Circulating Estrogen Metabolites and Risk for Breast Cancer in Premenopausal Women

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Abstract

Background: **It has been proposed that a shift toward 2-hydroxyestrone from 16α-hydroxyestrone metabolic pathway may be inversely associated with breast cancer risk because 2-hydroxyestrone is thought to be less genotoxic and estrogenic than 16α-hydroxyestrone.**

Methods: **We examined the associations of invasive breast cancer risk with circulating 2-hydroxyestrone, 16α-hydroxyestrone, and the 2-hydroxyestrone:16α-hydroxyestrone ratio in a case-control study on premenopausal women nested within a prospective cohort the New York University Women's Health Study. The serum levels of 2-hydroxyestrone and 16α-hydroxyestrone were measured in 377 incident premenopausal breast cancer cases and 377 premenopausal controls, who were matched on age at enrollment, number and dates of blood donations, and day and phase of menstrual cycle.**

Results: **Overall, no significant associations were observed between breast cancer risk and serum levels of 2-hydroxyestrone, 16α-hydroxyestrone, or their ratio. The 2-hydroxyestrone:16α-hydroxyestrone ratio was positively associated with risk for estrogen receptor–positive breast cancer in the analyses controlling for matching factors. However, the association was attenuated and not significant after adjustment for potential confounders (odds ratio for the highest versus the lowest quartile, 2.15; 95% CI, 0.88-5.27; P_{trend} = 0.09).**

Conclusions: **The results of the current study do not support the hypothesis that a metabolic shift from 16α-hydroxyestrone toward 2-hydroxyestrone in premenopausal women is associated with reduced risk for breast cancer. The association between the 2-hydroxy:16α-hydroxyestrone ratio and estrogen receptor–positive breast cancer needs to be explored in future studies.**

(Cancer Epidemiol Biomarkers Prev 2009;18(8):2273–9)

Introduction

A substantial body of experimental and epidemiologic evidence supports an important role of endogenous estrogens in the development of breast cancer (1-3). Circulating estradiol, estrone, and estrone sulfate have been shown to be positively associated with breast cancer risk in postmenopausal women in prospective studies (4-9). However, in premenopausal women, most prospective studies found no significant association of breast cancer risk with estrogens (10-14), which may be due to the marked cyclic variation of estrogens throughout the menstrual cycle. In the Nurses' Health Study, analyses taking into account menstrual cycle phase suggested that women with high total and free estradiol during the follicular phase are at a significantly increased risk, with stronger associations for invasive and estrogen- and progesterone receptor–positive tumors (15).

Distinct pathways in estrogen metabolism may affect breast cancer risk (16, 17). One hypothesis proposes that two important metabolites, 2-hydroxyestrone and 16α-hydroxyestrone, affect breast cancer risk differently because of their specific estrogenic and genotoxic properties (17-19). The 2-hydroxyestrogens bind to the estrogen receptor with affinity comparable with that of estradiol (20, 21) but have limited mitogenic effect (22, 23) and possibly even antiestrogenic effects (18, 24). In contrast, 16α-hydroxyestrone binds to the estrogen receptor with lower affinity than estradiol but has stronger estrogenic effects than 2-hydroxyestrone in terms of cell proliferation (25, 26). Based on differences in the estrogenic and genotoxic activity of 2-hydroxyestrone and 16α-hydroxyestrone in cell lines and animal models, Bradlow et al. (27) proposed that a shift toward 2-hydroxyestrone from the 16α-hydroxyestrone metabolic pathway, as indexed by the 2-hydroxyestrone:16α-hydroxyestrone ratio, is inversely associated with breast cancer risk.

The existing retrospective case-control studies on the association between 2-hydroxyestrone and 16α-hydroxyestrone levels and breast cancer are difficult to interpret because existing breast cancer may alter the estrogen metabolism (28-33). To date, only two prospective studies on estrogen metabolites and breast cancer risk in premenopausal women have been published (34, 35). Both studies examined urinary estrogen metabolites and reported that elevations in the 2-hydroxyestrone:16α-hydroxyestrone ratio were associated with a reduced risk for invasive breast cancer, although the results were not statistically significant (34, 35).

