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Reproducibility of Fifteen Urinary Estrogens and Estrogen Metabolites over a 2- to 3-Year Period in Premenopausal Women

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Abstract

Endogenous estrogens play an integral role in the etiology of breast, endometrial, and, possibly, ovarian cancers. Estrogen metabolism yields products that are potentially both estrogenic and genotoxic, yet individual metabolic patterns are just beginning to be explored in epidemiologic studies. Within the Nurses' Health Study II, we examined reproducibility of 15 urinary estrogens and estrogen metabolites (EM) among 110 premenopausal women with three luteal-phase urine samples collected over 3 years. EM were measured by a recently developed high-performance liquid chromatography-tandem mass spectrometry (LC-MS^2) method with high sensitivity, specificity, and precision. We assessed Spearman correlations and intraclass correlation coefficients (ICC) across the three samples. Correlations between urinary estrone or estradiol and EM were only modest (r = 0.1-0.5). The 2- and 4-hydroxylation pathways were highly correlated (r = 0.9) but weakly inversely correlated with the 16-hydroxylation pathway (r = −0.2). Within-woman reproducibility over time was fairly high for the three pathways, with ICCs ranging from 0.52 (16-hydroxylation pathway) to 0.72 (2-hydroxylation pathway). ICCs were similarly high for 2-catechols and the individual catechols (ICCs = 0.58-0.72). Individual and grouped methylated 2-catechols had fairly high ICCs (0.51-0.62), but methylated 4-catechols had low ICCs (0.14-0.27). These data indicate that, in general, urinary EM levels vary substantially among individuals compared with intraindiviual variability. Within-person reproducibility over time for most EM measures is comparable to or better than that for well-vetted biomarkers such as plasma cholesterol and, in postmenopausal women, estradiol. (Cancer Epidemiol Biomarkers Prev 2009;18(11):2860–8)

Introduction

Estrogens play an integral role in the etiology of breast, endometrial, and, possibly, ovarian cancers (1). The role of circulating estrogens in the etiology of breast cancer is well established in postmenopausal women (2-5), and estrogen level may be important among premenopausal women, although evidence is not entirely consistent (6-13). The metabolism of estrone and estradiol yields products that are potentially both estrogenic and genotoxic (14-19). Oxidation of estrone and estradiol occurs at the C-2 and C-4 positions to yield catechol estrogens (2-hydroxyestrone, 2-hydroxyestradiol, and 4-hydroxyestrone) and at the C-16 position to yield 16α-hydroxyestrone (see Fig. 1; refs. 14, 20). With further metabolism, the catechol estrogens are methylated into 2-methoxyestrone, 2-methoxyestradiol, 2-hydroxyestrone-3-methyl ether, 4-methoxyestrone, and 4-methoxyestradiol. Metabolites in the 16-hydroxy pathway are further metabolized into 17-epiestriol, estriol, 16-ketoestradiol, and 16-epiestriol. Based on experimental studies, metabolites along these pathways are hypothesized to have differential estrogenic and genotoxic activities. It has been hypothesized that metabolism favoring the 2-hydroxylation over the 16-hydroxylation pathway may be inversely associated with breast cancer risk (21). Although some studies have analyzed the association of the 2- and 16α-hydroxyestrone metabolites with breast cancer risk in humans (22-34), very little evidence exists regarding other metabolites or groups of metabolites.

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 (35, 36). These EM can be quantitated in 0.5 mL of urine, and the assay is sufficiently rapid and robust for epidemiologic research. Although we have assessed the within-woman reproducibility over time of plasma estrogens (37), no one has assessed the

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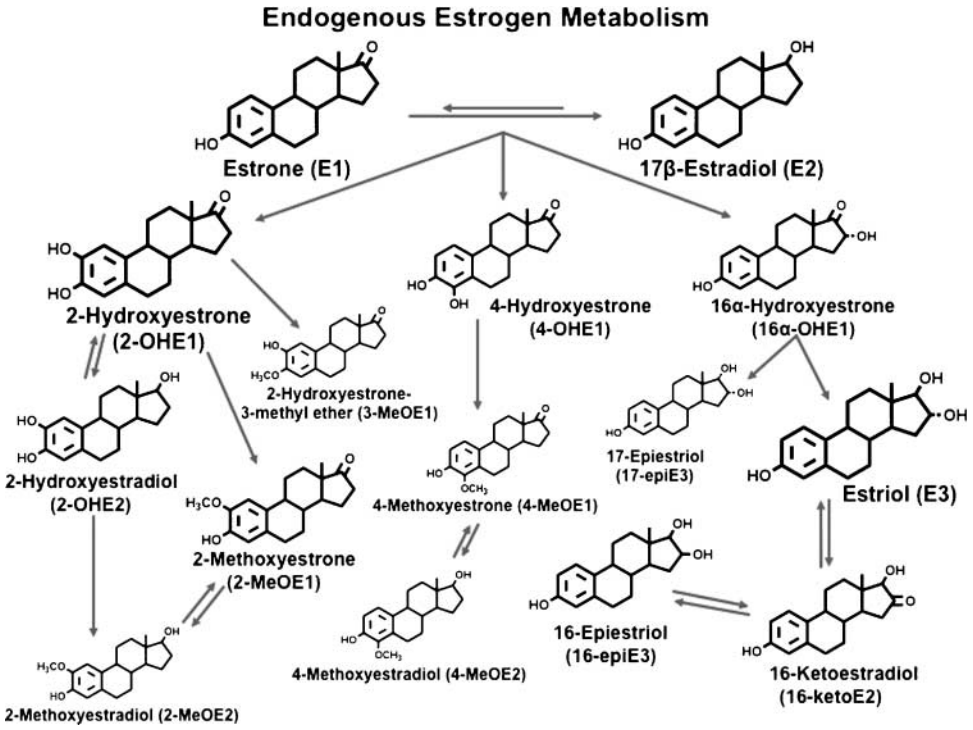

