Research Article

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Association between Reproductive Factors and Urinary Estrogens and Estrogen Metabolites in Premenopausal Women

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Abstract

Background: Little is known about relationships among reproductive factors, estrogens and estrogen metabolites (jointly referred to as EM), and estrogen metabolism patterns.

Methods: In a cross-sectional analysis, we examined the associations of age at menarche, menstrual cycle length and regularity, parity, age at first and last birth, breastfeeding, and duration of and time since use of oral contraceptives with mid-luteal phase urinary EM in a sample of 603 premenopausal women, ages 33 to 51 years, within the Nurses' Health Study II (NHSII). Fifteen individual urinary EMs were measured with liquid chromatography/tandem mass spectrometry (LC/MS-MS) and analyzed both individually and in metabolic pathways.

Results: Compared with women with extremely regular cycles, those with irregular cycles had lower levels of total EM (percent difference = 24%; $P_{trend} = 0.01$), estradiol (23%; $P_{trend} = 0.02$), and 16-hydroxylation pathway EM (32%; $P_{trend} < 0.01$). Longer menstrual cycles were associated with higher levels of estrone (percent difference ≥32 vs. <26 days: 25%; $P_{trend} = 0.03$), estradiol (24%; $P_{trend} = 0.01$), and 16-hydroxylation pathway EM (22%; $P_{trend} = 0.02$). Among parous women, older age at first birth was associated with lower 16-hydroxylation pathway EM (percent difference age at first birth >35 vs. ≤25 years: 20%; $P_{trend} = 0.02$). The other reproductive factors were not statistically significantly associated with individual urinary EM or EM pathways.

Conclusions and Impact: These data, based on a LC/MS-MS assay with high specificity and precision, provide an initial, comprehensive evaluation of the associations between reproductive factors and estrogen metabolism patterns. *Cancer Epidemiol Biomarkers Prev; 21(6); 959–68. ©2012 AACR.*

Introduction

Prior research suggests inverse associations between parity, breastfeeding, and age at menarche and breast cancer risk, and a positive association between age at first birth, current oral contraceptive (OC) use, and risk (1, 2). Data also suggest a decrease in risk associated with an irregular menstrual cycle pattern and longer cycles (1), though these data are not consistent. The positive association between endogenous parent estrogens (estrone and estradiol) and breast cancer is established in postmenopausal women (3, 4), and suggested in premenopausal women in some (5), but not all (6, 7) studies.

Despite the hormonal nature of these reproductive factors and the observed associations between endogenous estrogens and breast cancer, lifestyle correlates of estrogen metabolism have been minimally explored in epidemiologic studies. The parent estrogens can be irreversibly hydroxylated at the 2-, 4-, or 16-positions of the steroid ring, and the reactive catechol estrogen metabolites formed by 2- and 4-hydroxylation can be stabilized by methylation of one of the 2 adjacent hydroxyl groups. Only 3 prior studies have presented reproductive correlates of a subset of urinary estrogen metabolites, with all 3 studies measuring only 2-hydroxyestrone and 16α-hydroxyestrone and the ratio of the 2 metabolites in urine (8–10). These studies found no association between these 2 estrogen metabolites and age at menarche, age at first birth, or parity in premenopausal and postmenopausal women.

The hypothesized effects of individual estrogens and estrogen metabolites (jointly referred to as EM) vary, given that EMs differ in their relative capacity to stimulate

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cell proliferation and mutagenicity (11–13). A higher 2-hydroxyestrone to 16α-hydroxyestrone ratio has been hypothesized to be protective against breast cancer (14, 15), though that association has not consistently been observed when these 2 metabolites are measured in urine (8, 10, 16, 17), plasma (18), or serum (19). In the Nurses' Health Study II (NHSII), we recently reported inverse relations of urinary parent estrogens and EM in the 2- and 4-pathways with breast cancer risk among premenopausal women (16).

Using the recently developed stable isotope dilution high-performance liquid chromatography/tandem mass spectrometry (LC/MS-MS) method, we measured concurrently all 15 EMs in urine with high sensitivity, specificity, and reproducibility (20–23). Within the NHSII, among premenopausal women, we evaluated the associations between reproductive characteristics and urinary EM individually, by metabolic pathway (e.g., 2-, 4-, and 16-hydroxylation; catechols and methylated catechols) and by metabolic pathway ratios.

Materials and Methods

Study population and biospecimen collection

The NHSII was initiated in 1989 when 116,430 registered nurses enrolled in the cohort by completing and returning a questionnaire. Since that time, participants have completed biennial questionnaires to update exposures and collect health and disease data. Between 1996 and 1999, 29,611 participants provided blood and urine samples. These women were between 32 and 54 years of age and were cancer free at the time of collection. A total of 18,521 premenopausal women who had not used OC, been pregnant, or breastfed during the past 6 months provided samples timed within the menstrual cycle. Women were asked to collect blood samples in the early follicular phase (days 3–5 of the menstrual cycle) and blood and urine samples in the mid-luteal phase (7–9 days before the expected onset of the next cycle). All participants who provided biospecimens completed a brief questionnaire at the time of specimen 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 to allow determination of the timing of the luteal phase collection. Luteal urine samples were shipped, via overnight courier with an ice pack, to our laboratory with approximately 93% of samples received within 26 hours of collection. Samples have been stored in liquid nitrogen freezers since collection.