Received 4/3/09; revised 5/4/09; accepted 6/9/09; published online 8/6/09.

Grant support: National Cancer Institute (grant numbers R01CA034588, R01CA098661, P30CA016087) and the National Institute of Environmental Health Sciences Center Grant (grant number ES000260).

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doi:10.1158/1055-9965.EPI-09-0312

We examined the associations of invasive breast cancer risk with circulating 2-hydroxyestrone, 16α-hydroxyestrone, and the 2-hydroxyestrone:16α-hydroxyestrone ratio in a case-control study on premenopausal women nested within a prospective cohort the New York University Women's Health Study.

Materials and Methods

Study Population. The New York University Women's Health Study has been described in detail previously (4). Briefly, between March 1985 and June 1991, 14,274 women 35 to 65 y old were enrolled in the New York University Women's Health Study at a mammography screening center in New York City. Cohort eligibility was restricted to women who had neither used hormonal medications of any type nor been pregnant in the preceding 6 mo. At enrollment and at annual screening visits thereafter, subjects provided 30 mL of nonfasting peripheral venous blood, drawn using collection tubes without anticoagulant. Serum samples were stored at -80°C for future analyses. At the time of enrollment, 7,220 women were classified as premenopausal because they had reported the following: (*a*) at least one menstrual cycle during the preceding 6 mo or (*b*) a hysterectomy without oophorectomy before natural menopause and age <52 y.

Ascertainment of vital status and disease incidence is obtained through active follow-up using questionnaires mailed every 2 to 4 y and telephone calls for nonrespondents and through linkages with the U.S. National Death Index and with the statewide tumor registries of New York, New Jersey, and Florida. When a new cancer is reported, written permission is solicited from the patient (or next of kin if deceased) to request medical and pathology reports from hospitals and physicians. Breast cancer ascertainment in the New York University Women's Health Study is estimated to be 95% complete (36).

All incident invasive breast cancers cases diagnosed as of July 1, 2003 (start date of last complete round of follow-up), who were premenopausal at the time of blood donation were eligible for inclusion in the current study. A total of 384 eligible cases were identified. Of those, five cases were excluded because they had *in situ* carcinoma, one case because an insufficient amount of serum was available, and one case because no detectable levels of estrogen metabolites were observed. As a result, 377 incident premenopausal cases were included in the current study.

One control was selected for each case at random from the appropriate risk set. The risk set consisted of women who were premenopausal at entry, alive, and free of cancer at the time of diagnosis of the case and who matched the case on age at entry (within 6 mo), date of blood donation (within 90 d), and number and dates of subsequent blood donations. In addition, controls were matched to cases on day and phase of menstrual cycle calculated from the date of next menstruation, which was obtained from mail-back calendars distributed at the time of blood drawing. The mail-back calendars were returned by 75% of women. If the calendar was not returned, the phase and day of cycle were estimated using usual length of menstrual cycle for women who had reported five or more cycles in the preceding 6 mo and set to unknown for others.

The institutional review board of the New York University School of Medicine has reviewed and approved this study.

Laboratory Analyses. Estrogen metabolites 2-hydroxyestrone and 16α-hydroxyestrone were measured using a monoclonal antibody-based enzyme assay (ESTRAMET 2/16, Immuna Care, Inc.). The enzyme immunoassays for estrogen metabolites 2-hydroxyestrone and 16α-hydroxyestrone in serum were developed from reagents and buffers previously designed for the measurement of these metabolites in urine (37-40).

Each case and her matched control were always analyzed in the same batch. Samples within each batch were placed in random order and labeled so that laboratory personnel were blinded to case-control status. The intra-assay coefficients of variation from masked duplicate samples were 1.9% (2-hydroxyestrone) and 0.9% (16α-hydroxyestrone); the interassay coefficients of variation were 4.2% (2-hydroxyestrone) and 2.3% (16α-hydroxyestrone).

