

# Urinary Estrogen Metabolites and Mammographic Parenchymal Patterns in Postmenopausal Women<sup>1</sup>

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## Abstract

It has been hypothesized that women who metabolize their endogenous estrogens predominantly via  $16_{\alpha}$ -hydroxylation rather than via 2-hydroxylation and, as a result, have a low ratio of 2-hydroxyestrone (2-OHE1): $16_{\alpha}$ -hydroxyestrone ( $16_{\alpha}$ -OHE1) are at an increased risk of breast cancer. Epidemiological evidence in support of this hypothesis is scarce and mostly based on measurements made after the onset of the disease. To gain insight into the role of these metabolites in the etiology of breast cancer, we assessed their relationship with high-density Wolfe mammographic parenchymal patterns (P2/DY), a recognized indicator of risk of this tumor. The study was nested within a large cross-sectional survey on determinants of mammographic patterns carried out in a population-based breast screening program in Northern Greece. Urinary levels of 2-OHE1 and  $16_{\alpha}$ -OHE1 were measured in a random sample of 70 postmenopausal women with P2/DY mammographic patterns and in a random sample of 70 women with N1 mammographic patterns, individually matched to the P2/DY women on year of birth, years since menopause and date of urine collection. Women with a P2/DY pattern had, on average, 58% higher levels of 2-OHE1 ( $P = 0.002$ ) and 15% higher levels of  $16_{\alpha}$ -OHE1 ( $P = 0.37$ ) than those with an N1 pattern. The ratio of 2-OHE1: $16_{\alpha}$ -OHE1 was 35% higher ( $P = 0.005$ ) in women with a P2/DY pattern. Women in the highest one-third of this ratio were six times more likely to have a P2/DY pattern than those in the lowest one-third after adjusting for potential confounders (prevalence odds

ratio, 6.2; 95% CI, 1.7–22.9; test for linear trend,  $P = 0.002$ ). These findings seem to suggest that a high, rather than a low, 2-OHE1: $16_{\alpha}$ -OHE1 ratio may be associated with an increase in breast cancer risk at postmenopausal ages, unless the pathway through which estrogen metabolites may affect breast cancer risk is unrelated to mammographic parenchymal patterns.

## Introduction

There is strong evidence that endogenous estrogens play an important role in the etiology of breast cancer. Bilateral oophorectomy at young ages protects against breast cancer and both age at menarche and age at menopause (which identify the start and the end of a woman's ovarian activity) affect the risk of this cancer. More direct evidence comes from prospective studies showing that high levels of serum estrogens are associated with increasing risks of subsequent breast cancer (1–3).

Estrogens are metabolized by two mutually exclusive main pathways: 2-hydroxylation and  $16_{\alpha}$ -hydroxylation. The 2- and  $16_{\alpha}$ -hydroxylated metabolites are believed to have different biological properties. Experimental evidence indicates that  $16_{\alpha}$ -hydroxylated metabolites are biologically active, being able to bind to the estrogen receptor (4). These metabolites seem to be genotoxic (5) and the extent of  $16_{\alpha}$ -hydroxylation has been correlated with the incidence of mammary tumors in mice (6). In contrast, the 2-hydroxylated metabolites are thought to be biologically inactive (6), although this has been questioned by some researchers (7, 8). On the basis of this experimental evidence, Bradlow *et al.* (9) have hypothesized that the relative levels of 2- and  $16_{\alpha}$ -hydroxylated metabolites may play a role in the etiology of breast cancer, with a low 2: $16_{\alpha}$  ratio associated with a high risk of breast cancer.

Clarification of the role of estrogen metabolites in the etiology of breast cancer is important because, in contrast to most known risk factors, there is some evidence that they may be amenable to modulation through changes in behavior (10). Thus, if confirmed to be associated with breast cancer, prevention strategies based on dietary modification and physical exercise could potentially be used to induce favorable changes in the levels of these metabolites.

Epidemiological data in support of the hypothesis that a low 2: $16_{\alpha}$  ratio is associated with a high breast cancer risk are scarce, however. Various case-control studies (10–16) have thus far examined the relationship between estrogen metabolites and breast cancer, but their results have been largely inconsistent. One of the main limitations of these studies is that they had to rely on measurements of estrogen metabolites made

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<sup>3</sup> The abbreviations used are: 2: $16_{\alpha}$  (ratio), 2-hydroxylated metabolites: $16_{\alpha}$ -hydroxylated metabolites (ratio); BMI, body mass index; 2-OHE1, 2-hydroxyestrone;  $16_{\alpha}$ -OHE1,  $16_{\alpha}$ -hydroxyestrone; OR, odds ratio; CI, confidence interval.

after the onset of breast cancer and, therefore, on levels that might have been altered by the disease itself and/or by its treatment. In only two studies (17, 18) were samples collected prospectively several years before the onset of breast cancer. In one (17), the number of breast cancer cases was regarded as too small (60 pre- and 42 postmenopausal cases) to allow any definite conclusions, whereas in the other (18), the effect of the  $2:16_{\alpha}$  ratio differed by menopausal status: a low ratio was associated with a subsequent high breast cancer risk at premenopausal ages but with a moderately decreased risk at postmenopausal ages.

Mammographic density is a well-established risk factor for breast cancer (19–22), which is being increasingly used as an intermediate marker for this tumor in many epidemiological studies (23–25). The qualitative classification originally developed by Wolfe (19) is a standard method for classifying mammographic parenchymal patterns that takes into account density as well as other mammographic features. Hence, to gain insight into the role of estrogen metabolites in the pathogenesis of breast cancer, we have examined their relationship with high-density Wolfe mammographic parenchymal patterns (P2/DY).

## Materials and Methods

**Study Subjects.** The Ormylia Screening Program, which comprises one single screening center located in the village of Ormylia, is a population-based screening program set up in 1992 with the aim of providing a mammographic examination every three years to all women aged 40–65 years resident in the region of Halkidiki, Northern Greece. This is a predominantly rural region with a total population of about 92,000. The total coverage of the program was estimated to be only about 65% in 1997–8, when our study was conducted, mainly because of its staggered introduction across the region.