The current analysis is limited to premenopausal women with timed, luteal urine samples who were selected as controls for a nested case–control study of breast cancer ($N = 493$; ref. 16), as well as premenopausal women who participated in a biomarker reproducibility substudy of the NHSII ($N = 110$; ref. 23). The study was approved by the Committee on the Use of Human Subjects in Research at Brigham and Women's Hospital.

Exposure and covariate assessment

Women provided data on reproductive factors on the biennial questionnaires. Data collected included (year of collection): age at menarche (1989), parity (1997), age at first and last birth (1997), use of OCs (1997), time since OC use (1997), and breastfeeding (1997; cumulative across pregnancies). For menstrual cycle length, women were asked "what is the current usual length of your menstrual cycle (interval from first day of period to first day of next period)?" and were provided categorical response options ranging from "<21 days" to "51+ days or too irregular to estimate" (1993). Similarly, for menstrual cycle regularity women were asked "what is the current usual pattern of your menstrual cycles (when not pregnant or lactating)?" with response options ranging from "extremely regular (no more than 1–2 days before or after expected)" to "no periods" (1993). Data on covariates, including alcohol use and physical activity, were also collected on the biennial questionnaires; height was reported in 1989. Data for reproductive factors and covariates was collected before urine collection (i.e., through the 1997 questionnaire), with the exception of alcohol and physical activity for which the average of the values on the 1995 and 1999 (alcohol) and 1997 and 2001 (physical activity) questionnaires were used for statistical adjustment. In addition, the specimen collection questionnaire provided data on current weight, age, blood, and urine collection date and time, and whether the urine was a first morning urine sample.

Laboratory assays

For the urinary EM assay, 500 μL of frozen urine was sent to the Laboratory of Proteomics and Analytical Chemistry, SAIC-Frederick, Inc., Frederick, MD. Endogenous estrogens and their metabolites are usually present in urine as glucuronide and sulfate conjugates; therefore, an initial enzymatic 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 5 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) was added, followed by 0.5 mL of 0.15 mol/L acetate buffer, pH 4.1, containing 2 mg of ascorbic acid and an enzymatic preparation from *Helix pomatia* with β-glucuronidase and sulfatase activity (Sigma-Aldrich). The isotopically labeled EMs are used to correct for loss of urinary EM during the hydrolysis, extraction, dansyl chloride derivatization, and LC/MS-MS steps of the assay procedure. Details of the assay have been published previously (21, 22). In brief, LC/MS-MS was carried out with a TSQ Quantum-AM triple quadrupole mass spectrometer coupled with a Surveyor high-performance liquid chromatography system (Thermo Scientific). Both the liquid chromatography system and mass spectrometer were controlled by Xcalibur software (Thermo Scientific). Quantification of each EM in urine was carried out with Xcalibur Quan Browser (Thermo Scientific). Calibration curves for the 15 EMs were

constructed by plotting EM/deuterium-labeled EM peak area ratios versus amounts of the EM. The amount of each EM in the urine sample was then interpolated with a linear function. Masked replicate quality control samples were placed in each batch to assess laboratory variability. The overall coefficients of variation (CV) were <7% except for 4-methoxyestrone (17%) and 4-methoxyestradiol (15%), the 2 EMs with the lowest concentrations. The lower level of quantitation for each EM is about 150 fmol/mL urine.

Creatinine was measured in 3 batches at the Endocrine Core Laboratory at Emory University (Atlanta, GA), Dr. Nader Rifai's laboratory at Boston Children's Hospital (Boston, MA), and Dr. Vincent Ricchiuti's laboratory at Brigham and Women's Hospital (Boston, MA). Overall CVs were $\leq$9.2% in all laboratories.

Statistical analysis

Urinary EM concentrations (pmol/mL) were adjusted for creatinine levels to account for urine volume, which resulted in units of picomoles EM per milligram of creatinine, and were further log transformed to achieve an approximately normal distribution. EMs were evaluated individually, as well as grouped by metabolic pathways (e.g., methylated catechols, 16-hydroxylation pathway) and as pathway ratios (e.g., 2-hydroxylation pathway/16-hydroxylation pathway). We also examined the 2-hydroxyestrone/16α-hydroxyestrone ratio given the prior hypothesized association between this ratio and breast cancer risk (14, 15). Statistical outliers were identified by the extreme studentized many deviate procedure (24). This resulted in the exclusion of up to 10 values for individual EM, with the exception 2-methoxyestradiol which had 16 outliers, and up to 9 values for ratio measures.