Covariate Data. At enrollment and at annual screening visits thereafter, subjects completed self-administered questionnaires on demographic, medical, anthropometric, reproductive, and dietary factors. Additional information was collected through periodic follow-up questionnaires. Information on potential confounders, including age at menarche, family history of breast cancer, past history of oral contraceptives use, weight, height, and body mass index (BMI), was available from the interview at baseline, and information on education, smoking, and ethnicity was obtained from the first follow-up questionnaire collected ~2 y after baseline.

Statistical Analysis. The distributions of known breast cancer risk factors in cases and controls were compared using the conditional logistic regression model to take into account the matching (41). To test for differences in estrogen metabolites levels between case and matched control subjects, we used a mixed-effects regression model; after logarithmic transformation ($\log_2$) to reduce departures from the normal distribution, the estrogen metabolites levels were modeled as function of a random effect (matched set) and a fixed effect (case/control status; ref. 42). The 2-hydroxyestrone:16α-hydroxyestrone ratio was computed using the original (not log transformed) values. A local regression model was used to plot the estrogen metabolites levels by day of cycle. Spearman *r* was used to calculate the correlations between continuous variables.

Conditional logistic regression analysis was used to assess the association between estrogen metabolites and breast cancer. To compute odds ratios, serum measurements were categorized into quartiles using the frequency distribution of the controls. Odds ratios were computed relative to the lowest quartile. Analyses were also done on the continuous ($\log_2$ transformed) scale. Multivariate models included potential confounders, that is, variables associated with estrogen metabolite levels and/or risk for breast cancer. The final multivariate model included first-degree family history of breast cancer (negative, one affected relative ≥45 y old, one affected relative <45 y, or more than one affected relative), ever smoking (no, yes), BMI (after $\log_2$ transformation), age at menarche (after log transformation), and a combination of parity and age at

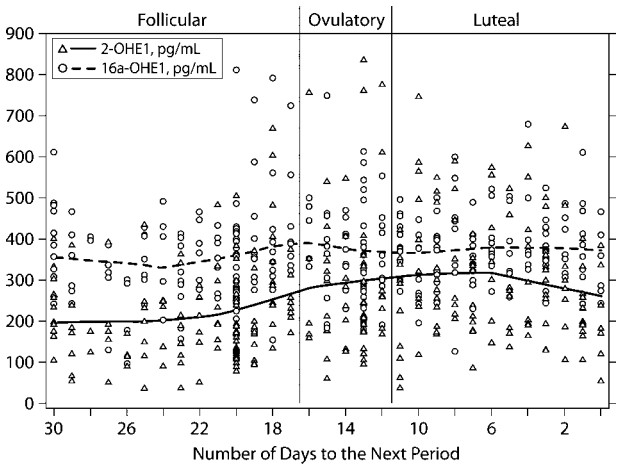

Figure 1. Variation of 2-hydroxyestrone and 16α-hydroxyestrone levels with day and phase of cycle among premenopausal controls, New York University Women's Health Study.

first full-term pregnancy (<20, 20-24, 25-29, 30+, nulliparous).

Analyses were also conducted after excluding cases diagnosed within 5 or 10 y of enrollment or women with unknown phase of cycle. Finally, we conducted analyses stratifying by phase of menstrual cycle (follicular/luteal) or estrogen receptor status.

Reported trend *P*s correspond to estrogen metabolites treated as ordered categorical variables. All *P*s are two sided. All analyses were done using SAS 9.1 (SAS Institute).

Results

Figure 1 shows the variation in serum 2-hydroxyestrone and 16α-hydroxyestrone by phase of cycle among control women. For 2-hydroxyestrone levels, we observed a steady increase throughout the follicular and ovulatory phases, reaching a peak at midluteal phase of the cycle. For 16α-hydroxyestrone, we observed a moderate increase during the follicular phase and fairly constant levels throughout the ovulatory and luteal phases (Fig. 1).