A large cross-sectional study was conducted within this program to identify determinants of mammographic parenchymal patterns in postmenopausal women. A random sample of 900 postmenopausal women who attended the screening program between November 1997 and June 1998 completed a detailed interviewer-administered questionnaire. The study subjects were randomly selected from all postmenopausal women resident in the catchment area who were invited to attend the screening program during the study period. Women were considered as postmenopausal if their menses had stopped for at least 12 months or they had undergone hysterectomy and/or bilateral oophorectomy. The random sample corresponded to 23% of the total eligible population. Only 3% of the women randomly selected refused to participate or failed to attend the program during the study period; these were replaced by other eligible women. The interviewer-administered questionnaire included questions on demographic and socioeconomic factors (*e.g.*, educational level, occupational history), general health and reproductive history. It did not contain specific questions on dietary habits although it included a question on whether women adhered to the fasting periods of the Christian-Orthodox religion. These involve complete abstinence from any type of meat and animal products (*e.g.*, eggs and dairy products) and, sometimes, fish for several periods, which may reach a total of 27 weeks per year. The interviewers were blind to the study hypothesis and to the mammographic results.

In addition to their routine mammographic examination, all of the participants had several anthropometric measurements taken and were asked to produce a spot urine sample. The anthropometric measurements were taken using standardized

procedures with women barefoot and wearing only underwear. Waist was measured at the smallest circumference between the costal margin and the iliac crest and the hips at the largest circumference over the maximum extension of the buttocks. Breast size was calculated as the difference between the largest breast circumference (mostly at the nipple level) and the chest circumference (thoracic cage). The measurements for each woman were performed independently by two interviewers and their average used in the analysis. Women were also asked to choose from a set of nine somatotype drawings (26), those that best reflected their body builds at ages 18 years and at interview. Recall data based on these drawings have been shown to be highly correlated with past measurements of BMI (27). In the analysis, drawings 1–3 were taken as representing a lean figure, drawings 4–6 a medium build figure, and drawings 7–9 an obese figure.

Each set of mammograms (two views per breast) was read by one of the two radiologists working in the Screening Program who assigned them to one of the four Wolfe patterns (N1, P1, P2, or DY; Ref. 28) using standard criteria (19). The radiologists were kept blind to the interview data and the study hypothesis. A reliability study conducted on a random sample of 100 mammograms showed high levels of intra- and interobserver agreement when they were recategorized into a low- (N1 or P1) or a high- (P2 or DY) density category ( $\kappa$  statistics equal to 95 and 90%, respectively). The intra- and interobserver agreement for the full four-category classification were slightly lower ( $\kappa$  statistics equal to 90 and 75%, respectively).

To examine the relationship between urinary estrogen metabolites and mammographic parenchymal patterns, a small study was conducted within this cross-sectional survey of 900 postmenopausal women. Women were not eligible for this study if they: (a) had undergone hysterectomy or oophorectomy ( $n = 97$ , 11% of the total 900 women); (b) had ever taken hormone replacement therapy, fertility drugs, oral contraceptives, or any other hormonal drugs ( $n = 105$ , 12%); (c) reported a previous history of any type of cancer or of any benign breast disease requiring hormonal treatment ( $n = 9$ , 1%); or (d) reported a family history of breast cancer ( $n = 37$ , 4%). Thus, a total of 652 women were eligible for the study, of whom 215 (33%) had an N1 pattern and 161 (25%) had a P2/DY pattern. A total of 70 women were randomly selected from all of the eligible women with a P2/DY mammographic pattern. A similar number of women were randomly selected from all those with an N1 pattern individually matched to the P2/DY women on year of birth (within 1 year), years since menopause (according to five categories: 1–2, 3–5, 6–10, 11–15, and  $\geq 16$  years), and date of urine collection (within 1 month). Women with an N1 mammographic pattern were taken as having low-density Wolfe patterns, whereas those with P2 or DY patterns as having high-density Wolfe patterns. The latter group comprised both P2 and DY patterns because the prevalence of women with DY patterns is low at postmenopausal ages (only nine eligible women had a DY pattern).

The study protocol was approved by the relevant ethical committees. Each subject was asked to give her informed consent before entering the study, and all appropriate measures were taken to ensure confidentiality of the data.

**Sample Collection and Laboratory Measurements.** Urine samples were collected throughout the study period, from November 1997 to June 1998, from all of the 900 women participating in the cross-sectional study on determinants of mammographic patterns. Vitamin C (5 mg/ml urine) was added to each sample immediately after collection to act as a preserva-

tive. The samples were stored at  $-20^{\circ}\text{C}$  within 4–5 h after collection and were never thawed prior to the estrogen metabolites assay. Aliquots were shipped (on dry ice) to the Strang-Cornell Cancer Research Laboratory in New York, where the levels of estrogen metabolites were measured using a newly improved immunoassay (29). This immunoassay has been described in detail elsewhere (29–33). In short, it involves the use of commercially available competitive enzyme immunoassay (EIA) kits (Immunacare, Inc., Bethlehem, PA) to measure 2-OHE1 and  $16_{\alpha}$ -OHE1 in urine samples. Because the estrogen metabolites are excreted in the urine as glucuronide and sulfate conjugates, a mixture of  $\beta$ -glucuronidase and arylsulfatase (glusulase from *Helix pomatia*, Sigma Chemical Co.) was used to cleave these conjugates prior to the assay. The enzyme digest was then neutralized and 10- $\mu\text{l}$  samples aliquoted and incubated at room temperature for 3 h to facilitate antibody binding. The assay was read kinetically using a Ceres 900 HDI plate reader (Biotek Instruments, Winnooski, VT) with analysis by Kineticale EIA Application software (Biotek Instruments). This newly developed immunoassay has been shown to be sensitive enough to measure the low levels of estrogen metabolites present in postmenopausal women (29, 33). The intraclass correlation coefficients for 2-OHE1,  $16_{\alpha}$ -OHE1, and their ratio, have been shown to range from 80 to 95% (30, 33). Validation of this immunoassay against gas chromatography-mass spectrometry (GC-MS) yielded correlation coefficients of  $\sim 90\%$  for both 2-OHE1 and  $16_{\alpha}$ -OHE1 (29, 30, 32, 33).

The samples were analyzed in matched pairs to minimize interbatch variation. For each sample, measurements were made in triplicate, and the average value used. Standards were also assayed in each microtiter plate, along with the urine samples being analyzed. Creatinine levels were measured to provide estrogen metabolite values corrected for volume. None of the samples had creatinine levels  $< 0.20$  mg/ml, concentrations at which the assay is believed to be less precise (29). The laboratory staff was kept blind to the interview data and the mammographic results. To assess the reliability of the measurements, a random subset of 10 samples were sent in duplicate, laboratory staff being unaware of their identity.