Reproductive factors evaluated include: duration of past OC use (never, 1–47 months, and $\geq$48 months), time since last OC use (never, 6–23 months, 24–47 months, 48–71 months, 48–71 months, 72–95 months, 96–119 months, and $\geq$120 months), age at menarche (<12, 12, 13, and $\geq$14 years), menstrual cycle pattern (extremely regular/no more than 1–2 days deviation from expected, very regular/within 3–4 days, regular/within 5–7 days, usually/always irregular), usual menstrual cycle length (<26, 26–31, $\geq$32 days), and parity (nulliparous; 1, 2, 3, or $\geq$4 children). Also evaluated among parous women were age at first and last birth ($\leq$25, >25–30, >30–35, and >35 years) and total duration of breastfeeding (0 to <1 month, 1 to 6 months, >6 to 12 months, >12 to 18 months, >18 to 24 months, >24 to 36 months, and >36 months).

We calculated geometric means with generalized linear models for each of the individual EM, metabolic pathway groups, and pathway ratios by categories of reproductive factors. Tests for trend were conducted by modeling continuous exposure measures (e.g., parity, age at first birth) or including the categorical variable in the model as an ordinal variable (e.g., menstrual cycle regularity). Multivariable models were adjusted for age at urine collection (years, continuous), luteal day at collection (<5, 6–7, 8–9, $\geq$10 days before next menstrual period), first morning urine (yes/no), body mass index (BMI) at collection (<25, 25–30, $\geq$30 kg/m^2), total physical activity (<3, 3 to <9, 9 to <18, 18 to <27, 27 to <42, $\geq$42 MET-h/wk), and alcohol consumption (nondrinker, up to 3 drinks per month, 3 drinks per month to 2 drinks per week, 2 drinks per week to 5 drinks per week, >5 drinks per weeks). Reproductive factors were mutually adjusted using combined age at first birth/parity (nulliparous, age at first birth <25 years/1–2 children, age at first birth 25 to 29 years/1–2 children, age at first birth $\geq$30 years/1–2 children, age at first birth <25 years/$\geq$3 children, age at first birth $\geq$25 years/$\geq$3 children) and, as categorized above, age at menarche, menstrual cycle regularity, and menstrual cycle length. In the age at first birth analysis, parity was included as a continuous variable.

All P values are 2 sided and considered statistically significant if $P < 0.05$. Analyses were conducted with SAS version 9 (SAS Institute).

Results

Participants had a mean age of 42.8 years at sample collection (Table 1). The majority of participants were parous (82%) and overall reported low consumption of alcohol (69% <2 drinks/wk). Mean BMI was 25 kg/m^2 (SD = 5.3). Nearly half of the women (49%) experienced menarche at age 12 years or younger and the majority had used OCs (84%). Among parous women, 45% had an age at first birth of 25 years or younger and 87% had breastfed for at least 1 month.

We observed several statistically significant associations between urinary EM levels and both regularity and length of menstrual cycles. In general, as compared with extremely regular menstrual cycles, irregular menstrual cycles were associated with lower urinary levels of total EM, estradiol, the 16-hydroxylation pathway, and four of the five 16-pathway EM; and the trends for these EM measures by cycle regularity were statistically significant (Table 2).

Specifically, relative to women with extremely regular menstrual cycles, women with irregular menstrual cycles had lower levels of total EM (24% difference; $P_{trend} = 0.01$), estradiol (23%; $P_{trend} = 0.02$), and 16-pathway EM (32%; $P_{trend} < 0.01$). Irregular menstrual cycles also were associated with a higher ratio of 4-/16-hydroxylation pathways (irregular vs. extremely regular cycles: 0.08 vs. 0.11; 45% difference; $P_{trend} = 0.03$). Menstrual cycle regularity was not associated with the 2- or 4-hydroxylation pathway EM, either individually or in groups.

Menstrual cycle length was statistically significantly positively associated with estrone, estradiol, the 16-hydroxylation pathway, and 3 of the 16-pathway EM, and nonsignificantly positively associated with total EM

Table 1. Characteristics of study population at urine collection: NHSII ($n = 603$)

Characteristic	Mean (SD)	
Age, y	42.8 (3.8)	
BMI, kg/m^2	25.1 (5.3)	
	n	%
Age at menarche		
<12	126	20.9
12	168	27.9
13	181	30.0
≥14	128	21.2
Menstrual cycle length		
<26 d	115	19.8
26–31 d	405	69.6
≥32 d	62	10.7
Menstrual cycle regularity[a]		
Extremely regular	160	27.5
Very regular	248	42.7
Regular	146	25.1
Usually/always irregular	27	4.7
Past oral contraceptive use		
Never	94	15.9
Ever, <4 y	266	44.9
Ever, ≥4 y	232	39.2
Parity		
Nulliparous	109	18.1
1 Child	76	12.6
2 Children	247	41.0
3 Children	119	19.7
≥4 Children	52	8.6
Age at first birth		
≤25	222	45.0
>25–30	177	35.9
>30–35	72	14.6
>35	22	4.5
Breastfeeding		
0 to <1 mo	65	13.2
1–6 mo	47	9.6
>6–12 mo	87	17.7
>12–18 mo	92	18.7
18–24 mo	51	10.4
>24–36 mo	84	17.1
>36 mo	65	13.2
Alcohol, ∼10 g serving		
Nondrinker	183	30.4
Up to 3 servings/mo	99	16.4
3/mo to 2/wk	135	22.4
2/wk to 5/wk	99	16.4
>5/wk	87	14.4
Race, Caucasian	571	94.7
First morning urine	472	79.6
Not perimenopausal[b]	529	87.7
Sample taken within 4–10 d of next menstrual period	516	85.6
Ovulatory cycle	542	89.9

[a]Extremely regular: no more than 1 to 2 days deviation from expected; very regular/within 3 to 4 days; regular/within 5 to 7 days; usually/always irregular.

