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Urinary estrogens and estrogen metabolites and subsequent risk of breast cancer among premenopausal women

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Abstract

Endogenous estrogens and estrogen metabolism are hypothesized to be associated with premenopausal breast cancer risk but evidence is limited. We examined 15 urinary estrogens/estrogen metabolites (EM) and breast cancer risk among premenopausal women in a case-control study nested within the Nurses' Health Study II (NHSII). In 1996–1999, urine was collected from 18,521 women during the mid-luteal menstrual phase. Breast cancer cases (N=247) diagnosed between collection and June 2005 were matched to 2 controls each (N=485). Urinary EM were measured by liquid chromatography-tandem mass spectrometry and adjusted for creatinine level. Relative risks (RRs) and 95% confidence intervals (CIs) were estimated by multivariate conditional logistic regression. Higher urinary estrone and estradiol levels were strongly significantly associated with lower risk (top vs. bottom quartile RR estrone=0.52, 95% CI=(0.30–0.88); estradiol=0.51, 95% CI=(0.30–0.86)). Generally inverse, though non-significant, patterns also were observed with 2- and 4-hydroxylation pathway EM. Inverse associations generally were not observed with 16-pathway EM and a significant positive association was observed with 17-epiestriol (top vs. bottom quartile RR=1.74, 95% CI=(1.08–2.81), p-trend=0.01). In addition, there was a significant increased risk with higher 16-pathway/parent EM ratio (comparable RR=1.61, 95% CI=(0.99–2.62), p-trend=0.04). Other pathway ratios were not significantly associated with risk except parent EM/non-parent EM (comparable RR=0.58, 95% CI=(0.35–0.96), p-trend=0.03). These data suggest that most mid-luteal urinary EM concentrations are not positively associated with breast cancer risk among premenopausal women. The inverse associations with parent EM and the parent EM/non-parent EM ratio suggest that women with higher urinary excretion of parent estrogens are at lower risk.

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Introduction

The positive association between levels of endogenous estrogen and breast cancer risk in postmenopausal women is well established (1) and estrogen levels also may be important among premenopausal women, although evidence is limited and not entirely consistent (2–9). Metabolism of the parent estrogens, estrone and estradiol, yields estrogen metabolites proposed to have varying estrogenic and genotoxic activities based on laboratory evidence (10–16). Irreversible oxidation of estrone and estradiol occurs at the C-2 or C-4 positions to yield catechol estrogens, with adjacent and reactive hydroxyl groups (2-hydroxyestrone, 2-hydroxyestradiol, 4-hydroxyestrone), or the C-16 position to yield 16α-hydroxyestrone (Figure 1) (10). With further metabolism, the catechol estrogens are irreversibly methylated into 2-methoxyestrone, 2-methoxyestradiol, 2-hydroxyestrone-3-methyl ether, 4-methoxyestrone and 4-methoxyestradiol. 16α-hydroxyestrone can be further metabolized into 17-epiestriol, estriol, 16-ketoestradiol, and 16-epiestriol. Metabolism favoring the 2-hydroxylation over the 16-hydroxylation pathway has been proposed to lower breast cancer risk (17). Although some studies have analyzed the relationship of 2-hydroxyestrone and 16α-hydroxyestrone with breast cancer risk in humans (18–31), other individual metabolites and estrogen metabolism pathways have not been evaluated systematically in human populations.

A high-performance liquid chromatography-tandem mass spectrometry (LC-MS^2) assay was developed to measure concurrently 15 estrogens and estrogen metabolites (EM) in urine with high sensitivity, specificity, accuracy and reproducibility (32). We previously reported increased breast cancer risks in premenopausal women with higher plasma free and total estradiol in the follicular phase (9). Although we did not observe statistically significant associations with plasma luteal phase estrogens, estrone and estrone sulfate in the luteal phase were suggestively inversely associated with breast cancer risk. Herein, we prospectively evaluate associations between 15 mid-luteal urinary EM and breast cancer risk among premenopausal women in a case-control study nested within the Nurses' Health Study II (NHSII).

Methods

Study population

The NHSII was established in 1989, when 116,430 female registered nurses, aged 25 to 42 years, completed and returned a questionnaire. The cohort has been followed biennially by questionnaire to update exposures and ascertain newly diagnosed disease.

Between 1996 and 1999, 29,611 cohort members who were cancer-free and between the ages of 32 and 54 years provided blood and urine samples. These women were similar to the overall cohort with respect to lifestyle factors, such as body mass index, parity, age at menarche, past oral contraceptive use, and only differed slightly in the prevalence of family history of breast cancer (19% vs. 15% in the overall cohort). Of the 29,611 women who gave blood, 18,521 were premenopausal (i.e., still having menstrual periods) participants who provided two blood samples and one urine sample timed within the menstrual cycle; the women had not used oral contraceptives, been pregnant, or breastfed within six months. Participants were sent a short questionnaire and a sample collection kit containing necessary supplies to have blood samples drawn by a local laboratory or a colleague. They provided blood samples drawn on the 3^{rd}–5^{th} day of their menstrual cycle (follicular samples) and blood and urine samples collected 7–9 days before the anticipated start of their next cycle (luteal samples). Follicular plasma was aliquotted by the participants 8–24 hours after collection and stored in their home freezer until the luteal collection. Urine samples were collected without preservatives, with 80% collected as first morning samples. The day of the

luteal collection, follicular and luteal blood samples and luteal urine samples were shipped, via overnight courier with an ice-pack, to our laboratory where the luteal blood sample was processed and separated into plasma, red blood cell, and white blood cell components. Approximately 93% of luteal samples were received within 26 hours of collection. Samples have been stored in liquid nitrogen freezers since collection. Women recorded the first day of the menstrual cycle during which the samples were collected and returned a postcard recording the first day of their next cycle. The study was approved by the Committee on the Use of Human Subjects in Research at Harvard School of Public Health and Brigham and Women's Hospital.

Cases

Breast cancer cases were identified on biennial questionnaires; the National Death Index was searched for nonresponders. Cases (N=253) had no previously reported cancer diagnosis and were diagnosed with breast cancer after sample collection but before June 1, 2005. Two cases were excluded because the urine samples of the matched controls were not available for assay, one case's urine sample vial broke prior to EM assay, and creatinine levels were not available for three cases. Therefore, 247 cases of breast cancer (N=168 invasive) were confirmed by medical record review (N=237) or verbal confirmation by the nurse (N=10). Given the greater than 99% confirmation rate on medical record review, these latter cases were included. Information on invasiveness and hormone receptor status was abstracted from the medical record. Mean time from sample collection to diagnosis was 47 months (range=1–100). Two controls were matched to each case (N=485) on age (±2 years); menopausal status at diagnosis; month/year of collection (±2 months); ethnicity (African-American, Asian, Hispanic, Caucasian, Other); luteal day ((date of next period-date of luteal collection) ±1 day). Two controls, from different case-control sets, were excluded because one was determined to be ineligible after selection and the other's urine sample vial broke prior to assay; seven controls were excluded due to missing creatinine measures.

