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 cancer. 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 three 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 (ICCs) across the three samples. Correlations between urinary estrone or estradiol and the estrogen metabolites 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). Reproducibility within woman 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 ICCs were low for the methylated 4-catechols (0.14–0.27). These data indicate that, in general, urinary EM levels vary substantially among individuals compared with intraindiviual variability. Reproducibility within person over time for most EM measures is comparable to or better than well-vetted biomarkers like plasma cholesterol and, in postmenopausal women, estradiol.

Estrogens play an integral role in the etiology of breast, endometrial, and possibly ovarian cancer (1). The role of circulating estrogens in the etiology of breast cancer is well established in postmenopausal women (2–5) and estrogen levels 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, 4-hydroxyestrone) and at the C-16 position to yield 16α-hydroxyestrone (see Figure 1) (14,20). With further metabolism, the catechol estrogens are

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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 activity. 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 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 0.5mL of urine, and the assay is sufficiently rapid and robust for epidemiologic research. Although we have assessed the reproducibility within-woman over time of plasma estrogens (37), no one has assessed intraindividual variability over time of urinary EM. We assessed 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 (NHSII).

Methods

Study population

The NHSII was established in 1989, when 116,678 female registered nurses, aged 25 to 42 years, 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 years 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 six months. They provided luteal phase urine samples collected 7–9 days 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 ($\leq -130°$ C). Approximately 93% of luteal samples were received within 26 hours 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-year period, 110 women with luteal samples collected between 3 and 11 days before the start of the next menstrual cycle for each of the 3 collections were selected for the urine reproducibility study. These women were included in the previously published paper on intraindividual reproducibility of plasma hormones (37). From the 330 possible samples (110 women x 3

collections), there were 3 women missing the second sample and 5 missing the third sample, for 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, 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. 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 (35,38). 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 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 (CVs) 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, the second with 95 samples at Dr. Nader Rifai's laboratory at the Boston Children's Hospital (Boston, MA). CVs were ≤4.5% in both labs.

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

Statistical Analysis

Absolute concentrations of individual EM were adjusted for creatinine to convert the data to pmol/mg creatinine. Individual EM were combined according to chemical characteristics (e.g., catechols and methylated catechols) and pathways (e.g., 2-hydroxylation, 4- hydroxylation, 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 (pmol/L) 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 5 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 (ICCs) 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 $\Phi^{-1}[i/(N+1)]$ 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-year period, we calculated ICCs by dividing the between-person variance by the sum of the within- and between-person variances; 95% confidence intervals (CI) were also calculated (42). To transform the probit score ICCs to rank correlations, we used the following formula: $ICC_{rank}=6/\pi*\sin^{-1}(ICC_{probit}/2)$ (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, 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 3 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 <400ng/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 to 10 days).

Geometric (or arithmetic for % 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 (catechol EM/methylated catechol EM ratios ranged from ~7 to 36). EM in the 16-hydroxylation pathway were more abundant than the 2-hydroxylation pathway (2-:16-hydroxylation pathway ratio=0.90) and EM in the 2-hydroxylation pathway were more abundant than the 4-hydroxylation pathway (4-:2-hydroxylation pathway ratio=0.11).