Figure 1. Endogenous estrogen metabolism.

intraindividual variability over time of urinary EM. We assessed the reproducibility over a 2- to 3-year period of the 15 EM in the luteal phase among 110 premenopausal women within the Nurses' Health Study II.

Materials and Methods

Study Population. The Nurses' Health Study II was established in 1989, when 116,678 female registered nurses, ages 25 to 42 y, completed and returned a questionnaire. The cohort continues to be 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 52 y provided blood and urine samples. Of these, 18,521 were premenopausal participants who provided blood and urine samples timed within the menstrual cycle; the women had not used oral contraceptives, been pregnant, or breastfed within the preceding 6 mo. They provided luteal-phase urine samples collected 7 to 9 d before the anticipated start of their next cycle. Samples were shipped, via overnight courier with an ice pack, to our laboratory where the urine was aliquoted and stored in liquid nitrogen freezers (≤−130°C). Approximately 93% of luteal samples were received within 26 h of collection.

Among the premenopausal women who provided samples timed within the menstrual cycle, a random sample of those who were not planning to be pregnant or lactating were invited to participate in the "hormone stability study." Details of the hormone stability study have been published previously (37). Briefly, second and third collection kits for blood and urine were mailed to women who returned the first kit without being reminded and who remained eligible to participate. Of the 412 women invited, 74% (n = 304) and 57% (n = 236) provided a second and third set of samples, respectively.

For each collection, women completed a questionnaire recording their weight, average menstrual cycle length, first day of the menstrual cycle during which the blood and urine samples were collected, and whether the urine was a first morning sample. In addition, women returned a postcard recording the first day of their next menstrual cycle, allowing us to back-date the luteal day of the urine collection.

Of the 236 women with collections for 3 different menstrual cycles over a 2- to 3-y period, 110 women with luteal samples collected between 3 and 11 d before the start of the next menstrual cycle for each of the three collections were selected for the urine reproducibility study. These women were included in the previously published article on intraindividual reproducibility of plasma hormones (37). From the 330 possible samples (110 women × 3 collections), there were 3 women missing the second sample and 5 missing the third sample, leaving a total of 322 samples. 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.

Laboratory Methods. All three of the urine samples from a single woman were assayed together; the samples were ordered randomly and labeled such that the laboratory could not identify samples from the same woman. For each collection for each woman, 500-μL frozen urine was sent to the Laboratory of Proteomics and Analytical Chemistry, Science Applications International Corporation-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. Each urine sample was thawed and mixed, and 400 μL were immediately aliquoted into a clean

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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, and 16-epiestriol-d_3) were added, followed by 0.5 mL of 0.15 mol/L acetate buffer (pH 4.1) containing 2 mg of ascorbic acid and β-glucuronidase/sulfatase from *Helix pomatia* (type HP-2, Sigma-Aldrich). 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 (35, 38). In brief, quantitative data were acquired using a TSQ Quantum-AM triple quadrupole mass spectrometer coupled with a Surveyor high-performance liquid chromatography system (Thermo). Both the HPLC and the mass spectrometer were controlled by Xcalibur software (Thermo). Quantitation of each EM in urine 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 from masked replicate quality control samples placed in each batch ranged from 1.0% (2-hydroxyestrone) to 6.5% (4-methoxyestrone).

Creatinine was measured in two batches: the first with 228 samples at the Endocrine Core Laboratory at Emory University (Atlanta, GA) using Sigma Diagnostics creatinine agents, and the second with 95 samples at Dr. Nader Rifai's laboratory at the Boston Children's Hospital (Boston, MA). Coefficients of variation were ≤4.5% in both laboratories.

Plasma follicular and luteal samples from each of the three collections were assayed at the same time for each woman. Estrogens and progesterones were measured at Quest Diagnostics-Nichols Institute (San Juan Capistrano, CA); details of the assay methods have been described in detail previously (39). Coefficients of variation were ≤14% for plasma hormones.

Statistical Analysis. Absolute concentrations of individual EM were adjusted for creatinine to convert the data to picomoles per milligram of creatinine. Individual EM were combined according to chemical characteristics (e.g., catechols and methylated catechols) and pathways (e.g., 2-hydroxylation, 4-hydroxylation, and 16-hydroxylation pathways), and absolute concentrations of these EM groups were calculated by summing the individual EM in the group. Parent EM was calculated as the sum of estrone and estradiol. Total EM was calculated as the sum of each of the 15 EM. Percent EM were obtained by dividing the individual or grouped EM by the total EM. Ratios of selected EM groups also were calculated. Although we did not assess most ratios of individual EM, we evaluated the 2-hydroxyestrone/16α-hydroxyestrone ratio, given the interest in this ratio as a potential predictor of breast cancer risk.

