

# Association of Genetic Polymorphisms with Serum Estrogens Measured Multiple Times During a 2-Year Period in Premenopausal Women

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## Abstract

There is evidence that circulating estrogens are associated with breast cancer risk. In this study of premenopausal women, we explored the association of polymorphisms in genes in the estrogen synthesis and metabolism pathways with serum and urinary levels of estrone ( $E_1$ ) and estradiol ( $E_2$ ) and with the urinary ratio of 2-hydroxyestrone (2-OHE<sub>1</sub>)/16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OHE<sub>1</sub>). This analysis included 220 women, who were participants in a 2-year randomized soy intervention. Blood specimens were collected in the luteal phase of the menstrual cycle an average of 4.4 times over 2 years. Overnight urinary specimens were collected on the same cycle day, only at baseline. Levels of  $E_1$ ,  $E_2$ , 2-OHE<sub>1</sub>, and 16 $\alpha$ -OHE<sub>1</sub> were measured by enzyme immunoassays. The DNA samples were analyzed by PCR/RFLP for the *COMT* Val<sup>158</sup>Met, *CYP1A1*\*2A, *CYP1A1*\*2B, *CYP1A2*\*1F,

*CYP1B1* Val<sup>432</sup>Leu, and *CYP17* T27C polymorphisms. We applied mixed models to investigate the relations between genotypes and repeated serum hormone measurements and generalized linear models to assess associations between genotypes and urinary estrogen metabolites. The *CYP1A2* C allele was significantly associated with lower serum  $E_2$  levels; in CC genotype carriers, serum  $E_2$  levels were 26.3% lower than in homo- and heterozygous common allele carriers combined ( $P = 0.01$ ). *CYP1A2*\*1F also affected the urinary 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio; carriers of the variant C allele had a markedly lower ratio than individuals with the AA genotype (1.37 versus 1.76;  $P = 0.002$ ). These data suggest that *CYP1A2*\*1F is associated with lower circulating levels of  $E_2$ , and that it may be a susceptibility locus for breast cancer. (Cancer Epidemiol Biomarkers Prev 2005;14(6):1521–7)

## Introduction

There is persuasive evidence from epidemiologic studies and experimental models for the role of endogenous estrogens and their metabolites in the pathogenesis of breast cancer. The most widely accepted theory states that estrogens increase the rate of mammary epithelial cell proliferation by stimulating estrogen receptor-mediated transcription (1–4). Another hypothesis is that estradiol increases risk through genotoxic metabolites that directly damage DNA (1, 2, 5–8). Epidemiologic studies showed that women with elevated circulating  $E_2$  levels are at higher risk for breast cancer (9–15). The major pathways of endogenous metabolism of estrogens involve hydroxylation at either C2 2-hydroxyestrone (2-OHE<sub>1</sub>) or C16 16 $\alpha$ -hydroxyestrone (16 $\alpha$ -OHE<sub>1</sub>). The 2-OHE<sub>1</sub> metabolite has little estrogenic activity, whereas 16 $\alpha$ -OHE<sub>1</sub> is an estrogen agonist (16–19). In a study by Ho et al. (20), 16 $\alpha$ -OHE<sub>1</sub> was shown to initiate neoplastic transformation of mammary epithelial cells. Other experimental studies showed an anti-estrogenic role for 2-OHE<sub>1</sub> (21) and a genotoxic effect for 16 $\alpha$ -OHE<sub>1</sub> (22–24). An inverse association between the 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio and breast cancer risk has also been reported in several epidemiologic studies (18, 19, 25).

Genes involved in estrogen synthesis and metabolism have been intensively studied in recent years in relation to breast cancer risk. However, few studies investigated associations between genetic polymorphisms and plasma estrogen levels,

