausal breast cancer cases and matched controls, the NYUWHS and the NSMSC (499 cases, 499 controls)

Characteristic	Cases	Controls	P[c]
Age at blood sampling (y), median (10th, 90th percentile)	60.0 (54.0, 65.1)	60.1 (53.8, 65.3)	Matched
Age at diagnosis, median (10th, 90th percentile)	68.5 (59.9, 75.9)	—	—
Age at menarche[a], median (10th, 90th percentile)	13.0 (11.0, 15.0)	13.0 (11.0, 15.0)	0.33
Age at menopause[b], median (10th, 90th percentile)	50.0 (43.0, 55.0)	50.0 (43.0, 54.0)	0.22
Age at first birth[a], median (10th, 90th percentile)	25.0 (20.0, 31.0)	24.0 (20.0, 32.0)	0.74
≤20 years	48 (13%)	53 (13%)	
21–25 years	183 (48%)	185 (47%)	0.76
26–30 years	107 (28%)	109 (28%)	
>30 years	41 (11%)	49 (12%)	
Nulliparous[a], *n* (%)	100 (21)	81 (17)	0.13
Complete oophorectomy, *n* (%)	48 (10)	47 (9)	0.91
Ever use of oral contraceptives[b], *n* (%)	116 (26)	114 (25)	0.70
Ever use of HRT[b], *n* (%)	57 (13)	59 (13)	0.97
Family history of breast cancer, *n* (%)	97 (19)	100 (20)	0.81
Height[a] (cm), median (10th, 90th percentile)	163.0 (155.0, 170.0)	162.0 (154.0, 168.0)	0.007
Weight[a] (kg), median (10th, 90th percentile)	67.0 (54.0, 84.0)	64.0 (54.0, 82.0)	0.006
BMI[a] (kg/m^2), median (10th, 90th percentile)	25.5 (20.8, 31.2)	24.8 (20.9, 30.9)	0.06
Estrone (pg/mL), median (10th, 90th percentile)	28.1 (18.6, 52.4)	26.5 (15.7, 41.9)	<0.0001

[a]≤3% missing data.

[b]≤10% missing data.

[c]Calculated using unconditional logistic regression with adjustment for age. Weight, BMI, and age at first birth (in parous only) were log-transformed.

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Table 2. Levels of 2-OHE1, 16α-OHE1, and the 2-OHE1:16α-OHE1 ratio by cohort and case–control status, postmenopausal women, the NYUWHS and the NSMSC cohorts (499 cases, 499 controls)

	NYUWHS			NSMSC		
Estrone metabolite	**Case ($n = 358$)**	**Control ($n = 358$)**	***P***	**Case ($n = 141$)**	**Control ($n = 141$)**	***P***
2-OHE1, pg/mL						
Median (10th, 90th percentile)	150.4 (80.0, 272.0)	148.0 (84.0, 269.0)	0.34	282.5 (221.5, 424.0)	284.3 (209.3, 415.5)	0.43
16α-OHE1, pg/mL						
Median (10th, 90th percentile)	296.0 (188.0, 442.0)	286.5 (198.0, 414.0)	0.79	420.0 (296.3, 534.0)	404.0 (275.0, 557.5)	0.17
2-OHE1:16α-OHE1 ratio						
Median (10th, 90th percentile)	0.51 (0.25, 0.95)	0.50 (0.29, 0.89)	0.48	0.70 (0.49, 1.15)	0.70 (0.48, 1.18)	0.68

0.0001). The correlation was positive but weaker for 2-OHE1 and estrone ($r_s = 0.20$; $P < 0.0001$) and 16α-OHE1 and estrone ($r_s = 0.18$; $P < 0.0001$).

