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Urinary estrogens and estrogen metabolites and mammographic density in premenopausal women

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Abstract

Mammographic density is a strong and independent risk factor for breast cancer and is considered an intermediate marker of risk. The major predictors of premenopausal mammographic density, however, have yet to be fully elucidated. To test the hypothesis that urinary estrogen metabolism profiles are associated with mammographic density, we conducted a cross-sectional study among 352 premenopausal women in the Nurses' Health Study II (NHSII). We measured average percent mammographic density using a computer-assisted method. In addition, we assayed 15 estrogens and estrogen metabolites (jointly termed EM) in luteal phase urine samples. We used multivariable linear regression to quantify the association of average percent density with quartiles of each individual EM as well as the sum of all EM (total EM), EM groups defined by metabolic pathway, and pathway ratios. In multivariable models controlling for body mass index (BMI) and other predictors of breast density, women in the top quartile of total EM had an average percent density 3.4 percentage points higher than women in the bottom quartile (95% confidence interval: -1.1, 8.0; p-trend=0.08). A non-significant positive association was noted for the 2-hydroxylation pathway catechols (breast density was 4.0 percentage points higher in top vs. bottom quartile; p-trend=0.06). In general, we observed no associations with parent estrogens or the 4- or 16-hydroxylation pathways or pathway ratios. These results suggest that urinary luteal estrogen profiles are not strongly associated with premenopausal mammographic density. If these profiles are associated with breast cancer risk, they may not act through influences on breast density.

Keywords

estradiol; estrogen metabolites; mammographic density; breast cancer; epidemiology

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Conflict of Interest

The authors declare that they have no conflict of interest.

Introduction

The hormonal etiology of breast cancer is well established, although the roles and relationships of specific hormones remain to be elucidated. Higher levels of circulating endogenous estrogens are associated with increased breast cancer risk among postmenopausal women [1, 2]; however, evidence for associations in premenopausal women is inconsistent, with some studies reporting no significant association with estrogens [3, 4] while others, including the Nurses' Health Study II (NHSII), report positive associations [5].

Mammographic density is one of the strongest risk factors for breast cancer and is considered an intermediate marker of risk [6, 7]. Mammographic density, a measure of the relative amount of fibroglandular tissue in the breast, which appears light on a mammogram (vs. fat, which appears dark), is associated with several breast cancer risk factors, including age, menopause, and body size but itself is a strong independent risk factor for breast cancer, with relative risks (RRs) ranging from 4 to 6 for women in the highest quartile of density vs. lowest [6]. The major predictors of premenopausal breast density and the biological mechanisms by which density influences cancer risk, however, have yet to be fully elucidated. Hypothesized mechanisms include increased cellular proliferation, cumulative exposure to growth factors and some hormones, including estrogens, and exposure to mutagenic factors [8].

The parent estrogens, estrone and estradiol, are metabolized along three pathways, by irreversible hydroxylation at the 2-, 4-, or 16-position of the steroid ring. Subsequently, the 2- and 4-pathway catechol estrogen metabolites, which have adjacent hydroxyl groups on the steroid ring, can be methylated at one of the hydroxyl groups. Specific patterns of estrogen metabolism may influence breast cancer risk given that the metabolites are hypothesized to have differential estrogenic and genotoxic activity. Specific metabolic patterns may also influence tissue bioavailability and excretion of estrogen. For example, based largely on experimental data, a shift from 16 α -hydroxyestrone production toward 2-hydroxyestrone production may be associated with reduced breast cancer risk [9]. Among premenopausal women in the NHSII, inverse associations with breast cancer risk were observed for luteal urinary levels of total estrogen metabolites, parent estrogens, 2- and 4-hydroxylation pathway metabolites, but not 16-hydroxylation pathway metabolites and for the ratio of parent estrogens to estrogen metabolites, while positive associations were noted for 17-epiestriol and the 16-pathway:parent estrogens ratio [10].

To date, only four studies have examined associations between estrogen metabolites and breast density. Among postmenopausal women, Riza et al. observed significantly higher levels of urinary 2-hydroxyestrone, and the ratio of 2-hydroxyestrone to 16 α -hydroxyestrone (2:16 α ratio) in those with high breast density (n=70) vs. those with low breast density (n=70) [11]. A significant positive association between the urinary 2:16 α ratio and mammographic density also was reported in a cross-sectional analysis of a predominantly premenopausal population with diverse ethnic backgrounds (n=305) [12]. Neither study investigated other individual estrogen metabolites. Among 194 postmenopausal women, Fuhrman et al. found that most individual urinary estrogen metabolites were not associated with breast density while positive associations were apparent for the ratio of parent estrogens to metabolites and inverse associations were noted for the ratios of 2-, 4-, and 16-pathways to parent estrogens [13]. In the only comprehensive study of individual estrogens and estrogen metabolites and mammographic density in premenopausal women to date, mammographic density was significantly positively associated with the percent of total urinary estrogens and estrogen metabolites in the 2-hydroxylation pathway and significantly inversely associated with the percent of the total in the 16-pathway, as well as percent estriol (n=188) [14]. Total urinary estrogens and estrogen

metabolites were not associated with mammographic density overall, but a significant positive association was observed for Asian women (n=74) [14]. Associations between polymorphisms in genes related to steroid hormone metabolism and breast density provide further suggestive evidence of a possible role for estrogen metabolites [15]. In the current study, including 352 premenopausal women with timed urine collections, we aimed to clarify relationships between 15 luteal urinary estrogens and estrogen metabolites (all 15 jointly referred to as EM) and mammographic density using data from the NHSII.