and only two studies explored polymorphisms in estrogen metabolism pathway genes and urinary 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio. Feigelson et al. (26) first reported an association between the *CYP17* T27C variant and increased plasma estradiol levels in 83 nulliparous, ovulating, young women sampled either during the luteal phase or the follicular phase of the menstrual cycle; although the association for the follicular samples was of borderline significance only. Significant associations between the *CYP17* C allele and increased serum estrogen levels were also found among postmenopausal women (27, 28). However, other studies failed to show a relation (29–32). The association between serum sex hormones and *CYP1B1* Val<sup>432</sup>Leu and *COMT* Val<sup>158</sup>Met was investigated among premenopausal women by Garcia-Closas et al. (29) and among postmenopausal women by Tworoger et al. (32) and Dunning et al. (33). Premenopausal hetero- and homozygous carriers of the Val<sup>432</sup>Leu variant allele had significantly increased luteal  $E_2$  levels; no association was found among postmenopausal women. The role of the *COMT* Val<sup>158</sup>Met polymorphism on serum  $E_2$  levels was also studied by Worda et al. (34) in an intervention study (oral administration of 2 mg of  $E_2$  valerate); 3 hours after administration, serum  $E_2$  levels were significantly higher in women with the Met/Met genotype.

Tworoger et al. (32) investigated the relationship between several polymorphisms and urinary estrogen metabolites and found significantly higher 16 $\alpha$ -OHE<sub>1</sub> levels in postmenopausal women with the *COMT* Met/Met genotype, compared with those with the Val/Val genotype. The *CYP1A1*\*2A polymorphism was found to alter 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio in the study by Taioli et al. *CYP1A2* activity was found to be negatively associated with free estradiol concentrations in the recent study by Hong et al. (35). In a recent study by our group (36), we reported lower mammographic density for women carrying the *CYP1A2* variant C allele; however, this was the opposite of our hypothesis as carriers of this allele have been shown to have lower *CYP1A2* inducibility (37). To our

Received 10/12/04; revised 3/1/05; accepted 3/14/05.

**Grant support:** This research was supported by an award from the U.S. Army Medical Research Materiel Command under DAMD17-00-1-0281 and by grant R01 CA80843 from the National Cancer Institute.

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knowledge, *CYP1A2\*1F* polymorphism has not previously been studied in relation to either breast cancer risk or estrogen concentrations.

The conflicting results of past studies on genetic polymorphisms and estrogen levels may be explained in part by the strong temporal fluctuation of serum estrone ( $E_1$ ) and estradiol ( $E_2$ ) levels (11, 38). In addition, the origin of estrogens differs between pre- and postmenopausal women and, therefore, different associations may be expected by menopausal status. In the present study, we investigated the association of several polymorphisms in estrogen synthesis and metabolism pathway genes (*COMT* Val<sup>158</sup>Met, *CYP1A1\*2A*, *CYP1A1\*2B*, *CYP1A2\*1F*, *CYP1B1* Val<sup>432</sup>Leu, and *CYP17* T27C) with serum  $E_1$  and  $E_2$  levels in healthy premenopausal women with regular menstrual cycles who participated in a dietary intervention trial and had their estrogen levels measured several times over 2 years. It is plausible that these polymorphisms can affect serum estrogen levels and distribution of estrogen metabolites in urine by changing the activity of important enzymes in estrogen synthesis and metabolism pathways. Because estrogens were measured multiple times over 2 years, we had a more reliable measure than the single sample uniformly used in past studies. We also investigated the association between these polymorphisms and the urinary 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio, which is thought to be a stable marker with relatively moderate within-person variation (39).

## Materials and Methods

**Study design and population.** This study included 220 healthy premenopausal women recruited at mammography clinics who participated in a 2-year randomized controlled soy intervention (40). The inclusion criteria were having a normal mammogram at baseline and no previous history of cancer, having regular menstrual cycles and an intact uterus and ovaries, not taking oral contraceptives or other hormones, not being pregnant or lactating, and consuming <7 servings of soy a week. All subjects completed a validated food frequency questionnaire (41), as well as a detailed questionnaire on reproductive, medical, and anthropometric factors at baseline. Subject recruitment, sample collection, and intervention results are described in detail elsewhere (40). Because the trial showed no significant intervention effect on any of the serum hormones during the 2-year period, we combined the data from both study groups for the current investigation. The protocol was approved by the Committee on Human Studies of the University of Hawaii and by the Institutional Review Boards of the three hospitals where women were recruited, and written informed consent was obtained from all participants.