Descriptive characteristics of premenopausal breast cancer cases and controls are presented in Table 1. Compared with controls, a higher proportion of cases had a positive family history of breast cancer (20% and 25%, respectively), and cases had a later mean age at first full-term pregnancy (24.8 years and 26.5, respectively; $P < 0.05$). Cases and controls were similar in terms of height, weight, and history of oral contraceptive use (Table 1).

The effects of age, menarche age, smoking, full-term pregnancy, history of oral contraceptives use, smoking, family history of breast cancer, and BMI on levels of estrogen metabolites among premenopausal controls are presented in Table 2. Serum levels of 2-hydroxyestrone and 16α-hydroxyestrone decreased with age ($P_{trend} = 0.002$ and 0.004, respectively). We observed a significant inverse relationship between age at menarche and serum levels of both 2-hydroxyestrone and 16α-hydroxyestrone ($P_{trend} = 0.02$ and 0.05, respectively). Women who had ever been pregnant had significantly lower levels of 2-hydroxyestrone compared with women who had never been pregnant (mean 250.3 versus 284.5, respectively; $P = 0.04$). Compared with never smokers, ever smokers tended to have lower mean levels of 16α-hydroxyestrone (372.3 and 355.0, respectively; $P = 0.06$) and higher mean 2-hydroxyestrone:16α-hydroxyestrone ratio (0.71 and 0.81, respectively; $P = 0.06$). Mean levels of estrogen metabolites did not vary significantly after stratification by past history of oral contraceptive use, family history of breast cancer, and BMI categories (Table 2).

Levels of 2-hydroxyestrone and 16α-hydroxyestrone were significantly correlated (Spearman $r = 0.34$ among controls; $P < 0.0001$ and 0.21 among cases; $P < 0.0001$). Overall, no associations were observed between serum levels of either 2-hydroxyestrone or 16α-hydroxyestrone and breast cancer risk (Table 3). Likewise, there were no associations in analyses done after excluding subjects with unknown phase of cycle ($n = 89$), diagnosed within 5 years of enrollment ($n = 57$) or within 10 years of enrollment ($n = 181$; data not shown). Results were similar for women who had donated blood in the follicular phase (88 matched sets) and women who had donated blood in the luteal phase (108 matched sets). In analyses stratified by estrogen receptor status, which was available for 48% of the cases (Table 4), we did not observe statistically significant trends for either 2-hydroxyestrone or 16α-hydroxyestrone, whether with or without adjustment for potential confounders (Table 4). However, the 2-hydroxyestrone:16α-hydroxyestrone ratio was positively associated with risk for estrogen receptor–positive breast cancer in the analyses controlling only for matching factors (odds ratio for the highest versus the lowest quartile, 2.24; $P_{trend} = 0.02$). After adjustment for potential confounders, the positive association between 2-hydroxyestrone:16α-hydroxyestrone ratio and risk for estrogen receptor–positive breast cancer remained but was only

Table 1. Characteristics of premenopausal breast cancer cases and matched controls, New York University Women's Health Study

Characteristics	Cases ($n = 377$)	Controls ($n = 377$)
Mean age (SD), y	44.4 (4.8)	44.3 (4.8)
Mean age at diagnosis (SD), y	54.4 (6.5)	—
Mean age at menarche (SD), y	12.5 (1.5)	12.4 (1.5)
Mean age at first full-term pregnancy* (SD), y	26.5 (6.0)	24.8 (5.4)
Nulliparous, *n* (%)	145 (38%)	134 (36%)
Family history of breast cancer, *n* (%)	95 (25%)	77 (20%)
Ever use of oral contraceptives, *n* (%)†	193 (57%)	206 (62%)
Ever smoke cigarettes, *n* (%)‡	197 (55%)	176 (50%)
Mean height (SD), cm	163.2 (6.8)	162.6 (6.8)
Mean weight (SD), kg	64.2 (11.8)	63.6 (12.9)
Mean BMI (SD), kg/m^2	24.1 (4.3)	24.0 (4.5)
Mean 2-OHE1 (SD), pg/mL	271.1 (161.8)	262.5 (154.1)
Mean 16α-OHE1 (SD), pg/mL	371.0 (117.1)	363.2 (108.7)
Mean 2-OHE1:16α-OHE1 ratio (SD)	0.79 (0.65)	0.76 (0.49)

