Estrogen Metabolism and Risk of Breast Cancer: A Prospective Study of the 2:16α-Hydroxyestrone Ratio in Premenopausal and Postmenopausal Women

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Experimental and clinical evidence suggests that 16α-hydroxylated estrogen metabolites, biologically strong estrogens, are associated with breast cancer risk, while 2-hydroxylated metabolites, with lower estrogenic activity, are weakly related to this disease. This study analyzes the association of breast cancer risk with estrogen metabolism, expressed as the ratio of 2-hydroxyestrone to 16α-hydroxyestrone, in a prospective nested case-control study. Between 1987 and 1992, 10,786 women (ages 35–69 years) were recruited to a prospective study on breast cancer in Italy, the "Hormones and Diet in the Etiology of Breast Cancer" (ORDET) study. Women with a history of cancer and women on hormone therapy were excluded at baseline. At recruitment, overnight urine was collected from all participants and stored at −80°C. After an average of 5.5 years of follow-up, 144 breast cancer cases and four matched controls for each case were identified among the participants of the cohort. Among premenopausal women, a higher ratio of 2-hydroxyestrone to 16α-hydroxyestrone at baseline was associated with a reduced risk of breast cancer: women in the highest quintile of the ratio had an adjusted odds ratio (OR) for breast cancer of 0.58 [95% confidence interval (CI) = 0.25−1.34]. The corresponding adjusted OR in postmenopausal women was 1.29 (95% CI = 0.53–3.10). Results of this prospective study support the hypothesis that the estrogen metabolism pathway favoring 2-hydroxylation over 16α-hydroxylation is associated with a reduced risk of invasive breast cancer risk in premenopausal women. (Epidemiology 2000;11: 635–640)

Keywords: estrogens, breast neoplasms, estrogen metabolites, cohort study

In 1979, Thomas Dao postulated that the way in which estrogens are metabolized might be important for breast cancer development.[1] The theory, known as "the unconventional estrogen hypothesis," proposed that the products of estrogen metabolism may be of etiological significance.[1] The theory originated from the evidence that "conventional" estrogens accounted only for less than half of total circulating estrogens[1] and from the inconsistent results observed, at that time, in epidemiologic studies based on "conventional" estrogen determinations. In 1982, Bradlow *et al.* hypothesized that hydroxylation of estradiol at the C-2 position produces metabolites with little or no estrogenic activity and thus it might be associated with decreased breast cancer risk. Conversely, hydroxylation of estradiol at the C-16 position produces metabolites with high estrogenic activity, and might be associated with increased breast cancer risk.[2] Several experimental, clinical, and epidemiologic studies support this hypothesis. In *in vitro* studies, 16α-hydroxylation has been shown to have strong biological estrogenic activity[3–5] and genotoxic characteristics,[6] while 2-hydroxylation metabolites had virtually no peripheral estrogenic effects.[7] In mice, 16α-hydroxylation of estrone was associated with increased spontaneous incidence of mammary tumors.[8] In clinical studies, the extent of biotransformation of radiolabeled E_2 via the 16α-hydroxylation pathway was higher in breast tissue (terminal duct lobular unit) from patients with breast cancer than it was for tissue from women who had undergone reductive mammoplasty.[9] In general, epidemiologic studies on estrogen metabolism support the proposed hypothesis. In case-control studies an increase in estrone 16α-hydroxylation in breast cancer cases was

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observed compared with healthy controls, in particular in postmenopausal women.[2,10–13] In other case-control studies, however, little or no association was found in premenopausal,[14] or in postmenopausal women.[15] Until now, only one prospective study has been conducted to investigate the role of estrogen metabolism as predictor of breast cancer. In that study, participants in the highest tertile of the 2-hydroxyestrone/16α-hydroxyestrone ratio had a 40% reduction in breast cancer risk compared with those in the lowest tertile.[16]

The present report analyzes the association between invasive breast cancer (ductal and lobular carcinoma) and prediagnostic estrogen metabolism. In particular, we tested the hypothesis that the pathway favoring 2-hydroxylation over 16α-hydroxylation may be associated with a decrease in breast cancer risk.