**Sample collection.** Fasting blood and overnight urine samples were collected between 7:30 and 10:00 a.m. for 208 and 184 women, respectively, during the luteal phase of a menstrual cycle, on the 5th day after ovulation, corresponding to approximately day 19 of the menstrual cycle. For <1% of women, blood samples were obtained on day 4 or 6. In addition to baseline, blood samples were obtained in the same manner at approximately 3, 6, 12, and 24 months. The sampling day was determined by using an ovulation test that detects the time of ovulation by measuring luteinizing hormone in a urine sample (42) and confirmed by serum progesterone values >5 ng/mL. During the 2-year study period, participants notified staff on the first day of each menstruation, and the menstruation dates were updated in the database. Two to three weeks before sample collection, study personnel reminded each study participant when she was supposed to start ovulation testing. After testing positive for ovulation, the blood collection was arranged 5 days later or in few cases on days 4 to 6.

**Hormone measurements.** Serum  $E_1$  and estradiol  $E_2$  levels were measured in all samples by RIA in the laboratory of Dr. Stanczyk (University of Southern California, Los Angeles, CA, USA) as described previously (43). The analyses were conducted in batches of 30 or 40 samples. Each batch contained all samples collected at baseline and at months 3, 6, 12, and 24 from the same woman. For quality control, two control samples containing known amounts of steroids were included for all the analysis steps in each analytic batch. Interassay coefficients of variation for the serum estrogens were 17.7% for  $E_1$  and 11.2% for  $E_2$ . Urinary  $E_1$ ,  $E_2$ , 2-OHE<sub>1</sub>, and 16 $\alpha$ -OHE<sub>1</sub> were measured in the laboratory of Dr. Kaaks (International Agency of Research on Cancer, Lyon, France), using a previously published protocol (42). All measurements were done in duplicate, including hydrolysis, solid phase extraction, HPLC, and RIA steps. The detection limits for urine  $E_1$  and  $E_2$  were 2.0 ng/100mL. Intra- and interbatch coefficients of variations were 4.7% and 16%, respectively, for  $E_1$  (at a concentration of 3,300 ng/L), and 1.7% and 14% for  $E_2$  (at 320 ng/L). The metabolites, 2-OHE-1 and 16 $\alpha$ -OHE-1, were measured by solid-phase enzyme immunoassays after enzymatic hydrolysis with Helix Pomatia (Estramet, Immunocare Corporation, Bethlehem, USA). Mean intrabatch and interbatch coefficients of variation were 10% and 15%, respectively, for both analytes.  $E_1$ ,  $E_2$ , 2-OHE<sub>1</sub>, and 16 $\alpha$ -OHE<sub>1</sub> in urine were adjusted for urinary creatinine levels.

**Genetic analysis.** DNA was purified from whole blood using Qiagen Midi Kits (Qiagen, Valencia, CA). The DNA samples were analyzed by PCR/RFLP for the *COMT* Val<sup>158</sup>Met, *CYP1A1\*2A*, *CYP1A1\*2B*, *CYP1A2\*1F* (rs762551), *CYP1B1* Val<sup>432</sup>Leu, and *CYP17* T27C (rs743572) polymorphisms, as reported previously (44, 45). In addition, we genotyped the samples for the rare *CYP1A1\*4* variant in order to correctly assign *CYP1A1\*2B* genotypes (46). Forty-two percent of the samples were assayed twice for quality control. The concordance rates among duplicates were 100% for *CYP1A1\*2*, *CYP1A2\*F*, and *COMT*, 99.9% for *CYP17*, and 91.4% for *CYP1A1\*3* and *CYP1B1*.

**Statistical analysis.** The SAS statistical software package version 8.2 (SAS Institute, Inc., Cary, NC) was used for the data analysis. In the questionnaire, subjects marked all ethnic backgrounds that applied to their parents. We assigned summary categories according to the following rules. A woman was classified as Caucasian if both of her parents were of Caucasian ancestry. In agreement with a common rule applied in the State of Hawaii (47), women with any Hawaiian heritage were classified as Native Hawaiian. If subjects reported two or three ethnic backgrounds, they were classified according to the ethnicity shared by both of the parents, or the ethnicity of the mother when parents did not share any ethnic background. Because of the similarity in variant allele frequencies, Japanese, Chinese, and Filipino women were combined into the Asian category. Because of their mixed ancestries, the Native Hawaiian women ( $n = 26$ ) were combined into the Mixed category that also included women with more than three ethnic backgrounds. Body mass index (BMI) was calculated as the ratio of weight in kilograms divided by the square of the height in meters. All outcome and dietary variables, as well as BMI, were transformed using their natural logarithm because they were not normally distributed. Relationships between levels of serum and urinary estrogens and their metabolites were examined by calculating the Pearson correlation coefficient ( $r$ ).