As expected, circulating estrone was positively associated with risk of breast cancer in the unadjusted model: OR for the highest versus lowest quartile = 2.37 (95% CI, 1.58–3.55; $P = 0.0004$) and the model adjusted for the potential confounders detailed in Statistical analysis: OR for the highest versus lowest quartile = 2.21 (95% CI, 1.43–3.41; $P = 0.003$). However, no significant trends in breast cancer risk were observed for increasing quartiles of 2-OHE1, 16α-OHE1, or the 2-OHE1:16α-OHE1 ratio overall or by cohort (Table 4). Likewise, no associations were observed when cases diagnosed within 5 years of enrollment were excluded ($n = 54$ in NYUWHS; $n = 74$ in the NSMSC, data not shown). Adjustment for estrone resulted in substantial changes in ORs compared with unadjusted models, although tests for trend remained nonsignificant (Table 4).

In analyses stratified by ER status (Table 5), we observed an inverse association between serum 2-OHE1 level and risk of ER+ breast cancer after adjustment for estrone (OR for a doubling in 2-OHE1, 0.72; 95% CI, 0.52–1.00; $P = 0.05$) and in the multivariate-adjusted model (OR, 0.67; 95% CI, 0.48–0.94; $P = 0.02$). There was an increase in ER− breast cancer risk with a doubling of levels of 2-OHE1. However, the CIs were very broad, and the *P* values were not significant for unadjusted or adjusted models (Table 5). Tests for heterogeneity of the 2-OHE1 associations with ER+ and ER− breast cancers were statistically significant ($P = 0.02$ for the model adjusted for estrone, and $P = 0.03$ for the multivariate model). We observed no significant associations for 16α-OHE1, or the 2-OHE1:16α-OHE1 ratio, in unadjusted or adjusted analyses (Table 5). We also observed no statistically significant associations in analyses of matched sets with missing ER status after adjustment for estrone (Supplementary Table S1).

Discussion

Results of this case–control study nested within two prospective cohorts showed that 2-OHE1, 16α-OHE1, and their ratio were not associated with overall breast cancer risk in postmenopausal women. However, for 2-OHE1, there was evidence of heterogeneity by tumor estrogen status, suggesting that the 2-OHE1 could have different effects on ER+ and ER− breast cancers. In analyses stratified by ER status that adjusted for circulating levels of the parent estrogen, estrone, we observed a significantly reduced risk of ER+ breast cancer for a doubling in serum levels of 2-OHE1. There was a suggested increase in risk of ER− breast cancer with increased levels of 2-OHE1. However, the results for ER− breast cancer were based on only 70 cases, the CIs were wide, and the *P* values were not significant. Therefore, we cannot exclude the role of chance in the results for the ER− subset. Given this possibility and because of the opposite directions of the effects for the ER+ and ER− subsets, interpretation of the results of the subset analyses should be cautious.

Table 3. Spearman correlation coefficients between estrogen measures, the NYUWHS and the NSMSC cohorts (499 cases, 499 controls)

	16α-OHE1	**2:16α-OHE1 ratio**	**Estrone**
2-OHE1	0.44	0.76	0.20
P	<0.0001	<0.0001	<0.0001
16α-OHE1	—	−0.18	0.18
P		<0.0001	<0.0001
2:16α-OHE1 ratio			0.09
P			0.0055

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Table 4 ORs (95% CIs) of invasive breast cancer according to cohort-specific quartiles of estrone metabolite or metabolite ratio, postmenopausal women, the NYUWHS and the NSMSC cohorts (499 cases, 499 controls)