**Statistical Methods.** The levels of the individual metabolites 2-OHE1 and  $16_{\alpha}$ -OHE1 were first corrected for urine volume by dividing their values by the corresponding creatinine level. The agreement between the 10 duplicate measurements of the metabolites was assessed by graphical inspection, as suggested by Bland and Altman (34), and by estimating intraclass correlation coefficients. Differences in metabolite levels between N1 and P2/DY women were described in terms of their medians and were initially assessed using the Wilcoxon matched-pairs signed-rank test (35).

The association between reproductive and anthropometric variables, which were regarded as potential confounding variables in this study, and P2/DY mammographic parenchymal patterns was investigated by first categorizing each exposure variable into thirds or fourths on the basis of its distribution among all 652 eligible women. Conditional logistic regression (36) was then used to take into account the matching factors, and a stepwise procedure was followed to identify the variables with the strongest independent association with P2/DY mammographic patterns. Similarly, the association with estrogen metabolites was assessed by first categorizing the values of the 2:16 $_{\alpha}$  ratio, and of each of the two individual metabolites, into thirds according to their distribution among women with an N1 pattern. Conditional logistic regression was used to calculate ORs for the top two-thirds of the metabolites *versus* the lowest

one-third with, and without, controlling for the reproductive and anthropometric factors identified by the stepwise procedure. These adjustments were performed by treating age at first birth and BMI as continuous variables to reduce the number of parameters required. Because of the nature of the study design, all of the estimated ORs are to be interpreted as prevalence ORs. Heterogeneity and linear trends among the category-specific estimates were assessed via likelihood ratio tests (36). Finally, to identify possible determinants of estrogen metabolism, the association between levels of estrogen metabolites (separately and as a ratio) and each of the reproductive and anthropometric variables available in this study was examined by calculating their rank correlations among women with an N1 pattern. All of the analyses were carried out in STATA.<sup>4</sup>

## Results

The mean difference between the first and the second measurements of the 2:16 $_{\alpha}$  ratio in the 10 duplicate samples was 0.014 with a SD of 0.321. All of the paired differences lay within the interval defined by mean difference  $\pm 2 \times \text{SD}$  ( $-0.656$  to  $0.628$ ) and there was no relationship between the magnitude of the differences and their mean. The intraclass correlation coefficients for the 2:16 $_{\alpha}$  ratio, 2-OHE1, and  $16_{\alpha}$ -OHE1 in this small sample were 76, 78, and 69%, respectively.

Table 1 shows the distribution of baseline characteristics by mammographic parenchymal patterns. Women with a P2/DY pattern had, as expected, similar ages at mammography and years since menopause (matching factors) as those with an N1 pattern and, hence, similar ages at menopause. P2/DY and N1 women had also similar ages at menarche, but P2/DY women had their first pregnancy and first birth later, had fewer children, although they reported slightly more abortions, and breastfed on average for shorter periods than N1 women. There were no differences between P2/DY and N1 women in relation to height, but P2/DY women weighed, on average, 4 kg less than N1 women and, hence, had lower average BMI. Although P2/DY and N1 women had similar waist:hip ratios, P2/DY women had smaller chest circumferences and smaller breasts than N1 women. A smaller proportion of P2/DY than N1 women reported marked changes in body build from a lean- or medium-build figure at age 18 years to an obese figure at the time of interview. P2/DY women had a 58% higher median level of 2-OHE1 (matched pairs test,  $P = 0.002$ ) and a 15% higher median level of  $16_{\alpha}$ -OHE1 ( $P = 0.37$ ) than N1 women. As a result, the 2:16 $_{\alpha}$  ratio was 35% ( $P = 0.005$ ) higher among P2/DY women.

Multivariate analyses identified age at first birth, current BMI, and self-reported increase in body build from age 18 to the time of interview as the strongest determinants of P2/DY mammographic parenchymal patterns among the reproductive and anthropometric variables available in this study (Table 2).

In agreement with the matched pairs test results, 54% (38 of 70) of the P2/DY women but only 31% (22 of 70) of the N1 women were in the top one-third of the 2:16 $_{\alpha}$  ratio (Table 3). The crude conditional logistic regression analysis showed that women in the top one-third of the 2:16 $_{\alpha}$  ratio were four times more likely to have a P2/DY Wolfe mammographic pattern than women in the lowest one-third, with a highly significant linear trend across the three categories. This association became even stronger (OR, 6.23; 95% CI, 1.70–22.89) after adjusting

<sup>4</sup> Stata Statistical Software, Release 6.0., 1999; Stata Corp., College Station, Texas.

Table 1 Baseline characteristics of women with P2/DY and N1 Wolfe mammographic parenchymal patterns

Variables	P2/DY women (n = 70)				N1 women (n = 70)			
	Mean <sup>a</sup>	(SD)	(Q1, Q3) <sup>b</sup>	%	Mean <sup>a</sup>	(SD)	(Q1, Q3) <sup>b</sup>	%
Matching variables								
Age at mammography	57.9	(5.3)	(53, 61)		57.8	(5.3)	(53, 61)	
Years since menopause	5.0		(2, 12)		5.0		(3, 12)	
Age at menopause	49.9	(4.3)	(48, 52)		49.5	(4.5)	(47, 52)	
Reproductive-related variables								
Age at menarche	13.3	(1.5)	(12, 14)		13.5	(2.3)	(12, 14)	
Age at first pregnancy <sup>c</sup>	24.7	(4.3)	(20, 24)		22.5	(3.9)	(22, 28)	
Age at first birth <sup>d</sup>	24.8	(4.0)	(22, 28)		22.8	(3.9)	(20, 24)	
Months ever breastfed <sup>d</sup>	12		(8, 20)		14.5		(9, 23)	
With 1+ children				94.3				98.6
With 1+ abortions				42.8				41.4
Anthropometric variables								
Height (cm)	155.2	(5.8)	(152, 158)		155.2	(5.6)	(151, 158)	
Weight (kg)	75.4	(10.3)	(68.5, 79)		79	(11.3)	(71.5, 86.5)	
BMI (kg/m <sup>2</sup> )	31.4	(4.7)	(28.2, 34.1)		32.8	(4.5)	(29.9, 35.7)	
Waist circumference (cm)	88.8	(9.1)	(84, 93)		92.0	(9.7)	(86, 96)	
Hip circumference (cm)	118.2	(8.3)	(112, 125)		120	(8.8)	(115, 127)	
Waist:hip ratio	0.75	(0.06)	(0.71, 0.79)		0.77	(0.09)	(0.72, 0.79)	
Chest circumference (cm)	91.5	(8.1)	(85, 97)		94	(7.4)	(89, 98)	
Breast size (cm)	12.1	(4.3)	(9, 14)		13.3	(4.3)	(10, 16)	
Large body build at age 18 <sup>e</sup>				0				3
Large body build at interview <sup>e</sup>				7				20
Marked increase in body build <sup>f</sup>				7				20
Urinary estrogen metabolites								
2:16 $\alpha$ -OHE1	1.12		(0.80, 1.73)		0.83		(0.59, 1.28)	
2-OHE1	8.53		(4.64, 11.87)		5.39		(3.91, 8.67)	
16 $\alpha$ -OHE1	7.50		(4.65, 11.10)		6.55		(4.63, 8.80)	

<sup>a</sup> Medians instead of means and SDs are reported when the distribution was too skewed.