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 16-pathway EM in the first, second, and third collections, respectively. Relative measures were very consistent across the three collections with the largest differences <1.2% for %estrone and %estradiol. Ratios of groups of metabolites were also very consistent. The largest changes were for 2-catechols/methylated 2-catechols (means=7.06, 6.92, 6.62 for the three collections, respectively) and 4-catechols/ methylated 4-catechols (comparable means=36.2, 35.2, 32.8). Finally, the 2-hydroxyestrone/ 16α-hydroxyestrone ratio decreased slightly across the three collections (means=4.12, 3.80, 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 estrogen metabolites 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), though 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 −0.20, respectively). On the relative scale, %estrone and %estradiol were modestly positively correlated (r=0.48), but were inversely correlated with %2- and %4-hydroxylation pathways (e.g., %estradiol and %2-hydroxylation pathway r=−0.38). The correlation between the %2- and %4-hydroxylation pathways was high (r=0.77). The %16-hydroxylation pathway was highly inversely correlated with the %2- and %4-hydroxylation pathways (r=−0.90 and −0.77, respectively) and unrelated to %estrone (r=−0.06) or %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 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= −0.11–0.26). Luteal urinary total EM was more weakly correlated with luteal plasma estrogens (r=0.26–0.33) than luteal urinary estrone and estradiol. There generally were not 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 %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 %estrone was higher (0.67). ICCs for the absolute concentrations of individual and grouped catechol estrogens were all ≥0.58, with higher ICCs for the % 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 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 % 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 % 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 % 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-/2-hydroxylation pathway). 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), <2 luteal days difference across collections (n=126), luteal days 6 to 9 prior to next menstrual cycle (n=189), average menstrual cycle length 26–31 days (n=222), and age at all three collections ≤45 years (n=211). Overall there was no single restriction that 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 % scale, the ICCs increased for parent EM (e.g., %estradiol increased from 0.52 to 0.60) and EM in the 16-hydroxylation pathway (e.g., %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., %4-hydroxyestrone from 0.51 to 0.60) and the low ICC of %4-methoxyestrone to become marginal (0.30 to 0.43); however the already low ICCs for absolute and %4-methoxyestradiol decreased.

Discussion

In this analysis of premenopausal luteal urinary EM, concentration of the 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 estrogen metabolites. However, estrone and estradiol were fairly highly correlated, as were the individual estrogen metabolites within each pathway. 2- 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, though 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 % 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) (43), blood pressure (ICC=0.60–0.64) (44), blood glucose (ICC=0.52) (45), pulse (ICC=0.49) (45), and plasma estradiol in postmenopausal women (ICC=0.68) (42), all of which are exposures considered to be reasonably well-measured and reliable predictors of disease in epidemiologic studies.

The relatively low correlation between parent estrogens and estrogen metabolites (e.g., estrone and estradiol correlations with other individual EM≤0.52) and between different estrogen metabolic pathways suggests 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 while the urine assay does detect all conjugates. For instance, estrone measured in plasma is unconjugated estrone, while 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 biologic reasons, or a combination of biologic differences and analytic differences in the assays. Although urinary estrogens include conjugated forms, the low correlations observed suggest 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 estrogen metabolites and breast cancer risk, and they have assessed only 2-hydroxyestrone, 16α-hydroxyestrone, and the 2:16α-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 estrogen metabolites and between urinary EM and plasma estrogens suggests that these EM may provide additional insight into the relationship between estrogen levels and risk of breast cancer.

While 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 estrogen metabolites derives from the fact that they exhibit differential estrogenic and genotoxic activity and may have different roles in breast carcinogenesis. 4-catechol EM and 16α-hydroxyestrone may have higher estrogenic activity than estradiol (46–52) while 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 risk of breast cancer 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 estrogen metabolites may provide information that is not available when only measuring parent estrogens, and that patterns of estrogen metabolism may vary substantially among individuals. Most estrogen metabolites, 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 utilized 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.