Case-control sets were assayed together, as were plasma follicular and luteal samples from each woman. Samples were ordered randomly and labeled to mask case-control status. Blinded replicate quality control samples were placed in each batch to assess laboratory variability.

Laboratory assays

For the urinary EM assay, 500μL of frozen urine was sent to the Laboratory of Proteomics and Analytical Chemistry, SAIC-Frederick, Inc., Frederick, MD. Given that endogenous estrogens and their metabolites are usually present in urine as glucuronide and sulfate conjugates, an initial hydrolysis step was included and the resulting free EM were measured. Each urine sample was thawed and mixed, and 400 μL was immediately aliquoted into a clean screw-cap glass tube and 20 μL of an internal standard solution containing 1.6 ng of each of five deuterated EM (17β-estradiol-d_4, estriol-d_3, 2-hydroxy-17β-estradiol-d_5, 2-methoxy-17β-estradiol-d_5, 16-epiestriol-d_3) was added, followed by 0.5 mL of 0.15 M acetate buffer, pH 4.1, containing 2 mg of ascorbic acid and β-glucuronidase/sulfatase from *Helix pomatia* (Type HP-2) (Sigma-Aldrich, St. Louis, MO). The deuterated EM are used to correct for loss of urinary EM during the hydrolysis, extraction, derivatization, and LC-MS^2 steps of the assay procedure. Details of the assay have been published previously (32). In brief, quantitative data were acquired using a TSQ Quantum-AM triple quadrupole mass spectrometer coupled with a Surveyor HPLC system (Thermo, San Jose, CA). Both the HPLC and the mass spectrometer were controlled by Xcalibur software (Thermo). Quantitation of each EM was carried out using Xcalibur Quan Browser (Thermo). Calibration curves for the 15 EM were constructed by plotting EM/deuterium labeled EM peak area ratios versus amounts of the EM. The amount of EM in the urine sample was then

interpolated using a linear function. The overall coefficients of variation (CVs) were <7% except for 4-methoxyestrone (17%) and 4-methoxyestradiol (15%), the two EM with the lowest concentrations.

Plasma hormone assay methods for estrogens have been described previously (9). In brief, samples for cases and matched controls through 2003 follow-up cycle were assayed at Quest Diagnostics (San Juan Capistrano, CA) by radioimmunoassay following extraction and celite column chromatography. Case and control samples for the 2005 follow-up cycle were assayed at the Mayo Clinic by liquid chromatography-tandem mass spectrometry (LC-MS^2) (ThermoFisher Scientific, Franklin, MA; Applied Biosystems-MDS Sciex, Foster City, CA). CVs for plasma estradiol and estrone were <11%. Progesterone was measured by chemiluminescent immunoassay with the Immulite auto-analyzer (Diagnostic Products, UK) at the Royal Marsden Hospital. CVs for progesterone were ≤17% overall (≤4% within-batch).

Creatinine was measured in two batches: the first at the Endocrine Core Laboratory at Emory University (Atlanta, GA) using Sigma Diagnostics creatinine agents, the second at Dr. Vincent Ricchiuti's laboratory at Brigham and Women's Hospital (Boston, MA). CVs were 9.2% and 2.4%, respectively.

Statistical Analysis

We identified and excluded statistical outliers for absolute and percent individual and grouped EM as well as EM ratios using the extreme studentized many deviate procedure (33). The number of outliers detected in each absolute EM ranged from 1 (methylated 4-catechols) to 19 (2-methoxyestradiol); no outliers were detected in percent or ratio measures. Women with luteal plasma progesterone levels <400 ng/dL were classified as anovulatory for the cycle during which the urine and blood were collected. We used mixed effects regression, by case-control set to account for matching, to test the paired differences in log-transformed hormone levels between cases and controls. Quartile cutpoints were based on control distributions. We used conditional logistic regression to estimate relative risks (RRs) and 95% confidence intervals (CIs). Multivariate models adjusted for body mass index (BMI) at age 18, family history of breast cancer, ages at menarche and first birth, history of benign breast disease, and parity. Multivariate results are presented as they were essentially the same as simple estimates. In stratified analyses, we used unconditional logistic regression, additionally adjusting for matching factors, since overall results were essentially the same from multivariate unconditional and conditional logistic regression models. We evaluated hormone receptor positive cases separately (N=125 estrogen receptor-positive (ER+), of which 111 were also progesterone receptor-positive (PR+)), but did not evaluate other hormone receptor subtypes because of low statistical power (N≤35 for each remaining subtype). Tests for interaction between stratification variables and hormones compared the slope of the quartile medians between groups (Wald test). Tests for trend were conducted by modeling quartile median concentrations and calculating the Wald statistic. The shape of the dose-response curves and tests for non-linearity were assessed using restricted cubic spline models (34). We corrected for random within-person and laboratory error (35), using within-person variability from our previously published reproducibility data (36) and between-person variability from the case-control data set. In these analyses, relative risks of breast cancer were calculated by comparing the median urinary EM level of women in the highest quartile with that of women in the lowest quartile. All p-values were based on two-sided tests and were considered statistically significant if ≤0.05.

We conducted a replacement analysis to estimate the effect of replacing one pathway with another, as is used with dietary components in nutritional epidemiology (37). In this model, total EM is held constant while each pathway's coefficient represents the effect of

substitution of that pathway for the pathway not included in the model. For example, in a model with the variables for the 2-, 4-, and 16-pathways included, the coefficient for 2-pathway estimates the effect of replacement of 2-pathway for parent estrogens (the component left out of the model).

We previously published our analysis of plasma estrogens in the cases and controls with follow-up through 2003 (9). More recently, we measured follicular and luteal plasma estrogens with follow-up through 2005. We examined the combined effects of plasma estrogens and urinary EM, restricted to women with ovulatory cycles, in a few ways. First, to determine if plasma levels modify associations with urinary EM, we stratified by plasma estrogen level (at the medians), using both follicular and luteal plasma measures. Second, to assess if relative differences between plasma and urine are important, we examined the ratio of plasma estrone and estradiol to urinary EM. Finally, to investigate the combined effects of plasma and urine, we cross-classified women by plasma estrogen and urinary EM using the medians as cut-points.