[b]Women considered perimenopausal at urine collection if report being menopausal within 4 years of urine collection.

and parent estrogens (Table 3). Longer menstrual cycles were associated with higher levels of estrone (≥32 vs. <26 days: 25% difference; $P_{trend} = 0.03$) and estradiol (24%; $P_{trend} = 0.01$; Table 3). Longer cycles were also associated with higher levels of the 16-hydroxylation pathway (≥32 vs. <26 days: 22% difference; $P_{trend} = 0.02$) and 3 of the 16-pathway EM (17-epiestriol, 16-ketoestradiol, and 16-epiestriol). The individual EMs in the 2- and 4-hydroxylation pathways were not associated with menstrual cycle length when considered individually or as groups, however there was a significant inverse association between cycle length and the ratio of 2-pathway/parent EM ($P_{trend} = 0.04$).

There were no statistically significant associations between parity and individual EM, metabolic pathway groups, or pathway ratios when comparing nulliparous with parous women (Table 4; all $P > 0.05$) or across increasing number of children (data not shown). Among parous women, older age at first birth was associated with lower levels of the 16-hydroxylation pathway, both grouped (>35 vs. ≤25 years: 20% difference; $P_{trend} = 0.02$), and individually, including 16-ketoestradiol (21%; $P_{trend} < 0.01$), and 16-epiestriol (28%; $P_{trend} < 0.01$; Table 4). Older age at first birth was positively associated with higher ratios when the 16-hydroxylation pathway served as the denominator, including the following ratios: catechols/16-hydroxylation pathway ($P_{trend} = 0.02$), 2-hydroxylation pathway/16-hydroxylation pathway ($P_{trend} = 0.01$), 4-hyrdroxylation pathway/16-hydroxylation pathway ($P_{trend} = 0.01$), 2-,4-hydroxylation pathway/16-hydroxylation pathway ($P_{trend} = 0.01$). Age at first birth was not associated with 2- and 4-hydroxylation pathways, with the exception of the 2-pathway/parent EM ratio ($P_{trend} = 0.03$). Age at last birth is significantly correlated with age at first birth ($r = 0.72$, $P < 0.01$), and results for age at last birth were similar to those for age at first birth (e.g., 16-hydroxlation pathway, >35 vs. ≤25 years: 18% difference, $P_{trend} = 0.05$; data not shown).

Breastfeeding, duration of OC use, years since OC use, and age at menarche were not associated with either total urinary concentration of EM or concentrations of individual EM or metabolic pathway groups, or pathway ratios (data not shown). For example, for total EM, the geometric mean for <1 month of breastfeeding was 151.2 pmol/mg creatinine and for >36 months of breastfeeding 171.9 pmol/mg creatinine, with no clear pattern across the intermediate categories ($P_{trend} = 0.28$).

We further evaluated these associations in 3 subgroups of participants: those who gave their urine samples during an ovulatory cycle (defined by plasma mid-luteal progesterone levels >400 ng/dL; $n = 542$), those whose urine sample was collected within 4 to 10 days of their next menstrual period ($n = 516$), and those who were not perimenopausal (i.e., women reporting they did not become menopausal within 4 years after sample collection; $n = 529$). Results among each of these subgroups were similar to the overall results (e.g., among women who were not perimenopausal, the association between

Table 2. Adjusted geometric means (pmol/mg creatinine) of estrogen/estrogen metabolite levels by menstrual cycle regularity: NHSII ($n = 603$)