Abbreviations: 16α-OHE1, 16α-hydroxyestrone; 2-OHE1, 2-hydroxyestrone.
*$P < 0.05$. All other, $P > 0.05$.
†Percentage of those whose oral contraceptives use history is known (89% of cases and 90% of controls).
‡Percentage of those whose smoking status is known (95% of cases and 93% of controls).

Table 2. Mean (SD) levels of estrogen metabolites by smoking status, family history of breast cancer, and BMI among premenopausal controls, New York University Women's Health Study

Characteristic	*n*	2-OHE1, pg/mL	16α-OHE1, pg/mL	2-OHE1:16α-OHE1 ratio
Age at sampling, y				
<45	212	273.1 (151.0)	376.4 (113.2)	0.77 (0.50)
45-49	111	268.7 (162.5)	355.5 (106.0)	0.78 (0.48)
≥50	54	207.8 (139.2)	327.4 (85.4)	0.65 (0.43)
P_{trend}		0.002	0.004	0.12
Age at menarche, y				
<12	92	283.1 (169.9)	375.8 (121.7)	0.81 (0.61)
12	112	274.1 (149.3)	371.7 (92.3)	0.77 (0.45)
13	88	255.3 (168.4)	341.9 (100.6)	0.75 (0.41)
>13	81	232.7 (120.8)	356.8 (120.4)	0.71 (0.47)
P_{trend}*		0.02	0.05	0.20
Full-term pregnancy				
Never	134	284.5 (162.6)	355.8 (89.2)	0.83 (0.50)
Ever	243	250.3 (148.2)	367.3 (118.0)	0.72 (0.47)
*P**		0.04	0.08	0.03
Age at first full term pregnancy, y				
<20	27	251.0 (184.0)	346.9 (107.7)	0.74 (0.53)
20-24	114	250.1 (145.0)	373.9 (122.2)	0.72 (0.52)
25-29	58	246.2 (140.1)	367.9 (125.9)	0.71 (0.43)
≥30	44	256.0 (147.2)	361.8 (103.2)	0.72 (0.38)
P_{trend}*		0.18	0.43	0.22
Use of oral contraceptives				
Never	127	248.8 (142.1)	362.6 (116.5)	0.73 (0.47)
Ever	207	272.8 (155.9)	365.0 (106.5)	0.79 (0.51)
*P**		0.23	0.96	0.23
Smoking status				
Never	173	254.3 (137.3)	372.3 (107.5)	0.71 (0.45)
Ever	176	270.6 (160.7)	355.0 (113.5)	0.81 (0.53)
*P**		0.43	0.06	0.06
Family history of breast cancer				
Negative	300	265.1 (152.9)	363.3 (106.0)	0.76 (0.46)
Positive	77	252.0 (159.6)	362.7 (119.2)	0.75 (0.58)
*P**		0.42	0.99	0.57
BMI categories				
Normal (<25.0 kg/m^2)	262	269.0 (155.0)	363.0 (109.6)	0.78 (0.50)
Overweight (25.0-29.9 kg/m^2)	85	249.3 (144.5)	361.4 (106.9)	0.70 (0.37)
Obese (≥30.0 kg/m^2)	29	238.6 (175.3)	370.3 (110.8)	0.70 (0.61)
P_{trend}*		0.42	0.47	0.16

*Age adjusted.

marginally significant (adjusted odds ratio for the highest versus the lowest quartile, 2.15; P_{trend} = 0.09).