	OR (95% CI)				
Estrone metabolite	**Quartile 1**	**Quartile 2**	**Quartile 3**	**Quartile 4**	***P trend***
All subjects					
2-OHE1, pg/mL					
Cases/controls	120/126	129/125	102/125	148/123	
Unadjusted[a]	1.00	1.12 (0.77–1.62)	0.87 (0.59–1.30)	1.34 (0.90–1.99)	0.29
Adjusted for estrone[b]	1.00	1.07 (0.73–1.55)	0.80 (0.54–1.21)	1.04 (0.68–1.58)	0.80
Multivariate-adjusted[c]	1.00	1.04 (0.71–1.53)	0.78 (0.52–1.18)	0.98 (0.64–1.51)	0.63
16α-OHE1, pg/mL					
Cases/controls	115/126	114/125	158/124	112/124	
Unadjusted[a]	1.00	1.01 (0.69–1.48)	1.42 (0.99–2.04)	1.02 (0.71–1.47)	0.53
Adjusted for estrone[b]	1.00	0.95 (0.64–1.40)	1.24 (0.85–1.80)	0.81 (0.55–1.19)	0.52
Multivariate-adjusted[c]	1.00	0.94 (0.63–1.40)	1.30 (0.88–1.90)	0.81 (0.54–1.20)	0.60
2-OHE1:16α-OHE1 ratio					
Cases/controls	127/129	119/121	105/125	148/124	
Unadjusted[a]	1.00	0.99 (0.69–1.42)	0.86 (0.59–1.26)	1.32 (0.88–1.97)	0.33
Adjusted for estrone[b]	1.00	1.01 (0.70–1.46)	0.83 (0.56–1.22)	1.17 (0.77–1.77)	0.75
Multivariate-adjusted[c]	1.00	1.02 (0.70–1.48)	0.82 (0.55–1.22)	1.13 (0.74–1.73)	0.88
NYUWHS					
2-OHE1, pg/mL					
Cases/controls	93/90	84/90	73/90	108/88	
Unadjusted[a]	1.00	0.93 (0.59–1.44)	0.78 (0.49–1.25)	1.21 (0.77–1.91)	0.46
Adjusted for estrone[b]	1.00	0.90 (0.57–1.42)	0.72 (0.45–1.16)	0.88 (0.54–1.44)	0.47
Multivariate-adjusted[c]	1.00	0.88 (0.55–1.40)	0.72 (0.44–1.17)	0.84 (0.51–1.37)	0.38
16α-OHE1, pg/mL					
Cases/controls	86/90	84/89	106/90	82/89	
Unadjusted[a]	1.00	1.00 (0.64–1.55)	1.24 (0.81–1.90)	0.98 (0.64–1.51)	0.84
Adjusted for estrone[b]	1.00	0.91 (0.58–1.44)	1.03 (0.66–1.60)	0.74 (0.47–1.16)	0.27
Multivariate-adjusted[c]	1.00	0.87 (0.54–1.40)	1.03 (0.65–1.63)	0.72 (0.45–1.15)	0.26
2-OHE1:16α-OHE1 ratio					
Cases/controls	98/93	77/86	74/90	109/89	
Unadjusted[a]	1.00	0.85 (0.55–1.30)	0.79 (0.50–1.25)	1.31 (0.80–2.15)	0.45
Adjusted for estrone[b]	1.00	0.86 (0.56–1.33)	0.74 (0.46–1.19)	1.13 (0.68–1.88)	0.90
Multivariate-adjusted[c]	1.00	0.88 (0.57–1.37)	0.74 (0.46–1.21)	1.11 (0.66–1.89)	0.96
NSMSC					
2-OHE1, pg/mL					
Cases/controls	27/36	45/35	29/35	40/35	
Unadjusted[a]	1.00	1.78 (0.88–3.60)	1.23 (0.57–2.65)	1.78 (0.77–4.08)	0.40
Adjusted for estrone[b]	1.00	1.65 (0.81–3.36)	1.14 (0.52–2.49)	1.54 (0.65–3.63)	0.62
Multivariate-adjusted[c]	1.00	1.48 (0.68–3.20)	0.86 (0.37–2.02)	1.28 (0.51–3.24)	0.99
16α-OHE1, pg/mL					
Cases/controls	29/36	30/36	52/34	30/35	
Unadjusted[a]	1.00	1.00 (0.47–2.13)	1.99 (0.99–3.98)	1.12 (0.54–2.34)	0.38
Adjusted for estrone[b]	1.00	0.98 (0.45–2.13)	1.87 (0.92–3.80)	0.95 (0.44–2.05)	0.64
Multivariate-adjusted[c]	1.00	1.04 (0.46–2.35)	1.87 (0.88–3.99)	0.88 (0.38–2.00)	0.82
2-OHE1:16α-OHE1 ratio					
Cases/controls	29/36	42/35	31/35	39/35	
Unadjusted[a]	1.00	1.51 (0.75–3.03)	1.17 (0.58–2.36)	1.43 (0.70–2.91)	0.54
Adjusted for estrone[b]	1.00	1.54 (0.76–3.12)	1.14 (0.56–2.32)	1.32 (0.64–2.74)	0.74
Multivariate-adjusted[c]	1.00	1.67 (0.78–3.57)	1.06 (0.49–2.29)	1.39 (0.62–3.13)	0.83

[a]Conditional logistic regression (matching variables: age, date of blood donation, and cohort).