<sup>b</sup> (Q1, Q3): interquartile range.

<sup>c</sup> Ever-pregnant women only.

<sup>d</sup> Parous women only.

<sup>e</sup> Large body build defined as self-reported obese figure (somatotypes 7–9; see “Materials and Methods”).

<sup>f</sup> Marked increases in body build defined as self-reported change from a lean (somatotypes 1–3)- or medium (somatotypes 4–6)-build figure at age 18 years to an obese figure (somatotypes 7–9) at the time of the interview (see “Materials and Methods”).

for age at first birth, current BMI, and self-reported increase in body build from age 18 (Table 3), the three independent determinants of P2/DY patterns identified in Table 2. Examination of the separate associations between the two metabolites and P2/DY patterns showed a statistically significant linear trend across the 2-OHE1 thirds but not across the 16 $\alpha$ -OHE1 thirds (Table 3). The magnitude of the stratum-specific estimates suggested, however, that the associations between levels of individual estrogen metabolites, and in particular 2-OHE1, and P2/DY patterns may be J-shaped rather than linear. The exclusion of five women who were current smokers and three who were ex-smokers at the time of interview did not affect the results (the test for linear trend for the 2:16 $\alpha$  ratio remained as statistically significant in both crude and adjusted analyses;  $P = 0.002$ ). Similarly, adjustments for adherence to the fasting periods of the Christian-Orthodox religion as a marker of traditional Mediterranean diet, or for educational level and type of occupation as crude markers of physical exercise, made no difference to the results.

The 2:16 $\alpha$  ratio among women with an N1 pattern was not associated with any of the measured baseline reproductive or anthropometric variables, apart from a positive correlation with age at menopause [rank correlation ( $r$ ) 0.27;  $P = 0.02$ ] and a negative correlation with years since menopause ( $r$ ,  $-0.31$ ;  $P = 0.008$ ). These findings were mainly determined by the correlation of these factors with 2-OHE1. The 16 $\alpha$ -OHE1 metabolite was only negatively correlated with waist:hip ratio ( $r$ ,  $-0.31$ ;  $P = 0.007$ ).

## Discussion

The main aim of this study was to assess whether the 2:16 $\alpha$  ratio was associated with mammographic density as a way of gaining insight into the role of these metabolites in the etiology of breast cancer. This approach seems justified because several epidemiological studies have reported the magnitude of the breast cancer risk associated with increased mammographic density to be about a 4- to 6-fold increase when density is measured on a continuous scale as percentage of breast density, and a 2- to 4-fold increase when, as in the present study, breast density is measured indirectly by P2/DY Wolfe patterns (37). Breast cancer and mammographic density are known to share many reproductive-related risk factors and are, therefore, likely to have a common, possibly hormone-related, etiology.

Our study found that a high 2:16 $\alpha$  ratio was associated with P2/DY Wolfe mammographic patterns. This finding contrasts with the hypothesis proposed by Bradlow *et al.* (9) that 2-hydroxylated metabolites are “good estrogens.” Our results were, however, broadly consistent with those from the largest case-control study conducted thus far (66 cases and 76 controls) to examine the relationship between estrogen metabolites and postmenopausal breast cancer (16). In that study, women in the highest third of the 2:16 $\alpha$  ratio were at a slightly increased risk of breast cancer, although not significant, relative to those in the lowest third (OR, 1.13; 95% CI, 0.46–2.78). This study, however, included only prevalent cancer cases (38). Thus, its results may have been distorted by the possible relationship between

Table 2 ORs and 95% CIs for P2/DY versus N1 Wolfe mammographic parenchymal patterns by baseline characteristics selected in the multivariate analysis

Variables	P2/DY women (n = 70)	N1 women (n = 70)	Univariate analysis <sup>a</sup>		Multivariate analysis <sup>b</sup>	
			OR	(95% CI)	OR	(95% CI)
Age at first birth (yr) <sup>c</sup>						
≤20	8	19	1		1	
21–23	21	26	1.54	(0.52–4.57)	1.34	(0.39–4.56)
24–26	18	14	2.81	(0.90–8.80)	3.75	(1.00–14.03)
≥27	19	10	4.51	(1.31–15.49)	4.10	(0.90–18.72)
Test for heterogeneity <sup>d</sup>			P = 0.06		P = 0.12	
Test for linear trend <sup>e</sup>			P = 0.006		P = 0.02	
BMI (kg/m <sup>2</sup> )						
<30	24	11	1		1	
30–31	17	18	0.42	(0.15–1.13)	0.35	(0.11–1.09)
32–34	15	22	0.33	(0.12–0.87)	0.27	(0.09–0.83)
≥35	14	19	0.31	(0.10–0.91)	0.40	(0.11–1.49)
Test for heterogeneity <sup>d</sup>			P = 0.07		P = 0.13	
Test for linear trend <sup>e</sup>			P = 0.02		P = 0.09	
Changes in body build from age 18 to interview						
No change	21	13	1		1	
Moderate increase in body build	44	43	0.58	(0.26–1.31)	0.49	(0.18–1.33)
Marked increase in body build	5	14	0.11	(0.02–0.60)	0.10	(0.01–0.72)
Test for heterogeneity <sup>d</sup>			P = 0.01		P = 0.07	
Test for linear trend <sup>e</sup>			P = 0.008		P = 0.02	

<sup>a</sup> ORs and 95% CI computed using conditional logistic regression to account for matching factors.

<sup>b</sup> ORs and 95% CI computed using conditional logistic regression to account for matching factors and adjusting for all of the other variables in the table.

<sup>c</sup> Among parous women only.

<sup>d</sup> Likelihood ratio test for heterogeneity among the stratum-specific ORs.

