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Research Article

Urinary Concentrations of Estrogens and Estrogen Metabolites and Smoking in Caucasian Women

Fangyi Gu[1], Neil E. Caporaso[1], Catherine Schairer[2], Renee T. Fortner[5,6], Xia Xu[4], Susan E. Hankinson[5,6,7], A. Heather Eliassen[5,6], and Regina G. Ziegler[3]

Abstract

Background: Smoking has been hypothesized to decrease biosynthesis of parent estrogens (estradiol and estrone) and increase their metabolism by 2-hydroxylation. However, comprehensive studies of smoking and estrogen metabolism by 2-, 4-, or 16-hydroxylation are sparse.

Methods: Fifteen urinary estrogens and estrogen metabolites (jointly called EM) were measured by liquid chromatography/tandem mass spectrometry (LC/MS-MS) in luteal phase urine samples collected during 1996 to 1999 from 603 premenopausal women in the Nurses' Health Study II (NHSII; 35 current, 140 former, and 428 never smokers). We calculated geometric means and percentage differences of individual EM (pmol/mg creatinine), metabolic pathway groups, and pathway ratios, by smoking status and cigarettes per day (CPD).

Results: Total EM and parent estrogens were nonsignificantly lower in current compared with never smokers, with estradiol significant ($P_{multivariate} = 0.02$). We observed nonsignificantly lower 16-pathway EM ($P = 0.08$) and higher 4-pathway EM ($P = 0.25$) and similar 2-pathway EM in current versus never smokers. EM measures among former smokers were similar to never smokers. Increasing CPD was significantly associated with lower 16-pathway EM (P-trend $= 0.04$) and higher 4-pathway EM (P-trend $= 0.05$). Increasing CPD was significantly positively associated with the ratios of 2- and 4-pathway to parent estrogens (P-trend $= 0.01$ and 0.002), 2- and 4-pathway to 16-pathway (P-trend $= 0.02$ and 0.003), and catechols to methylated catechols (P-trend $= 0.02$).

Conclusions: As hypothesized, we observed lower urinary levels of total EM and parent estrogens in active smokers. Our results also suggest smoking is associated with altered estrogen metabolism, specifically increased 2- and 4-hydroxylation, decreased 16-hydroxylation, and decreased catechol methylation.

Impact: Our study suggests how smoking might influence estrogen-related cancers and conditions. *Cancer Epidemiol Biomarkers Prev; 22(1); 58–68. ©2012 AACR.*

Introduction

An antiestrogenic effect of smoking has been suggested by reduced risk of endometrial cancer (1), earlier age at natural menopause (2), and increased risk of osteoporosis (3, 4) among smokers. Underlying hypotheses may include decreased biosynthesis of the parent estrogens, estrone, and estradiol (3, 5); increased 2-hydroxylation of the parent estrogens, which is hypothesized to reduce estrogenic activity (3, 6); and competitive binding of the 2-hydroxy-benzo[a]pyrene and possibly other compounds in cigarette smoke to sex hormone binding globulin (SHBG) or estrogen receptors (3).

Estrone and estradiol are interconverted via the 17β-hydroxysteroid dehydrogenases (17β-HSD). Each can be irreversibly hydroxylated at the 2-, 4-, or 16-positions of the steroid ring by cytochrome P450 enzymes (7; Fig. 1). The reactive catechol estrogens, with adjacent hydroxyl groups on the steroid ring, in the 2-hydroxylation and 4-hydroxylation pathways can be stabilized by methylation of a hydroxyl group. 16α-Hydroxyestrone can be further metabolized by reduction and oxidation at the 17- and 16-positions of the steroid ring. Practically all estrogens and estrogen metabolites (jointly referred to as EM) in urine are conjugated with sulfate or glucuronide moieties.

Early studies evaluating estrogen excretion in relationship to smoking focused on the 2 parent estrogens and estriol, the most abundant estrogen metabolite in the 16-hydroxylation pathway (8–10). Results of these studies

Authors' Affiliations: [1]Genetic Epidemiology Branch, [2]Biostatistics Branch, and [3]Epidemiology and Biostatistics Program, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda; [4]Laboratory of Proteomics and Analytical Technologies, Advanced Technology Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, Frederick, Maryland; [5]Channing Laboratory, Department of Medicine, Brigham and Women's Hospital and Harvard Medical School; [6]Department of Epidemiology, Harvard School of Public Health, Boston; and [7]Division of Biostatistics and Epidemiology, School of Public Health and Health Sciences, University of Massachusetts, Amherst, Massachusetts

A.H. Eliassen and R.G. Ziegler contributed equally to this work.

Corresponding Author: Fangyi Gu, National Cancer Institute, 6120 Executive Blvd, Room 7110, Bethesda, MD 20892. Phone: 301-594-0749; Fax: 301-402-4489; E-mail: guf@mail.nih.gov

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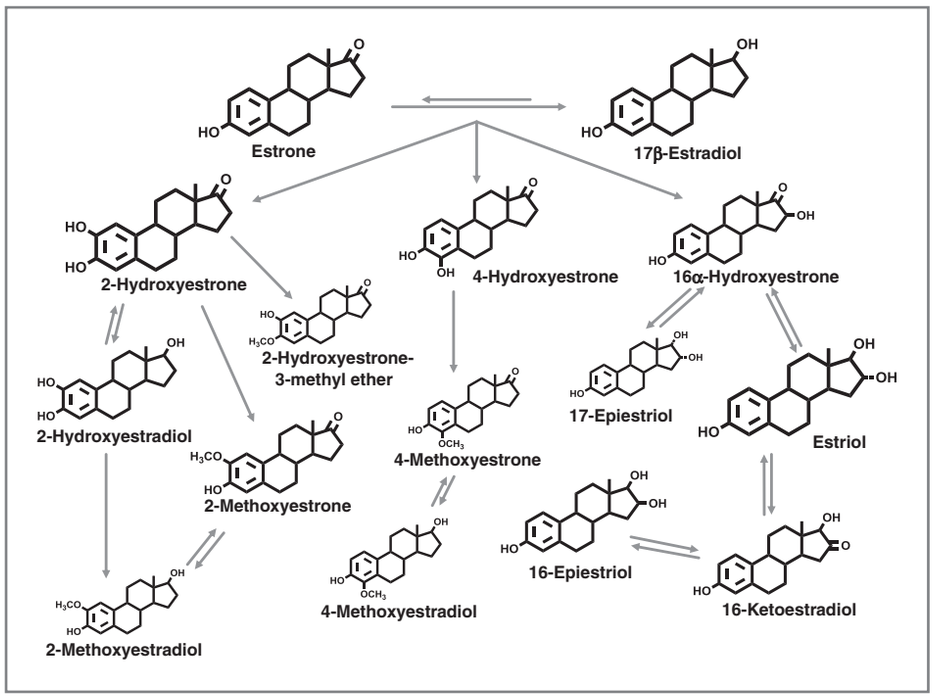