Methods

Study population

The NHSII is an ongoing cohort study of 116,430 women who were ages 25–42 at baseline in 1989. Self-administered questionnaires are collected every two years to update information on diseases and risk factors such as weight, family history of breast cancer, age at menarche, parity, alcohol consumption, and use of oral contraceptives. Between 1996 and 1999, 29,611 women, including 18,521 premenopausal women ages 32–52 years who had not used oral contraceptives, been pregnant, or breastfed within the preceding 6 months and who had no personal history of cancer provided blood and urine samples timed with their menstrual cycle. Luteal-phase urine and blood samples were collected 7–9 days before the anticipated start of their next period. Women returned a postcard with the actual start date of their next period, which allowed for backward counting for accurate luteal timing. Urine samples were collected with no preservatives and shipped with a frozen water bottle to our laboratory via overnight carrier. Samples have been stored in liquid nitrogen freezers since collection.

Within this subcohort, a nested case-control study of breast cancer was established to investigate various biomarkers as potential predictors of breast cancer risk [10]. In addition, regular screening mammograms were collected for these women. Screening mammograms were obtained as close as possible to the time of urine collection (median time from urine to mammogram: 7 months; interquartile range: –2 to 25 months) and we successfully obtained mammograms from approximately 80% of eligible women in the case-control study. Women from whom we did and did not receive mammograms were similar with regard to breast cancer risk factors including body mass index (BMI), parity and family history of breast cancer. We conducted cross-sectional analyses among controls from this nested case-control study. EM were measured in premenopausal women with timed samples; we further restricted the present analyses to women who were also premenopausal at mammography. The final analytic sample included 352 women.

This study was approved by the institutional review board of Brigham and Women's Hospital.

Mammographic density measurements

To assess mammographic density, the craniocaudal views of both breasts were digitized at 261 m/pixel with a Lumysis 85 laser film scanner, which covers a range of 0 to 4.0 absorbance. Film screen images were digitized and viewed on the computer screen and total breast area and total dense area were assessed using Cumulus software [16]. Percent mammographic density was calculated as absolute dense area divided by total breast area. All images were read by a single reader in two batches of mammograms approximately three years apart. Although there was high reproducibility within batch (within-person intraclass correlation coefficients 0.90; [17]), there was evidence of batch-to-batch variability in density measurements. Therefore, for the larger case-control dataset, we fit separate multivariable linear regression models to estimate the effect of batch on density

measurements, adjusting for age, menopausal status, body mass index, and case-control status [18]. We then adjusted density measurements in the second batch by adding the coefficient for mammogram batch to the raw value to estimate the measurements that would have been obtained if the mammogram had been included in the first batch.

We used the average percent density of both breasts for our main analyses [19, 20]. However, recent evidence suggests that absolute dense and non-dense areas may be independently associated with breast cancer risk [17, 21, 22], so we also examined these as separate outcomes in secondary analyses.

Laboratory analyses

Detailed laboratory methods have been previously described [10]. Briefly, we measured 15 EM concurrently in 500 µl mid-luteal phase urine samples using stable isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Laboratory of Proteomics and Analytical Chemistry, SAIC-Frederick, Inc. [23–25]. Specifically, we measured two parent estrogens (estrone and estradiol); five metabolites in the 2-hydroxylation pathway (2-hydroxyestrone, 2-hydroxyestradiol, 2-methoxyestrone, 2-methoxyestradiol, 2-hydroxyestrone-3-methyl ether), three metabolites in the 4-hydroxylation pathway (4-hydroxyestrone, 4-methoxyestrone, 4-methoxyestradiol), and five metabolites in the 16-hydroxylation pathway (16-hydroxyestrone, 17-epiestriol, estriol, 16-ketoestradiol, and 16-epiestriol). Five deuterated EM (17β-estradiol-d₄, estriol-d₃, 2-hydroxy-17β-estradiol-d₅, 2-methoxy-17β-estradiol-d₅, 16-epiestriol-d₃) were added as the urine samples were thawed to correct for EM loss during all steps of the assay procedure and permit accurate quantitation. Since endogenous estrogens and their metabolites are present in urine primarily as glucuronide and sulfate conjugates, an initial enzymatic hydrolysis step with β-glucuronidase/sulfatase from *Helix pomatia* was included. Therefore, for each EM, the EM concentration includes the unconjugated, glucuronidated, and sulfated forms. Masked replicate quality control samples were placed in each batch to assess laboratory variability. Overall coefficients of variation (CVs) were <7%, except for 4-methoxyestrone (CV=17%) and 4-methoxyestradiol (CV=15%), the two EM with the lowest concentrations [10]. The lower level of quantitation for each EM is approximately 150 fmol/mL urine.