Estrogen/estrogen metabolite measures	Extremely regular	Very regular	Regular	Usually/always irregular	P_{trend}
n	160	248	146	27	
Total estrogens and estrogen metabolites	227.9	189.0	199.0	173.8	0.01
Parent estrogens	46.8	36.6	42.5	38.0	0.10
Estrone	31.1	24.9	28.3	26.3	0.14
Estradiol	15.9	13.0	14.0	12.2	0.02
Catechols	62.8	54.6	60.6	62.7	0.77
2-Catechols	55.1	46.4	52.8	54.4	0.73
2-Hydroxyestrone	48.3	40.7	46.9	48.2	0.82
2-Hydroxyestradiol	6.1	4.9	5.6	5.3	0.29
4-Catechols					
4-Hydroxyestrone	5.9	5.2	6.2	5.8	0.72
Methylated catechols	11.6	8.8	10.8	10.0	0.32
Methylated 2-catechols	11.2	8.5	10.4	9.8	0.34
2-Methoxyestrone	8.9	6.7	8.1	7.9	0.28
2-Methoxyestradiol	0.73	0.64	0.71	0.77	0.93
2-Hydroxyestrone-3-methyl ether	1.3	1.1	1.3	1.0	0.77
Methylated 4-catechols	0.21	0.19	0.21	0.15	0.51
4-Methoxyestrone	0.14	0.12	0.13	0.09	0.30
4-Methoxyestradiol	0.05	0.05	0.06	0.04	0.64
2-Hydroxylation pathway	68.0	56.0	64.5	65.5	0.60
4-Hydroxylation pathway	6.3	5.7	6.6	6.1	0.74
16-Hydroxylation pathway	81.3	67.0	68.2	55.3	<0.01
16α-Hydroxyestrone	14.2	11.4	11.9	9.4	0.02
Estriol	37.0	31.3	30.7	24.8	0.01
17-Epiestriol	1.7	1.5	1.9	1.1	0.76
16-Ketoestradiol	16.3	13.4	14.2	11.3	0.01
16-Epiestriol	7.3	6.3	6.3	5.4	0.01
Ratios of metabolic pathways					
4-Catechols/2-catechols	0.10	0.12	0.11	0.10	0.65
2-Catechols/16-pathway	0.68	0.67	0.78	0.98	0.09
Catechols/16-pathway	0.78	0.77	0.89	1.1	0.08
4-Pathway/2-pathway	0.09	0.11	0.10	0.09	0.29
2-Pathway/16-pathway	0.84	0.79	0.95	1.2	0.12
4-Pathway/16-pathway	0.08	0.09	0.10	0.11	0.03
2,4-Pathway/16-pathway	0.94	0.91	1.1	1.3	0.09
2-Pathway/4,16-pathway	0.75	0.71	0.82	1.0	0.17
2-Catechols/methylated 2-catechols	4.9	5.2	5.2	5.6	0.38
4-Catechols/methylated 4-catechols	26.2	26.9	29.7	37.3	0.27
Catechols/methylated catechols	5.4	5.9	5.7	6.2	0.31
Parent estrogens/estrogen metabolites	0.27	0.26	0.28	0.26	0.67
2-Pathway/parent estrogens	1.5	1.4	1.5	1.7	0.37
4-Pathway/parent estrogens	0.13	0.15	0.16	0.16	0.11
16-Pathway/parent estrogens	1.7	1.8	1.6	1.4	0.13
2-Hydroxyestrone/16α-hydroxyestrone	3.4	3.5	3.9	5.1	0.09

NOTE: Adjusted for age, luteal day, first morning urine, BMI, alcohol consumption, physical activity, age at menarche, cycle length, breastfeeding, age at first birth/parity.

≥32 vs. <26 days for the 16-hydroxylation pathway was 69.2 vs. 59.2 pmol/mg creatinine, $P_{trend} = 0.06$, as compared with 72.5 vs. 59.6 pmol/mg creatinine, $P_{trend} = 0.02$ among all women).

Discussion

In this first study of 15 urinary EMs and reproductive factors among premenopausal women, we observed

Table 3. Adjusted geometric means (pmol/mg creatinine) of estrogen/estrogen metabolite levels by menstrual cycle length: NHSII ($n = 603$)

	Menstrual cycle length, d			
Estrogen/estrogen metabolite measures	**<26**	**26–31**	**32$^+$**	**P_{trend}**
N	115	405	62	
Total estrogens and estrogen metabolites	176.7	186.3	199.9	0.17
Parent estrogens	36.5	41.7	43.4	0.06
Estrone	23.8	27.6	29.8	0.03
Estradiol	12.3	14.0	15.2	0.01
Catechols	52.3	55.7	55.0	0.94
2-Catechols	45.4	48.8	46.5	0.97
2-Hydroxyestrone	40.1	43.3	40.6	0.93
2-Hydroxyestradiol	4.7	4.9	5.3	0.67
4-Catechols				
4-Hydroxyestrone	5.0	5.2	5.9	0.69
Methylated catechols	9.3	9.7	9.2	0.92
Methylated 2-catechols	9.0	9.4	9.0	0.97
2-Methoxyestrone	7.1	7.4	7.1	0.92
2-Methoxyestradiol	0.64	0.71	0.66	0.67
2-Hydroxyestrone-3-methyl ether	1.0	1.2	1.1	0.39
Methylated 4-catechols	0.17	0.18	0.18	0.93
4-Methoxyestrone	0.10	0.11	0.10	0.76
4-Methoxyestradiol	0.05	0.04	0.05	0.88
2-Hydroxylation pathway	56.0	60.0	56.7	0.95
4-Hydroxylation pathway	5.5	5.5	6.4	0.83
16-Hydroxylation pathway	59.6	66.2	72.5	0.02
16α-Hydroxyestrone	10.3	11.0	12.0	0.15
Estriol	30.8	30.1	32.4	0.54
17-Epiestriol	1.2	1.5	1.9	0.01
16-Ketoestradiol	11.5	13.0	14.8	0.01
16-Epiestriol	5.6	6.3	7.3	<0.01
Ratios of metabolic pathways				
4-Catechols/2-catechols	0.11	0.11	0.13	0.75
2-Catechols/16-pathway	0.76	0.72	0.64	0.10
Catechols/16-pathway	0.86	0.80	0.75	0.13
4-Pathway/2-pathway	0.10	0.10	0.11	0.70
2-Pathway/16-pathway	0.92	0.87	0.78	0.10
4-Pathway/16-pathway	0.09	0.08	0.09	0.23
2,4-Pathway/16-pathway	1.0	1.0	0.90	0.10
2-Pathway/4,16-pathway	0.80	0.79	0.68	0.13
2-Catechols/methylated 2-catechols	5.0	5.1	5.1	0.95
4-Catechols/methylated 4-catechols	30.1	27.9	33.7	0.88
Catechols/methylated catechols	5.5	5.6	5.9	0.86
Parent estrogens/estrogen metabolites	0.27	0.29	0.29	0.19
2-Pathway/parent estrogens	1.5	1.4	1.3	0.04
4-Pathway/parent estrogens	0.15	0.13	0.15	0.26
16-Pathway/parent estrogens	1.6	1.6	1.7	0.61
2-Hydroxyestrone/16α-hydroxyestrone	3.9	3.8	3.4	0.19