Methods

Between June 1987 and June 1992, 10,786 healthy women, ages 35 to 69 years, residents of Varese province, northern Italy, participated in a prospective study of hormones, diet, and breast cancer risk: the "Ormoni E Dieta Nella Etiologia Dei Tumori Della Mammella" (ORDET) study.[17] All members of the cohort were volunteers recruited from the general population through radio, television, and newspaper advertising. Women were also invited to participate in the study through meetings organized by municipalities, local offices of the Italian National Health System, women's associations, churches, and unions. There were 162,700 women between 35 and 69 years of age (the age-range of the cohort study)[18] in Varese province during the recruitment phase of the study. Thus, the total number of women recruited in the cohort represents approximately 7% of the general population of women in that age range.

Women with a history of cancer and bilateral oophorectomy, and those currently pregnant or breast feeding, with chronic or acute liver disease, or on hormone therapy within 3 months before recruitment into the study were not eligible to participate. At baseline, questionnaires on menstrual history and reproductive characteristics, and data on height, weight, and other anthropometric measures were collected. Anthropometric measurements were made by nurses based on a standardized protocol. We calculated body mass index (BMI) as weight in kilograms divided by height in meters squared. Postmenopausal status was defined as the absence of menstrual bleeding for at least 12 months.

Case Ascertainment

On June 1995, after an average of 5.5 years of follow-up, the ORDET file was linked with the local cancer registry (Lombardy Cancer Registry) files to identify breast cancer cases. The ORDET file was also linked with the regional file of Varese residents to determine the vital status of the cohort members. Thirty-seven women were diagnosed with breast cancer before enrollment in the cohort, four were diagnosed with breast cancer *in situ*, and 10 were lost to follow-up. Thus, there were 10,735 women eligible for this study. Among those, 89 died from causes other than breast cancer and 144 were identified by the cancer registry as cases of invasive breast cancer; of them 71 were postmenopausal at the time of recruitment.

Controls

For each breast cancer case, four matched controls were randomly chosen from members of the cohort who were alive at the time of diagnosis of the matched case without having developed breast cancer. Controls were matched to cases on age ($\pm$5 years), menopausal status, time of day at blood draw (each participant donated 40 ml of whole blood), recruitment center (participants were recruited in two centers, 25 km apart, and specimens collected in the second center were transported by car every morning to the laboratory located in the first center), and recruitment date ($\pm$180 days).

Sample Collection

All women participating in the study were asked to urinate before bedtime (7:00 PM) and then to collect all overnight urine including the first-morning urine (7:00 AM) in a standard container provided by the study. At the completion of the urine collection, samples were transported to the laboratory of the study center. Within 3 hours after the last void, urine samples were filtered and stored in freezers at -80°C. Urine containers were tightly capped to prevent evaporation. Urine was not removed from the freezers, and the temperature was kept constant throughout storage, with no freeze-thaw cycles.

In premenopausal women, urine was collected in the luteal phase of the menstrual cycle between the 20th and the 24th day.

Estrogen metabolism was compared in cases and in controls using the ratio of 2:16α-hydroxyestrone. An increase in 2-hydroxylation (or a decrease in 16α-hydroxylation), reflected by a high ratio value, was interpreted as a shift of estrogen metabolism toward a less estrogenic hormonal milieu.

Laboratory Analysis

Stored urinary samples from breast cancer cases and related controls were handled identically and assayed together in the same batch. All laboratory personnel were blinded with regard to case-control status.