Figure 1. Pathways of endogenous estrogen metabolism. The size of the chemical structures generally reflects the relative urinary concentrations for each estrogen or estrogen metabolite.

were mixed. To our knowledge, only 1 group has explored the influence of smoking on estrogen metabolism (6, 11). Urinary estriol was used as a measure of 16-hydroxylation and 2-hydroxyestrone as a measure of 2-hydroxylation.

We explored the influence of smoking status and intensity on luteal phase urinary concentrations of estrone, estradiol, estriol, and 12 additional estrogen metabolites representing 2-, 4-, and 16-pathway metabolism in 603 premenopausal women from the Nurses' Health Study II (NHSII), a large cohort with extensive information on smoking history. We used a high-performance liquid chromatography/tandem mass spectrometry (LC/MS-MS) assay to measure concurrently 15 EM with high sensitivity, specificity, accuracy, and reproducibility (12).

Materials and Methods

Study design and population

The NHSII, a cohort including 116,430 female registered nurses, ages 25 to 42 years at enrollment, was established in 1989 and has been followed up biennially to update exposures and disease status. A subcohort of 29,611 cancer-free women, ages 32 to 54, provided biospecimens between 1996 and 1999 (13). A total of 18,521 were premenopausal, provided 1 midluteal urine sample (7–9 days before the anticipated start of their next cycle), and had not used oral contraceptives, been pregnant, or breastfed within the past 6 months.

Urine samples, 80% as first morning urine, were collected without preservatives, chilled immediately, and shipped on the day of collection, via overnight courier with an ice-pack, to the Channing Laboratory where they were aliquoted and stored in liquid nitrogen (at −130°C or less; ref. 14). Approximately 93% of the samples were received within 26 hours of collection. The first day of the next cycle was reported by returning a postcard and used to determine the actual luteal day of the urine collection. The study was approved by the Committee on the Use of Human Subjects in Research at Harvard School of Public Health (Boston, MA) and Brigham and Women's Hospital (Boston, MA).

Our analyses included 603 premenopausal women. Of these, 493 were control subjects for a nested case–control study of breast cancer (14). The additional 110 samples were from the first of 3 repeated urine samples in a NHSII biomarker reproducibility study (15). Current, former, and never smokers were similarly distributed in these 2 populations: the corresponding percentages were 6.4%, 26.4%, and 67.3% for the nested case–control study and 5.7%, 22.5%, and 71.8% for the biomarker study $\left(P_{\chi^2} = 0.6\right)$.

Smoking history and covariate assessment

Smoking variables were derived using information from the baseline and biennial follow-up questionnaires up through 1997, the year closest to the period of urine collection (1996–1999). A woman who reported smoking at least 100 cigarettes was defined as a smoker. Smoking status and average number of cigarettes per day (CPD) category (1–4, 5–14, 15–24, 25–34, 35–44, and 45+) were based on the 1997 questionnaire. Former smokers were defined as those quitting more than 6 months before the 1997 questionnaire.

Covariates in this study included: age at menarche (assessed in 1989), usual menstrual cycle length (1993),

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menstrual cycle pattern (1993), parity (1997), age at first birth (1997), family history of breast cancer (1989 and 1997 questionnaires), history of benign breast disease (1997), alcohol use (average of 1995 and 1999 questionnaires), and physical activity (average of 1997 and 2001 questionnaires). In addition, the biospecimen collection questionnaire provided data on age, current weight, urine collection date and time, fasting status, and whether the urine was a first morning sample. Height reported on the 1989 questionnaire was used to calculate body mass index (BMI).

EM measurements

EM assays were conducted at the Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc. (Frederick, MD). The glucuronide and sulfate conjugates of the EM were hydrolyzed enzymatically. The details of the assay have been published previously (12, 16). The overall laboratory coefficients of variation (CV), including within- and between-batch variation and all steps of the assay, were less than 7% except for 4-methoxyestrone (17%) and 4-methoxyestradiol (15%), the 2 EM with the lowest concentrations. The lower level of quantitation for each EM is approximately 150 fmol/mL urine. Current, former, and never smokers were randomly assigned, and similarly distributed, across assay batches for EM and creatinine (both $P_{\text{Fisher}} > 0.2$).

Urinary creatinine was measured to adjust EM concentration for urine volume. Creatinine was measured in 3 batches at Emory University (Atlanta, GA), Boston Children's Hospital (Boston, MA), and Brigham and Women's Hospital. Total laboratory CVs were 9.2% or less.

Statistical analyses

Total EM was calculated by summing all 15 individual EM, expressed as moles. EM were grouped by metabolic pathway (Fig. 1), and molar quantities of the appropriate EM summed. Total EM, individual EM, and metabolic pathway groups were adjusted for creatinine (pmol/mg creatinine). We also created ratios of selected metabolic pathway groups (pmol/pmol). The individual EM, metabolic pathway groups, and pathway ratios were log transformed, and statistical outliers were identified using the extreme studentized many-deviate procedure (17). Up to 10 values were excluded for each EM, except for 2-methoxyestradiol with 16 outliers.