Acknowledgments

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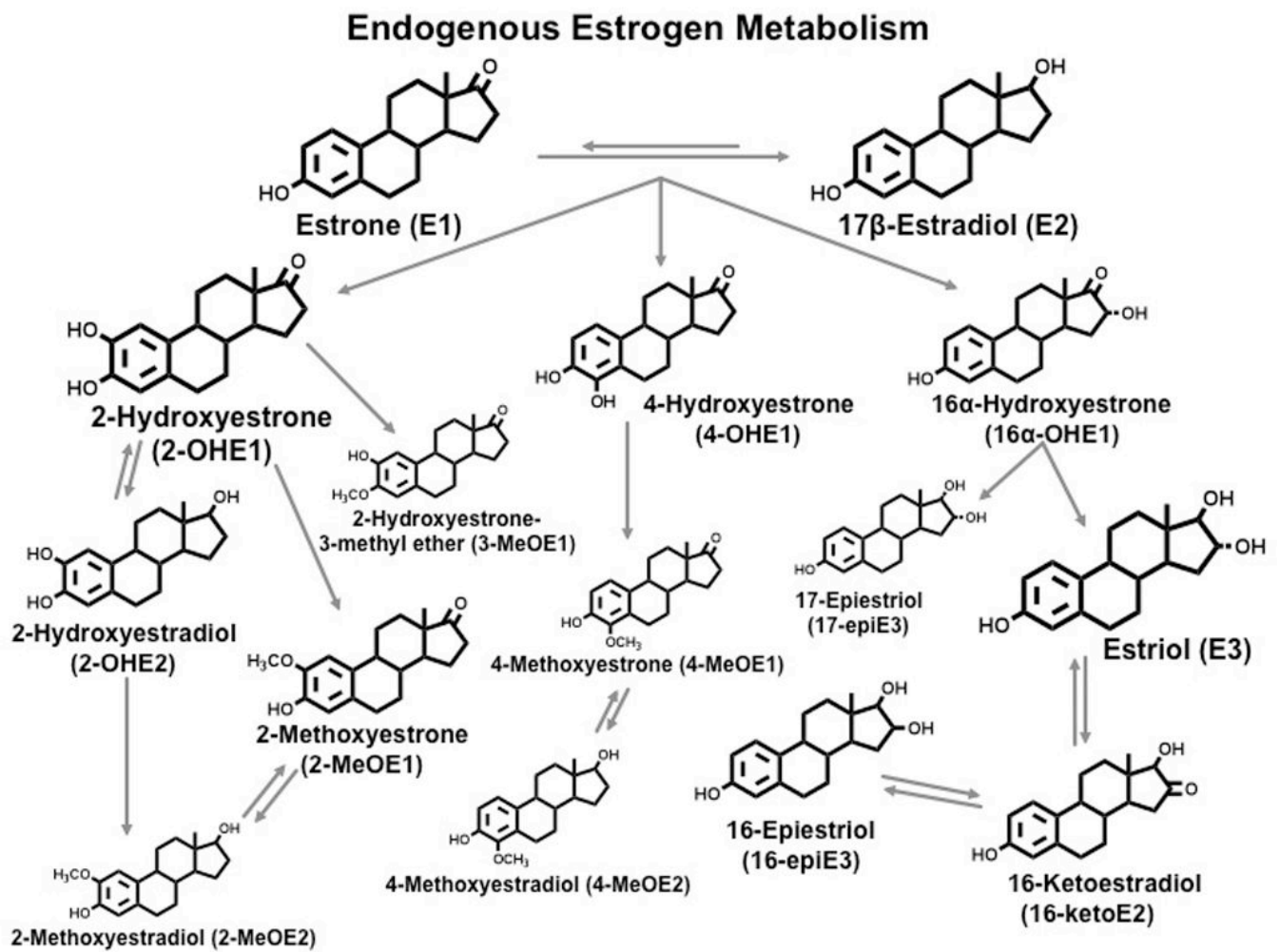
Figure 1.
Endogenous Estrogen Metabolism

Table 1

Means[*] and ranges of individual and grouped urinary estrogens and estrogen metabolites (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)	21.1 (12.0-33.9)
	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 EM4-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	
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)	

[*] Means/ranges are geometric for absolute and ratio measures, arithmetic for % measures.

Table 2

Spearman correlations of individual and grouped urinary estrogens and estrogen metabolites (EM), expressed as absolute concentrations and percent of total EM for the average of 3 collections.