Creatinine was measured in two batches at the Endocrine Core Laboratory at Emory University (Atlanta, GA) (CV=9.2%) and at the laboratory of Dr. Vincent Ricchiuti at Brigham and Women's Hospital (CV=2.4%). Progesterone was measured in plasma by chemiluminescent immunoassay using the Immulite Auto-Analyzer (Diagnostic Products). The overall CV was 17% and the within-batch CV was 4%.

Statistical analyses

Urinary EM concentrations (pmol/mL) were adjusted for creatinine levels to account for urine volume, which resulted in units of picomoles EM per milligram of creatinine (pmol/mg). There was no association between urinary creatinine concentrations and mammographic density in our study population (data not shown). We examined individual EM, the sum of all 15 EM (hereafter referred to as “total EM”), groups defined by metabolic pathway, and metabolic pathway ratios. Our main analyses considered absolute concentrations of EM and EM groups; we also evaluated individual and grouped EM as a percentage of total EM in secondary analyses. We fit multivariable linear regression models with percent mammographic density as the dependent variable and quartiles of each EM measure as the independent variable to quantify the relationship between EM and density. Generalized estimating equations were used to take into account the correlation between matched controls. Statistical tests for trend were from a Wald test using the median of each quartile as a continuous variable.

Multivariable models adjusted for ages at urine collection and mammography (continuous), first morning urine sample (yes, no), age at menarche (<12, 12, 13, 14 years), parity and age at first birth (nulliparous; 1–2 children, <25 years; 1–2 children, 25–29 years; 1–2 children, 30 years; 3+ children, <25 years; 3+ children, 25 years, missing), family history of breast cancer (yes, no), alcohol intake (0 g/d, 0.1–4.9 g/d, 5–14.9 g/d, 15+g/d, missing), and BMI at urine collection (continuous). Results from multivariable models without BMI were generally similar to those from models that adjusted for age alone, so age-adjusted models are not presented. Previous analyses in this population have also identified physical activity [26], height [27], and menstrual cycle regularity and length [28] as predictors of EM. Addition of these variables did not appreciably change effect estimates and therefore these covariates were not included in final multivariable models.

We conducted stratified analyses according to BMI (< vs. ≥ 25 kg/m²) and tested for statistical interaction by modeling the cross-product of continuous BMI and quartile medians of the EM measure (Wald test). Because of smaller sample sizes, we adjusted only for ages at urine collection and mammography, first morning urine sample, and continuous BMI in stratified analyses.

Several sensitivity analyses were also performed. Specifically, we excluded potential high outliers identified using the extreme Studentized many outlier procedure [29], resulting in exclusion of up to two observations for individual EM measured in absolute concentrations and up to five observations for group or ratio measures. We also performed analyses excluding women with an anovulatory cycle as defined by luteal plasma progesterone levels less than 400 ng/dL (n=42). Finally, we restricted analyses to women with dates of mammograms within 24 months of urine collection (n=258).

Analyses were conducted with SAS version 9.1 for UNIX (SAS Institute, Cary, NC). All *P* values were based on two-sided tests and were considered statistically significant if 0.05.

Results

Basic characteristics of the 352 premenopausal women included in this analysis are summarized in Table 1. The mean ages at urine collection and mammography were 42.6 and 44.0 years, respectively, and mean average percent breast density was 43.5. Mean BMI at urine collection was 25.0 kg/m². The majority of women were parous (82%), with an average age at first birth of 26.8 years. Seven percent reported a family history of breast cancer in a first-degree relative. Alcohol consumption was generally low.

In a multivariable model without adjustment for BMI, women in the top quartile of total EM had an average percent density 4.6 percentage points higher than women in the bottom quartile [95% confidence interval (CI): −0.7, 10.0; p-trend: 0.11 (Model 1)] (Table 2a). EM in the 2-hydroxylation pathway initially appeared to be the strongest predictors of mammographic density [difference between top and bottom quartile: 6.5; 95% CI: 1.3, 11.7; p-trend: 0.01 (Model 1)]; however, further adjustment for BMI (Model 2) greatly attenuated the observed associations and rendered the association with the 2-pathway EM non-statistically significant (difference between top and bottom quartile: 2.8; 95% CI: −1.6, 7.1; p-trend: 0.18) (Table 2a). Similar patterns were observed for catechol EM as a group and particularly for catechols in the 2-pathway, with significant positive associations that were substantially attenuated upon adjustment for BMI (Table 2b). However, even after adjustment for BMI, women with the highest concentrations of 2-hydroxyestrone had noticeably higher percent breast densities and this result was of borderline statistical significance (difference between top and bottom quartile: 4.2; 95% CI: −0.1, 8.5; p-trend: 0.06) (Table 2b).

There were no clear patterns of association for parent estrogens (Table 2a) or for 4- or 16-hydroxylation pathway EM when evaluated as a group (Table 2a) or individually (Tables 2b, 2c, and 2d). The methylated catechol EM in both the 2- and 4-pathways generally displayed suggestive positive associations with average percent mammographic density in multivariable models that did not include BMI, with similar effect sizes as in the combined catechol analyses, but the observed associations tended to be null upon adjustment for BMI (Table 2c). Findings generally were similar for individual or group EM expressed as a percentage of total EM (data not shown).