<sup>e</sup> Likelihood ratio test for linear trend among the stratum-specific ORs.

high levels of the metabolites ratio and longer survival of the cases (39). At an ecological level, our results are consistent with observed differences in estrogen metabolite levels across population groups with different underlying breast cancer risks. In one study (40), the ratio of 2-hydroxylated to 16 $\alpha$ -hydroxylated metabolites was 4–5 times higher in healthy premenopausal Finnish women (a high breast cancer-risk population) than in healthy premenopausal Oriental women (a low-risk population).

By contrast, other case-control studies (10, 11, 13–15) have supported the hypothesis that a low 2:16 $\alpha$  ratio may be associated with an increased risk of breast cancer. These case-control studies, however, suffer from various methodological problems. Similarly to the case-control study by Ursin *et al.* (16) described above, the retrospective nature of their design implied that the measurement of estrogen metabolites was done after the onset of breast cancer and, therefore, the levels could have been affected by the disease and its treatment. Further, most of these studies were based on small convenience samples and in very few were any attempts made to control for the effect of any potential confounders.

The association between estrogen metabolites and breast cancer has been evaluated prospectively in only two studies (17, 18). In one (17), the findings pointed to an inverse relation to the one we have found in the present study, but the number of cases was regarded as too small (60 premenopausal and 42 postmenopausal cases) to allow any definite conclusions. In the other (18), similarly to ours, the data were consistent with a high 2:16 $\alpha$  ratio being associated with a high risk of breast cancer in postmenopausal women.

Little is known about the relationship between levels of endogenous estrogens and mammographic features, but preliminary results from the Nurses' Health Study showed a positive

correlation between free percentage of estradiol and percentage of breast density (41). Indirect support for a role of endogenous estrogens in determining high-density patterns is also provided by studies showing that mammographic density increases with the use of estrogen replacement therapy (42, 43) and decreases with the administration of tamoxifen (25) as well as with hormonal contraceptive regimens designed to suppress ovarian function (23). Our study showed that women with P2/DY patterns had, on average, higher levels of 2-OHE1 and 16 $\alpha$ -OHE1 than women with N1 patterns, although this difference was statistically significant only for 2-OHE1. These findings are consistent with those reported for breast cancer in the prospective study by Muti *et al.* (18). In their study, both pre- and postmenopausal breast cancer cases had higher levels of each of these individual metabolites prior to the onset of the disease relative to healthy age-matched controls (18). These findings, taken together with those from our study, seem to suggest that estrogen metabolism may play a role in the etiology of postmenopausal breast cancer and that this effect may be mediated, at least in part, by mammographic parenchymal patterns. Additional studies are needed, however, to establish whether this potential effect of estrogen metabolism is independent of that of serum levels of estrogens. The lack of a clear relationship between levels of urinary estrogen metabolites and serum concentrations of estradiol in postmenopausal women, observed in one study (17), would suggest that the effect of estrogen metabolites on the etiology of breast cancer would be independent of the etiology of estrogens.

Our study has several strengths. The results are unlikely to have been affected by selection bias because the study was nested within a large cross-sectional survey on determinants of mammographic patterns in postmenopausal women conducted in a population-based screening program, and the level of

Table 3 Crude and adjusted ORs and 95% CIs of P2/DY versus N1 Wolfe mammographic parenchymal patterns by thirds of urinary levels of estrogen metabolites and their ratio

Variable	P2/DY women (n = 70)	N1 women (n = 70)	Crude <sup>a</sup>		Adjusted <sup>b</sup>	
			OR	(95% CI)	OR	(95% CI)
2:16 $\alpha$ -OHE1 ratio						
≤0.66	11	23	1		1	
0.67–1.08	21	25	1.67	(0.68–4.14)	1.79	(0.54–5.95)
≥1.09	38	22	4.07	(1.52–10.91)	6.23	(1.70–22.89)
Test for heterogeneity <sup>c</sup>				P = 0.008		P = 0.005
Test for linear trend <sup>d</sup>				P = 0.002		P = 0.002
2-OHE1 (ng/ml creatinine)						
≤5.00	19	22	1		1	
5.01–6.58	4	25	0.21	(0.06–0.81)	0.12	(0.02–0.71)
≥6.59	47	23	2.21	(0.91–5.35)	2.17	(0.71–6.67)
Test for heterogeneity <sup>c</sup>				P < 0.001		P = 0.0001
Test for linear trend <sup>d</sup>				P = 0.008		P = 0.03
16 $\alpha$ -OHE1 (ng/ml creatinine)						
≤5.37	22	23	1		1	
5.38–7.85	16	24	0.72	(0.30–1.73)	0.56	(0.17–1.82)
≥7.86	32	23	1.69	(0.68–4.21)	1.09	(0.37–3.24)
Test for heterogeneity <sup>c</sup>				P = 0.16		P = 0.44
Test for linear trend <sup>d</sup>				P = 0.26		P = 0.78

<sup>a</sup> ORs and 95% CIs computed separately for each variable using conditional logistic regression to account for matching factors.

<sup>b</sup> ORs and 95% CIs computed separately for each variable using conditional logistic regression to account for matching factors and controlling for parity, age at first birth (continuous), current measured BMI (continuous), and changes in body build from age 18 to interview.

<sup>c</sup> Likelihood ratio test for heterogeneity among the stratum-specific ORs.

<sup>d</sup> Likelihood ratio test for linear trend among the stratum-specific ORs.

participation in the survey was very high (97%). The reliability of the laboratory assays is known to be high with intraclass correlation coefficients ranging from 80 to 95% (33); the reliability values observed in the present study were somewhat lower but were based on a rather small sample (only 10 duplicate measurements). However, as laboratory staff was kept blind to the mammographic results, any exposure misclassification would have been nondifferential, and, hence, the observed results would be an underestimation of the true association between estrogen metabolites and mammographic patterns. Classification of mammograms into Wolfe patterns is subjective but the levels of intra- and interagreement that we observed were high and of a magnitude that was similar to those reported in other studies (21); moreover, blind evaluation of mammograms eliminated the possibility of bias in the way the outcome was ascertained. Confounding was controlled as well as possible through multiple logistic regression. The reproductive and anthropometric variables identified as being the main determinants of P2/DY patterns in the present analyses were similar to those identified in the main cross-sectional study,<sup>5</sup> and in agreement with those reported by other studies (44, 45).