Generalized linear models (GLM) were used to calculate age-adjusted geometric means of individual EM, metabolic pathway groups, and pathway ratios according to smoking status (never, former, and current) and associated P values for tests of the difference in means. Percentage difference in geometric means for current compared with never smokers was calculated by $[\exp(\beta) - 1] \times 100\%$, with β being the age-adjusted GLM regression coefficient. For CPD analyses, we included 4 categories: never smokers and current smokers with CPD < 5, 5 ≤ CPD < 15, and CPD ≥ 15. Percentage difference in geometric means per CPD category was calculated by $[\exp(\beta) - 1] \times 100\%$,with β being the age-adjusted regression coefficient. Age-adjusted linear trend P values were calculated by F tests, using a continuous variable for the 4 CPD categories.

The following variables, when added individually, did not substantially modify the age-adjusted associations [changes of exp(β) were less than 10%]: current BMI (continuous), height (continuous), age at menarche (<12, 12–13, or >13 years), menstrual cycle pattern (regular or not), usual menstrual cycle length (≤25, 26–31, or ≥32 days), parity (nulliparous, 1, 2, or ≥3 births), age at first birth (nulliparous, ≤25, 26–35, or >35 years), family history of breast cancer, history of benign breast disease, alcohol consumption (never, 1–3 drinks/mo, 1 drink/wk, 2–4 drinks/wk, or ≥5 drinks/wk), and physical activity (<3, 3 to <9, 9 to <18, 18 to <27, ≥27 MET-h/wk). Subjects with missing values were assigned to the largest category of each variable. Duration of past oral contraceptive use and time since last use were not associated with EM measures (18).

Sensitivity analyses were conducted restricting to first morning urine samples (26 current and 341 never smokers), to luteal days 4 to 10 before next cycle (33 current and 370 never smokers), or to ovulatory cycles, based on plasma progesterone levels measured at the time of urine collection (33 current and 370 never smokers).

To fully adjust for several potentially important confounding factors at the same time, and remove variation in EM contributed by other covariates, we calculated multivariate-adjusted P values by fitting residual models in 2 steps (19). First, we fitted GLM models by regressing individual EM, metabolic pathway groups, and pathway ratios on age (continuous), BMI (continuous), alcohol consumption (continuous), physical activity (continuous), first morning urine (yes/no), and luteal day at urine collection (<5, 6–7, 8–9, or ≥10 days before next menstrual period), to obtain residuals of the EM measures for each person. Then we regressed the residuals for each EM measure on smoking status or CPD (4 categories).

SAS software version 9.1.3 (SAS Institute) was used for the analyses.

Results

Our study included 428 never smokers, 140 former smokers, and 35 current smokers. The age at urine collection ranged from 33 to 51 years.

Current smokers were slightly taller than never smokers, more likely to have irregular menses, more likely to have children, and less physically active and drank more alcohol (Table 1). Adding these variables individually to age-adjusted models for smoking status and intensity did not change results substantially. There were no statistically significant differences between former and never smokers for most covariates, except that former smokers tended to be older at urine collection and drink more heavily (data not shown).

In general, the age-adjusted geometric means of urinary levels of individual EM, metabolic pathway groups, and

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Table 1. Characteristics of study population: NHSII ($n = 603$)[a]

	Never smoker ($n = 428$)	Former smoker ($n = 140$)	Current smoker ($n = 35$)	*P*-current vs. never[b]
Age at urine collection, mean (SD), y	42.7 (3.9)	43.4 (3.6)	42.9 (2.9)	0.66
CPD, mean (SD)		13.4 (7.6)	11.3 (6.1)	
Years of smoking, mean (SD)		12.8 (5.4)	22.5 (7.1)	
Age at starting smoking, mean (SD), y		16.4 (3.0)	18.2 (5.3)	
Age at stopping smoking, mean (SD), y		28.1 (6.9)		
BMI, mean (SD), kg/m^2	25.0 (5.3)	25.1 (4.9)	25.8 (6.4)	0.43
Height, mean (SD), in	65.1 (2.7)	64.9 (2.8)	66.1 (2.4)	0.04
Age at menarche, *n* (%)				
<12 y	91 (21.3)	27 (19.3)	8 (22.9)	0.23
12–13 y	251 (58.6)	82 (58.6)	16 (45.7)	
>13 y	86 (20.1)	31 (22.1)	11 (31.4)	
Menstrual cycle pattern, *n* (%)[c]				
Regular	398 (96.1)	130 (94.9)	31 (88.6)	0.06
Nonregular	16 (3.9)	7 (5.1)	4 (11.4)	
Usual menstrual cycle length, *n* (%)[c]				
≤25 d	79 (19.0)	28 (20.4)	8 (26.7)	0.56
26–31 d	292 (70.4)	96 (70.1)	17 (56.7)	
≥32 d	44 (10.6)	13 (9.5)	5 (16.6)	
Age at first birth, *n* (%)				
≤25 y	157 (46.3)	51 (42.5)	14 (43.7)	0.14
>25–30 y	125 (36.9)	42 (35.0)	8 (25.0)	
>30–35 y	44 (13.0)	21 (17.5)	7 (21.9)	
>35 y	13 (3.8)	6 (5.0)	3 (9.4)	
Parity, *n* (%)[c]				
Nulliparity	86 (20.2)	20 (14.3)	3 (8.6)	<0.01
1	46 (10.8)	19 (13.6)	11(31.4)	
2	176 (41.3)	58 (41.4)	11(31.4)	
≥3	118 (27.7)	43 (30.7)	10(28.6)	
Family history of breast cancer, *n* (%)	35 (8.2)	16 (11.4)	1 (2.9)	0.50
History of benign breast disease, *n* (%)	65 (15.2)	21(15.0)	6 (17.1)	0.76
Physical activity, median (interquartile range), MET h/wk	15.4 (6.9–30.3)	16.2 (7.8–31.4)	15.0 (5.4–26.2)	0.05
Alcohol use, median (interquartile range), drinks/mo	1.0 (0–3.8)	2.8 (0.9–9.3)	3.7 (0.6–9.8)	0.02

[a]Age and weight were reported at urine sample collection, which took place between 1996 and 1999. Other characteristics were based on biennial NHSII questionnaires.