Results

Parent estrogens were, on average, 22% of total EM among controls (Figure 2). The largest proportions of total EM were in the 2- and 16-pathways, with 36% and 38%, respectively. The 4-pathway made up a small mean proportion of EM at 4%. Of the individual EM, the most abundant was 2-hydroxyestrone, with a mean of 27% of the total EM. The next highest were estriol (18%) and estrone (15%). Four of the five methylated catechol EM were the least abundant EM, at <1% each; 2-methoxyestrone was 5%.

Comparisons of urine collection characteristics and breast cancer risk factors between cases and controls are presented in Table 1. Luteal samples collected in an anovulatory cycle were more common among controls (11.6%) than cases (9.4%). Controls had gained slightly more weight since age 18 compared with cases (11.2 vs. 10.2 kg). Cases were more likely to be nulliparous (21.5 vs. 19.4%), have an older age at first birth (27.4 vs. 26.7 y), and less likely to have breast fed (79.4 vs. 83.6%). Cases had a higher prevalence of family history of breast cancer (13.4 vs. 9.1%) and personal history of benign breast disease (20.2 vs. 14.9%). Of the individual EM, controls had significantly higher absolute levels of estradiol (median 13.7 vs. 12.2 pmol/mg creatinine, p=0.02), methylated catechols and methylated 2-catechols (e.g., median methylated catechols 10.7 vs. 10.1, p=0.04), and 2-hydroxyestrone-3-methyl ether (median 1.13 vs. 1.01, p=0.01) (Supplemental Table). Cases had significantly higher levels of 17-epiestriol (median 1.70 vs. 1.48, p=0.03). For the ratios of metabolic pathways, controls had significantly higher parents/estrogen metabolite ratios (0.27 vs. 0.26, p=0.04) while cases had higher ratios of 16-pathway/parent EM (1.67 vs. 1.56, p=0.01).

Women with higher levels of urinary parent EM were at significantly lower risk of breast cancer compared to those with the lowest levels (top vs. bottom quartile RR=0.50, 95% CI (0.29–0.85); estrone RR=0.52, 95% CI (0.30–0.88); estradiol RR=0.51, 95% CI (0.30–0.86), p-trend=0.005) (Figure 3A). The associations with parent EM and estrone were significantly non-linear (p-curvature=0.01, 0.01 respectively). Non-significant inverse trends were observed with the 2- and 4-pathways but not the 16-pathway EM (Figure 3A). Inverse trends were suggestive in the catechol and methylated catechol EM within the 2- and 4-pathways (Figures 3B & 3C), particularly for combined methylated catechols (top vs. bottom quartile RR=0.62, 95% CI (0.40–0.98), p-trend=0.06), methylated 2-catechols (RR=0.63, 95% CI (0.40–1.00), p-trend=0.07), 2-hydroxyestrone-3-methyl ether (RR=0.64, 95% CI (0.41–1.01), p-trend=0.08), and 4-methoxyestradiol (RR=0.61, 95% CI (0.37–0.99), p-trend=0.07). Individual EM in the 16-pathway generally were not inversely associated with breast cancer risk, and one, 17-epiestriol, was significantly positively associated with risk (top vs. bottom

quartile RR=1.74, 95% CI (1.08–2.81), p-trend=0.01) (Figure 3D). Results generally were similar when EM were expressed as a % of total (results not shown).

To contrast EM in different metabolic pathways we investigated several ratios. The ratios of catechols to methylated catechols were not significantly associated with breast cancer risk, either within or across the 2- and 4-pathways (Figure 4A). Comparing parent EM and estrogen metabolites, a significant inverse association was observed for the ratio of parent EM to all other EM (top vs. bottom quartile RR=0.58, 95% CI (0.35–0.96), p-trend=0.03). The 2-pathway:parent EM ratio was not related to risk, the 4-pathway:parent EM ratio was positively, but nonsignificantly, associated with risk, and the 16-pathway:parent EM ratio was positively associated with risk (top vs. bottom quartile RR=1.61, 95% CI (0.99–2.62), p-trend=0.04) (Figure 4B). Breast cancer risk tended to increase with the 4- to 2-pathway ratio and decrease with the 2- to 16-pathway and 4- to 16-pathway ratios, although none of the tests for trend were significant. The 2-hydroxyestrone to 16α-hydroxyestrone ratio was not associated with risk (comparable RR=0.90, 95% CI (0.57–1.41), p-trend=0.86) (data not shown in figure).

We conducted several secondary analyses restricting to subsets of cases, including invasive, ER+, ER+/PR+, and diagnosed >2 years after urine collection. Restricting to invasive cases yielded slightly attenuated results for parent EM, estrone, and estradiol (e.g., top vs. bottom quartile parent EM RR=0.74, 95% CI (0.42–1.31), p-trend=0.02) (Supplemental Table 2); otherwise results were similar to the overall findings (e.g., 17-epiestriol RR=1.80, 95% CI (1.07–3.04), p-trend=0.01). Results also were similar to the overall results when restricted to ER+ and ER+/PR+ cases. No differences were observed when we excluded cases diagnosed within the first two years of urine collection. Similarly, results were comparable among cases diagnosed 0-</≤4 years and 4–8 years after urine collection with the exception of 17-epiestriol where stronger results were observed in later cases, although the interaction was not significant (RR (95% CI) 0–4 years: 1.47 (0.83–2.61), p-trend=0.08; 4–8 years: 2.18 (1.15–4.12), p-trend=0.04; p-heterogeneity=0.82).

Restricting analyses to first morning urine or to women whose urine was collected in an ovulatory cycle did not substantially affect the results. Restricting to women whose urine was collected 4–10 days prior to the onset of the next menstrual cycle, in the mid-luteal phase, resulted in slightly stronger associations for methylated catechols (top vs. bottom quartile RR=0.49, 95% CI (0.29–0.81), p-trend=0.01) and the 16-pathway/parent EM ratio (top vs. bottom quartile RR=1.62, 95% CI (0.99–2.67), p-trend=0.03). Among women premenopausal at diagnosis (N=220 cases) results were similar.