[b]Conditional logistic regression adjusting for log-transformed estrone levels.

[c]Conditional logistic regression adjusting for log-transformed estrone levels, log-transformed BMI, height (continuous), age at menarche (continuous), age at menopause (continuous), family history of breast cancer (negative, positive), age at first birth/parity (≤20, 21–25, 26–30, >30 years, or nulliparous), oral contraceptive use (never, ever), HRT use (never, ever), and complete oophorectomy (no, yes).

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Table 5. ORs (95% CIs) of invasive breast cancer for a doubling in estrone metabolite or metabolite ratio by ER status, postmenopausal women, the NYUWHS and the NSMSC

	ER status				
	ER+		ER−		
Estrone metabolite	**OR (95% CIs)**	***P***	**OR (95% CIs)**	***P***	***P* for heterogeneity**
Cases/controls	254/254		70/70		
2-OHE1, pg/mL					
Unadjusted[a]	0.91 (0.68–1.22)	0.52	1.82 (0.80–4.18)	0.16	0.12
Adjusted for estrone[b]	0.72 (0.52–1.00)	0.05	2.19 (0.90–5.33)	0.08	0.02
Multivariate-adjusted[c]	0.67 (0.48–0.94)	0.02	2.83 (0.83–9.70)	0.10	0.03
16α-OHE1, pg/mL					
Unadjusted[a]	0.95 (0.65–1.38)	0.77	1.32 (0.60–2.93)	0.49	0.45
Adjusted for estrone[b]	0.89 (0.60–1.32)	0.56	1.46 (0.63–3.39)	0.37	0.29
Multivariate-adjusted[c]	0.86 (0.57–1.29)	0.46	1.60 (0.59–4.36)	0.35	0.26
2-OHE1:16α-OHE1 ratio					
Unadjusted[a]	0.95 (0.74–1.23)	0.72	1.22 (0.66–2.24)	0.53	0.47
Adjusted for estrone[b]	0.84 (0.64–1.10)	0.20	1.23 (0.67–2.27)	0.50	0.26
Multivariate-adjusted[c]	0.81 (0.61–1.07)	0.14	1.17 (0.53–2.56)	0.70	0.39

[a]Conditional logistic regression (matching variables: age, date of blood donation, cohort).

[b]Conditional logistic regression adjusting for log-transformed estrone levels.

[c]Conditional logistic regression adjusting for log-transformed estrone levels, log-transformed BMI, height (continuous), age at menarche (continuous), age at menopause (continuous), family history of breast cancer (negative, positive), age at first birth/parity (≤ 20, 21–25, 26–30, >30 years, or nulliparous), oral contraceptive use (never, ever), HRT use (never, ever), and complete oophorectomy (no, yes).

When comparing the result of different studies, it is important to consider the laboratory assays used for measurement of estrogen metabolites. The current study used an EIA method to measure estrogen metabolites, similar to assays used in earlier studies (25–27, 29–31). Several recent studies (28, 34–36) used the LC/MS method, which is considered more accurate. One study compared the levels of 2-OHE1 and 16α-OHE1 measured by immunoassays and LC/MS (43), and reported that the two methods were highly correlated in premenopausal women (r_s for 2-OHE1 and 16α-OHE1 were 0.8 and 0.9, respectively) but only moderately correlated in postmenopausal women ($r_s = 0.37$ for 2-OHE1, $r_s = 0.62$ for 16α-OHE1, and $r_s = 0.17$ for the 2-OHE1:16α-OHE1 ratio; ref. 43). These data suggest that future studies should take into account that immunoassays for estrogen metabolites may be less accurate compared with LC/MS, particularly at the low estrogen levels observed in postmenopausal women.