From among the 322 total samples, we identified and excluded statistical outliers using the extreme studentized deviate many-outlier procedure (40) for each of the absolute and percent EM measures and the EM ratios. This resulted in the removal of up to five values in several of the EM.

For absolute measures and ratios, we calculated geometric means and 5th and 95th percentiles on the natural log scale and exponentiated the values back to the original scale; means and percentiles for percent measures were calculated on the original scale. To examine Spearman correlations and intraclass correlation coefficients (ICC) among the EM, we first calculated probit scores for each individual at each collection. The advantage of probit scores is that they have a normal distribution even if the original data are skewed and confidence intervals are more accurate (41). Scores were calculated as Φ^{-1} $[i/(N + 1)]$, where Φ is the cumulative distribution function for a standard normal distribution, i is the rank of the participant within the collection, and N is the number of participants in the collection (41). We averaged the probit scores over the three collections to calculate Spearman correlation coefficients. Between-person and within-person variances were estimated from the three sets of probit scores using a linear mixed model. To assess reproducibility over the 2- to 3-y period, we calculated ICCs by dividing the between-person variance by the sum of the within- and between-person variances; 95% confidence intervals were also calculated (42). To transform the probit score ICCs to rank correlations, we used the following formula: $ICC_{rank} = 6/\pi \times \sin^{-1}(ICC_{probit}/2)$ (ref. 41). ICCs calculated using log-transformed EM data were similar to probit-transformed data. We tried adjusting for variables assessed at each urine collection, including age, date of collection, first morning urine, luteal day, body mass index (BMI), and menstrual cycle length. Adjustment for these factors did not change our results; therefore, we did not include these variables in the final model.

Results

A total of 110 women were included in these analyses; 102 women contributed all three urine samples, 3 women were missing the second sample, and 5 were missing the third sample. The three urine collections were conducted over an average of 34 months (range, 24-46 months). At the first collection, women ranged in age from 34 to 49 years (mean, 41 years) with a mean BMI of 24.6 kg/m^2 and weight ranging from 41 to 116 kg. A total of 83% of samples were first morning urine. Two women each contributed three samples from anovulatory cycles (defined as progesterone levels <400 ng/dL); 15 women contributed one sample from an anovulatory cycle. Samples were collected an average of 7 days before the first day of the woman's next menstrual cycle (5th-95th percentile, 4-10 days).

Geometric (or arithmetic for percent measures) means and 5th-95th percentile ranges of the individual EM, EM groups, and selected EM ratios are presented in Table 1. Mean total EM was 219 pmol/mg creatinine, with the main contributors being 2-hydroxyestrone (28% of the total), estriol (17% of the total), and estrone (15% of the total). The EM with the lowest concentrations were four of the methylated catechol EM (2-methoxyestradiol, 2-hydroxyestrone-3-methyl ether, 4-methoxyestrone, and 4-methoxyestradiol), each contributing <1% to the total. Catechol EM concentrations were higher than methylated catechol EM concentrations (catechol EM/methylated catechol EM ratios ranged from ~7 to 36). EM in the 16-hydroxylation pathway were more abundant than EM in the 2-hydroxylation pathway (2-hydroxylation/16-hydroxylation pathway ratio = 0.90), and EM in the 2-hydroxylation pathway were more abundant than EM in

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the 4-hydroxylation pathway (4-hydroxylation/2-hydroxylation pathway ratio = 0.11).

The absolute concentrations of most EM were fairly consistent across the three collections (data not shown). The largest difference in levels was for 2-hydroxyestrone and therefore 2-catechols, catechols, and 2-pathway EM, with higher levels in the first collection (mean, 53.3 pmol/mg creatinine) than in the second and third collections (means, 46.4 and 45.0 pmol/mg creatinine). The levels in the 16-pathway also decreased slightly in subsequent collections, with means of 76.9, 71.4, and 72.3 pmol/mg creatinine for the 16-pathway EM in the first, second, and third collections, respectively. Relative measures were very consistent across the three collections, with the largest difference of <1.2% for percent estrone and percent estradiol. Ratios of groups of metabolites were also very consistent. The largest changes were for 2-catechols/methylated 2-catechols (means of 7.06, 6.92, and 6.62 for the three collections, respectively) and 4-catechols/methylated 4-catechols (comparable means of 36.2, 35.2, and 32.8). Finally, the 2-hydroxyestrone/16α-hydroxyestrone ratio decreased slightly across the three collections (means of 4.12, 3.80, and 3.59, respectively).

Correlations among absolute and percent measures for individual and grouped EM are shown in Table 2. On an absolute scale, estrone and estradiol were moderately correlated (r = 0.62). However, correlations between estrone and most of the other individual EM were more modest (r = 0.07-0.52). Estradiol correlations with most individual metabolites were even lower; r's for most metabolites were <0.35. However, individual EM within each pathway were generally moderately to highly correlated with one another (five EM within the 2-hydroxylation pathway, r = 0.41-0.87; five EM in the 16-hydroxylation pathway, r = 0.35-0.79), although there was less correlation among the three EM in the 4-hydroxylation pathway (r = 0.22-0.30). The 2- and 4-hydroxylation pathways were highly correlated (r = 0.87), but the 16-hydroxylation pathway was weakly inversely correlated with the 2- and 4-hydroxylation pathways (r = −0.19 and r = −0.20, respectively). On the relative scale, percent estrone and percent estradiol were modestly positively correlated (r = 0.48), but were inversely correlated with the percent 2- and 4-hydroxylation pathways (e.g., percent estradiol and percent 2-hydroxylation pathway, r = −0.38). The correlation between the percent 2- and 4-hydroxylation