We further investigated possible associations with ratios of pathway groups, as well as the 2-hydroxyestrone:16 α -hydroxyestrone ratio (Table 2e). In multivariate models without BMI, percent mammographic density was 6.0 percentage points lower (p-trend=0.01) when women with a ratio of parent estrogens:estrogen metabolites in the highest quartile were compared to women in the lowest quartile, 9.1 percentage points higher (p-trend <0.01) when comparing extreme quartiles of the 2-pathway:parent estrogens ratio; and 5.9 percentage points higher (p-trend <0.01) when comparing extreme quartiles of the 2-pathway:16-pathway ratio. However, after adding BMI, none of the trends was significantly associated with average percent mammographic density nor were any suggestive trends noted.

In secondary analyses that considered absolute measures of mammographic density, we found associations between group and individual EM and ratio measures with absolute dense area to be generally null (Supplementary Table S1). In contrast, statistically significant inverse associations between total EM, 2-pathway, 4-pathway, and 16-pathway EM and many individual EM, particularly those in the 2-hydroxylation pathway, and absolute non-dense area were noted based on results from multivariable models that controlled for BMI and other predictors of breast density (Supplementary Table S2).

Because BMI is a strong predictor of mammographic density and was a strong confounder in analyses of EM measures, we evaluated associations of individual EM, EM groups, and EM ratios with percent mammographic density among women with BMI <25 kg/m² (n=224) and BMI $\geq$ 25 kg/m² (n=128) separately in stratified analyses. Overall, results were generally similar in both strata and there was no evidence of meaningful effect modification by BMI. Significant statistical interactions with BMI were observed for some individual EM based on continuous cross-product terms, but closer examination of effects within strata defined by BMI suggested that these were generally null associations in each strata, but with opposite signs for parameter estimates which were close to zero (data not shown). For example, the interaction with BMI was statistically significant for 2-methoxyestrone (p-interaction = 0.03), but there was no apparent association with percent density among women with BMI <25 kg/m² (beta=0.26; p-trend = 0.19) or BMI $\geq$ 25 kg/m² (beta=-0.15; p-trend = 0.56).

For absolute dense area, no consistent patterns were observed when analyses were stratified by BMI (data not shown). For absolute non-dense area, results were similar across strata defined by BMI, with similarly suggestive inverse trends as seen in main analyses. Associations appeared slightly stronger for women with BMI $\geq$ 25 kg/m², but confidence intervals were generally wider in this group due to a smaller sample size and there was no evidence of effect modification by BMI (all p-interaction >0.05) (data not shown).

Results of sensitivity analyses excluding potential high outliers for the EM measures, anovulatory women, or women whose mammogram was more than 24 months before or after urine collection were not materially different from primary analyses (data not shown).

Discussion

In summary, total EM, individual EM and EM pathways were not strongly associated with premenopausal percent mammographic density in this cross-sectional analysis. In general, positive associations for total EM, the 2-hydroxylation pathway EM, 2-pathway catechol EM, 2- and 4-pathway methylated catechol EM and individual EM in these groups were observed in multivariable models that did not include BMI. Much of the apparent effect, however, was explained by BMI, which is a strong negative predictor of mammographic density [30–32]. In addition, in this group of women, BMI was inversely related to total EM and many individual EM, particularly in the 2-hydroxylation pathway [27]. Adjustment for BMI substantially attenuated estimates of the effect of EM on mammographic density and rendered associations weak and possibly null. We found no clear evidence that EM-density associations varied by level of BMI.

Our findings are in general agreement with two prior studies of premenopausal women [12, 14]. Maskarinec et al. [14] reported inverse associations of mammographic density with 16-hydroxylation pathway EM, including estriol, and positive associations with the 2-hydroxylation pathway, but, with the exception of estriol, significant associations were restricted to Asian women (n=74) and not observed in the Caucasian group, which is more similar to our study. We observed a borderline statistically significant positive association for 2-hydroxyestrone (p-trend: 0.06), which was also similarly associated with mammographic density among premenopausal women in the prior analysis [14]. This finding should be interpreted with caution, however, because these analyses were exploratory and we evaluated several EM. Further, in contrast to the prior report [14], we found no association with the ratio of 2-hydroxyestrone to 16-hydroxyestrone.

Absolute measures of mammographic density may be independently associated with breast cancer risk [17, 21, 22] and absolute dense and non-dense breast area may have different risk profiles. In general, we found no associations between individual EM or pathway groups and absolute dense area in multivariable models that controlled for BMI and other predictors of breast density. In contrast, total EM, each of the three estrogen hydroxylation pathways and many individual EM, particularly those in the 2-hydroxylation pathway, were significantly inversely associated with absolute non-dense breast area (i.e., the adipose component of breast tissue). Because BMI is strongly correlated with absolute non-dense breast area (Spearman correlation coefficient: 0.65), it is difficult to disentangle these effects from observed associations between BMI and EM profiles [27]. Although we adjusted for BMI continuously in multivariable models, residual confounding by adiposity remains a concern. In stratified analyses, inverse associations were apparent for both leaner and heavier women.