Analyses of 2-hydroxyestrone (2-OHE1) and 16α-hydroxyestrone (16α-OHE1) were performed using a competitive solid-phase enzyme immunoassay (IMMUNA CARE Corporation, Bethlehem, PA).[19] The urinary forms of these estrogen metabolites are found as glucuronide conjugates and require the removal of the sugar moiety before recognition by the monoclonal antibodies. A mixture of β-glucuronidase and arylsulfatase (glusulase from *Helix pomatia*, Sigma Chemical Co., St. Louis, MO) was used for this purpose. The enzyme digest was then neutralized. Assay incubation time was 3 hours at room temperature. The assay was read kinetically using a Ceres 900 HDI plate reader (Biotek Instruments, Winooski, VT), and the data were reduced using Kineticalc EIA Application software (Bio-Tek® Instruments). Both

assays have been shown to demonstrate 100% recovery of metabolites with serial dilution and "spiking" of exogenous estrogens into urine samples. The EIA kits have been evaluated for validity and reproducibility and the values for each metabolite were compared with values obtained by gas chromatography-mass spectrometry.[19–24] As a measure of reproducibility, control samples were included and their values had to fall within two standard deviations from the mean of a continuous Levy-Jennings control plot. In addition, 10% duplicates were included with each batch of samples to determine reproducibility.

The within-assay coefficient of variation was 6% for samples at the urinary concentration levels for premenopausal women, and 13% for levels characteristic of postmenopausal women.

Statistical Analysis

2-hydroxyestrone and 16α-hydroxyestrone urinary levels were standardized by the total volume of urine collection. We used conditional logistic regression to obtain the odds ratios of breast cancer in relation to estrogen metabolites and their ratio.[25] The independent variables of interest were 2-OHE1, 16α-OHE1, and the ratio of 2-OHE1 and 16α-OHE1 by quintiles of serum concentration. We based the cutoff points for each quintile on the distribution of the estrogen metabolites in controls. Premenopausal women had approximately fourfold higher urinary 2-OHE1 and 16α-OHE1 than postmenopausal women (median of 40.3 ng/ml for 2-OHE1 and 17.5 ng/ml for 16α-OHE1 in premenopausal and 9.7 ng/ml and 4 ng/ml in postmenopausal women). Because of these differences in estrogen metabolite levels in urine, premenopausal and postmenopausal women were analyzed separately.

There were 73 eligible premenopausal breast cancer cases and 292 matched controls. We excluded six breast cancer cases and the 24 related controls because they were perimenopausal [mean age of 51, with a maximum of two menstrual bleedings in the year before recruitment into the study, with estrogen metabolite levels lower than premenopausal, and higher than postmenopausal women (median of 26.85 ng/ml for 2-OHE1, and 8.38 ng/ml for 16α-OHE1)]. In addition, we excluded four controls because of missing urine collection. Finally, 67 premenopausal breast cancer cases and 264 controls were available for the present analysis. In postmenopausal women, there were 71 breast cancer cases and 284 matched controls. We excluded 10 controls because of missing urine collection; the final analysis included 71 breast cancer cases and 274 controls.

We identified age, BMI, waist-to-hip ratio, age at menarche, age at first birth, parity, and age at menopause as