NOTE: Adjusted for age, luteal day, first morning urine, BMI, alcohol consumption, physical activity, age at menarche, cycle regularity, breastfeeding, age at first birth/parity.

statistically significant associations of menstrual cycle regularity, menstrual cycle length, and age at first birth with parent estrogens and EM in the 16-hydroxylation pathway. We observed higher levels of estrone and estradiol among women with longer menstrual cycle than with shorter menstrual cycle length and lower levels of

Table 4. Adjusted geometric means (pmol/mg creatinine) of estrogen/estrogen metabolite levels by parity and age at first birth: Nurses' Health Study II ($n = 603$)

	Parity[b]		Age at first birth				
Estrogen/estrogen metabolite measures	**Nulliparous**	**Parous**	**≤25**	**26–30**	**31–35**	**>35**	**P_{trend}[a]**
N	109	494	222	177	72	22	
Total estrogens and estrogen metabolites	203.1	198.3	193.9	195.4	189.6	172.7	0.29
Parent estrogens	43.6	40.3	42.3	41.7	39.9	34.2	0.28
Estrone	29.4	27.0	28.1	28.2	26.0	21.8	0.31
Estradiol	14.2	13.5	13.8	14.2	13.3	12.4	0.58
Catechols	64.1	58.5	53.3	56.9	55.1	58.6	0.84
2-Catechols	55.9	50.6	45.5	50.0	47.3	48.0	0.83
2-Hydroxyestrone	49.6	44.6	40.1	44.6	41.7	41.8	0.83
2-Hydroxyestradiol	5.6	5.3	4.8	5.0	4.9	5.7	0.73
4-Catechols							
4-Hydroxyestrone	5.7	5.7	5.5	5.5	6.0	7.0	0.47
Methylated catechols	10.8	9.9	9.1	9.6	10.1	9.1	0.34
Methylated 2-catechols	10.4	9.5	8.8	9.3	9.6	8.8	0.42
2-Methoxyestrone	8.0	7.5	6.9	7.4	7.5	6.9	0.42
2-Methoxyestradiol	0.73	0.69	0.65	0.66	0.80	0.52	0.58
2-Hydroxyestrone-3-methyl ether	1.3	1.2	1.1	1.1	1.2	0.95	0.59
Methylated 4-catechols	0.22	0.20	0.18	0.18	0.18	0.17	0.90
4-Methoxyestrone	0.13	0.12	0.11	0.11	0.10	0.09	0.48
4-Methoxyestradiol	0.06	0.05	0.04	0.05	0.05	0.06	0.11
2-Hydroxylation pathway	67.5	61.4	55.9	60.7	58.0	59.0	0.73
4-Hydroxylation pathway	6.1	6.1	5.8	5.8	6.3	7.7	0.36
16-Hydroxylation pathway	67.5	69.2	71.8	67.7	62.6	57.2	0.02
16α-Hydroxyestrone	11.3	12.0	12.1	11.8	10.4	11.4	0.09
Estriol	29.8	31.5	33.4	30.2	29.4	25.6	0.06
17-Epiestriol	1.6	1.6	1.7	1.4	1.5	1.3	0.05
16-Ketoestradiol	13.5	14.1	14.4	13.1	12.5	11.3	<0.01
16-Epiestriol	6.5	6.4	6.9	6.4	6.2	4.9	<0.01
Ratios of metabolic pathways							
4-Catechols/2-catechols	0.09	0.11	0.12	0.10	0.12	0.15	0.75
2-Catechols/16-pathway	0.83	0.73	0.64	0.75	0.79	0.83	0.05
Catechols/16-pathway	0.95	0.83	0.73	0.85	0.92	1.0	0.02
4-Pathway/2-pathway	0.09	0.10	0.11	0.09	0.11	0.13	0.70
2-Pathway/16-pathway	1.0	0.87	0.76	0.91	0.97	1.0	0.01
4-Pathway/16-pathway	0.09	0.09	0.08	0.09	0.10	0.13	0.01
2,4-Pathway/16-pathway	1.1	1.0	0.87	1.0	1.1	1.2	0.01
2-Pathway/4,16-pathway	0.87	0.77	0.69	0.80	0.82	0.85	0.05
2-Catechols/methylated 2-catechols	5.4	5.2	5.2	5.4	4.9	5.4	0.50
4-Catechols/methylated 4-catechols	25.5	28.6	31.2	29.4	31.5	42.2	0.71
Catechols/methylated catechols	5.9	5.8	5.9	6.0	5.5	6.4	0.40
Parent estrogens/estrogen metabolites	0.28	0.27	0.28	0.28	0.28	0.24	0.81
2-Pathway/parent estrogens	1.6	1.5	1.3	1.4	1.5	1.8	0.03
4-Pathway/parent estrogens	0.14	0.15	0.14	0.14	0.16	0.23	0.05
16-Pathway/parent estrogens	1.6	1.7	1.7	1.6	1.5	1.7	0.08
2-Hydroxyestrone/16α-hydroxyestrone	4.4	3.7	3.4	3.8	4.2	3.6	0.09