[b]The *P* value comparing current versus never smokers was calculated using a *t* test for continuous variables, a Fisher exact test for menstrual cycle pattern and family history of breast cancer (more than 25% of cells with expected number less than 5), and a χ^2 test for the other categorical variables.

[c]Numbers do not sum to total due to missing values; in analyses, the subjects missing values for menstrual cycle pattern, usual menstrual cycle length, age at first birth, and parity were assigned to the largest category for each variable.

pathway ratios in the former smokers were similar to those in the never smokers (Table 2). The majority of former smokers had stopped smoking years before urine sample collection (collected 1996–1999). Only 2% had quit within the year preceding the 1997 questionnaire, and only 10% had quit within the 5 preceding years. For all these reasons, we decided to focus on the differences between current and never smokers in our additional analyses.

Comparing current with never smokers, we observed lower levels of total EM (age-adjusted geometric means = 182.5 and 199.1 pmol/mg creatinine, respectively), parent estrogens (36.3 and 41.4 pmol/mg creatinine, respectively), estrone, estradiol, 16-pathway EM (58.3 and 69.7 pmol/mg creatinine, respectively), and each of the individual EM in the 16-pathway; the multivariate-adjusted *P* values for estradiol, estriol, and 16-epiestriol were statistically significant (Table 2). In contrast, 4-pathway EM and

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Table 2. Age-adjusted geometric means by smoking status: NHSII ($n = 603$)

EM measures	Geometric mean[a] Current smoker ($n = 35$)	Former smoker ($n = 140$)	Never smoker ($n = 428$)	*P* current vs. never[b]	$P_{multivariate}$ current vs. never[c]	*P* former vs. never[b]
Individual EM and metabolic pathway groups (pmol/mg creatinine)						
Total EM	182.5	206.3	199.1	0.32	0.32	0.47
Parent estrogens	36.3	42.0	41.4	0.21	0.11	0.82
Estrone	24.2	28.9	27.4	0.25	0.18	0.40
Estradiol	11.6	13.6	13.8	0.10	**0.02**	0.88
2-Hydroxylation pathway EM	64.5	65.1	64.7	0.99	0.92	0.94
2-Pathway catechols	55.4	53.8	53.2	0.78	0.66	0.89
2-Hydroxyestrone	49.4	47.7	47.3	0.77	0.64	0.91
2-Hydroxyestradiol	5.5	5.3	5.3	0.79	0.91	0.91
2-Pathway methylated catechols	8.4	9.8	9.9	0.21	0.19	0.89
2-Methoxyestrone	6.3	7.7	7.7	0.13	0.14	0.89
2-Methoxyestradiol	0.56	0.68	0.72	0.06	**0.03**	0.52
2-Hydroxyestrone-3-methyl ether	1.3	1.2	1.2	0.50	0.61	0.95
4-Hydroxylation pathway EM	7.1	6.1	5.8	0.19	0.25	0.60
4-Hydroxyestrone[d]	6.5	5.7	5.5	0.30	0.34	0.71
4-Pathway methylated catechols	0.27	0.24	0.21	0.14	0.06	0.15
4-Methoxyestrone	0.18	0.15	0.13	0.08	**0.03**	0.15
4-Methoxyestradiol	0.05	0.05	0.05	0.83	0.94	0.81
16-Hydroxylation pathway EM	58.3	73.7	69.7	0.11	0.08	0.37
16α-Hydroxyestrone	11.0	13.6	11.9	0.57	0.63	0.11
Estriol	23.3	32.1	30.8	**0.03**	**0.02**	0.58
17-Epiestriol	1.5	1.5	1.7	0.48	0.65	0.21
16-Ketoestradiol	12.7	14.8	14.3	0.28	0.39	0.61
16-Epiestriol	5.0	6.8	6.4	**0.01**	**0.004**	0.23
Catechols	63.2	61.9	60.3	0.73	0.63	0.73
Methylated catechols	8.8	10.2	10.2	0.22	0.21	1.00
Metabolic pathway ratios						
Estrogen metabolites:parent estrogens	0.8	0.7	0.7	0.17	**0.05**	0.8
2-Pathway EM:parent estrogens	1.8	1.5	1.5	0.13	**0.04**	1.00
4-Pathway EM:parent estrogens	0.20	0.14	0.14	**0.02**	**0.01**	0.60
16-Pathway EM:parent estrogens	1.6	1.7	1.7	0.73	0.89	0.56
2-Pathway EM:16-pathway EM	1.1	0.9	0.9	0.21	0.16	0.70
4-Pathway EM:16-pathway EM	0.12	0.08	0.08	**0.03**	**0.03**	0.93
2-Pathway EM:4-pathway EM	9.1	10.4	10.9	0.16	0.23	0.56
2-Pathway catechols:methylated catechols	6.6	5.3	5.4	**0.04**	**0.02**	0.96
4-Pathway catechols:methylated catechols	24.4	23.3	25.8	0.82	0.54	0.44
Catechols:methylated catechols	7.2	5.9	5.9	**0.04**	**0.02**	0.96

[a]GLMs regressing ln(EM measure) on smoking status (current, former, and never smoker) were adjusted for age at urine collection; geometric means were calculated as exp(least square mean).

[b]*P* values are adjusted for age only. *P* values less than 0.05 are in boldface.

[c]Multivariate *P* values are adjusted for age, BMI, alcohol use, physical activity, first morning urine, and luteal day at urine collection by fitting residual models. *P* values less than 0.05 are in boldface.

[d]4-Hydroxyestrone is the only 4-pathway catechol.

each of the individual EM in the 4-pathway, except 4-methoxyestradiol, tended to be higher among current smokers than never smokers (age-adjusted geometric means for 4 pathway EM = 7.1 and 5.8, respectively), but only the difference for 4-methoxyestrone was statistically significant. We observed similar 2-pathway EM levels in current and never smokers, with 2-pathway catechols higher in current smokers and 2-pathway methylated catechols lower; but these differences were not statistically significant except for 2-methoxyestradiol.