Various issues should, however, be taken into account in the interpretation of our findings. First, the Wolfe classification categorizes mammographic parenchymal patterns by taking into account breast density as well as other mammographic features. Thus, the use of a continuous measure of density, such as percentage of breast density, would have allowed a more precise quantification of the role of estrogen metabolites on mammographic density and should be pursued in future studies. Second, because of the nature of the outcome of interest and the

cross-sectional design of our study, all of the ORs should be interpreted as prevalence ORs. As a result, it is not possible to establish whether the increase in the 2:16 $\alpha$  ratio preceded, or not, high-density Wolfe patterns. The presence of a dose-response relationship between the thirds of the 2:16 $\alpha$  ratio and the prevalence of P2/DY patterns, however, seems to strengthen the evidence in favor of the ratio being a determinant and not *vice versa*. Third, similarly to other researchers, we relied on “spot” urine samples rather than 24-h collections, but we measured creatinine levels to estimate metabolite levels corrected for volume (although, of course, such corrections made no difference to the levels of the 2:16 $\alpha$  ratio). Fourth, we aimed at obtaining morning urine samples from all of the participants, but because of the logistics of the screening program, this was not possible for 24% of them. Adjustment in the analysis for time of urine collection did not affect our results, however. This is consistent with findings from previous studies showing little diurnal variation in the urinary excretion of these metabolites (29, 46). Fifth, P2/DY and N1 women were individually matched on date of urine collection to avoid any effect that storage time might have had on the levels of estrogen metabolites, and interbatch variation was minimized by analyzing the samples in matched pairs.

Little is known about the determinants of estrogen metabolism. In our study, the 2:16 $\alpha$  ratio was positively associated with age at menopause and negatively associated with years since menopause. It is interesting to note that, with the exception of the prospective study by Meilahn *et al.* (17), none of the other studies took into account the potential confounding effect of these two variables. There is some evidence that cigarette smoking (47) may increase the 2:16 $\alpha$  ratio. Very few women in our study were current or past smokers, and their exclusion from the analyses did not alter the findings. Results from small studies have shown that intensive physical exercise (48), con-

<sup>5</sup> E. Riza, I. dos Santos Silva, B. De Stavola. Determinants of high risk mammographic parenchymal patterns in post-menopausal women, manuscript in preparation.

sumption of cruciferous vegetables (49), and supplementation with omega-3 fatty acids (10) may increase the 2:16 $\alpha$  ratio whereas dietary fat may decrease it (50). These studies were conducted under experimental conditions, however, with women being exposed to unusual high levels of physical exercise or unusual high, or low, levels of intake of the particular dietary factor under study. In contrast, no differences in the levels of 2-OHE1 and 16 $\alpha$ -OHE1 were found between Finnish women who followed a lactovegetarian diet and Finnish women who followed an omnivorous diet (12). We collected limited information on dietary habits or levels of physical exercise, but the study was conducted in a rural area of northern Greece, where the population tends to follow a traditional Mediterranean diet. As a crude marker of traditional dietary habits, we asked the women whether they adhered to the fasting periods of the Christian-Orthodox religion. Adjustment for this marker of traditional diet did not affect the results presented here. Similarly, adjustments for educational level or type of occupation, as crude markers of levels of physical exercise, made no difference to the results, partly because most of the women were engaged in rural activities.

A high BMI is known to be associated with an increased breast cancer risk in postmenopausal women (51) despite being associated with low mammographic density (22). For women with similar BMI, however, there is a gradual increase in breast cancer risk from N1 to DY patterns (22); conversely, for women with a similar Wolfe pattern, breast cancer risk increases with increasing BMI (19). These findings seem to suggest that each factor is a distinct determinant of breast cancer risk. The mean BMI of our study population was higher than those reported for other Western populations (52). It was, however, consistent with the mean BMI (as estimated from self-reported weights and heights) for the >3000 postmenopausal women who had ever attended the Ormylia Screening Program. It is also consistent with surveys showing that the prevalence of obesity (BMI equal or greater than 30 kg/m<sup>2</sup>) among Greek women is about 48% (52). Similarly to other studies (16, 17), however, there was no association between BMI and levels of estrogen metabolites in the present study, and adjustment for BMI in the analysis, either as a categorical or as a continuous variable, made little difference to the estimates of the association between estrogen metabolites and Wolfe mammographic patterns. The lack of association between levels of estrogen metabolites and BMI suggests that any potential effect estrogen metabolites might have on mammographic patterns and, possibly, on breast cancer risk are likely to be independent of those of BMI.

In summary, it has been hypothesized that a low 2:16 $\alpha$  ratio is associated with a high risk of breast cancer. In the present study, however, we found a high 2:16 $\alpha$  ratio to be associated with P2/DY Wolfe mammographic parenchymal patterns, a recognized risk factor for breast cancer. Although it is conceivable that estrogen metabolism, similarly to BMI, may have opposite effects on mammographic patterns and on breast cancer risk, a more straightforward interpretation of our findings would point to a high, rather than a low, 2:16 $\alpha$  ratio being implicated in the etiology of this tumor at postmenopausal ages.