Table 1. Means and ranges of individual and grouped urinary EM), expressed as absolute concentrations (pmol/mg creatinine), percent of the total EM, and selected EM ratios; first collection

EM measure	Mean (5th-95th percentile)	
	pmol/mg creatinine	% of total
Total EM	219 (117-454)	
Parent EM	44.0 (21.6-84.4)	21.11 (11.95-33.86)
Estrone	31.0 (13.7-62.3)	14.97 (7.85-25.03)
Estradiol	12.3 (5.40-27.3)	6.15 (2.54-12.67)
Catechol EM	68.2 (22.9-214)	35.36 (11.79-64.57)
2-Catechol EM	59.7 (18.1-198)	31.22 (10.09-55.34)
2-Hydroxyestrone	53.3 (16.4-179)	28.02 (9.18-51.10)
2-Hydroxyestradiol	5.94 (1.80-20.8)	3.20 (0.96-6.09)
4-Catechol EM		
4-Hydroxyestrone	7.57 (2.24-25.7)	4.14 (0.99-8.39)
Methylated catechol EM	8.72 (2.91-26.5)	4.65 (1.47-9.27)
Methylated 2-catechol EM	8.46 (2.76-24.8)	4.54 (1.38-9.23)
2-Methoxyestrone	5.93 (1.93-19.7)	3.25 (0.86-7.36)
2-Methoxyestradiol	0.79 (0.30-2.14)	0.40 (0.17-0.74)
2-Hydroxyestrone-3-methyl ether	1.41 (0.42-4.39)	0.80 (0.25-1.55)
Methylated 4-catechol EM	0.20 (0.08-0.57)	0.11 (0.04-0.24)
4-Methoxyestrone	0.16 (0.06-0.47)	0.09 (0.03-0.21)
4-Methoxyestradiol	0.04 (0.01-0.14)	0.02 (0.00-0.07)
2-Hydroxylation pathway EM	69.6 (22.0-209)	35.76 (12.08-61.51)
4-Hydroxylation pathway EM	7.85 (2.37-26.2)	4.25 (1.11-8.44)
16-Hydroxylation pathway EM	76.9 (32.6-184)	38.88 (17.16-67.15)
16α-Hydroxyestrone	12.9 (4.70-40.1)	7.09 (1.70-14.59)
Estriol	32.6 (11.1-93.2)	17.44 (5.68-34.12)
17-Epiestriol	2.19 (0.52-10.5)	1.51 (0.24-5.28)
16-Ketoestradiol	18.2 (7.99-43.9)	9.21 (3.47-15.57)
16-Epiestriol	7.30 (3.50-16.3)	3.65 (1.60-6.33)
	Ratios	
4-Catechol/2-catechols	0.13 (0.06-0.29)	
2-Catechols/16-pathway	0.78 (0.15-3.38)	
Catechols/16-pathway	0.89 (0.18-3.96)	
4-Pathway/2-pathway	0.11 (0.05-0.24)	
2-Pathway/16-pathway	0.90 (0.19-3.80)	
4-Pathway/16-pathway	0.10 (0.02-0.46)	
2,4-Pathway/16-pathway	1.01 (0.21-4.27)	
2-Pathway/4,16-pathway	0.78 (0.18-2.63)	
2-Catechols/methylated 2-catechols	7.06 (2.73-18.3)	
4-Catechol/methylated 4-catechols	36.2 (7.98-146)	
Catechols/methylated catechols	7.80 (3.06-20.6)	
Parent estrogens/estrogen metabolites	0.26 (0.14-0.51)	

NOTE: Means and ranges are geometric for absolute and ratio measures and arithmetic for percent measures.

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Table 2. Spearman correlations of individual and grouped urinary estrogens EM, expressed as absolute concentrations and percent of total EM for the average of three collections