There were no substantial differences in analyses stratified by age at urine collection, BMI at urine collection, or family history of breast cancer. Stratifying by whether women's menstrual cycle patterns had changed compared with the pattern in their 20s did not affect the results. However, the associations between some EM and breast cancer did vary by menstrual cycle regularity between ages 18 and 20 years, with stronger associations observed among those who did not have regular cycles at those ages (N=62 cases). For example, estradiol was not significantly associated with breast cancer risk among those with regular cycles (top vs. bottom quartile RR=0.84, 95% CI (0.50–1.42), p-trend=0.27) but was strongly and significantly inversely associated among those who did not have regular cycles (comparable RR=0.19, 95% CI (0.06–0.59), p-trend=0.008, p-heterogeneity=0.06). Associations with parent EM and estrone also were suggestively stronger among those who did not have regular cycles in early adulthood (p-heterogeneity=0.07 and 0.11, respectively). Stratifying by history of benign breast disease generally yielded similar results except for stronger associations among those who had a history of the disease (N=50 cases) with estrone (top vs. bottom quartile positive history RR=0.07, 95% CI (0.01–0.41); negative

history RR=0.80, 95% CI (0.47–1.37); p-heterogeneity=0.04) and 2-pathway EM (comparable RR=0.31, 95% CI (0.09–1.03); RR=1.03, 95% CI (0.62–1.70); p-heterogeneity=0.04).

In the replacement model analysis, we found lower risks of breast cancer when parent estrogens replaced EM in any of the pathways. For example, in a model with the parent estrogens and the 4- and 16-pathways (leaving out the 2-pathway), each 10nmol/mg creatinine decrease in 2-pathway EM and increase in parent estrogens was associated with a 19% reduction in risk (RR=0.81, 95% CI (0.73–0.91)); a 10nmol/mg creatinine increase in parent estrogens is comparable to a one quartile change. Similar associations were observed when we modeled parent estrogens as a replacement for 4-pathway EM and 16-pathway EM. No significant associations were observed for one pathway replacing another (e.g., 10 nmol/mg creatinine decrease in 2-pathway EM and increase in 16-pathway EM RR=1.03, 95% CI (0.98–1.08)).

Extending our previously published results of plasma estrogens and breast cancer risk (9) with another cycle of follow-up, with a total of 249 cases, we observed similar results, although the association with follicular free estradiol was attenuated (top vs. bottom quartile RR=1.51, 95% CI (0.88–2.61), p-trend=0.21). Although the trend with luteal estrone was not statistically significant, the association was inverse (comparable RR=0.61, 95% CI (0.38–0.98), p-trend=0.12), similar to our previously published results. When we combined these updated plasma measures with the urinary measurements from the current analysis, for each combination it appeared that higher urinary estrone and estradiol were beneficial regardless of plasma level (Table 2). For instance, women with high urinary luteal estrone and either low or high plasma luteal estrone were at significantly lower risk compared with women with low plasma and low urinary estrone (urine/plasma high/low RR=0.56, 95% CI (0.32–0.97), high/high RR=0.65, 95% CI (0.43–0.99)). Similar associations were observed for urinary and plasma luteal estradiol. We also observed a similar pattern for both estrone and estradiol when we examined the combination of luteal urinary levels and follicular plasma levels (e.g., estradiol high/low RR=0.60, 95% CI (0.36–1.01), high/high RR=0.58, 95% CI (0.34–0.97)).

Next, we calculated the ratio of urinary:plasma luteal estrone (and estradiol) levels. We observed that women with a higher urinary luteal estrone to plasma luteal estrone ratio (i.e., higher urinary excretion relative to plasma levels) were at lower risk of breast cancer compared to those in the highest quartile of the ratio (bottom vs. top quartile RR=0.52, 95% CI (0.31–0.87), p-trend=0.02). Although the association with luteal urinary estradiol to plasma estradiol ratio was in the same direction, it was weaker and not statistically significant (comparable RR=0.78, 95% CI (0.46–1.33), p-trend=0.29). When we stratified the EM analyses by median plasma estrone or estradiol level, using both follicular and luteal plasma measures, we did not observe meaningful differences in the associations between urinary EM and breast cancer risk (data not shown).

Results corrected for measurement error were strengthened but the magnitude of difference between the uncorrected and corrected analyses varied given the range of intraclass correlation coefficients (ICCs). For example, with an ICC of 0.71, estradiol results were fairly similar (median of top vs. bottom quartile uncorrected RR=0.55, 95% CI (0.37–0.83); corrected RR=0.43, 95% CI (0.24–0.77)). However, the difference between uncorrected and corrected was larger for 17-epiestriol (ICC=0.42) (uncorrected RR=1.71, 95% CI (1.13–2.57); corrected RR=3.69, 95% CI (1.33–10.2)).

Discussion

In this study, which focuses systematically on estrogen metabolism patterns and subsequent breast cancer risk in premenopausal women, we did not observe increased risks with any individual EM or estrogen metabolism pathway except 17-epiestriol. Luteal urinary estrone and estradiol each were significantly inversely associated with breast cancer risk while total EM as well as EM in the 2- and 4-hydroxylation pathways were generally suggestively inversely associated while EM in the 16-pathway were not inversely associated with risk. Ratios of the three hydroxylation pathways were not significantly associated with risk although the 2:16-pathway and 4:16-pathway ratios were suggestively inversely associated. We observed a significant inverse association with the ratio of parent estrogens to estrogen metabolites. Plasma concentration of parent estrogens did not appear to modify these associations and high urinary concentrations of parent estrogens were inversely associated with risk regardless of plasma parent estrogen levels.

Three prior prospective studies of estrogen metabolites and breast cancer risk in premenopausal women have only investigated 2-hydroxyestrone and 16α-hydroxyestrone (26, 27, 31). In two small (<70 cases) studies of premenopausal women (matched on menstrual cycle phase or mid-luteal), non-significant inverse associations were observed for the urinary 2-hydroxyestrone:16α-hydroxyestrone ratio (top vs. bottom tertile RR=0.75, 95% CI (0.35–1.62); top vs. bottom quintile RR=0.55, 95% CI (0.23–1.32)) (38, 39). In the recent New York University Women's Health Study, these two metabolites were measured in serum and cases (N=377) were matched to controls on day of the menstrual cycle (31). No significant associations were observed overall (e.g., top vs. bottom quartile 2-hydroxyestrone:16α-hydroxyestrone ratio RR=1.13, 95% CI (0.68–1.87) p-trend=0.51) but a suggestive increased risk was observed in ER+ cases (comparable RR=2.15, 95% CI (0.88–5.27), p-trend=0.09). In our study, the associations with both 2-hydroxyestrone and 16α-hydroxyestrone were non-significantly inverse and we did not observe a consistent trend or significant associations between the 2-hydroxyestrone:16α-hydroxyestrone ratio and breast cancer risk.