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#### References

1. Toniolo, P. G., Levitz, M., Zeleniuch-Jacquotte, A., Banerjee, S., Koenig, K. L., Shore, R. E., Strax, P., and Pasternack, B. S. A prospective study of endogenous estrogens and breast cancer in postmenopausal women. *J. Natl. Cancer Inst. (Bethesda)*, 87: 190–197, 1995.
2. Berrino, F., Muti, P., Micheli, A., Gianfranco, B., Krogh, V., Raffaella, S., Pisani, P., Panico, S., and Secreto, G. Serum sex hormones levels after menopause and subsequent breast cancer. *J. Natl. Cancer Inst. (Bethesda)*, 88: 291–296, 1996.
3. Thomas, H. V., Key, T. J., Allen, D. S., Moore, J. W., Dowsett, M., Fentiman, I. S., and Wang, D. Y. A prospective study of endogenous hormone concentrations and breast cancer risk in postmenopausal women. *Br. J. Cancer*, 76: 401–405, 1997.
4. Fishman, J., and Martucci, C. Biological properties of 16 $\alpha$ -hydroxyestrone: implications in estrogen physiology and pathophysiology. *J. Clin. Endocrinol. Metab.*, 51: 611–615, 1980.
5. Telang, N. T., Suto, A., Wong, G. Y., Osborne, M. P., and Bradlow, H. L. Induction by estrogen metabolite 16 $\alpha$ -hydroxyestrone of genotoxic damage and aberrant proliferation in mouse mammary epithelial cells. *J. Natl. Cancer Inst. (Bethesda)*, 84: 634–638, 1992.
6. Bradlow, L. H., Hershcopf, R. E., Martucci, C. P., and Fishman, J. Estradiol 16 $\alpha$ -hydroxylation in the mouse correlates with mammary tumour incidence and presence of murine mammary tumor virus: a possible model for hormonal etiology of breast cancer in humans. *Proc. Natl. Acad. Sci. USA*, 82: 6295–6299, 1985.
7. Lemon, H. M., Heidel, J. W., and Rodriguez-Sierra, J. F. Increased catechol estrogen metabolism as a risk factor for nonfamilial breast cancer. *Cancer (Phila.)*, 69: 457–465, 1992.
8. Lottering, M. L., Haag, M., and Seegers, J. C. Effects of 17 $\beta$ -estradiol metabolites on cell cycle events in MCF-7 cells. *Cancer Res.*, 52: 5926–5932, 1992.
9. Bradlow, L. H., Hershcopf, R. E., and Fishman, J. F. Oestradiol 16 $\alpha$ -hydroxylase: a risk marker for breast cancer. *Cancer Surv.*, 5: 573–583, 1986.
10. Osborne, M. P., Karmali, R. A., Hershcopf, R. J., Bradlow, H. L., Kourides, I. A., Williams, W. R., Rosen, P. P., and Fishman, J.  $\omega$  3 fatty acids: modulation of estrogen metabolism and potential for breast cancer prevention. *Cancer Invest.*, 6: 629–631, 1988.
11. Schneider, J., Kinne, D., Fracchia, A., Pierce, V., Anderson, K. E., Bradlow, H. L., and Fishman, J. Abnormal oxidative metabolism of estradiol in women with breast cancer. *Proc. Natl. Acad. Sci. USA*, 79: 3047–3051, 1982.
12. Adlercreutz, H., Fotsis, T., Hockerstedt, K., Hamalainen, E., Bannwart, C., Bloigu, S., Valtonen, A., and Ollus, A. Diet and urinary estrogen profile in premenopausal omnivorous and vegetarian women and in premenopausal women with breast cancer. *J. Steroid Biochem.*, 34: 527–530, 1989.
13. Kabat, G. C., Chang, C. J., Sparano, J. A., Sepkovic, D. W., Hu, X-P., Khalil, A., Rosenblatt, R., and Bradlow, H. L. Urinary estrogen metabolites and breast cancer: a case-control study. *Cancer Epidemiol. Biomark. Prev.*, 6: 505–509, 1997.
14. Ho, G. H., Luo, X. W., Ji, C. Y., and Foo, S. C. Urinary 2/16 $\alpha$ -hydroxyestrone ratio: correlation with serum insulin-like growth factor binding protein-3 and a potential biomarker for breast cancer risk. *Ann. Acad. Med. Singapore*, 27: 294–299, 1998.
15. Zheng, W., Dunning, L., Jin, F., and Holtzman, J. Correspondence re: G. C. Kabat *et al.*, Urinary estrogen metabolites and breast cancer: a case-control study. *Cancer Epidemiol. Biomark. Prev.*, 7: 85–86, 1997.
16. Ursin, G., London, S., Stanczyk, F. Z., Gentzschlein, E., Paganini-Hill, A., Ross, R. K., and Pike, M. C. Urinary 2-hydroxyestrone/16 $\alpha$ -hydroxyestrone ratio and risk of breast cancer in postmenopausal women. *J. Natl. Cancer Inst. (Bethesda)*, 91: 1067–1072, 1999.
17. Meilahn, E. N., De Stavola, B., Allen, D. S., Fentiman, I., Bradlow, H. L., Sepkovic, D. W., and Kuller, L. H. Do urinary oestrogen metabolites predict breast cancer? Guernsey III cohort follow-up. *Br. J. Cancer*, 78: 1250–1255, 1998.
18. Muti, P., Bradlow, H. L., Micheli, A., Krogh, V., Freudenheim, J. L., Schunemann, H. J., Stanulla, M., Yang, J., Sepkovic, D. W., Trevisan, M., and Berrino, F. Estrogen metabolism and risk of breast cancer: a prospective study of the 2:16 $\alpha$  ratio in pre- and postmenopausal women. *Epidemiology*, 11: 635–640, 2000.
19. Whitehead, J., Carlile, T., Kopecky, K. J., Thompson, D. J., Gilbert, F. I. Jr., Present, A. J., Threatt, B. A., Krook, P., and Hadaway, E. The relationship between Wolfe's classification of mammograms, accepted breast cancer risk factors, and the incidence of breast cancer. *Am. J. Epidemiol.*, 122: 994–1006, 1985.
20. Saftlas, A. F., and Szklo, M. Mammographic parenchymal patterns and breast cancer risk. *Epidemiol. Rev.*, 9: 146–174, 1987.