Discussion

Results of this nested case-control study on 2-hydroxyestrone and 16α-hydroxyestrone metabolites do not support previous hypotheses that overall breast cancer risk is associated with either 16α-hydroxyestrone or 2-hydroxyestrone in premenopausal women. Similarly, there was no association of overall breast cancer risk with the 2-hydroxyestrone:16α-hydroxyestrone ratio. In analyses stratified by estrogen receptor status, the risk for estrogen receptor–positive breast cancer seemed to be elevated with increasing 2-hydroxyestrone:16α-hydroxyestrone ratio.

Table 3. Odds ratios (95% CIs) of invasive breast cancer according to quartiles of estrogen metabolites, premenopausal women, New York University Women's Health Study (377 cases, 377 controls)

Characteristic	OR (95% CI)				P_{trend}
	Quartile 1	Quartile 2	Quartile 3	Quartile 4	
2-OHE1, pg/mL	<158	158-235	236-333	>333	
Unadjusted*	1.00 (reference)	1.22 (0.79-1.89)	0.89 (0.57-1.39)	1.37 (0.84-2.25)	0.48
Adjusted†	1.00 (reference)	1.25 (0.78-2.02)	0.93 (0.56-1.53)	1.27 (0.74-2.18)	0.65
16α-OHE1, pg/mL	<293	293-356	357-415	>415	
Unadjusted*	1.00 (reference)	1.17 (0.78-1.75)	0.89 (0.60-1.34)	1.31 (0.87-1.99)	0.42
Adjusted†	1.00 (reference)	1.27 (0.80-2.02)	1.14 (0.72-1.78)	1.41 (0.89-2.24)	0.21
2-OHE1:16α-OHE1 ratio	<0.453	0.453-0.635	0.636-0.936	>0.936	
Unadjusted*	1.00 (reference)	0.91 (0.59-1.38)	1.07 (0.70-1.65)	1.18 (0.74-1.88)	0.38
Adjusted†	1.00 (reference)	0.83 (0.53-1.31)	1.04 (0.65-1.65)	1.13 (0.68-1.87)	0.51

Abbreviation: OR, odds ratio.
*Conditional logistic regression (matching variables: age, date of blood donation, day and phase of menstrual cycle).
†Conditional logistic regression (matching variables: age, date of blood donation, day and phase of menstrual cycle) adjusting for first-degree family history of breast cancer (negative, one affected relative ≥45 years old, one affected relative <45, or more than one affected relative), ever smoking (no, yes), BMI ($\log_2$ transformed), age at menarche (log transformed), parity/age at first full-term pregnancy (<20, 20-24, 25-29, 30+, nulliparous).

Table 4. Odds ratios (95% CIs) of invasive breast cancer by estrogen receptor status, premenopausal women, New York University Women's Health Study (145 estrogen receptor–positive cases/145 controls and 52 estrogen receptor–negative cases/52 controls)