	Parent	E1	E2	Cat	2Cat	2OHE1	2OHE2	4Cat	4OHE1	Me Cat	Me-2Cat	2MeE1	2MeE2	2OH3Me	Me-4Cat	4MeE1	4MeE2	2Path	4Path	16Path	16aOHE1	E3	17EpiE3	16KetoE2	16EpiE3	Total EM
Parent EM	_1.0_	**0.96**	**0.79**	0.32	0.32	0.33	0.27	0.32	0.27	**0.48**	**0.48**	**0.45**	**0.42**	**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**
E1	**0.93**	_1.0_	**0.62**	**0.40**	**0.40**	**0.41**	0.36	0.39	0.36	**0.52**	**0.52**	**0.48**	**0.50**	**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**
E2	**0.73**	**0.48**	_1.0_	0.06	0.06	0.07	0.01	0.10	0.01	0.27	0.26	0.26	0.12	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**
Catechol EM	-0.30	-0.24	**-0.41**	_1.0_	_1.0_	**0.99**	**0.89**	**0.89**	**0.89**	**0.64**	**0.63**	**0.64**	**0.66**	**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**
2-Catechol EM	-0.30	-0.24	**-0.42**	**0.99**	_1.0_	**0.99**	**0.90**	**0.86**	**0.87**	**0.62**	**0.62**	**0.64**	**0.67**	**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**
2OHE1	-0.29	-0.23	**-0.41**	**0.99**	**1.00**	_1.0_	**0.87**	**0.87**	**0.87**	**0.64**	**0.64**	**0.66**	**0.67**	**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**
2OHE2	-0.31	-0.23	**-0.42**	**0.99**	**1.00**	**0.79**	_1.0_	**0.76**	**0.58**	**0.56**	**0.56**	**0.57**	**0.67**	**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**
4-Catechol EM	-0.14	-0.11	-0.25	**0.82**	**0.76**	**0.77**	**0.58**	_1.0_	_1.0_	**0.60**	**0.56**	**0.60**	**0.56**	**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**
4OHE1	0.28	0.26	0.12	0.37	0.36	0.37	0.30	**0.76**	_1.0_	0.38	0.37	**0.40**	0.30	0.18	0.37	0.39	0.20	**0.70**	**0.61**	-0.11	-0.11	-0.27	-0.05	-0.05	-0.07	**0.45**
Me Catechol EM	0.28	0.27	0.12	0.37	0.36	0.37	0.30	0.38	0.37	_1.0_	**0.97**	**0.70**	**0.81**	**0.59**	0.36	0.36	0.21	**0.46**	0.39	**-0.53**	-0.35	**-0.55**	-0.24	-0.35	**-0.46**	**0.45**
Me 2-Catechol EM	0.28	0.27	0.12	**0.41**	**0.40**	**0.41**	0.33	**0.44**	**0.40**	**0.97**	_1.0_	**0.98**	**0.76**	**0.84**	**0.41**	0.32	0.19	**0.70**	**0.61**	**-0.55**	-0.11	**-0.57**	-0.27	-0.36	**-0.44**	**0.44**
2MeE1	0.24	0.21	0.12	**0.41**	**0.40**	**0.41**	0.33	**0.40**	**0.40**	**0.70**	**0.98**	_1.0_	**0.73**	**0.82**	0.33	0.32	0.17	**0.50**	**0.40**	**-0.56**	-0.28	**-0.57**	-0.28	-0.39	-0.39	**0.50**
2MeE2	0.13	0.21	-0.12	0.39	**0.40**	**0.40**	**0.44**	**0.40**	0.30	**0.81**	**0.76**	**0.73**	_1.0_	**0.46**	0.21	0.25	0.09	**0.47**	0.31	**-0.47**	-0.30	**-0.48**	-0.28	-0.34	-0.23	0.32
2OH3Me	0.24	0.14	0.17	0.15	0.13	0.14	0.15	0.18	0.18	**0.59**	**0.84**	**0.82**	**0.46**	_1.0_	**0.41**	0.36	0.19	0.19	0.22	-0.28	-0.16	-0.33	-0.05	-0.11	-0.27	0.36
Me 4-Catechol EM	0.24	0.14	0.29	0.03	0.03	0.03	-0.01	-0.05	0.37	0.36	**0.41**	0.33	0.21	**0.41**	_1.0_	**0.93**	**0.48**	0.36	0.35	0.12	0.06	0.06	-0.01	0.16	0.19	0.36