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The ratios of estrogen metabolites, 2-pathway EM, and 4-pathway EM to parent estrogens were each statistically significantly elevated, based on the multivariate model, in current compared with never smokers. The ratio of 16-pathway EM to parent estrogens was slightly, but not significantly, lower in current smokers compared with never smokers (Table 2). Both the 2-pathway EM:16-pathway EM and the 4-pathway EM:16-pathway EM ratios were elevated among current smokers, with the difference for the 4:16 ratio statistically significant. The ratio of catechols to methylated catechols, in the 2-pathway and in the 2-pathway and 4-pathway combined, were statistically significantly elevated in current smokers.

The percentage difference [and 95% confidence interval (CI)] in the age-adjusted geometric means for selected EM measures are presented in Fig. 2 to provide an overview of the strength of the differences in EM measures between current and never smokers. Compared with individual EM and metabolic pathway groups, the percentage differences for metabolic pathway ratios were stronger and more often statistically significant. The ratios of 2-pathway EM and 4-pathway EM to parent estrogens were 8.5% (multivariate-adjusted $P = 0.04$) and 19% ($P = 0.01$)

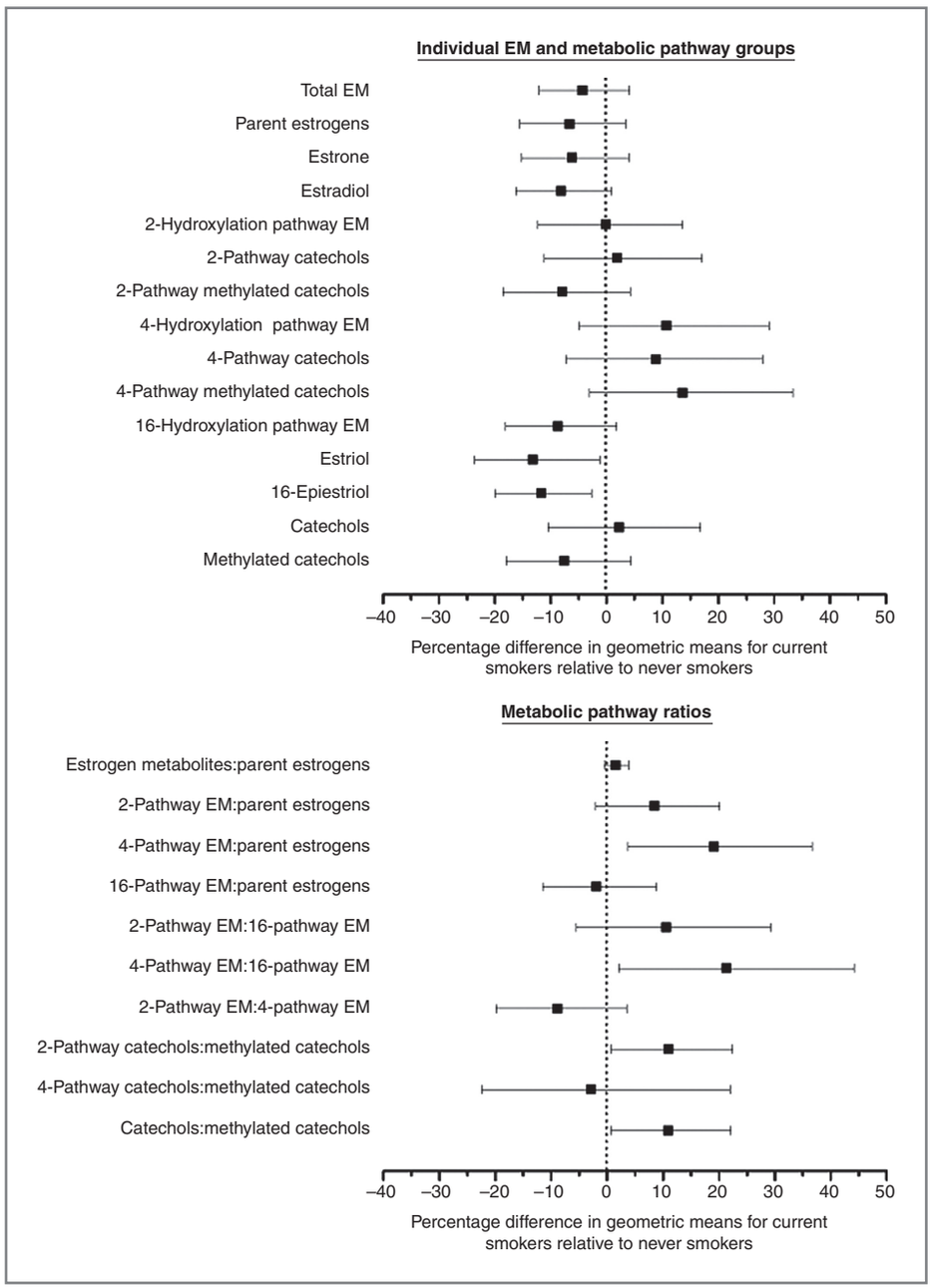

Figure 2. Percentage difference in age-adjusted geometric means (and 95% CI), comparing current ($n = 35$) with never ($n = 428$) smokers, for selected individual estrogens and estrogen metabolites (jointly referred to as EM), metabolic pathway groups, and metabolic pathway ratios. GLMs regressing ln(EM measure) on current and never-smoking status were adjusted for age at urine collection. The percentage difference was calculated as $[\exp(\beta) - 1] \times 100$, where β is the regression coefficient.

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higher, whereas 16-pathway EM:parent estrogens was slightly lower ($P = 0.89$) in current smokers, compared with never smokers. The 2-pathway EM:16-pathway EM and the 4-pathway EM:16-pathway EM ratios were 11% ($P = 0.16$) and 21% ($P = 0.03$) higher in current smokers, whereas the 2-pathway EM: 4-pathway EM ratio was 8.9% lower ($P = 0.23$). Finally, the ratio of catechols to methylated catechols in the 2-pathway was 11% higher ($P = 0.02$), but the equivalent ratio for the 4-pathway was similar in current and never smokers.