	Parent			Cat	2Cat			4Cat	Me Cat	Me-2Cat		
		E1	E2			2OHE1	2OHE2	4OHE1			2MeE1	2MeE2
Parent EM	*1.0*	**0.96**	**0.79**	0.32	0.32	0.33	0.27	0.32	**0.48**	**0.48**	**0.45**	**0.42**
E1	**0.93**	*1.0*	**0.62**	**0.40**	**0.40**	**0.41**	0.36	0.39	**0.52**	**0.52**	**0.48**	**0.50**
E2	**0.73**	**0.48**	*1.0*	0.06	0.06	0.07	0.01	0.10	0.27	0.26	0.26	0.12
Catechol EM	−0.30	−0.24	**−0.41**	*1.0*	**1.00**	**0.99**	**0.89**	**0.89**	**0.64**	**0.63**	**0.65**	**0.66**
2-Catechol EM	−0.30	−0.24	**−0.42**	**0.99**	*1.0*	**0.99**	**0.90**	**0.86**	**0.62**	**0.62**	**0.64**	**0.67**
2OHE1	−0.29	−0.23	**−0.41**	**0.99**	**1.00**	*1.0*	**0.87**	**0.87**	**0.64**	**0.64**	**0.66**	**0.67**
2OHE2	−0.31	−0.23	**−0.42**	**0.81**	**0.83**	**0.79**	*1.0*	**0.76**	**0.56**	**0.56**	**0.57**	**0.67**
4-Catechol EM												
4OHE1	−0.14	−0.11	−0.25	**0.82**	**0.76**	**0.77**	**0.58**	*1.0*	**0.60**	**0.60**	**0.60**	**0.56**
Me Catechol EM	0.28	0.26	0.12	0.37	0.36	0.37	0.30	0.38	*1.0*	**1.00**	**0.98**	**0.76**
Me 2-Catechol EM	0.28	0.27	0.12	0.37	0.36	0.37	0.30	0.37	**1.00**	*1.0*	**0.98**	**0.76**
2MeE1	0.24	0.21	0.12	**0.41**	**0.40**	**0.41**	0.33	**0.40**	**0.97**	**0.97**	*1.0*	**0.73**
2MeE2	0.13	0.21	−0.12	0.39	**0.40**	**0.40**	**0.44**	0.30	**0.70**	**0.70**	**0.65**	*1.0*
2OH3Me	0.31	0.30	0.17	0.15	0.13	0.14	0.15	0.18	**0.81**	**0.82**	**0.73**	**0.46**
Me 4-Catechol EM	0.24	0.14	0.29	0.03	0.02	0.03	−0.01	0.02	0.36	0.36	0.33	0.21
4MeE1	0.26	0.17	0.27	0.03	0.02	0.03	0.01	0.03	0.36	0.36	0.32	0.25
4MeE2	0.04	−0.01	0.07	0.02	0.03	0.04	−0.05	−0.02	0.21	0.21	0.22	0.09
2-Pathway EM	−0.27	−0.22	**−0.38**	**0.98**	**0.99**	**0.99**	**0.82**	**0.77**	**0.46**	**0.46**	**0.50**	**0.47**
4-Pathway EM	−0.13	−0.11	−0.25	**0.82**	**0.76**	**0.77**	**0.59**	**1.00**	0.39	0.38	**0.40**	0.31
16-Pathway EM	−0.03	−0.06	0.12	**−0.88**	**−0.87**	**−0.88**	**−0.69**	**−0.76**	**−0.53**	**−0.53**	**−0.56**	**−0.47**
16αOHE1	−0.01	0.04	−0.01	**−0.75**	**−0.74**	**−0.74**	**−0.63**	**−0.65**	−0.35	−0.35	−0.38	−0.30
E3	−0.08	−0.13	0.11	**−0.82**	**−0.81**	**−0.82**	**−0.63**	**−0.71**	**−0.55**	**−0.55**	**−0.57**	**−0.48**
17EpiE3	0.07	0.04	0.11	**−0.48**	**−0.47**	**−0.47**	−0.39	**−0.43**	−0.24	−0.23	−0.28	−0.28
16KetoE2	0.04	0.03	0.16	**−0.78**	**−0.77**	**−0.77**	**−0.64**	**−0.70**	−0.35	−0.35	−0.39	−0.34
16EpiE3	0.00	−0.02	0.11	**−0.63**	**−0.63**	**−0.64**	**−0.41**	**−0.55**	**−0.46**	**−0.46**	**−0.49**	−0.23

NOTE: Correlations in bottom left of the table are EM expressed as percent of total EM; correlations in top right are expressed as absolute concentrations of EM. Correlations $\geq |0.40|$ are in boldface.

pathways was high ($r = 0.77$). The percent 16-hydroxylation pathway was highly inversely correlated with the percent 2- and 4-hydroxylation pathways ($r = -0.90$ and $r = -0.77$, respectively) and unrelated to percent estrone ($r = -0.06$) or percent estradiol ($r = 0.12$).

Correlations between luteal urinary estrogens and follicular and luteal plasma estrogens are presented in Table 3. The strongest correlations were between luteal urinary estrone and luteal plasma estrone and estrone sulfate ($r = 0.56$ and $r = 0.57$, respectively) as well as follicular plasma estrone sulfate ($r = 0.49$). Luteal urinary estradiol was modestly correlated with all three luteal plasma estrogens ($r = 0.36$-0.42), but not consistently related to follicular plasma estrogens ($r = -0.11$ to 0.26). Luteal urinary total EM was more weakly correlated with luteal plasma estrogens ($r = 0.26$-0.33) than with luteal urinary estrone and estradiol. There were generally no high correlations between the luteal urinary individual EM and plasma estrogens (data not shown).