While 2-hydroxyestrone and 16α-hydroxyestrone have long been hypothesized to have differential effects on breast cancer risk (17), these are only two of many EM created endogenously from the metabolism of estrogens. Experimental evidence suggests several potentially estrogenic and genotoxic mechanisms by which specific EM may be differentially associated with breast cancer risk. Although both 2- and 4-catechol EM bind to the ER with affinities comparable to estradiol, 4-catechol EM have lower dissociation rates than estradiol and an enhanced ability to upregulate ER-dependent processes (40), while 2-catechol EM act as either weak mitogens (41) or weak inhibitors of cell proliferation (42). While 16α-hydroxyestrone binds to the ER with lower affinity than estradiol, it binds covalently (43) and leads to a constitutively activated ER (44). Laboratory evidence supports the estrogenic role of these EM in breast cancer cell lines, with 4-hydroxyestradiol and 16α-hydroxyestrone increasing proliferation and decreasing apoptosis in a manner similar to estradiol; however these effects were achieved only at concentrations 10-fold higher than estradiol (41). In contrast, 2-hydroxyestradiol did not have substantial proliferative or anti-apoptotic effects.

EM also can be genotoxic, but the individual EM vary in their ability to induce DNA damage. Catechol estrogens can be oxidized into quinones and induce DNA damage directly through the formation of DNA adducts, or indirectly via redox cycling and generation of reactive oxygen species (10). However, the oxidized forms of the catechol estrogens differ in their ability to damage DNA through adducts, with oxidized 2-catechols forming stable and reversible DNA adducts and oxidized 4-catechols forming unstable adducts, which lead

to depurination and mutations (13, 16, 45). In human breast epithelial cells, 2- and 4-catechols have been shown to produce reactive oxygen species and induce oxidative DNA damage (46). These catechols also induce neoplastic transformation in ER-cells, and thus act independently from the ER (15). In normal breast tissue, women with breast cancer have higher amounts of 4-hydroxyestradiol and catechol estrogen quinone conjugates compared with women without breast cancer (45). Two studies have shown higher levels of urinary depurinating estrogen-DNA adducts in women at high risk of breast cancer and those with prevalent breast cancer compared with control women (47, 48). 16α-hydroxyestrone also may be genotoxic, as it has been shown to increase unscheduled DNA synthesis in mouse mammary cells (49).

While the catechol estrogens have estrogenic and genotoxic potential, the methylated catechol estrogens, which are catechol estrogens with one hydroxyl group methylated, have been hypothesized to lower risk of breast cancer. The suggested mechanisms are indirect, by decreasing circulating levels of catechol estrogens and thereby the opportunity for catechols to exert genotoxic or proliferative effects, or direct, by inhibiting tumor growth and inducing apoptosis (15, 50). This latter effect has been observed in both ER+ and ER− breast cancer cell lines (50). Thus, the balance between phase I (oxidation) and phase II (methylation) metabolism of estrogen may be important in hormonally-related cancer development.

Despite the estrogenic and genotoxic potential of many of the EM, we only observed a significantly increased breast cancer risk with one EM, 17-epiestriol, which has particularly strong estrogenic activity and binds to both ERα and ERβ with an affinity comparable to estradiol (51). To our knowledge, there is no experimental or epidemiologic evidence for a role of this EM in breast carcinogenesis. We did not observe reduced risk for higher concentrations of 2-pathway EM relative to 16-pathway EM, nor did we observe a consistent benefit of higher concentrations of methylated catechol EM compared with catechol EM.

The significant inverse associations observed with higher levels of parent estrogens, estrone and estradiol, and higher levels of the ratio of parent estrogens to estrogen metabolites are provocative. To our knowledge, only one small prior study (N=38 cases) examined urinary parent estrogens and breast cancer risk in premenopausal women and found non-significant inverse associations with both estrone and estradiol (top vs. bottom tertile RR=0.4, 95% CI (0.2–1.1) for each) (52). It is possible that the benefit we observed with urinary estrone and estradiol reflects greater excretion of parent estrogens prior to metabolism to other more estrogenic and/or genotoxic forms of estrogen or just greater excretion of all forms of estrogens. Indeed, when we analyzed plasma and urine estrogen levels simultaneously, we observed that women with higher levels of urinary estrone and estradiol, regardless of plasma levels, were at lower risk of breast cancer.

In contrast to our plasma estrogen results in a subset of the women included in this analysis (9), we found stronger associations between several EM and breast cancer risk among women who did not have regular menstrual cycles between ages 18 and 22. Given that the NHSII urine sample collections were timed within the menstrual cycle and therefore initiated only among women who were regularly cycling at the time of collection, this is perhaps a unique subset of women who had irregular cycles in early adulthood but regular cycles later. This association could be due to chance, as there is no obvious biologic reason for this finding.

The comparison of these results with our plasma estrogens and breast cancer analysis in the same case-control set raises a few issues. First, it is possible that urinary parent estrogens and their metabolites are a further step removed from what is happening in the breast tissue,

and plasma may be a more relevant proxy of this breast tissue activity, making the interpretation of our urinary results more complex. In a small study comparing breast tissue and urinary EM, using a different assay, within women with breast cancer (N=9), higher levels of parent estrogens, but lower levels of estrogen metabolites, were observed in breast tissue compared with urine (53). Second, in our original plasma analysis we observed significantly increased risk of breast cancer with higher levels of follicular, but not luteal, estradiol. In the present study we only measured EM in luteal urine samples. Since we do not know the correlation between follicular and luteal urinary EM, it is possible that associations with breast cancer may differ for follicular and luteal EM. The fact that higher levels of luteal plasma estrone appeared inversely associated with breast cancer risk, albeit not significantly so (9), suggests estrogen levels at different times in the menstrual cycle may represent different sources and breast tissue bioactivity. Finally, we primarily have measured unconjugated estrogens in the plasma whereas EM in the urine generally are conjugated. While conjugated estrone and estradiol in circulation act as a reservoir and are not as biologically active as their unconjugated counterparts (54), it is unclear how conjugated estrogens in the urine might reflect estrogenic activity in the breast tissue.