There are several important limitations to this study. First, analyses were cross-sectional in nature. We measured EM in a single urine sample collected close to the time of mammogram. While a single EM measurement may not accurately reflect long-term profiles, reproducibility of urinary EM measures in NHSII is fairly good over 2–3 years [33]. Second, urinary EM profiles may not be a good proxy for estrogen activity in breast tissue. In a small study of breast cancer patients (n=9), parent estrogens were detected in higher concentrations in breast tissue than in urine, EM in the 2- and 16-hydroxylation pathway were detected in lower concentrations in breast tissue than in urine, and EM in the 4-hydroxylation pathway were detected in urine only [34]. Finally, the majority of women included in this analysis (98%) were white. Therefore, our findings may not be generalizable to other ethnic groups. In particular, stronger associations between EM measures and mammographic density were reported in Asian women than in non-Asian women [14].

The current study is the largest systematic investigation of all 15 EM and mammographic density in premenopausal women to date (n=352). Additional strengths of this study include the use of a high-performance LC-MS/MS assay to measure urinary EM with high sensitivity, specificity, accuracy, and reproducibility [24, 35]; urine samples that were carefully timed within the luteal phase of the menstrual cycle; quantitative assessments of mammographic density from screening mammograms with high intra-reader reliability; and detailed information on potential confounders, including predictors of breast density and breast cancer risk factors.

Mammographic density is an intermediate marker of breast cancer risk [6]. We previously reported significant inverse associations of urinary estrone and estradiol and suggestive inverse associations of total EM and 2- and 4-hydroxylation pathway EM with breast cancer risk in this study population [10]. The lack of association between EM and percent mammographic density suggests that if the previously observed associations with breast cancer are causal, the mechanism of action may be independent of mammographic density. Further research is warranted to assess the joint effects of urinary estrogen metabolism profiles and mammographic density on breast cancer risk.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of the study population (NHSII, n=352).

	Mean (SD) or %
Age at urine collection, y	42.6 (4.0)
Age at mammography, y	44.0 (3.9)
Average percent mammographic density	43.5 (19.1)
Average dense area, cm ²	97.7 (49.4)
Average nondense areas, cm ²	144.2 (86.2)
Body mass index at urine collection, kg/m ²	25.0 (5.3)
Age at menarche 12, %	46.6
Nulliparous, %	18.2
Number of children *	2.3 (0.9)
Age at first birth, y *	26.8 (4.5)
Family history of breast cancer, %	7.4
Alcohol consumption, g/d	3.5 (5.6)

NHSII; Nurses' Health Study II

*
among parous women only.

Table 2

Difference in average percent mammographic density [β (95% confidence interval)] by quartile of EM measure (pmol/mg creatinine) (n = 352).

a) Total EM, parent estrogens, and estrogen hydroxylation pathways

Grouped EM		Quartiles				p-trend
		1	2	3	4	
Total EM	cutpoints	<142.7	142.7–194.1	195.0–266.4	>266.4	
	Model 1	ref	2.3 (–2.9, 7.6)	2.5 (–2.8, 7.8)	4.6 (–0.7, 10.0)	0.11
	Model 2	ref	–1.2 (–5.6, 3.2)	–1.1 (–5.7, 3.5)	3.4 (–1.1, 8.0)	0.08
Parent estrogens	cutpoints	<27.4	27.4–42.7	42.9–61.4	>61.4	
	Model 1	ref	–0.1 (–5.2, 5.1)	–0.9 (–6.3, 4.5)	–1.9 (–7.2, 3.5)	0.46
	Model 2	ref	–0.3 (–4.3, 3.8)	–1.0 (–5.3, 3.3)	0.2 (–4.3, 4.7)	0.96
Estrone	cutpoints	<17.8	17.8–27.7	28.1–40.9	>40.9	
	Model 1	ref	2.8 (–2.0, 7.6)	0.5 (–4.6, 5.7)	–0.4 (–5.7, 4.8)	0.63
	Model 2	ref	0.2 (–4.0, 4.4)	–1.1 (–5.2, 3.0)	0.0 (–4.6, 4.5)	0.90
Estradiol	cutpoints	<9.3	9.3–13.6	13.6–19.9	>19.9	
	Model 1	ref	–2.2 (–7.5, 3.1)	–0.2 (–6.1, 5.7)	–4.4 (–10.2, 1.3)	0.16
	Model 2	ref	–2.3 (–6.5, 2.0)	–0.2 (–4.9, 4.6)	–0.7 (–5.7, 4.3)	0.99
2-Hydroxylation pathway	cutpoints	<44.5	44.5–67.4	67.7–106.2	>106.2	
	Model 1	ref	2.8 (–2.8, 8.5)	5.7 (0.4, 11.0)	6.5 (1.3, 11.7)	0.01
	Model 2	ref	0.1 (–4.8, 5.1)	0.3 (–4.2, 4.8)	2.8 (–1.6, 7.1)	0.18
4-Hydroxylation pathway	cutpoints	<3.2	3.2–6.0	6.0–9.7	>9.7	
	Model 1	ref	–0.3 (–5.7, 5.2)	–2.8 (–7.9, 2.3)	1.2 (–4.1, 6.5)	0.65
	Model 2	ref	0.1 (–4.3, 4.5)	–2.8 (–6.9, 1.4)	0.8 (–3.5, 5.2)	0.74
16-Hydroxylation pathway	cutpoints	<42.7	42.7–71.0	71.6–106.4	>106.4	
	Model 1	ref	–5.8 (–10.9, –0.8)	–2.9 (–8.2, 2.5)	–1.3 (–6.1, 3.6)	0.82
	Model 2	ref	–4.0 (–8.1, 0.1)	–1.6 (–6.3, 3.1)	0.3 (–3.7, 4.3)	0.39