We also calculated the percentage difference (and 95% CI) in age-adjusted geometric means of the individual EM, metabolic pathway groups, and pathway ratios by CPD, categorized as 0, <5, 5 to <15, and ≥15 CPD (Table 3). *P* values for trend are presented for both age- and multivariate-adjusted models. The patterns we observed with CPD were similar to those noted when we compared current with never smokers. For EM measures that were higher in current smokers, we observed positive associations with increasing CPD; and for EM measures that were lower in current smokers, we observed inverse associations with CPD. The statistical significance was generally strengthened for the association with CPD, Specifically, the positive associations of 4-pathway EM and 4-pathway methylated catechols and the inverse association of 16-pathway EM with current smoking became statistically significant when CPD information was considered. The positive association for the 2-pathway EM:16-pathway EM ratio became statistically significant.

Sensitivity analyses restricting the comparison of EM measures in current and never smokers with first morning urine samples, ovulatory menstrual cycles, or luteal urine collections 4 to 10 days before the next menstrual cycle did not change the observed patterns.

Discussion

We explored the luteal phase urinary concentrations of 15 individual EM, EM grouped by metabolic pathways, and the ratios of selected metabolic pathways in relation to smoking status and intensity (CPD) among 603 premenopausal, predominately Caucasian women. Overall, urinary levels of individual EM, metabolic pathway groups, and pathway ratios were similar in never and former smokers, 90% of whom had quit smoking more than 5 years earlier. Urinary levels of total EM, parent estrogens, and 16-pathway EM tended to be lower and 4-pathway EM tended to be higher in current, compared with never, smokers; 2-pathway EM was similar between the 2 groups. The ratios, estrogen metabolites:parent estrogens, 2-pathway EM:parent estrogens, 4-pathway EM:parent estrogens, 4-pathway EM:16-pathway EM, and catechols:methylated catechols, were all significantly higher in current, compared with never, smokers. When we examined the associations of these EM measures with CPD, the same patterns were observed; the statistical significance, based on trend tests, was strengthened; and the positive association with the 2-pathway EM:16-pathway EM ratio became statistically significant.

An antiestrogenic effect of smoking has been suggested by its inverse associations with endometrial cancer risk, earlier age at natural menopause, and increased osteoporosis risk. Three hypotheses about the underlying mechanism have been developed (3). One is that smoking inhibits estrogen biosynthesis (3, 5); another is that smoking induces the 2-hydroxylation pathway (3, 6); the third is that 2-hydroxy-benzo[a]pyrene, and possibly other compounds in cigarette, binds competitively to SHBG or estrogen receptors (3). We were able to address the first 2 hypotheses in this analysis.

Smoking and estrogen biosynthesis

Decreased biosynthesis of endogenous estrogen due to smoking was proposed by MacMahon and colleagues (10) and based on their findings of 30% lower levels of urinary estrone, estradiol, and estriol in premenopausal female smokers ($n = 39$), compared with former ($n = 22$) or never ($n = 43$) smokers, during the luteal phase, but not follicular phase, of their cycle. However, this study did not consider other estrogen metabolites abundant in urine, such as the 2-pathway EM, and therefore, did not accurately measure total estrogen production. We also found lower urinary levels of estrone, estradiol, and estriol during the luteal phase in current smokers, relative to former and never smokers, as well as decreasing levels of these 3 EM with increasing smoking intensity. Furthermore, we observed modest, but nonsignificant lower levels of total EM among current and higher intensity smokers, which is consistent with the hypothesis that smoking might reduce total estrogen biosynthesis. However, Key and colleagues (9) and Berta and colleagues (8) did not find comparable differences in estrone and estradiol by smoking status in 24-hour urine samples from premenopausal women. In the Key and colleagues' study, only estriol was reduced (by about 8%) in current smokers ($n = 53$), compared with nonsmokers ($n = 114$), with the decrease most apparent in the luteal phase; whereas none of these 3 EM was reduced among current smokers ($n = 237$), relative to nonsmokers ($n = 447$), in the Berta study, which, similar to our study, collected only luteal phase samples.

The hypothesis that smoking reduces estrogen biosynthesis is also supported by an *in vitro* study in humans (5). Nicotine (a major component of tobacco smoke), cotinine (a major metabolite of nicotine), and anabasine (a minor component of cigarette smoke) all reduced androstenedione conversion to estrone in choriocarcinoma cell cultures, and reduced the conversion of testosterone to estradiol in preparations of placental microsomes. These findings suggest some nicotinic alkaloids directly inhibit aromatase activity (5), the major source of endogenous estrogen in postmenopausal women.

The similar geometric mean levels of individual EM, metabolic pathway groups, and pathway ratios in never and former smokers (Table 2) suggests that the influence of smoking on estrogen production and metabolism lasts

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Table 3. Age-adjusted geometric means and percentage difference by categories of CPD among never ($n = 428$) and current smokers ($n = 35$): NHSII