There are several limitations to this analysis including the fact that we are measuring EM in urine, which is likely only partially correlated with estrogen activity in the breast tissue. In addition, we cannot rule out modest effects for some of the EM, given the wide confidence intervals. We only measured EM in a single urine sample, which may not accurately reflect long-term exposure. However, our prior work suggests that the reproducibility is fairly good for most of these 15 urinary EM (e.g., 3-year ICCs were 0.52 for parent EM, 0.72 for 2-pathway EM, 0.57 for 4-pathway EM, 0.52 for 16-pathway EM) (36). Further, we only have luteal and not follicular urine samples, and the associations with follicular concentrations may not be similar to the associations we observed between luteal samples and breast cancer risk, as we observed in our previous plasma analyses (9). Although the data on ER/PR status of the tumors were collected from medical records and were not standardized by a single lab. However, we have directly compared hormone receptor status from pathology reports with central laboratory testing and found a very high level of concordance (87.3%) (55). In addition, we previously have found important differences in associations by ER/PR status in the NHS and NHSII cohorts (9, 56, 57). Finally, although our assay of 15 urinary EM allowed for an assessment of overall effects of these EM, it did not allow us to distinguish between different mechanisms.

Our study also has several important strengths, including the fact that our mid-luteal urine samples were carefully timed, and matched between cases and controls by counting backwards from the onset of the next menstrual cycle, which is more accurate than forward counting (58). In addition, the measurement of all the estrogens and estrogen metabolites in urine allowed for a thorough investigation of individual EM as well as comparisons between metabolic pathways. Finally, the prospective nature of the study, measuring EM in urine samples collected prior to diagnosis, avoided the possibility that disease may alter EM levels and yield spurious associations.

In summary, in this first comprehensive nested case-control study of estrogen metabolism and risk of breast cancer in premenopausal women, we observed significant inverse associations with luteal urinary levels of the parent estrogens and non-significant inverse associations with levels of total EM, 2- and 4-pathway EM, but not 16-pathway EM. The reduced risk associated with increased urinary excretion of parent estrogens was present in women with low and high plasma levels of parent estrogens. Women with higher urinary ratios of parent estrogens to estrogen metabolites also were at a significantly reduced risk of breast cancer. These data suggest that women who excrete more parent estrogens are at reduced breast cancer risk. The only significant positive association observed was with 17-

epiestriol, a metabolite in the 16-hydroxylation pathway at relatively low concentrations. Further research is necessary to confirm the associations observed, investigate the role of genotoxic adducts in these results, explore relationships with circulating and breast tissue EM levels, and identify the determinants of estrogen metabolism patterns.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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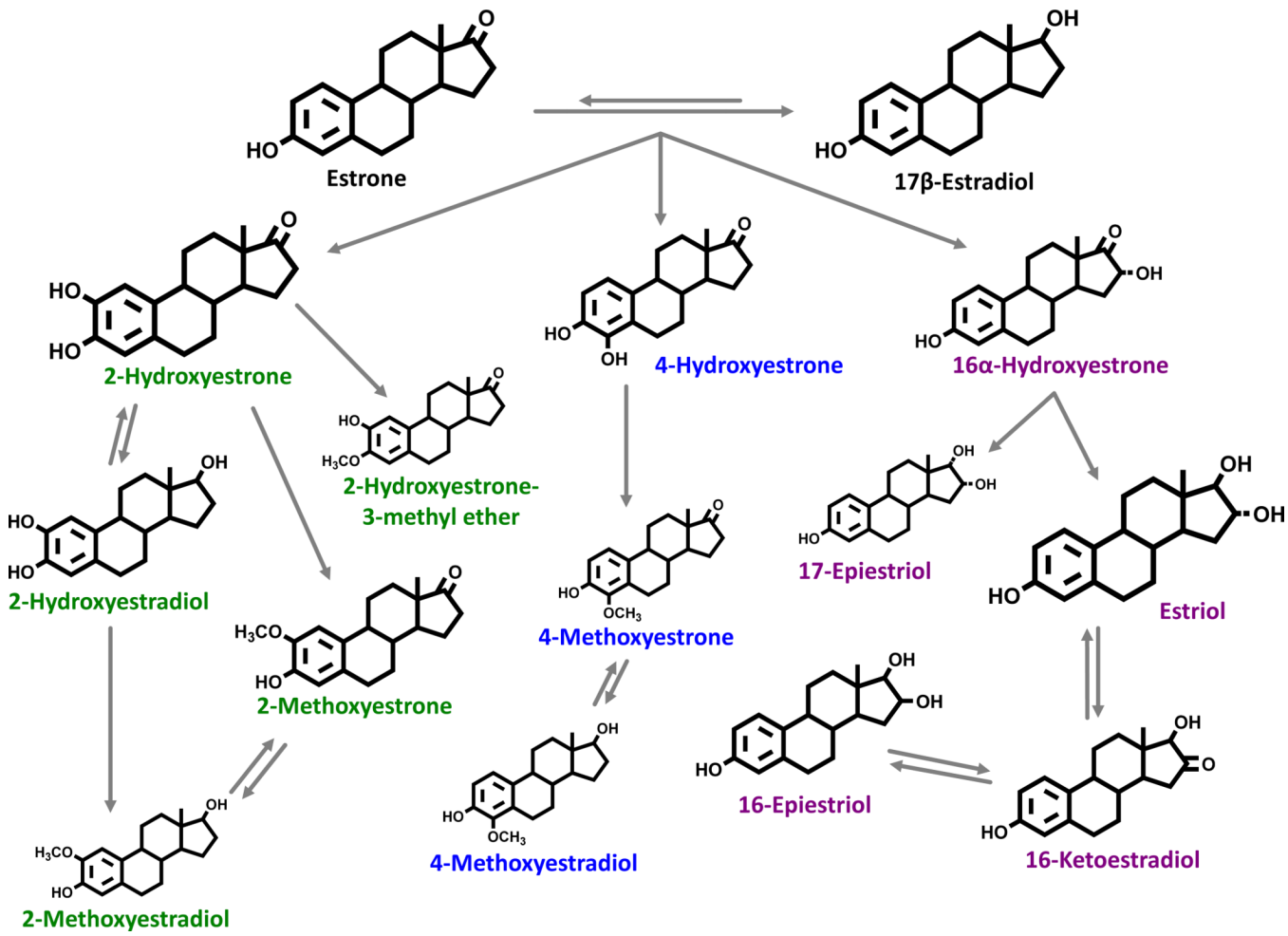

Figure 1. Pathways of endogenous estrogen metabolism
Figure first published in Eliassen et al, Cancer Epidemiol Biomarkers Prev 2009 (ref 42)