TABLE 1. Characteristics of the Study Participants

	Premenopause					Postmenopause			
	Cases		Controls			Cases		Controls	
	N	%	N	%		N	%	N	%
Age (yrs)					Age (yrs)				
35–41	23	34.3	72	27.1	43–55	16	22.5	66	24
42–45	10	14.9	74	28.4	56–58	21	29.6	79	29
46–48	16	23.9	66	24.9	59–62	12	16.9	58	21.2
49–57	18	26.9	52	19.6	63–70	22	31.0	71	25.8
BMI (kg/m^2)					BMI (kg/m^2)				
≤21.5	15	22.3	67	25.2	≤23.8	22	31.0	63	23.1
21.6–24.0	17	25.4	67	25.2	23.9–26.0	15	21.2	72	26.2
24.1–27.8	17	25.4	65	24.8	26.01–29.0	17	23.9	69	25.1
>27.8	18	26.9	65	24.8	>29.0	17	23.9	70	25.6
Waist-to-hip ratio					Waist-to-hip ratio				
≤0.74	14	20.9	72	27.4	≤0.77	21	29.6	59	21.4
0.75–0.78	12	17.9	73	27.9	0.78–0.81	16	22.5	65	23.8
0.79–0.81	19	28.4	51	19.1	0.82–0.85	15	21.2	78	28.4
>0.81	22	32.8	68	25.6	>0.85	19	26.7	72	26.4
Menarche (yrs)					Menarche (yrs)				
<12	13	19.4	56	21.2	<12	10	14.1	37	13.4
12	15	22.4	62	23.5	12	13	18.3	47	16.9
13	23	34.3	56	21.2	13	15	21.1	59	21.5
≥14	16	23.9	90	34.1	≥14	33	46.5	131	47.2
Age at 1st birth (yrs)					Age at 1st birth (yrs)				
≤22	19	32.2	56	23.6	≤23	18	29.5	76	30.8
23–24	12	20.4	60	25.3	24	6	9.9	25	10.1
25–27	10	16.9	63	26.6	25–27	21	34.3	78	31.6
≥28	18	30.5	58	24.5	≥28	16	26.3	68	27.5
Parity					Parity				
0	8	11.9	28	10.6	0	7	9.9	35	12.8
1	15	22.4	53	20.1	1	22	31.0	56	20.4
2	32	47.8	128	48.5	2	26	36.6	102	37.3
≥3	12	17.9	55	20.8	≥3	16	22.5	81	29.5
					Menopause (yrs)				
					≤46	17	23.9	84	30.6
					47–48	18	25.4	67	24.5
					49–52	24	33.8	72	26.3
					≥53	12	16.9	51	18.6

potential covariates according to their potential biologic relevance and logistic regression was used to control for these covariates. In the initial regression model, we examined all variables. We evaluated each covariate for confounding by removing each from the fully adjusted model. Age, BMI, waist-to-hip ratio, age at menarche, age at first birth, parity, and age at menopause did not substantially modify the results. None of the potential covariates was a confounder of the association between breast cancer and estrogen metabolites and their ratio. Nevertheless, we included them in further analysis to provide fully adjusted estimates for comparison with those reported in the published literature, in particular with the previous prospective cohort study.[16]

As last step of the analysis, we investigated the shape of the dose-response relation of estrogen metabolism, reflected by the 2-OHE1/16α-OHE1 ratio, and breast cancer risk using spline regression estimates in premenopausal and postmenopausal women. We included age, BMI, waist-to-hip, and reproductive variables in the model as parametric covariates (just as in the logistic model), while the 2-OHE1/16α-OHE1 ratio was included as a term smoothed by a smoothing spline. The fitted odds ratio from this model for each person was plotted as a function of the estrogen metabolite ratio. We used the median of the lowest quintile of the 2-OHE1/16α-OHE1 ratio as a reference.

Results

Characteristics of the study population are reported in Table 1. Among premenopausal women, cases were more likely to have higher BMI, and a higher waist-to-hip ratio, and to report an earlier age at menarche, later age at first full-term pregnancy, and nulliparity. For postmenopausal women, cases were similar to controls in age at menarche, and age at first full-term pregnancy. Cases reported lower number of children and older age at menopause and presented lower BMI, and waist-to-hip ratio.

In premenopausal women, the crude and the adjusted estimates for 2-OHE1 and for 16α-OHE1 were slightly higher than in postmenopausal women (Tables 2 and 3). Within each group of women, the risk ratio levels for both estrogen metabolites were similar, with wide confidence intervals. In premenopausal women (Table 2), the increase in 2-OHE1/16α-OHE1 ratio was associated with a reduction in odds ratios for breast cancer across quintiles: the highest quintile of 2-OHE1/16α-OHE1 ratio had the lowest risk estimate, even after adjustment for covariates.