EM measures	Geometric means[a]				Percentage change per category (95% CI)[a]	*P* for trend[b]	$P_{multivariate}$ for trend[c]
	Never smokers ($n = 428$)	CPD<5 ($n = 16$)	5≤CPD<15 ($n = 8$)	CPD≥15 ($n = 11$)			
Individual EM and metabolic pathway groups (pmol/mg creatinine)							
Total EM	199.10	177.57	203.94	174.41	−3.8 (−11.4, 4.3)	0.35	0.49
Parent estrogens	41.46	35.34	44.10	32.55	−6.2 (−15.0, 3.4)	0.2	0.27
Estrone	27.44	23.59	29.81	21.60	−5.9 (−14.8, 3.9)	0.23	0.4
Estradiol	13.74	11.37	13.74	10.59	−7.5 (−15.5, 1.2)	0.09	0.07
2-Hydroxylation pathway EM	64.68	46.45	97.42	76.88	5.2 (−7.2, 19.2)	0.43	0.32
2-Pathway catechols	53.21	39.43	86.17	65.58	7.2 (−6.2, 22.5)	0.31	0.2
2-Hydroxyestrone	47.33	34.60	77.17	59.64	7.7 (−6.1, 23.5)	0.29	0.18
2-Hydroxyestradiol	5.29	4.47	8.11	5.58	4.3 (−8.8, 19.1)	0.54	0.54
2-Pathway methylated catechols	9.85	6.52	10.12	10.44	−2.5 (−13.5, 9.9)	0.68	0.6
2-Methoxyestrone	7.74	4.76	8.06	7.94	−3.7 (−15.1, 9.2)	0.55	0.55
2-Methoxyestradiol	0.72	0.55	0.49	0.64	−8.9 (−19.5, 3.1)	0.14	0.04
2-Hydroxyestrone-3-methyl ether	1.17	1.02	1.38	1.68	9.0 (−3.6, 23.3)	0.17	0.26
4-Hydroxylation pathway EM	5.81	4.94	10.91	8.95	16.2 (0.2, 34.7)	**0.05**	**0.05**
4-Hydroxyestrone[d]	5.49	4.41	9.86	8.50	15.1 (−1.4, 34.5)	0.08	0.07
4-Pathway methylated catechols	0.21	0.21	0.39	0.29	16.0 (−0.6, 35.3)	0.06	**0.04**
4-Methoxyestrone	0.13	0.14	0.26	0.19	20.0 (0.5, 43.3)	**0.04**	**0.04**
4-Methoxyestradiol	0.05	0.04	0.06	0.04	−1.0 (−18.6, 20.4)	0.92	0.74
16-Hydroxylation pathway EM	69.83	74.27	44.41	49.78	−11.3 (−20.2, −1.4)	**0.03**	**0.04**
16α-Hydroxyestrone	11.98	12.31	9.68	10.28	−5.4 (−17.5, 8.5)	0.43	0.76
Estriol	30.88	29.74	17.22	20.30	−15.0 (−24.9, −3.7)	**0.01**	**0.01**
17-Epiestriol	1.68	2.20	0.82	1.27	−10.8 (−24.6, 5.5)	0.18	0.42
16-Ketoestradiol	14.34	17.63	9.75	9.63	−10.6 (−19.2, −1.0)	**0.03**	0.09
16-Epiestriol	6.37	6.54	3.74	4.08	−14.4 (−22.1, −6.1)	**0.001**	**0.002**
Catechols	60.30	44.97	98.38	74.91	7.6 (−5.4, 22.3)	0.27	0.19
Methylated catechols	10.20	6.79	10.78	10.84	−2.1 (−12.9, 10.1)	0.72	0.64
Metabolic pathway ratios							
Estrogen metabolites:parent estrogens	1.34	1.31	1.32	1.26	1.7 (−0.4, 3.9)	0.11	0.06
2-Pathway EM:parent estrogens	1.51	1.31	2.23	2.37	13.8 (3.1,25.7)	**0.01**	**0.01**
4-Pathway EM:parent estrogens	0.14	0.14	0.25	0.28	24.4 (8.9, 42.1)	**0.001**	**0.002**
16-Pathway EM:parent estrogens	1.67	2.10	1.01	1.53		0.32	0.33
2-Pathway EM:16-pathway EM	0.91	0.62	2.21	1.55	19.8 (3.0, 39.4)	**0.02**	**0.02**
4-Pathway EM:16-pathway EM	0.08	0.07	0.25	0.18	31.0 (10.9, 54.6)	**0.002**	**0.003**
2-Pathway EM:4-pathway EM	10.89	9.38	8.98	8.62	−8.5 (−19.2, 3.5)	0.16	0.23
2-Pathway catechols:methylated catechols	5.37	6.04	8.53	6.29	10.3 (0.3, 21.2)	**0.04**	**0.01**
4-Pathway catechols:methylated catechols	25.95	21.49	25.16	29.23	0.5 (−19.3, 25.1)	0.96	0.96
Catechols:methylated catechols	5.9	6.6	9.1	6.9	10.2 (0.4, 21.0)	**0.04**	**0.02**

[a]GLMs regressing ln(EM measure) on CPD (0 for never smokers; 1 for CPD < 5; 2 for 5 ≤ CPD < 15; 3 for CPD ≥ 15) were adjusted for age at urine collection; geometic means were calculated as exp(least square mean). The percentage change was calculated as $[\exp(\beta) - 1] \times 100$, where β is the regression coefficient.

[b]*P* values are adjusted for age only. *P* values less than 0.05 are in boldface.

[c]Multivariate *P* values are adjusted for age, BMI, alcohol use, physical activity, first morning urine, and luteal day at urine collection by fitting residual models. *P* values less than 0.05 are in boldface.

[d]4-Hydroxyestrone is the only 4-pathway catechol.

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for a limited period. We do not have enough women who quit smoking in the years just before urine collection to determine how quickly the influence of smoking disappears. Similar geometric means of urinary estrone, estradiol, and estriol between never and former smokers were also reported by MacMahon and colleagues (10). These results are consistent with the observation that the age at menopause of former smokers is found to be much closer to that of never smokers than that of current smokers (3). Taken together, all these results suggest that the influence of smoking on the estrogen-related physiologic processes is limited in time and reversible after smoking cessation.

Smoking and estrogen metabolism

To our knowledge, only 2 epidemiologic publications, both from the same group (6), have focused on smoking and estrogen metabolism patterns, including the 2-hydroxylation pathway. Using follicular phase urine samples from 14 smokers and 13 nonsmokers, Michnovicz and colleagues showed that the extent of estrogen 2-hydroxylation was higher ($P < 0.001$), and the estriol:estrone ratio lower ($P < 0.01$) in smokers (6). Using the same samples, they later reported the 2-hydroxyestrone:estriol ratio was higher ($P < 0.001$) among smokers, and the ratios of metabolites did not vary substantially through the menstrual cycle (11).