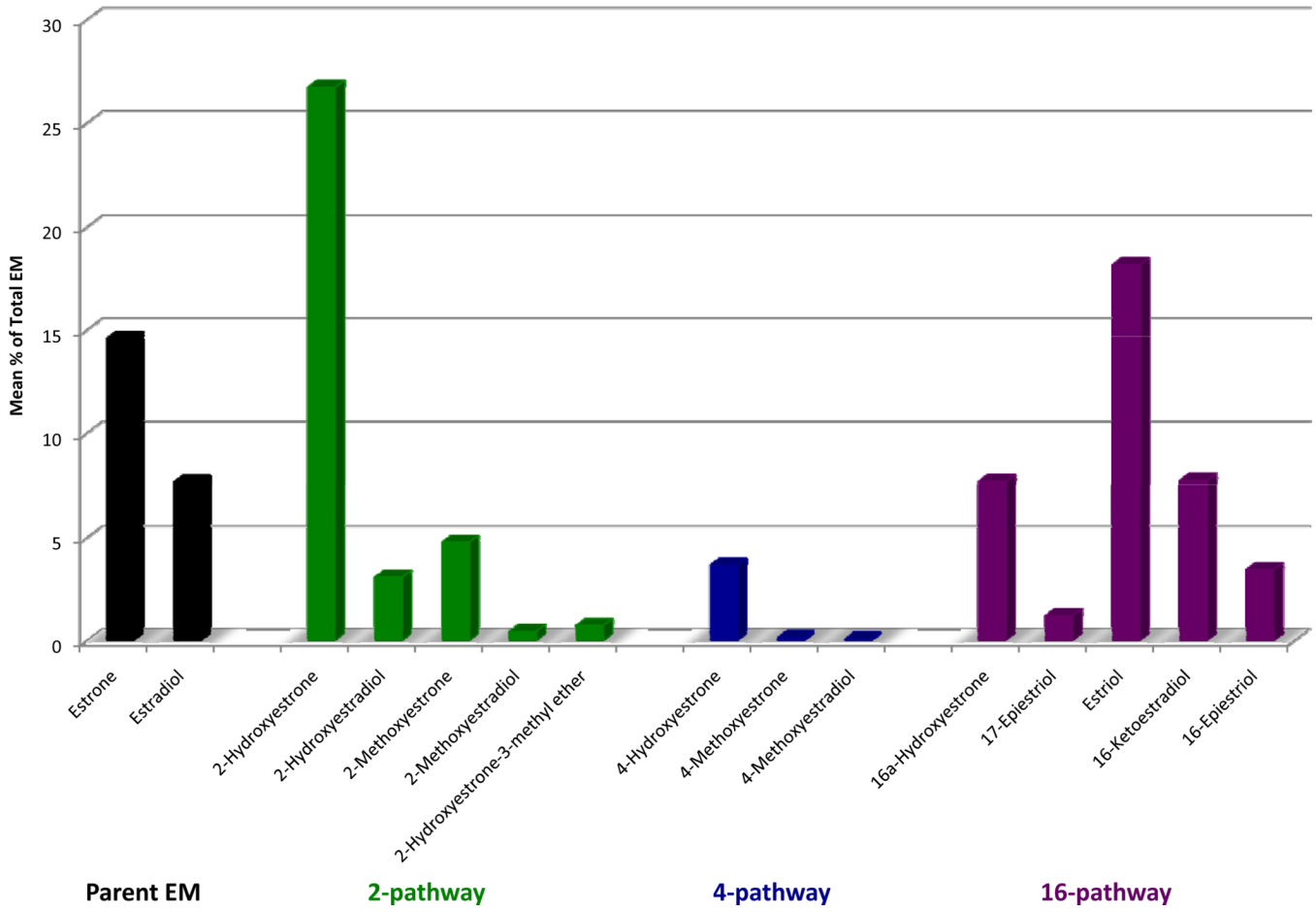

Figure 2. Mean percent of summed total of 15 urinary estrogens and estrogen metabolites (EM) among controls