Figure 1 shows the ORs for breast cancer as a function of the 2-OHE1/16α-OHE1 ratio estimated by smoothed regression splines. The ORs of breast cancer decreased with increases in the estrogen metabolites ratio.

In postmenopausal women (Table 3), there was no clear evidence of decreased breast cancer risk with increasing levels of 2-OHE1/16α-OHE1 ratio. In Figure 2, the plot of ORs, as a function of 2-OHE1/16α-OHE1 ratio, indicates that there was an increase in breast cancer risk in the second, third, and fourth quintile of 2-OHE1/16α-OHE1 ratio and a tendency toward a decrease in risk in the fifth quintile of the ratio.

Discussion

Results of this prospective nested case-control study suggest that the estrogen metabolism pathway favoring 2-hydroxylation over 16α-hydroxylation is associated with a reduced risk of invasive breast cancer risk, in particular in premenopausal women. In postmenopausal women, the association of estrogen metabolites and their ratio with breast cancer risk was weaker and the risk reduction was not evident.

There are a number of potential explanations for these findings, including physiological differences between pre-

TABLE 2. Breast Cancer Risk by Quintiles of Estrogen Metabolite Levels and Their Ratio in Premenopausal Women

	Cases/Controls	Crude RR	Adjusted RR*
2-hydroxyestrone (2-OHE1)			
I†	9/53	1.00	1.00
II	15/52	1.70 (0.68–4.22)	1.83 (0.73–4.60)
III	18/54	1.96 (0.81–4.75)	2.08 (0.85–5.09)
IV	15/53	1.67 (0.67–4.14)	1.85 (0.73–4.66)
V	10/52	1.13 (0.43–3.01)	1.23 (0.45–3.35)
16α-hydroxyestrone (16α-OHE1)			
I†	7/52	1.00	1.00
II	18/54	2.48 (0.96–6.42)	2.52 (0.97–6.58)
III	13/52	1.86 (0.69–5.03)	2.01 (0.74–5.52)
IV	17/53	2.38 (0.91–6.22)	2.62 (0.99–6.96)
V	12/53	1.68 (0.61–4.61)	1.90 (0.68–5.31)
2-OHE1/16α-OHE1 Ratio			
I†	19/54	1.00	1.00
II	14/51	0.78 (0.35–1.72)	0.76 (0.34–1.69)
III	11/52	0.60 (0.26–1.39)	0.60 (0.25–1.44)
IV	12/54	0.63 (0.28–1.43)	0.62 (0.27–1.45)
V	11/53	0.59 (0.26–1.36)	0.55 (0.23–1.32)

* ORs-adjusted for age, body mass index, waist-to-hip, and reproductive variables.
† Referent category.
Quintile definition for 2-OHE1: I, ≤18.94 ng/ml; II, 18.94–32.90 ng/ml; III, 32.90–53.91 ng/ml; IV, 53.91–86.88 ng/ml; V, ≥86.88 ng/ml.
Quintile definition for 16α-OHE1: I, ≤7.95 ng/ml; II, 7.95–14.51 ng/ml; III, 14.51–21.58 ng/ml; IV, 21.58–36.35 ng/ml; V, ≥36.35 ng/ml.
Quintile definition for 2-OHE1/16α-OHE1 Ratio: I, ≤1.80; II, 1.80–2.30; III, 2.30–2.72; IV, 2.72–3.29; V, ≥3.29.