b) Catechol estrogen metabolites

Grouped EM	Individual EM	Quartiles				p-trend
		1	2	3	4	
Catechol EM	cutpoints	<40.0	40.0–62.4	62.5–94.8	>94.8	
	Model 1	ref	0.3 (–5.4, 6.1)	3.9 (–1.2, 9.1)	7.3 (2.6, 12.1)	<0.01

b) Catechol estrogen metabolites

Grouped EM	Individual EM	Quartiles				p-trend
		1	2	3	4	
2-pathway catechol EM	Model 2	ref	-0.9 (-5.9, 4.1)	-0.3 (-4.6, 4.0)	3.4 (-0.9, 7.7)	0.08
	cutpoints	<37.0	37.0-54.8	55.9-87.9	>87.9	
	Model 1	ref	2.2 (-3.4, 7.7)	4.4 (-0.8, 9.6)	8.0 (3.1, 13.0)	<0.01
	Model 2	ref	0.3 (-4.6, 5.2)	0.0 (-4.4, 4.3)	4.0 (-0.4, 8.4)	0.06
2-Hydroxyestrone	cutpoints	<32.5	32.5-50.2	50.6-77.8	>77.8	
	Model 1	ref	3.2 (-2.3, 8.7)	5.2 (0.1, 10.3)	8.3 (3.4, 13.3)	<0.01
	Model 2	ref	0.8 (-4.0, 5.5)	0.2 (-4.1, 4.5)	4.2 (-0.1, 8.5)	0.06
	cutpoints	<3.3	3.3-5.3	5.4-8.8	>8.8	
2-Hydroxyestradiol	Model 1	ref	-0.7 (-5.9, 4.6)	3.5 (-2.1, 9.0)	4.6 (-0.4, 9.6)	0.03
	Model 2	ref	-2.0 (-6.4, 2.4)	1.1 (-3.5, 5.7)	2.2 (-2.4, 6.7)	0.16
	cutpoints	<2.9	2.9-5.7	5.7-9.4	>9.4	
	Model 1	ref	-0.9 (-6.4, 4.6)	-2.7 (-7.9, 2.5)	0.6 (-4.7, 5.8)	0.78
4-pathway catechol EM	Model 2	ref	0.5 (-3.9, 4.9)	-2.3 (-6.5, 2.0)	0.6 (-3.7, 4.9)	0.90

c) Methylated catechol estrogen metabolites

Grouped EM	Individual EM	Quartiles				p-trend
		1	2	3	4	
Methylated catechol EM	cutpoints	<7.4	7.4–10.8	10.9–15.6	>15.6	
	Model 1	ref	1.6 (–3.8, 7.0)	4.9 (–0.4, 10.3)	4.5 (–0.7, 9.6)	0.07
	Model 2	ref	–2.2 (–7.2, 2.8)	0.1 (–4.6, 4.9)	0.7 (–3.5, 4.8)	0.51
	cutpoints	<7.0	7.0–10.5	10.6–15.3	>15.3	
Methylated 2-pathway Catechol EM	Model 1	ref	3.3 (–1.9, 8.6)	5.2 (–0.1, 10.6)	5.6 (0.3, 10.8)	0.04
	Model 2	ref	–0.2 (–5.1, 4.8)	0.9 (–3.8, 5.6)	2.0 (–2.2, 6.2)	0.28
	cutpoints	<5.7	5.7–8.5	8.5–12.2	>12.2	
	Model 1	ref	1.2 (–4.0, 6.5)	6.9 (1.6, 12.3)	3.8 (–1.7, 9.2)	0.13
2-Methoxyestrone	Model 2	ref	–1.4 (–6.2, 3.4)	2.0 (–2.7, 6.7)	1.2 (–3.0, 5.5)	0.39
	cutpoints	<0.45	0.45–0.74	0.74–1.09	>1.09	
	Model 1	ref	3.0 (–2.5, 8.4)	4.2 (–0.8, 9.2)	2.8 (–2.6, 8.2)	0.34
	Model 2	ref	0.1 (–4.6, 4.8)	–0.7 (–5.3, 3.8)	1.4 (–3.0, 5.8)	0.58
2-Hydroxyestrone-3-methyl ether	cutpoints	<0.75	0.75–1.1	1.1–1.7	>1.7	