Three recent studies using an LC/MS method (34–36) reported significant inverse associations between the ratio of the 2-hydroxylation to the 16α-hydroxylation pathways, and also the ratio of the 2-hydroxylation pathway to parent estrogens, and breast cancer risk in postmenopausal women (34–36). These results are consistent with the hypothesis that more extensive hydroxylation along the 2-pathway may be associated with a reduced risk of postmenopausal breast cancer. These previous studies (34–36) have not presented the results by tumor receptor status. Our study further suggests that the reduced risk in these women may be limited to ER+ breast cancer.

When comparing estrogen metabolite levels in serum samples (NYUWHS cohort) and EDTA-plasma samples (NSMSC cohort), we observed that estrogen metabolites were higher in EDTA-plasma than in serum samples. One potential explanation of this result is that addition of chelating agents such as EDTA may inhibit the enzymatic reactions and metabolism of estrogen compounds *in vitro*. Another possibility is that EDTA, a potent metal chelator, inactivates the alkaline phosphatase (44), which is used for conjugation of estrogen metabolites in the ESTRAMET 2/16 EIA. This may result in higher EIA-measured estrogen metabolite levels in EDTA plasma compared with serum and suggests that EDTA-free samples should be preferable for the EIA. Nevertheless, the analyses stratified by cohort (type of sample) showed similar results, suggesting that the type of sample did not affect the conclusions. Furthermore, a previous validation study comparing levels of estrogen metabolites in serum and in EDTA plasma samples collected at the same visit in 17 NSMSC subjects showed that Pearson correlation coefficients were high between serum and EDTA plasma levels (0.96 for 2-OHE1, 0.99 for 16α-OHE1, and 0.81 for the 2-OHE1:16α-OHE1 ratio; ref. 45).

The strengths of this study include its prospective design with blood samples collected years before the

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diagnosis (median, 8.1 years; 10th–90th percentile range, 2.2–14.9 years). In addition, we reported previously that estrogen metabolites have relatively high temporal reliability over a 2-year period, with estimated intraclass correlation coefficients of 0.62, 0.95, and 0.69 for 2-OHE1, 16α-OHE1, and the 2-OHE1:16α-OHE1 ratio, respectively (45). These results indicate that a single measurement is a relatively good measure of an individual woman's average level over several years.

Additional strengths of this study include taking into account tumor ER status and adjusting for the levels of parent estrogen. The inverse association between 2-OHE1 and risk of ER+ breast cancer was strengthened after adjusting for estrone, which is positively associated with both ER+ breast cancer risk and levels of 2-OHE1, suggesting negative confounding by estrone. The positive association between 2-OHE1 and risk of ER− breast cancer was also stronger after adjusting for estrone levels, although none of the estimates were statistically significant. Among limitations, the sample size for subset analyses, particularly for ER− breast cancer, was small and the results of subset analyses by ER status should be tested in future studies.

In summary, the results suggest that the association of estrogen metabolites with breast cancer risk may differ by ER status of the tumor. In this study, metabolism favoring 2-hydroxylation of parent estrogens was associated with reduced risk of ER+ breast cancer in postmenopausal women after adjustment for estrone. Therefore, it will be important to consider level of parent estrogen and tumor receptor status in future studies on the role of estrogen metabolites in breast cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: A.A. Arslan, K.L. Koenig, R.E. Shore, E. Lundin, G. Hallmans, A. Zeleniuch-Jacquotte

Development of methodology: A.A. Arslan, K.L. Koenig, R.E. Shore, G. Hallmans, A. Zeleniuch-Jacquotte

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K.L. Koenig, P. Lenner, E. Lundin, P. Toniolo, G. Hallmans, A. Zeleniuch-Jacquotte

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): A.A. Arslan, K.L. Koenig, Y. Afanasyeva, Y. Chen, G. Hallmans, A. Zeleniuch-Jacquotte

Writing, review, and/or revision of the manuscript: A.A. Arslan, K.L. Koenig, P. Lenner, Y. Afanasyeva, R.E. Shore, Y. Chen, E. Lundin, G. Hallmans, A. Zeleniuch-Jacquotte

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.A. Arslan, P. Lenner

Study supervision: R.E. Shore, P. Toniolo

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