TABLE 3. Breast Cancer Risk by Quintiles of Estrogen Metabolite Levels and Their Ratio in Postmenopausal Women

	Cases/Controls	Crude RR	Adjusted RR*
2-hydroxyestrone (2OHE1)			
I†	10/54	1.00	1.00
II	20/55	1.96 (0.84–4.58)	1.77 (0.75–4.20)
III	14/56	1.35 (0.55–3.30)	1.30 (0.52–3.21)
IV	11/54	1.10 (0.43–2.80)	0.94 (0.36–2.46)
V	16/55	1.57 (0.65–3.77)	1.61 (0.66–3.94)
16α-hydroxyestrone (16αOHE1)			
I†	11/53	1.00	1.00
II	17/57	1.44 (0.62–3.35)	1.35 (0.57–3.18)
III	13/55	1.14 (0.47–2.77)	1.01 (0.40–2.50)
IV	15/54	1.34 (0.56–3.18)	1.18 (0.48–2.87)
V	15/55	1.31 (0.55–3.12)	1.34 (0.55–3.27)
2OHE1/16α-OHE1 Ratio			
I†	12/54	1.0	1.0
II	16/55	1.31 (0.56–3.02)	1.42 (0.60–3.33)
III	17/54	1.42 (0.62–3.25)	1.41 (0.60–3.33)
IV	12/57	0.95 (0.39–2.29)	1.02 (0.41–2.53)
V	14/54	1.17 (0.49–2.75)	1.31 (0.53–3.18)

* ORs-adjusted for age, BMI, waist-to-hip and reproductive variables.
† Referent category.
Quintile definition for 2OHE1: I, ≤4.32 ng/ml; II, 4.32–7.86 ng/ml; III, 7.86–12.77 ng/ml; IV, 12.77–21.11 ng/ml; V, ≥21.11 ng/ml.
Quintile definition for 16α-OHE1: I, ≤1.81 ng/ml; II, 1.81–3.28 ng/ml; III, 3.28–5.08 ng/ml; IV, 5.08–8.15 ng/ml; V, ≥8.15 ng/ml.
Quintile definition for 2OHE1/16α-OHE1 Ratio: I, ≤1.77; II, 1.77–2.26; III, 2.26–2.80; IV, 2.80–3.66; V, ≥3.66.

menopausal and postmenopausal women in relation to the effect of estrogen metabolism on breast cancer risk. We can speculate that in women with the highest levels of biologically active estrogens, characteristic of premenopausal women, the weaker activity of 2-OHE1 may prevail in protecting target tissues from the action of the major active estrogens (2-OHE1 estrogen antagonist action). Conversely, in postmenopausal women with low estrogen concentration, estrogens with weak biological activity may support epithelial cell proliferation and neoplastic transformation (2-OHE1 estrogen agonist action).

Furthermore, reasons for the observed discrepancy between premenopausal and postmenopausal women may be related to exposure to breast cancer risk factors occurring after urine collection in postmenopausal women characterized by lower 2OHE1/16α-OHE1 ratio, such as increase in body weight or initiation of hormone replacement therapy. Unfortunately, up-dated information on exposure to breast cancer risk factors after recruitment is presently not available to support this interpretation of the study results. Finally, the lack of association of estrogen metabolism and breast cancer risk in postmenopausal women, might also be explained, at least in part, by the high technical variability of estrogen metabolite determinations observed in urine from this group of women. Postmenopausal women are characterized by fourfold lower urine estrogen metabolite levels than premenopausal women. At postmenopausal estrogen metabolite levels, we observed larger laboratory variability than at the level detected in premenopausal women (coefficients of variation were 13% and 6%, respectively). Biochemical analyses were conducted in blind fashion, and since the distribution of urinary levels of estrogen metabolites and their ratio was similar for breast cancer cases and related controls, the errors in hormone measurements were likely to be randomly distributed between breast cancer cases and controls resulting in an attenuation of the point estimates, in particular in postmenopausal women.[26]