NOTE. Adjusted for age, luteal day, first morning urine, BMI, alcohol consumption, physical activity, age at menarche, cycle length and regularity, breastfeeding, parity.

[a]Test for trend across age at first birth; excludes nulliparous women.

[b]Differences between nulliparous and parous all $P > 0.05$ with the exception of 4-catechols/2-catechols where $P = 0.05$.

estradiol among women with irregular compared with regular menstrual cycles. Of the 3 major pathways (2-, 4-, and 16-hydroxylation) of estrogen metabolism, most of our statistically significant findings were for the 16-hydroxylation pathway, with longer menstrual cycle length associated with higher levels of these EM, and menstrual cycle irregularity and older age at first birth associated with lower levels. There were no statistically significant associations between these reproductive factors and 2- and 4-hydroxylation pathway EM. In addition, we observed no statistically significant associations of age at menarche, duration of or time since OC use, parity, or breastfeeding with individual EM or estrogen metabolism pathways.

Three prior studies have evaluated the association between reproductive factors and urinary EM, but each of these included only 2-hydroxyestrone and 16α-hydroxyestrone (8–10) and only one study presented results separately for premenopausal women (8). In the Guernsey III cohort, Meilahn and colleagues found no statistically significant associations between age at menarche, parity, or age at first birth and the 2-hydroxyestrone/16α-hydroxyestrone ratio in premenopausal ($n = 139$), as well as postmenopausal ($n = 184$), women (8). The remaining 2 studies presented data on pre- and postmenopausal women combined (ref. 9; % premenopausal, cases = 26%; controls = 34.4%) or postmenopausal women (10). In these studies, no statistically significant associations were observed between parity (9, 10), age at menarche (10), or age at first birth (9) and 2-hydroxyestrone, 16α-hydroxyestrone, or the ratio of the 2 metabolites. These studies were limited by small sample size and limited representation of premenopausal women. In this analysis, we found no association between parity, age at menarche, or age at first birth and 2-hydroxyestrone, 16α-hydroxyestrone, or their ratio, in agreement with these prior findings. To our knowledge, there is no prior epidemiologic data on the association between OC use, breastfeeding, or menstrual cycle regularity and length and urinary measures of estrogen metabolism.

Experimental data suggest that individual EM vary in their estrogenic and genotoxic potential. EMs in the 2- and 4-hydroxylation pathways bind to the estrogen receptor, but have different rates of dissociation, which lead to potentially differential effects. EMs in the 2-hydoxylation pathway show a faster rate of dissociation than EMs in the 4-hydroxylation pathway (25–27), which suggests a higher estrogenic potential of 4-hydroxylation pathway EM than 2-hydroxylation pathway EM. Data also suggest higher genotoxicity for 4-hydroxylation pathway EM (11). In addition, neoplastic tissue, both benign and malignant, has higher levels of 4-hydroxylation pathway EM than normal breast tissue while 2-hydroxylation pathway EMs are similar in neoplastic and normal breast tissue (11). The 16-hydroxylation pathway EMs also exhibit estrogenic and genotoxic potential. Specifically, 16α-hydroxyestrone can bind covalently to the estrogen receptor, which leads to a constitutively activated receptor (28). In addition, animal data show unscheduled DNA synthesis in mouse mammary cells (29) associated with 16α-hydroxyestrone, suggesting genotoxicity. However, these hypotheses about the roles of specific EM in human carcinogenesis are all based on experimental model systems.