ICCs are presented in Table 4; absolute measures of individual and grouped EM are adjusted for creatinine. Overall, the ICCs for absolute and percent EM were fairly high, although the ICC for total EM was only moderate (ICC = 0.39). ICCs for absolute concentrations of the parent estrogens were similar to one another (estrone ICC = 0.52, estradiol ICC = 0.49) but the ICC for percent estrone was higher (0.67). ICCs for the absolute concentrations of individual and grouped catechol estrogens were all $\geq$0.58, with higher ICCs for the percent measures for catechols, 2-catechols, and 2-hydroxyestrone (ICCs increased from 0.72, 0.72, and 0.71 on the absolute scale to 0.85, 0.84, and 0.83 on the relative scale). However, reproducibility of 4-hydroxyestrone was somewhat reduced on the relative scale (ICC = 0.51) compared with the absolute scale (ICC = 0.58). The methylated 2-catechols had moderate to high ICCs on both the absolute (0.51-0.62) and relative (0.50-0.67) scales. Methylated 4-catechols, which are the EM with the lowest concentrations, had very low ICCs (0.27 and 0.14 for 4-methoxyestrone and 4-methoxyestradiol, and 0.25 for methylated 4-catechols); the ICCs for 4-methoxyestrone and methylated 4-catechols increased slightly on the relative scale, but were still very low (comparable ICCs of 0.30 and 0.27). For the pathways, ICCs on both scales were very high for the 2-hydroxylation pathway (0.72 and 0.85 on absolute and percent scales), moderate for the 4-hydroxylation pathway with a decline on the relative scale (0.57 and 0.51), and moderate on the absolute scale for the 16-hydroxylation pathway (0.52) but high on the percent scale (0.82). ICCs for individual metabolites in the 16-hydroxylation pathway ranged from 0.42 (16-ketoestradiol) to 0.54 (estriol); each was substantially improved on the percent scale, ranging from 0.56 (17- and 16-epiestriol) to 0.77 (estriol). ICCs for the EM ratios were very low for ratios comparing the 4- and 2-hydroxylation pathway EM (ICC = 0.21 for both 4-catechols/2-catechols and 4-hydroxylation/2-hydroxylation pathways). With the exception of the 4-catechol/methylated 4-catechol (ICC = 0.42), the ICCs for other ratios were moderate to high (range, 0.58-0.85). The 2-hydroxyestrone/16α-hydroxyestrone ratio had high reproducibility (ICC = 0.76; data not shown).

We conducted several sensitivity analyses to assess the robustness of our overall ICCs, including restricting analyses to first morning urine samples ($n = 258$), ovulatory cycles ($n = 301$), <1 kg/m^2 change in BMI across collections ($n = 137$), difference of <2 luteal days across collections ($n = 126$), luteal days 6 to 9 before next menstrual cycle ($n = 189$), average menstrual cycle length of 26 to 31 days ($n = 222$), and age ≤45 years at all three collections ($n = 211$). Overall, there was no single restriction that

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Table 2. Spearman correlations of individual and grouped urinary estrogens EM, expressed as absolute concentrations and percent of total EM for the average of three collections (Cont'd)

2OH3Me	Me-4Cat	4MeE1	4MeE2	2Path	4Path	16Path	16aOHE1	E3	17EpiE3	16KetoE2	16EpiE3	Total EM
0.45	0.38	0.37	0.11	0.36	0.33	0.33	0.28	0.25	0.26	0.36	**0.43**	**0.64**
0.49	0.32	0.33	0.07	**0.44**	**0.40**	0.30	0.29	0.20	0.25	0.35	**0.41**	**0.66**
0.23	**0.40**	0.35	0.12	0.10	0.10	0.32	0.19	0.28	0.21	0.30	0.35	**0.46**
0.44	0.31	0.30	0.15	**0.99**	**0.89**	−0.19	−0.20	−0.24	−0.10	−0.10	0.03	**0.65**
0.43	0.29	0.29	0.15	**0.99**	**0.86**	−0.18	−0.20	−0.24	−0.10	−0.09	0.03	**0.65**
0.45	0.29	0.29	0.15	**0.99**	**0.87**	−0.18	−0.19	−0.24	−0.09	−0.09	0.02	**0.65**
0.41	0.25	0.25	0.06	**0.89**	**0.77**	−0.13	−0.19	−0.17	−0.06	−0.06	0.09	**0.60**
0.43	0.30	0.30	0.06	**0.87**	**1.00**	−0.21	−0.21	−0.25	−0.10	−0.16	−0.01	**0.58**
0.85	**0.46**	**0.43**	0.20	**0.70**	**0.61**	−0.18	−0.11	−0.26	−0.04	−0.05	−0.07	**0.45**
0.84	**0.44**	**0.41**	0.18	**0.70**	**0.61**	−0.18	−0.11	−0.27	−0.05	−0.06	−0.07	**0.45**
0.77	**0.41**	0.39	0.19	**0.71**	**0.62**	−0.20	−0.12	−0.28	−0.07	−0.09	−0.09	**0.44**
0.59	0.32	0.32	0.10	**0.71**	**0.57**	−0.11	−0.04	−0.18	−0.06	0.00	0.12	**0.50**
1.0	**0.41**	0.37	0.19	**0.50**	**0.45**	−0.08	−0.01	−0.17	0.04	0.03	0.01	0.32
0.36	*1.0*	**0.93**	**0.48**	0.33	0.32	0.12	0.06	0.06	−0.01	0.16	0.19	0.36
0.35	**0.94**	*1.0*	0.22	0.32	0.32	0.11	0.03	0.08	0.03	0.13	0.18	0.33
0.18	**0.51**	0.24	*1.0*	0.17	0.07	0.05	0.08	−0.03	−0.09	0.08	0.08	0.17
0.22	0.07	0.07	0.05	*1.0*	**0.87**	−0.19	−0.19	−0.25	−0.10	−0.09	0.03	**0.66**
0.19	0.06	0.06	0.00	**0.77**	*1.0*	−0.20	−0.20	−0.25	−0.10	−0.15	0.00	**0.58**
−0.28	−0.14	−0.15	−0.04	**−0.90**	**−0.77**	*1.0*	**0.85**	**0.95**	**0.55**	**0.87**	**0.82**	**0.48**
−0.16	−0.16	−0.20	0.05	**−0.76**	**−0.66**	**0.83**	*1.0*	**0.74**	**0.49**	**0.79**	**0.66**	0.37
−0.33	−0.18	−0.16	−0.11	**−0.84**	**−0.71**	**0.95**	**0.70**	*1.0*	**0.45**	**0.77**	**0.78**	**0.40**
−0.05	−0.09	−0.08	−0.07	**−0.48**	**−0.43**	**0.52**	**0.47**	0.39	*1.0*	**0.53**	0.35	0.30
−0.11	0.00	−0.02	0.04	**−0.79**	**−0.71**	**0.86**	**0.77**	**0.74**	**0.48**	*1.0*	**0.72**	**0.48**
−0.27	−0.03	−0.05	0.02	**−0.66**	**−0.55**	**0.74**	**0.53**	**0.72**	0.25	**0.60**	*1.0*	**0.57**