Intra-individual variability and the long-term effect of cryopreservation are additional sources of variability in the determination of estrogen metabolites potentially affecting the study results. In fact, the present study was based on one single urine collection and data from a previously published study[27] showed that 2-hydroxyestrone and 16α-hydroxyestrone are characterized by a certain level of measurement error due to intra-individual variability (intraclass correlation coefficients were R = 0.79 for 2-OHE1 and R = 0.62 for 16α-OHE1), which may contribute to the attenuation of the observed risks. Sample degradation might be expected to introduce a source of variability in prospective cohort studies; however, data from a previous prospective study

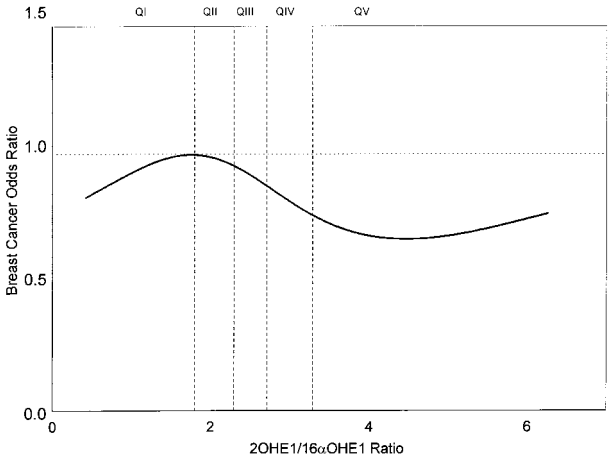

FIGURE 1. Premenopausal women: Spline curve of the semi-parametric fit of the 2-OHE1/16α-OHE1 ratio after adjustment for age, BMI, waist-to-hip ratio, and reproductive variables with the 10th percentile of the 2-OHE1/16α-OHE1 ratio as reference. Quintiles of the ratio are indicated by the letters Q1 to Q5.

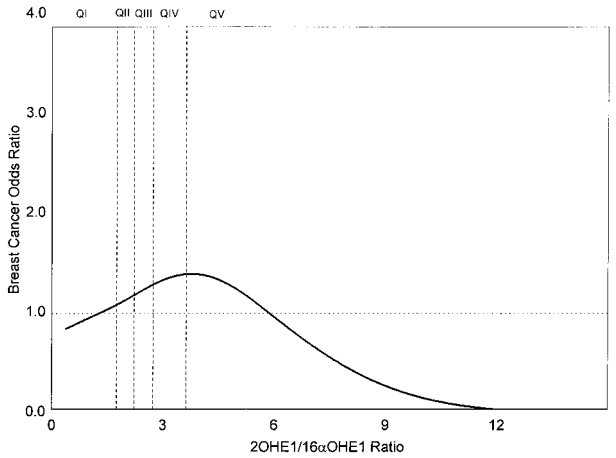

FIGURE 2. Postmenopausal women: Spline curve of the semi-parametric fit of the 2-OHE1/16α-OHE1 ratio after adjustment for age, BMI, waist-to-hip ratio, and reproductive variables with the 10th percentile of the 2-OHE1/16α-OHE1 ratio as reference. Quintiles of the ratio are indicated by the letters Q1 to Q5.

showed stability of estrogen metabolites over time.[16] Furthermore, cases and controls were matched on date at sample collection. Thus, any degradation effect should be minimal and similar for cases and controls.

The present results may also be compatible with a potential role of estrogen metabolism as an indicator of undiagnosed breast cancer rather than a precursor. The 40% decrease in breast cancer risk observed in our study for the highest quintile of premenopausal women, however, was similar to the results of the Guernsey prospective study after 19 years of follow-up.[16]

The present prospective study and the previous one differed in number of breast cancer cases in postmenopausal status (42 in the Guernsey study *vs* 71 in the ORDET study), and in the findings concerning postmenopausal women: the Guernsey study found similar results in premenopausal and in postmenopausal women. Participants of the two prospective studies were similar in age, BMI, and in the distribution of classical breast cancer risk factors. The concordant findings, in particular among premenopausal women, are suggestive of an effect of estrogen metabolism on breast cancer risk.

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