To date, our prior analysis of these 15 EMs and breast cancer risk, the controls from which are included in this analysis, is the first and only comprehensive epidemiologic study of these 15 EMs in urine and breast cancer risk among premenopausal women (16). We observed a decreased risk with higher urinary levels of estrone and estradiol (RR estrone = 0.52, 95% CI, 0.30–0.88; estradiol = 0.51, 95% CI, 0.30–0.86), inverse, but not statistically significant patterns with 2- and 4-hydroxylation pathway EM, no clear relationship with urinary 16-pathway EM, and an increased risk associated with higher urinary 17-epiestriol levels (top vs. bottom quartile RR, 1.74, 95% CI, 1.08–2.81; $P_{trend} = 0.01$).

The importance of excreted versus circulating estrogens is unclear. Circulating estrogens may not reflect all of the activity in the breast tissue and urinary estrogens are an additional step removed. Considering plasma levels alone, data from the NHSII suggest an increased risk with high levels of estradiol in the follicular phase, but not in the luteal phase (5). In analyses in our EM case–control study including both urine and plasma measures, high urinary estrone and estradiol (i.e., high excretion) was consistently associated with decreased breast cancer risk, regardless of plasma levels. Therefore, from our data it appear that estrogen excretion may be an important factor.

Age at menarche, parity, and breastfeeding are inversely associated and age at first birth positively associated with risk of breast cancer (1, 2). The associations between irregular and/or long menstrual cycles and breast cancer risk have not been consistent, with either no association (30, 31) or an inverse association (32–34) reported. In the NHSII cohort, cycles >32 days long or too irregular to estimate at ages 18 to 22 (but not in later adulthood) were associated with decreased risk of breast cancer only in the subgroup of women diagnosed before age 40 (HR, 0.71; 95% CI, 0.53–0.97; ref. 32). Given experimental data suggesting the 4- and 16-hydroxylation pathways may have the highest genotoxic and proliferative potential, we hypothesized that these EM would be positively associated with hormonally related breast cancer risk factors; and 2-hydroxylation pathway EM inversely associated with these risk factors (e.g., higher 4- and 16-pathway EM and lower 2-pathway EM associated with the higher risk group). Our findings for menstrual cycle regularity were in the hypothesized direction with more irregular menstrual cycles associated with lower 16-hydroxylation pathway EM. However, for menstrual cycle length, our results indicating a positive association with higher 16-pathway levels are contrary to the hypothesized direction based on experimental evidence. Similarly, our finding of older age at first birth being

associated with lower levels of EM in the 16-hydroxylation pathway, including 17-epiestriol, is contrary to the hypothesized direction, given the consistent, positive association between older age at first birth and breast cancer risk (1, 2) and the positive association between 17-epiestriol and breast cancer in the NHSII case–control study (16). Despite our hypotheses, based on the experimental literature, that 4-pathway EM would be positively associated with hormonally related breast cancer risk factors and 2-pathway EM would be inversely related, we found no association between these pathways and menstrual cycle characteristics or age at first birth.

There are several limitations to this study. This is a cross-sectional study, which limits our ability to infer causality. Although the reproductive factors studied were all measured before the specimen collection, it is possible that reverse causality was in part responsible for these associations, given that a woman's pattern of estrogen metabolism may affect reproductive events. We used data on usual menstrual cycle length and regularity, collected in 1993, and cycle length and regularity may change over time. This would result in some nondifferential classification of the exposure and bias our results toward the null. Second, data from a single urine collection were used for this analysis. However, although multiple measures would be preferable, a within-person stability analysis in a subset of the samples included in this analysis ($n = 110$) revealed moderately high intraclass correlation coefficients (ICC) over 3 years, with ICC $\geq$ 0.50 observed for most of the individual EM, estrogen metabolic pathways, and pathway ratios (23). Finally, although we made multiple comparisons, we interpret our results cautiously and in the context of biologically driven hypotheses and what is currently known about estrogen biochemistry and metabolism. Our study has important strengths including a sample size substantially larger than previous studies, a more comprehensive investigation of reproductive factors than earlier work, and a comprehensive examination of individual and grouped EM representing the 3 main pathways of estrogen metabolism. Prior analyses of urinary estrogen metabolites used enzyme immunoassays (8–10), while this study used an LC/MS-MS assay, a method that concurrently measures all 15 urinary EM and provides improved reproducibility, specificity, and accuracy, compared with previous methods (35).

In conclusion, this first analysis in premenopausal women of the association between reproductive factors and 15 urinary luteal EMs yielded some statistically significant associations between menstrual cycle length and regularity and age at first birth and patterns of estrogen metabolism; no associations were observed with age at menarche, OC use, parity, and breastfeeding. Regular menstrual cycles were associated with higher levels of 16-hydroxylation pathway EM, which have estrogenic and genotoxic potential, but the opposite was observed for longer menstrual cycle length and older age at first birth. Similarly, estradiol was associated in opposite directions with menstrual cycle irregularity and length. Further work is needed to confirm our observed associations between reproductive factors and estrogen metabolism patterns and determine additional correlates of urinary EM in premenopausal women.

Disclosure of Potential Conflicts of Interest

The content of this publication does not necessarily reflect the views or policies of the U.S. Department of Health and Human Services; nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government. No potential conflicts of interest were disclosed by the other authors.

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