Using a comprehensive measure of the EM profile that includes all 2-, 4-, and 16-pathway estrogen metabolites, our observations support the initial finding. Absolute levels of 2-pathway EM did not increase in smokers, compared with nonsmokers, despite decreased levels of parent estrogens in smokers. The absolute concentration of 16-pathway EM was lower in current smokers, with the percentage difference decrease with CPD about twice that of the percentage difference decrease in parent estrogen levels. Thus, the 2-pathway EM:parent estrogens ratio and the 2-pathway EM:16-pathway EM ratio were higher in current smokers and, additionally, in smokers with higher CPD. These observations suggest smoking favors the metabolism of parent estrogens into the 2-pathway compared with the 16-pathway.

In addition, the higher ratios of 4-pathway EM: parent estrogens, 4-pathway EM:2-pathway EM, and 4-pathway EM:16-pathway EM among current and higher intensity smokers in our study suggests smoking also favors metabolism of parent EM into the 4-pathway compared with the 2- and 16-pathways. The increased ratio of catechols to methylated catechols in the 2-pathway in current and higher intensity smokers suggests smoking reduces methylation of catechol EM in this pathway. The modestly higher ratios of estrogen metabolites to parent estrogens in current and higher intensity smokers suggest smoking may induce metabolism of parent estrogens. These additional observations have not been previously reported because of the limited number of estrogen metabolites measured in earlier studies of smoking and estrogen metabolism.

Our observations are biologically interpretable. Several constituents of cigarette smoke, including nicotine, 3-methylcholanthrene, and benzo[a]pyrene, have been shown to induce the activity of hepatic cytochrome P450 enzymes, which metabolize a variety of chemicals, including steroid hormones (7, 20). For example, CYP1A2, induced by smoking, affects metabolism of parent estrogens to catechol estrogens (21, 22). In addition, a functional SNP rs4680 (also known as *V158M*) of *COMT*, which codes for the enzyme that methylates catechol estrogens, is associated with smoking intensity (22, 23).

Smoking and estrogen-related cancers

The associations between smoking and estrogen-related cancers are complex. An inverse association has been reported for endometrial cancer (1), whereas the relationships with breast and ovarian cancers are controversial (24, 25). In addition, although there are many hypotheses based on experimental research and animal and *in vitro* models about which individual estrogen metabolites and which profiles of estrogen metabolism are associated with risk of breast, endometrial, or ovarian cancer, the epidemiologic research is extremely limited, and not yet conclusive. In the only comprehensive study of urinary EM and risk of premenopausal breast cancer published to date (17), low urinary levels of estrone, estradiol, 2-pathway EM, and 4-pathway EM in the luteal phase were associated with increased breast cancer risk. In the current cross-sectional analysis, low urinary estrone and estradiol, but high 4-pathway EM, were associated with smoking, in premenopausal women. Given the multiple effects of smoking on estrogen biosynthesis and metabolism (some effects may negate but others may favor carcinogenesis), as well as the uncertainty whether estrogen profiles measured in urine accurately represent those of serum or target tissues, further work will be required to understand the interrelationships of smoking, estrogen production and metabolism, and carcinogenesis.

Strengths and limitations

Our study has several strengths. The LC/MS-MS assay has high sensitivity, accuracy, and precision for all 15 EM found in urine, which allows us to evaluate, in detail, the individual patterns of estrogen metabolism. We used carefully timed luteal urine samples from a large number of premenopausal women. We had information on not only smoking status but also CPD close to the time of urine collection and were able to examine the dose–response relationship between smoking intensity and EM measures.

There are also limitations to our study. A small number of current smokers (only 35) limited the statistical power to detect weak associations and effect modification by alcohol use and BMI. However, even with this limitation, we still detected associations of smoking status and intensity with urinary metabolic pathway groups and pathway ratios that were consistent and

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statistically significant. Using a residual model, we were able to adjust concurrently for several potential confounders.

Because our study was cross-sectional, we could not conclusively determine the causal relationship between smoking and estrogen production and metabolism. We are cautious in interpreting our findings as we only measured EM concentrations in urine and do not know exactly how these concentrations relate to circulating and target tissue EM levels. Passive smoking has been associated with lower serum estrone, estradiol, and estriol levels, similar to active smoking (26). Unfortunately, we did not collect detailed information on passive smoking exposure at the time of urine collection. The association of passive smoking with estrogen metabolism will need to be examined in another study.

Conclusions

Our results suggest an association between smoking and estrogen levels and patterns of metabolism in luteal phase urine samples from premenopausal women. We observed in current and heavier smokers modestly lower urinary levels of total EM, which supports the hypothesis of decreased biosynthesis of parent estrogens in smokers. We also observed, as has been hypothesized, a relative increase in 2-hydroxylation and decrease in 16-hydroxylation among current and heavier smokers. For the first time, we report evidence that smoking might be associated with increased metabolism of parent estrogens, increased 4-hydroxylation, and decreased methylation of catechol estrogens. Additional studies are warranted to replicate our results.

Disclosure of Potential Conflicts of Interest

R.G. Ziegler has ownership interest (including patents).

No potential conflicts of interest were disclosed by the other authors. 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.

Authors' Contributions

Conception and design: N.E. Caporaso, S.E. Hankinson, A.H. Eliassen, R.G. Ziegler

Development of methodology: A.H. Eliassen, R.G. Ziegler

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N.E. Caporaso, X. Xu, S.E. Hankinson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): F. Gu, N.E. Caporaso, C. Schairer, R.T. Fortner, S.E. Hankinson, A.H. Eliassen, R.G. Ziegler

Writing, review, and/or revision of the manuscript: F. Gu, N.E. Caporaso, C. Schairer, R.T. Fortner, S.E. Hankinson, A.H. Eliassen, R.G. Ziegler

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Gu, N.E. Caporaso, R.T. Fortner, A.H. Eliassen

Study supervision: F. Gu, N.E. Caporaso, A.H. Eliassen, R.G. Ziegler

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Fangyi Gu, Neil E. Caporaso, Catherine Schairer, et al.

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