c) Methylated catechol estrogen metabolites						
Grouped EM	Individual EM	Quartiles				p-trend
		1	2	3	4	
Methylated 4-pathway Catechol EM	Model 1	ref	7.3 (1.6, 13.0)	4.8 (-0.6, 10.2)	7.3 (2.1, 12.5)	0.04
	Model 2	ref	1.3 (-4.0, 6.6)	0.8 (-3.9, 5.6)	3.6 (-1.0, 8.1)	0.11
	cupoints	<0.11	0.11-0.20	0.20-0.41	>0.41	
	Model 1	ref	2.0 (-3.8, 7.8)	0.8 (-4.4, 6.1)	5.8 (0.2, 11.3)	0.04
	Model 2	ref	-0.1 (-4.7, 4.6)	-2.0 (-6.6, 2.7)	0.8 (-3.7, 5.2)	0.67
	cupoints	<0.06	0.06-0.13	0.14-0.27	>0.27	
4-Methoxyestrone	Model 1	ref	1.1 (-5.1, 7.3)	1.0 (-4.5, 6.5)	5.6 (0.2, 10.9)	0.02
	Model 2	ref	-1.3 (-6.2, 3.6)	-4.0 (-8.8, 0.8)	-0.9 (-5.4, 3.5)	0.99
	cupoints	<0.02	0.02-0.04	0.04-0.12	>0.12	
	Model 1	ref	3.3 (-2.1, 8.8)	1.3 (-4.7, 7.3)	5.8 (0.0, 11.6)	0.06
	Model 2	ref	1.6 (-2.8, 6.1)	-0.7 (-5.5, 4.1)	3.5 (-1.1, 8.1)	0.12
	cupoints	<0.02	0.02-0.04	0.04-0.12	>0.12	
d) 16-hydroxylation pathway estrogen metabolites						
Individual EM		Quartiles				p-trend
		1	2	3	4	
16 α -Hydroxyestrone	cupoints	<7.1	7.1-11.8	11.8-20.0	>20.0	
	Model 1	ref	-10.0 (-15.4, -4.6)	-6.5 (-11.8, -1.3)	-1.8 (-6.8, 3.3)	0.34
	Model 2	ref	-7.2 (-11.8, -2.7)	-5.9 (-10.4, -1.4)	-2.1 (-6.6, 2.3)	0.66
17-Epiestriol	cupoints	<0.74	0.74-1.5	1.5-2.8	>2.8	
	Model 1	ref	-5.2 (-10.3, -0.2)	-2.0 (-7.6, 3.7)	-4.8 (-10.0, 0.5)	0.26
	Model 2	ref	-5.0 (-9.4, -0.5)	-1.7 (-6.8, 3.4)	-1.8 (-5.9, 2.2)	1.00
Estril	cupoints	<18.4	18.4-30.3	30.5-46.8	>46.8	
	Model 1	ref	-1.7 (-6.7, 3.2)	-3.4 (-8.5, 1.8)	-4.8 (-10.1, 0.4)	0.07
	Model 2	ref	-0.2 (-4.5, 4.1)	-1.9 (-6.4, 2.5)	-0.9 (-5.3, 3.5)	0.61
16-Ketoestradiol	cupoints	<8.9	8.9-14.2	14.3-20.3	>20.3	
	Model 1	ref	-3.5 (-8.2, 1.2)	-2.6 (-8.1, 2.9)	-0.7 (-5.8, 4.4)	0.87
	Model 2	ref	-2.6 (-6.5, 1.4)	-1.4 (-5.8, 3.0)	-1.1 (-5.4, 3.2)	0.91
16-Epiestriol	cupoints	<4.2	4.2-6.3	6.4-9.0	>9.0	
	Model 1	ref	-6.9 (-12.0, -1.7)	-1.0 (-6.4, 4.4)	-6.5 (-11.8, -1.1)	0.14
	Model 2	ref	-5.7 (-10.1, -1.4)	0.4 (-4.4, 5.2)	-2.5 (-7.1, 2.0)	0.94