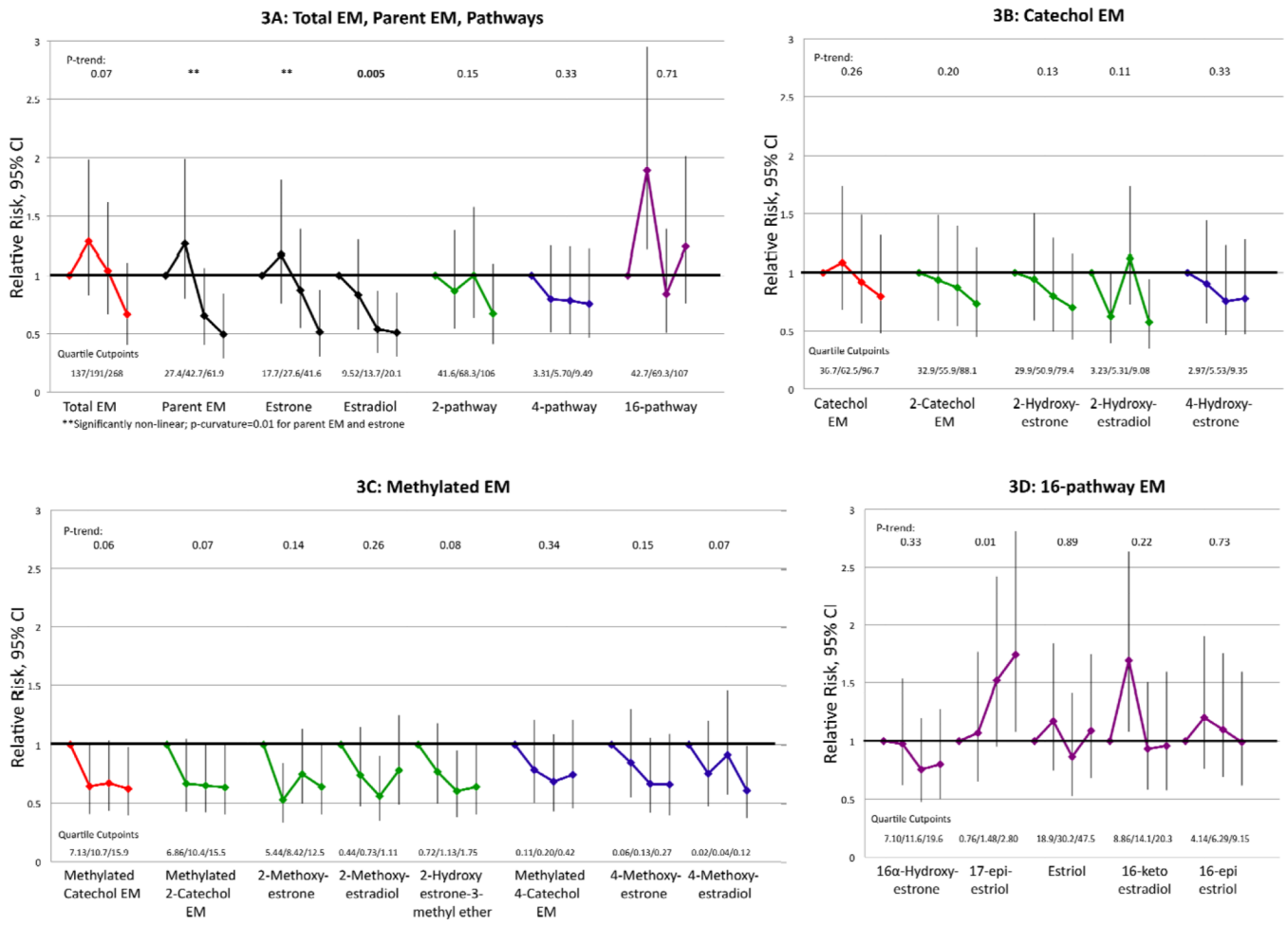

Figure 3. A–D. Multivariate* RRs (95% CI) of breast cancer according to quartiles (pmol/mg creatinine) of Estrogens and Estrogen Metabolites in the Nurses' Health Study II

*Multivariate models adjusted for: first morning urine (yes, no), BMI at age 18 (<21, 21–<23, ≥23), 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), family history of breast cancer (yes, no), history of benign breast disease (yes, no)

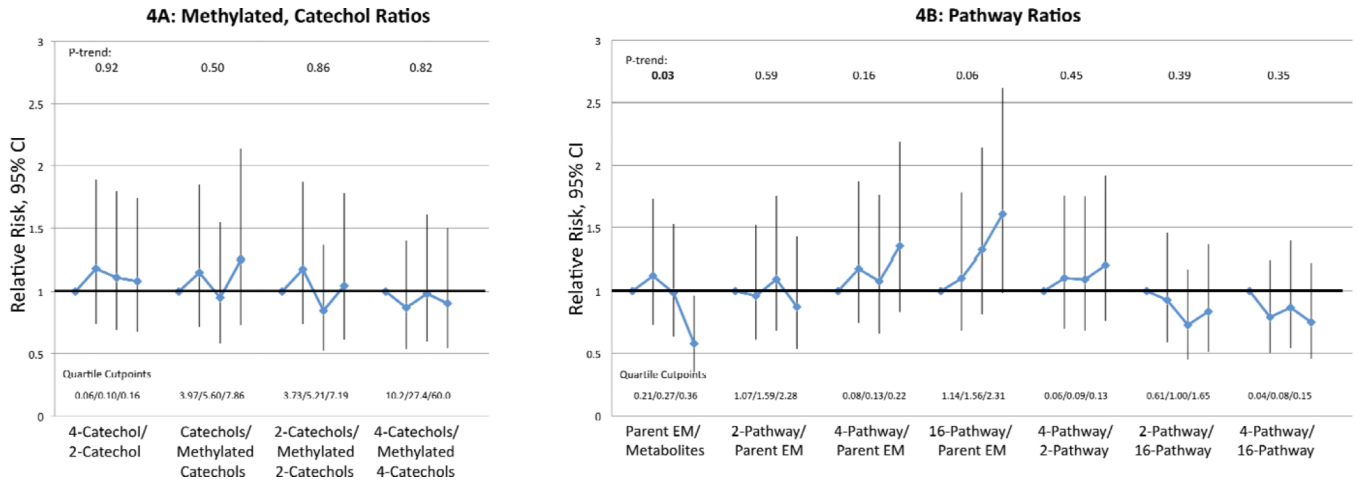

Figure 4. A–B. Multivariate* RRs (95% CI) of breast cancer according to quartiles of EM ratios in the Nurses' Health Study II

*Multivariate models adjusted for: first morning urine (yes, no), BMI at age 18 (<21, 21–<23, ≥23), 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), family history of breast cancer (yes, no), history of benign breast disease (yes, no)

Table 1

Characteristics of breast cancer cases and matched controls in the Nurses' Health Study II; mean (SD) or %

	Cases (N=247)	Controls (N=485)
Age at urine collection, y	43.4 (4.0)	43.1 (3.8)
Days to next menstrual period	7.7 (3.1)	7.6 (2.9)
Collected in anovulatory cycle, %	9.4	11.6
Age at menarche, y	12.5 (1.4)	12.5 (1.4)
BMI at age 18, kg/m^2	20.7 (3.0)	21.0 (2.9)
BMI at urine collection, kg/m^2	24.5 (4.9)	25.2 (5.5)
Weight change since age 18, kg	10.2 (10.1)	11.2 (11.5)
Ever used OCs, %	85.4	85.6
Duration of past OC use[#], y	2.6 (0.6)	2.5 (0.5)
Nulliparous, %	21.5	19.4
Parity[*], children	2.2 (0.8)	2.3 (1.0)
Age at first birth[*], y	27.4 (4.7)	26.7 (4.5)
Ever breast fed[*], %	79.4	83.6
Family history of breast cancer, %	13.4	9.1
History of benign breast disease, %	20.2	14.9

[#]Among ever OC users only

[*]Among parous women only

Table 2

Multivariate RRs (95% CI) of breast cancer according to cross-classified luteal urinary EM and luteal plasma estrogens, among ovulatory women only in the Nurses' Health Study II

	Urine/Plasma levels*			
	Low/Low	**Low/High**	**High/Low**	**High/High**
Estrone: luteal urine & luteal plasma				
	1.00 (ref)	0.85 (0.52–1.39)	0.56 (0.32–0.97)	0.65 (0.43–0.99)
Estradiol: luteal urine & luteal plasma				
	1.00 (ref)	1.19 (0.74–1.90)	0.63 (0.35–1.13)	0.67 (0.43–1.05)
Estrone: luteal urine & follicular plasma				
	1.00 (ref)	0.97 (0.61–1.53)	0.68 (0.41–1.14)	0.64 (0.40–1.04)
Estradiol: luteal urine & follicular plasma				
	1.00 (ref)	0.97 (0.61–1.54)	0.60 (0.36–1.01)	0.58 (0.34–0.97)

Multivariate models adjusted for: first morning urine (yes, no), BMI at age 18 (<21, 21–<23, ≥23), 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), family history of breast cancer (yes, no), history of benign breast disease (yes, no)

* Cutpoints based on medians of urine and plasma levels