21. Oza, A. M., and Boyd, N. F. Mammographic parenchymal patterns: a marker of breast cancer risk. *Epidemiol. Rev.*, *15*: 196–208, 1993.
22. Byrne, C., Schairer, C., Wolfe, J., Parekh, N., Salane, M., Brinton, L. A., Hoover, R., and Haile, R. Mammographic features and breast cancer risk: effects with time, age, and menopausal status. *J. Natl. Cancer Inst. (Bethesda)*, *87*: 1622–1629, 1995.
23. Spicer, D. V., Ursin, G., Parisky, Y. R., Pearce, J. G., Shoupe, D., Pike, A., and Pike, M. C. Changes in mammographic densities by a hormonal contraceptive designed to reduce breast cancer risk. *J. Natl. Cancer Inst. (Bethesda)*, *86*: 431–436, 1994.
24. Ekblom, A., Thurffjell, E., Hsieh, C.-C., Trichopoulos, D., and Adami, H.-O. Perinatal characteristics and adult mammographic patterns. *Int. J. Cancer*, *61*: 177–180, 1995.
25. Ursin, G., Pike, M. C., and Spicer, D. V. Can mammographic densities predict effects of tamoxifen on the breast? *J. Natl. Cancer Inst. (Bethesda)*, *88*: 128–129, 1996.
26. Sørensen, T. I. A., Stunkard, A. J., Teasdale, T. W., and Higgins, M. W. The accuracy of reports of weight: children's recall of their parents' weights 15 years earlier. *Int. J. Obes.*, *7*: 115–122, 1982.
27. Must, A., Willett, W. C., and Dietz, W. H. Remote recall of childhood height, weight, and body build by elderly subjects. *Am. J. Epidemiol.*, *138*: 56–64, 1993.
28. Wolfe, J. N. Breast patterns as an index of risk for developing breast cancer. *Am. J. Roentgenol.*, *126*: 1130–1137, 1976.
29. Bradlow, H. L., Sepkovic, D. W., Klug, T., and Osborne, M. P. Application of an improved ELISA assay to the analysis of urinary estrogen metabolites. *Steroids*, *63*: 406–413, 1998.
30. Klug, T. L., Bradlow, H. L., and Sepkovic, D. W. Monoclonal antibody-based enzyme immunoassay for simultaneous quantitation of 2- and 16 $\alpha$ -hydroxyestrone in urine. *Steroids*, *59*: 648–655, 1994.
31. Pasagian-Macaulay, A., Meilahn, E. N., Bradlow, H. L., Sepkovic, D. W., Buhari, A. M., Simkin-Silverman, L., Wing, R. R., and Kuller, L. H. Urinary markers of estrogen metabolism 2- and 16 $\alpha$ -hydroxylation in premenopausal women. *Steroids*, *61*: 461–467, 1996.
32. Ziegler, R. G., Rossi, S. C., Fears, T. R., Bradlow, H. L., Adlercreutz, H., Sepkovic, D., Kiuru, P., Wahala, K., Vaught, J. B., Donaldson, J. L., Falk, R. T., Fillmore, C. M., Siiteri, P. K., Hoover, R. N., and Gail, M. H. Quantifying estrogen metabolism: an evaluation of the reproducibility and validity of enzyme immunoassays for 2-hydroxyestrone and 16 $\alpha$ -hydroxyestrone in urine. *Environ. Health Perspect.*, *105* (Suppl. 3): 607–614, 1997.
33. Falk, R. T., Rossi, S. C., Fears, T. R., Sepkovic, D. W., Migella, A., Adlercreutz, H., Donaldson, J., Bradlow, H. L., and Ziegler, R. G. A new ELISA kit for measuring urinary 2-hydroxyestrone, 16 $\alpha$ -hydroxyestrone, and their ratio: reproducibility, validity, and assay performance after freeze-thaw cycling and preservation by boric acid. *Cancer Epidemiol. Biomark. Prev.*, *9*: 81–87, 2000.
34. Bland, J. M., and Altman, G. A. Measuring agreement in method comparison studies. *Stat. Methods Med. Res.*, *8*: 135–160, 1999.
35. Altman, D. G. (ed.). *Practical Statistics for Medical Research*. London: Chapman and Hall, Ltd., 1991.
36. Clayton, D., and Hills, M. (eds.). *Statistical Methods in Epidemiology*, pp. 290–297 and 237–253. Oxford: Oxford University Press, 1993.
37. Byrne, C. Studying mammographic density: implications for understanding breast cancer. *J. Natl. Cancer Inst. (Bethesda)*, *89*: 531–533, 1997.
38. Castagnetta, L. A. M., and Carruba, G. Urinary 2-hydroxyestrone/16 $\alpha$ -hydroxyestrone ratio and risk of breast cancer in postmenopausal women. *J. Natl. Cancer Inst. (Bethesda)*, *91*: 1893, 1999.
39. Dupont, E., Klug, T., McCann, C., Vincent, D., Shons, A., Teng, S., Reintgen, D., Cantor, A., and Cox, C. The prognostic value of altered estrogen metabolism. *Ann. Surg. Oncol.*, *7* (Suppl. 1): 35, 2000.
40. Adlercreutz, H., Gorbach, S. L., Goldin, B. R., Woods, M. N., Dwyer, J. T., and Hämäläinen, E. Estrogen metabolism and excretion in Oriental and Caucasian Women. *J. Natl. Cancer Inst. (Bethesda)*, *86*: 1076–1082, 1994.
41. Byrne, C., Hankinson, S., Colditz, G., Pollack, M., and Speizer, F. A cross-sectional study of mammographic density and plasma hormones. *Am. J. Epidemiol.*, *149*: 190, 1999.
42. Kaufman, Z., Garstin, W. I., Hayes, R., Michell, M. J., and Baum, M. The mammographic parenchymal patterns of women on hormonal replacement therapy. *Clin. Radiol.*, *43*: 389–392, 1991.
43. Berkowitz, J. E., Gatewood, O. M., Goldblum, L. E., and Gayler, B. W. Hormonal replacement therapy: mammographic manifestations. *Radiology*, *174*: 199–201, 1990.
44. De Stavola, B. L., Gravelle, I. H., Wang, D. Y., Allen, D. S., Bulbrook, R. D., Fentiman, I. S., Hayward, J. L., and Chaudary, M. C. Relationship of mammographic parenchymal patterns with breast cancer risk factors and risk of breast cancer in a prospective study. *Int. J. Epidemiol.*, *19*: 247–254, 1990.
45. Gram, I. T., Funkhouser, E., and Tabar, L. Reproductive and menstrual factors in relation to mammographic parenchymal patterns among perimenopausal women. *Br. J. Cancer*, *71*: 647–650, 1995.
46. Westerlind, K. C., Gibson, K. G., and Wolfe, P. The effect of diurnal and menstrual cyclicity and menopausal status on estrogen metabolites: implications for disease-risk assessment. *Steroids*, *64*: 233–243, 1999.
47. Michnovicz, J. J., Hershcopf, R. J., Naganuma, H., Bradlow, H. L., and Fishman, J. Increased 2-hydroxylation of estradiol as a possible mechanism for the anti-estrogenic effect of cigarette smoking. *N. Engl. J. Med.*, *315*: 1305–1309, 1986.
48. Snow, R. C., Barbieri, R. L., and Frisch, R. E. Estrogen 2-hydroxylase oxidation and menstrual function among elite oarswomen. *J. Clin. Endocrinol. Metab.*, *69*: 369–376, 1989.
49. Tiwari, R. K., Guo, L., Bradlow, H. L., Telang, N. T., and Osborne, M. P. Selective responsiveness of human breast cancer cells to indole-3-carbinol, a chemopreventive agent. *J. Natl. Cancer Inst. (Bethesda)*, *86*: 126–131, 1994.
50. Longcope, C., Gorbach, S., Goldin, B., Woods, M., Dwyer, J., Morrill, A., and Warram, J. The effect of a low fat diet on estrogen metabolism. *J. Clin. Endocrinol. Metab.*, *64*: 1246–1250, 1987.
51. Hunter, D. J., and Willett, W. C. Diet, body size, and breast cancer. *Epidemiol. Rev.*, *15*: 110–132, 1993.
52. de Groot, L. C., Sette, S., Zajkas, G., Carbajal, A., and Amorim, J. A. (on behalf of the Euronut SENECA investigators). Nutritional status: anthropometry. *Eur. J. Clin. Nutr.*, *45* (Suppl. 3): 31–42, 1991.