Characteristic	Quartile 1	Quartile 2	Quartile 3	Quartile 4	P_{trend}
2-OHE1, pg/mL					
ER+					
Unadjusted*	1.00 (reference)	2.29 (1.13-4.66)	1.66 (0.77-3.57)	2.56 (1.11-5.89)	0.12
Adjusted†	1.00 (reference)	2.74 (1.17-6.41)	1.91 (0.76-4.76)	2.38 (0.84-6.74)	0.41
ER-					
Unadjusted*	1.00 (reference)	0.76 (0.24-2.38)	0.85 (0.30-2.40)	0.66 (0.14-3.12)	0.66
Adjusted†	1.00 (reference)	1.26 (0.26-6.06)	1.02 (0.27-3.91)	2.39 (0.25-22.49)	0.70
16α-OHE1, pg/mL					
ER+					
Unadjusted*	1.00 (reference)	1.29 (0.68-2.44)	1.12 (0.61-2.06)	0.83 (0.41-1.70)	0.65
Adjusted†	1.00 (reference)	1.21 (0.59-2.52)	0.92 (0.45-1.90)	0.59 (0.26-1.34)	0.18
ER-					
Unadjusted*	1.00 (reference)	1.91 (0.61-5.94)	1.04 (0.33-3.33)	1.15 (0.36-3.71)	0.92
Adjusted†	1.00 (reference)	2.44 (0.50-11.93)	1.70 (0.36-7.94)	1.38 (0.28-6.78)	0.64
2-OHE1:16α-OHE1 ratio					
ER+					
Unadjusted*	1.00 (reference)	1.24 (0.63-2.43)	1.83 (0.91-3.66)	2.24 (1.07-4.68)	0.02
Adjusted†	1.00 (reference)	1.44 (0.65-3.18)	1.79 (0.80-4.00)	2.15 (0.88-5.27)	0.09
ER-					
Unadjusted*	1.00 (reference)	1.06 (0.34-3.28)	1.13 (0.32-4.00)	0.62 (0.14-2.80)	0.64
Adjusted†	1.00 (reference)	1.12 (0.29-4.41)	1.69 (0.34-8.40)	1.18 (0.21-6.49)	0.74

Abbrevaitions: ER+, estrogen receptor positive; ER-, estrogen receptor negative.
*Conditional logistic regression (matching variables: age, date of blood donation, day and phase of menstrual cycle).
†Conditional logistic regression (matching variables: age, date of blood donation, day and phase of menstrual cycle) adjusting for first-degree family history of breast cancer (negative, one affected relative ≥45 years old, one affected relative <45 years old, or more than one affected relative), ever smoking (no, yes), BMI (log_2 transformation), age at menarche (log transformed), or parity/age at first full-term pregnancy (<20, 20-24, 25-29, 30+, nulliparous).

In a recent prospective study on circulating estrogen metabolites in postmenopausal women from the Nurses' Health Study, Eliassen et al. (43) also observed that neither 2-hydroxyestrone nor 16α-hydroxyestrone were associated with breast cancer risk overall. Interestingly, they also observed a significant positive association of breast cancer risk with the 2-hydroxy:16α-hydroxyestrone ratio but only among women with estrogen receptor–negative/progesterone receptor–negative tumors. Future studies should clarify whether the association between 2-hydroxyestrone:16α-hydroxyestrone ratio and breast cancer risk varies by estrogen receptor status and by menopausal status.

To date, only two small prospective epidemiologic studies have examined the association between 2-hydroxyestrone and 16α-hydroxyestrone metabolites and breast cancer risk among premenopausal women, both measuring metabolites in urine samples. Meilahn et al. (34) studied 60 breast cancer cases and 184 control women age ≥35 years from the Guernsey III prospective cohort and found that women in the highest tertile of the 2-hydroxyestrone:16α-hydroxyestrone ratio had 25% lower risk for breast cancer compared with those in the lowest tertile. However, these results were not statistically significant, with an odds ratio of 0.75 (95% CI = 0.35-1.62; $P = 0.46$). In another study, Muti et al. (35) analyzed 67 premenopausal breast cancer cases and 264 matched controls from the Italian study of Hormones and Diet in the Etiology of Breast Tumors (ORDET) prospective cohort with urine collected in the luteal phase of menstrual cycle between the 20th and 24th day. They reported that a higher 2-hydroxyestrone:16α-hydroxyestrone ratio at baseline was associated with about 40% reduced risk for breast cancer. Similarly, the results were not statistically significant, with an odds ratio of 0.58 (95% CI, 0.25-1.34) for women in the highest quintile of the ratio compared with those in the lowest quintile.