resulted in consistent substantial improvements in the ICCs (data not shown). The restrictions that resulted in the largest changes in ICCs were BMI and luteal day. Excluding women with BMI changes over the collections generally resulted in small increases in ICCs (e.g., ICC for estradiol increased from 0.49 to 0.53), with a few larger increases (e.g., 16α-hydroxyestrone increased from 0.46 to 0.65). On the percent scale, the ICCs increased for parent EM (e.g., percent estradiol increased from 0.52 to 0.60) and EM in the 16-hydroxylation pathway (e.g., percent 16α-hydroxyestrone increased from 0.64 to 0.75). Excluding luteal day differences ≥2 days modestly increased some ICCs that were fairly high to begin with (e.g., methylated catechols ICC changed from 0.61 to 0.68). This exclusion also increased a few modest ICCs (e.g., percent 4-hydroxyestrone from 0.51 to 0.60) and the low ICC of percent 4-methoxyestrone to marginal level (0.30 to 0.43); however, the already low ICCs for absolute and percent 4-methoxyestradiol decreased.

Discussion

In this analysis of premenopausal luteal urinary EM, 16-hydroxylation pathway EM are most abundant, followed by 2-hydroxylation pathway EM, parent EM, and 4-hydroxylation pathway EM, which make up only a very small proportion of total EM. We observed limited correlation between either estrone or estradiol and the individual EM. However, estrone and estradiol were fairly highly correlated, as were the individual EM within each pathway. 2-Hydroxylation and 4-hydroxylation pathway EM were highly correlated, but weakly inversely correlated with 16-hydroxylation pathway EM. We also observed fairly low correlations between plasma estrogens and urinary EM, although correlations with urinary estrone and estradiol were modest. ICCs were generally very high, except for 4-methylated catechols, and generally improved when based on the percent concentration rather than the absolute concentrations. In addition, results were robust with no substantial changes in sensitivity analyses. Given our use of mid-luteal urine samples in this study, it is not clear whether these results may also apply to follicular EM.

These comprehensive data on premenopausal luteal urinary EM in general show excellent reproducibility over time, with many ICCs >0.60, suggesting that one measure may adequately represent longer-term (i.e., at least 3 years) exposure. These ICCs compare favorably with the reproducibility over a several-year period of serum cholesterol (ICC = 0.65; ref. 43), blood pressure (ICC = 0.60-0.64; ref. 44), blood glucose (ICC = 0.52; ref. 45), pulse (ICC = 0.49; ref. 45), and plasma estradiol in postmenopausal women (ICC = 0.68; ref. 42), all of which are exposures considered to be reasonably well-measured and reliable predictors of disease in epidemiologic studies.

Table 3. Spearman correlation coefficients for urinary luteal estrogens with plasma follicular and luteal estrogens; average of three collections

	Plasma estrogens					
	Luteal			Follicular		
	Estradiol	Estrone	Estrone sulfate	Estradiol	Estrone	Estrone sulfate
Urinary luteal estrogens						
Estrone	0.34	**0.56**	**0.57**	−0.07	0.28	**0.49**
Estradiol	0.36	0.38	**0.42**	−0.11	0.01	0.26
Total EM	0.33	0.27	0.26	0.08	0.13	0.21

NOTE: *n* ranges from 87 (follicular estrone) to 106. Correlations ≥ |0.40| are in boldface. Luteal urine and luteal plasma were collected on the same day.

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Table 4. ICCs (95% confidence intervals) for individual and grouped EM, expressed as absolute concentrations, percent of total EM, and selected EM ratios