4MeE1	0.26	0.17	0.30	0.03	0.03	0.03	0.01	-0.02	0.39	0.36	0.32	0.32	0.25	0.36	**0.93**	_1.0_	0.22	0.32	0.32	0.11	0.03	0.08	0.03	0.13	0.18	0.33
4MeE2	0.04	-0.01	0.07	0.02	0.02	0.03	-0.05	-0.02	0.20	0.21	0.19	0.17	0.09	0.19	**0.48**	0.22	_1.0_	0.17	0.07	0.05	0.08	-0.03	-0.09	0.08	0.08	0.17
2-Pathway EM	-0.27	-0.22	-0.38	**0.98**	**0.99**	**0.99**	**0.82**	**0.77**	**0.70**	**0.46**	**0.70**	**0.50**	**0.47**	0.19	0.07	0.07	0.05	_1.0_	**0.87**	-0.19	-0.19	-0.25	-0.09	-0.10	0.03	**0.66**
4-Pathway EM	-0.13	-0.11	-0.25	**0.82**	**0.82**	**0.77**	**0.59**	**1.00**	**0.61**	0.39	**0.61**	**0.40**	0.31	0.22	0.35	0.32	0.08	**0.77**	_1.0_	-0.20	-0.20	-0.25	-0.10	-0.15	0.00	**0.58**
16-Pathway EM	-0.03	-0.06	0.12	**-0.88**	**-0.87**	**-0.88**	**-0.69**	**-0.76**	**-0.76**	**-0.53**	**-0.55**	**-0.56**	**-0.47**	-0.28	-0.14	-0.15	-0.04	**-0.77**	**-0.77**	_1.0_	**0.85**	**0.95**	**0.55**	**0.87**	**0.82**	**0.48**
16aOHE1	-0.01	0.04	0.11	**-0.75**	**-0.74**	**-0.74**	**-0.63**	**-0.65**	**-0.65**	-0.35	-0.35	-0.38	-0.30	-0.16	-0.16	-0.20	0.05	**-0.66**	**-0.66**	**0.83**	_1.0_	**0.70**	**0.47**	**0.77**	**0.66**	0.37
E3	-0.08	-0.13	0.11	**-0.82**	**-0.81**	**-0.82**	**-0.63**	**-0.71**	**-0.71**	**-0.55**	**-0.55**	**-0.57**	**-0.48**	-0.33	-0.18	-0.16	-0.11	**-0.84**	**-0.71**	**0.95**	**0.70**	_1.0_	0.39	**0.74**	**0.78**	**0.40**
17EpiE3	0.07	0.04	0.16	**-0.48**	**-0.47**	**-0.47**	-0.39	**-0.43**	**-0.43**	-0.24	-0.23	-0.28	-0.28	-0.05	-0.08	-0.08	-0.07	**-0.48**	**-0.43**	**0.52**	**0.47**	0.39	_1.0_	**0.53**	0.35	0.30
16KetoE2	0.04	0.03	0.11	**-0.78**	**-0.77**	**-0.77**	**-0.64**	**-0.70**	**-0.70**	-0.35	-0.34	-0.39	-0.34	-0.11	-0.02	-0.02	0.04	**-0.79**	**-0.71**	**0.86**	**0.77**	**0.74**	**0.53**	_1.0_	**0.72**	**0.48**
16EpiE3	0.00	-0.02	0.11	**-0.63**	**-0.63**	**-0.64**	**-0.41**	**-0.55**	**-0.55**	**-0.46**	**-0.46**	**-0.49**	-0.23	-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**

Note: Correlations in bottom left of table are EM expressed as % of total EM; Correlations in top right are expressed as absolute concentrations of EM. Correlations ≥|0.40| are **bolded**.

Table 3

Spearman correlation coefficients for urinary luteal estrogens with plasma follicular and luteal estrogens, average of 3 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

N ranges from 87 (follicular estrone) to 106.

Correlations ≥|0.40| are **bolded**.

Luteal urine and luteal plasma collected the same day.

Table 4

Intraclass correlation coefficients (95% confidence intervals) for individual and grouped estrogens and estrogen metabolites (EM), expressed as absolute concentrations, percent of total EM, and selected EM ratios.

Analyte	ICC (95% CI)	
	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)