e) Ratios of metabolic pathway groups

Ratios of EM	Quartiles				p-trend
	1	2	3	4	
4-pathway catechol/2-pathway catechols	cutpoints <0.06	0.06–0.10	0.10–0.15	>0.15	
Model 1	ref	–4.0 (–9.5, 1.5)	–7.7 (–12.9, –2.5)	–5.6 (–10.7, –0.6)	0.05
Model 2	ref	–1.0 (–5.5, 3.5)	–3.6 (–7.9, 0.7)	–1.7 (–6.1, 2.6)	0.45
Catechols/Methylated catechols	cutpoints <4.0	4.0–5.6	5.6–7.7	>7.7	
Model 1	ref	2.5 (–3.0, 8.1)	3.3 (–2.4, 9.1)	1.2 (–3.5, 6.0)	0.73
Model 2	ref	1.7 (–2.6, 6.0)	1.5 (–3.5, 6.4)	2.2 (–1.7, 6.1)	0.34
2-pathway catechols/Methylated 2-pathway catechols	cutpoints <3.75	3.75–5.2	5.2–7.0	>7.0	
Model 1	ref	5.0 (–0.5, 10.4)	3.7 (–2.3, 9.7)	4.2 (–0.7, 9.1)	0.18
Model 2	ref	0.9 (–3.6, 5.4)	0.5 (–4.4, 5.5)	2.4 (–1.9, 6.6)	0.29
4-pathway catechol/Methylated 4-pathway catechols	cutpoints <11.5	11.5–26.6	26.7–59.4	>59.4	
Model 1	ref	–0.8 (–6.2, 4.6)	–4.2 (–9.7, 1.4)	–3.1 (–8.2, 1.9)	0.21
Model 2	ref	1.1 (–3.6, 5.8)	–1.0 (–5.4, 3.5)	0.3 (–3.9, 4.5)	0.98
Parent estrogens/Estrogen metabolites	cutpoints <0.20	0.20–0.27	0.27–0.36	>0.36	
Model 1	ref	–1.7 (–7.2, 3.8)	–6.5 (–11.7, –1.2)	–6.0 (–11.3, –0.6)	0.01
Model 2	ref	–1.2 (–5.8, 3.4)	–3.8 (–8.1, 0.5)	–1.3 (–5.5, 2.8)	0.44
2-pathway/Parent estrogens	cutpoints <1.08	1.08–1.6	1.6–2.3	>2.3	
Model 1	ref	4.7 (–0.8, 10.1)	8.3 (2.9, 13.6)	9.1 (4.0, 14.2)	<0.01
Model 2	ref	–0.2 (–5.1, 4.7)	1.1 (–3.7, 5.8)	1.4 (–3.1, 5.9)	0.44
4-pathway/Parent estrogens	cutpoints <0.08	0.08–0.13	0.13–0.21	>0.21	
Model 1	ref	2.2 (–3.0, 7.5)	4.3 (–1.3, 9.9)	3.5 (–1.4, 8.3)	0.22
Model 2	ref	–1.1 (–5.4, 3.3)	2.4 (–2.1, 6.9)	–0.7 (–4.6, 3.2)	0.85
16-pathway/Parent estrogens	cutpoints <1.13	1.13–1.57	1.58–2.3	>2.3	
Model 1	ref	2.0 (–3.5, 7.5)	–0.9 (–6.4, 4.5)	1.3 (–3.9, 6.6)	0.79
Model 2	ref	1.4 (–3.5, 6.3)	–1.6 (–6.4, 3.1)	1.8 (–2.6, 6.2)	0.49
4-pathway/2-pathway	cutpoints <0.06	0.06–0.09	0.09–0.13	>0.13	
Model 1	ref	–3.1 (–8.3, 2.2)	–8.0 (–13.1, –2.9)	–6.1 (–11.3, –1.0)	0.02
Model 2	ref	–0.4 (–4.8, 4.0)	–4.6 (–9.0, –0.2)	–1.3 (–5.4, 2.8)	0.48
2-pathway/16-pathway	cutpoints <0.61	0.61–1.0	1.0–1.67	>1.67	
Model 1	ref	1.0 (–4.8, 6.8)	9.5 (4.1, 15.0)	5.9 (0.9, 10.9)	<0.01

e) Ratios of metabolic pathway groups

Ratios of EM	Quartiles					p-trend
	1	2	3	4		
4-pathway/16-pathway	Model 2	ref	-2.0 (-6.6, 2.7)	4.9 (0.3, 9.5)	1.1 (-3.3, 5.4)	0.23
	cupoints	<0.05	0.05-0.09	0.09-0.15	>0.15	
	Model 1	ref	0.6 (-5.0, 6.1)	2.1 (-3.8, 7.9)	3.3 (-2.1, 8.6)	0.17
2-hydroxyestosterone/16a-hydroxyestosterone	Model 2	ref	-1.2 (-5.6, 3.3)	-1.2 (-5.9, 3.5)	0.2 (-4.5, 4.9)	0.75
	cupoints	<2.4	2.4-4.3	4.3-7.0	>7.0	
	Model 1	ref	1.7 (-4.1, 7.4)	5.8 (0.3, 11.2)	3.8 (-1.3, 8.9)	0.09
EM; estrogens and estrogen metabolites	Model 2	ref	-0.2 (-5.2, 4.8)	1.4 (-3.4, 6.1)	0.8 (-3.8, 5.4)	0.62

EM; estrogens and estrogen metabolites

Model 1: Adjusted for age at urine collection, age at mammogram, first morning urine sample (yes, no), age at menarche (<12, 12, 13, 14), parity & age at first birth (nulliparous, 1-2 children & <25 yrs, 1-2 children & 25-29 yrs, 1-2 children & 30 yrs, 3+ children & <25 yrs, 3+ children & 25 yrs, missing), family history of breast cancer (yes, no), alcohol intake (0 g/d, 0.1-4.9 g/d, 5-14.9 g/d, 15+g/d, missing)

Model 2: Additionally adjusted for body mass index at urine collection (continuous, kg/m²)