Analyte	ICC (95% confidence interval)	
	Absolute concentration	% of total EM
Parent EM	0.52 (0.41-0.62)	0.64 (0.54-0.72)
Estrone	0.52 (0.42-0.63)	0.67 (0.58-0.75)
Estradiol	0.49 (0.39-0.60)	0.52 (0.42-0.62)
Catechol EM	0.72 (0.64-0.79)	0.85 (0.80-0.89)
2-Catechol EM	0.72 (0.64-0.78)	0.84 (0.79-0.88)
2-Hydroxyestrone	0.71 (0.63-0.78)	0.83 (0.78-0.87)
2-Hydroxyestradiol	0.67 (0.59-0.75)	0.68 (0.60-0.76)
4-Catechol EM		
4-Hydroxyestrone	0.58 (0.48-0.67)	0.51 (0.41-0.62)
Methylated catechol EM	0.61 (0.51-0.69)	0.64 (0.55-0.73)
Methylated 2-catechol EM	0.62 (0.52-0.70)	0.65 (0.56-0.73)
2-Methoxyestrone	0.62 (0.52-0.71)	0.67 (0.59-0.75)
2-Methoxyestradiol	0.51 (0.41-0.62)	0.53 (0.42-0.63)
2-Hydroxyestrone-3-methyl ether	0.51 (0.40-0.61)	0.50 (0.40-0.61)
Methylated 4-catechol EM	0.25 (0.15-0.39)	0.27 (0.17-0.40)
4-Methoxyestrone	0.27 (0.17-0.41)	0.30 (0.20-0.43)
4-Methoxyestradiol	0.14 (0.06-0.30)	0.13 (0.05-0.29)
2-Pathway EM	0.72 (0.64-0.78)	0.85 (0.80-0.89)
4-Pathway EM	0.57 (0.47-0.67)	0.51 (0.40-0.61)
16-Pathway EM	0.52 (0.41-0.62)	0.82 (0.76-0.86)
16α-Hydroxyestrone	0.46 (0.35-0.57)	0.64 (0.55-0.72)
Estriol	0.54 (0.44-0.64)	0.77 (0.70-0.83)
17-Epiestriol	0.50 (0.39-0.60)	0.56 (0.46-0.65)
16-Ketoestradiol	0.42 (0.31-0.53)	0.59 (0.49-0.68)
16-Epiestriol	0.45 (0.34-0.56)	0.56 (0.46-0.66)
Total EM	0.39 (0.28-0.51)	
	EM ratios	
4-Catechol/2-catechols	0.21 (0.11-0.35)	
2-Catechols/16-pathway	0.83 (0.78-0.88)	
Catechols/16-pathway	0.83 (0.77-0.87)	
4-Pathway/2-pathway	0.21 (0.11-0.35)	
2-Pathway/16-pathway	0.83 (0.77-0.87)	
4-Pathway/16-pathway	0.70 (0.62-0.77)	
2,4-Pathway/16-pathway	0.85 (0.80-0.89)	
2-Pathway/4,16-pathway	0.84 (0.79-0.88)	
2-Catechols/methylated 2-catechols	0.58 (0.48-0.67)	
4-Catechol/methylated 4-catechols	0.42 (0.31-0.54)	
Catechols/methylated catechols	0.60 (0.50-0.69)	
Parent estrogens/estrogen metabolites	0.65 (0.55-0.73)	

The relatively low correlations between parent estrogens and EM (e.g., estrone and estradiol correlations with other individual EM ≤0.52) and between different estrogen metabolic pathways suggest that these EM convey additional information about patterns of estrogen metabolism beyond assessing estrogen exposure with urinary estrone and estradiol. Some of the low correlations we observed between plasma and urinary estrogens could be due to a combination of factors. Assays conducted in plasma did not include measurement of conjugates, whereas the urine assay does detect all conjugates. For instance, estrone measured in plasma is unconjugated estrone, whereas estrone measured in urine detects glucuronides and sulfates as well as unconjugated forms. Thus, it is unclear whether the low correlations are due entirely to biological reasons or a combination of biological differences and analytic differences in the assays. Although urinary estrogens include conjugated forms, the low correlations observed suggest that it is possible that EM may provide additional insight into the estrogen-breast cancer relationship beyond what epidemiologic studies of plasma estrogens can provide.

Few epidemiologic studies have examined EM and breast cancer risk, and they have assessed only 2-hydroxyestrone, 16α-hydroxyestrone, and the 2-hydroxyestrone/16α-hydroxyestrone ratio, with mixed results (22-34). To our knowledge, there are no epidemiologic studies to date of the associations between methoxyestrogens or any metabolites in the 4-hydroxylation pathway and breast cancer risk. The lack of strong correlations between urinary parent estrogens and individual EM and between urinary EM and plasma estrogens suggests that these EM may provide additional insight into the relationship between estrogen level and risk of breast cancer.

Whereas circulating estrogens are established risk factors for postmenopausal breast cancer (2-5) and may be associated with risk among premenopausal women, although studies are not consistent (6-13), interest in EM derives from the fact that they exhibit differential estrogenic and genotoxic activities and may have different roles in breast carcinogenesis. 4-Catechol EM and 16α-hydroxyestrone may have higher estrogenic activity than estradiol (46–52), whereas 2-catechol EM may act as either weak mitogens (53, 54) or inhibitors of proliferation (55, 56). 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 (20). The methoxy estrogens, which are methylated catechol estrogens, have been hypothesized to lower the risk of breast cancer

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indirectly by decreasing circulating levels of catechol estrogens or directly by inhibiting tumor growth and inducing apoptosis (57-61).

In summary, these data suggest that measuring individual EM may provide information that is not available when only measuring parent estrogens, and that patterns of estrogen metabolism may vary substantially among individuals. Most EM, when measured in premenopausal women in the mid-luteal phase, had high reproducibility over time, suggesting that one measure is enough to reflect longer-term exposure. In addition, the LC-MS^2 assay we used is highly sensitive and specific, offers relatively high-throughput and robust results, and requires minimal volume. These characteristics all support the investigation of EM in epidemiologic studies of hormonal carcinogenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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