It is important to point out that, in our study, serum samples were analyzed as opposed to the urinary samples used in previous studies on premenopausal women. The assays for urinary estrogen metabolites have been validated against gas chromatography–mass spectroscopy methods (37, 38), and the assays for 2-hydroxyestrone and 16α-hydroxyestrone in serum were validated against urine assays by adding quality controls with known amounts of urinary metabolites to serum samples and then doing the serum assay. Although serum levels of estrogen metabolites in premenopausal women are relatively low compared with the major estrogens (estrone and estradiol; ref. 44), we believe that serum measurements are preferable because urine is less proximal to breast tissue and urine measurements may reflect variability in catabolism and excretion to a larger degree than serum levels. Serum levels of estrone have been shown to be strongly correlated with its breast levels during follicular and luteal phases of the cycle (45); however, data on the correlations between serum and breast tissue levels of estrogen metabolites are lacking.

As expected, both 2-hydroxyestrone and 16α-hydroxyestrone levels decreased with increasing age. Age at menarche was inversely associated with 2-hydroxyestrone and 16α-hydroxyestrone levels, confirming that menarche onset is strongly related to estrogen metabolite levels later in life. Among the findings of the current study was the observation that parous women had significantly lower levels of 2-hydroxyestrone and lower 2-hydroxyestrone:16α-hydroxyestrone ratio compared with nulliparous women. Our data also support the previous observation by Jernström et al. (46) that smokers have a higher 2-hydroxyestrone:16α-hydroxyestrone ratio compared with never smokers among premenopausal women, although the results were borderline significant ($P = 0.06$).

Excessive underlying intra-individual variability of measurements may potentially affect the results of epidemiologic studies based on sampling at a single point in time. This is of particular concern for hormones that vary according to phase of the menstrual cycle, such as estradiol. 2-Hydroxyestrone and 16α-hydroxyestrone do vary during the menstrual cycle, as documented by Jernström et al. (46) and our data (Fig. 1). However, the magnitude of changes during the menstrual cycle is quite small. Moreover, in our study, cases and controls were matched according to phase and day of menstrual cycle, thus limiting the potential effect of these variations on risk estimates.

Current use of oral contraceptives or recent pregnancy may affect the circulating levels of estrogen metabolites (46). However, eligibility for this study was restricted to women who had neither used hormonal medications of any type nor been pregnant in the preceding 6 months. In addition, cases and controls were similar in terms of past history of oral contraceptive use and parity, and adjusting for these variables did not substantially affect our results.

Sample degradation might introduce a source of variability in prospective cohort studies that store biological specimens for many years or decades, such as the current study. To minimize this effect, samples used in the current study had been stored constantly at -80°C and never defrosted until laboratory analyses, and cases and controls were matched on sample time in storage. In addition, a previous study showed the stability of estrogen metabolites over time (47).

The strengths of the current study include its fairly large sample size, the use of serum rather than urine for estrogen metabolite measurements, and careful control for the effect of day and phase of menstrual cycle by using the starting date of the next successive menstrual period, which is generally considered a more accurate index of cycle day than is the time since the last cycle, which had been used in previous studies.

In conclusion, the results of the current study do not support the hypothesis that a metabolic shift from 16α-hydroxyestrone toward 2-hydroxyestrone is associated with reduced risk for premenopausal breast cancer. On the contrary, our findings suggest that shift toward 2-hydroxyestrone and particularly an elevated 2-hydroxyestrone:16α-hydroxyestrone ratio may be associated with an increased risk for estrogen receptor–positive premenopausal breast cancer. Future studies should confirm and expand these observations by including measurements of other estrogen metabolites, in particular, 4-hydroxyestrone, a catecholestrogen. Catecholestrogens, which include 2- and 4-hydroxyestrogens, may induce DNA damage through redox cycling, which generates reactive oxygen species and form reactive semiquinones and quinones capable of forming adducts with glutathione and purines in DNA. Measurement of 4-hydroxyestrone, which is thought to have greater estrogenic and genotoxic potential than 2-hydroxyestrone (48-50), would be of particular interest.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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We thank the participants of the New York University Women's Health Study, whose invaluable help and dedication made this project possible, and Dr. Thomas L. Klug for laboratory analyses and support of this study.

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Cancer Epidemiol Biomarkers Prev 2009;18:2273-2279.