We used mixed linear models (MIXED SAS procedure) with repeated measures of  $E_1$  and  $E_2$  to model the fixed effects of genotype, time of blood draw, study group assignment (intervention versus control), interaction between genotype and study group, and interaction between study group and time (to explore differences in the intervention effect on

hormone levels at different times during the 2-year study period). We included subject as a random effect in the model, accounting for the covariance structure between the repeated measures (48). Given the lack of an intervention effect and no indication of effect modification by genotype or time on circulating hormones, study group, time, and both interaction variables were dropped from the final models. The PROC GLM procedure was used to assess associations of urinary  $E_1$ ,  $E_2$ , 2-OHE<sub>1</sub>, and 16 $\alpha$ -OHE<sub>1</sub> with genotype. The PROC MIXED SAS procedure was also used to calculate intraclass correlations between the repeated measures of serum  $E_1$  and  $E_2$  levels.

In preliminary analyses, we assessed the relationships between each of the outcome variables with age, ethnicity, BMI, age at menarche, age at first live birth, parity, family history of breast cancer, total daily calories, total daily calories from fat, daily alcohol, fat, saturated fat, and caffeine intakes and explored associations between each polymorphism and ethnicity, age at menarche, and BMI. We also introduced these variables into the mixed models to look for potential confounders. Our final models included covariates that were associated with at least one of the predictor or outcome variables, namely: ethnicity, age, BMI, age at menarche, and number of children. As no significant changes occurred in BMI over time, a baseline BMI measurement was included into the models. We also explored associations between genotypes and hormonal variables within each ethnic group.

For all models, genotype was successively treated as a nonordered categorical variable to obtain least-square means and to test for heterogeneity, and as an ordered categorical variable (0, 1, and 2, for the homozygous common genotype and the heterozygous and homozygous variant genotype, respectively) to test for a gene dosage effect. In addition, we evaluated genotype as a dichotomous variable, combining heterozygous and homozygous variant allele carriers for a pair-wise comparison with homozygous carriers of the common allele (testing a dominant pattern of inheritance) and combining homozygous and heterozygous common allele carriers for a pair-wise comparison with homozygous variant allele carriers (testing a recessive genetic model).

## Results

**Characteristics of the study population.** The subject characteristics are presented in Table 1 by ethnicity. The mean age of the study participants was 43.0 years (SD, 2.8; range 34-47). The sample included an almost equal number of Caucasian and Asian women. On average, women of mixed ethnicity were slightly younger, and Caucasians had a somewhat older age at menarche, lower parity, and higher age at first live birth. BMI was lowest among Asian women, intermediate in Caucasians, and highest in women of mixed ethnicity ( $P = 0.048$ ). Few women (6%) reported to be current smokers.

The distributions of the genotypes were all consistent with Hardy-Weinberg equilibrium within each ethnic group. The variant allele frequencies were significantly different by ethnicity for all polymorphisms under study, except for *CYP17* T27C and *CYP1A2\*1F*.

Out of the 208 women who donated blood samples during the 2-year study period, 116 women had serum  $E_1$  and  $E_2$  levels measured five times, whereas 56 women had four measurements, 24 had three measurements, and 9 women had two measurements. Overall, serum estrogens were measured 4.4 times. The variability in number of measurements was due to women dropping out of the study (31 subjects) and to sample exclusions due to wrong timing (16 samples), or occasional use of exogenous hormones (72 samples). Overall, we had 894 measurements for each  $E_1$  and  $E_2$ , representing 86% of the maximum possible number of measurements ( $n = 1,040$ ).

The within-person intraclass correlation for  $E_1$  and  $E_2$  over time was 0.55 and 0.41, respectively. In Table 1, these hormonal measurements were averaged for each subject over the number of collections (Table 1). Overall, mean serum  $E_1$  and  $E_2$  levels were lowest in Asian women and highest in women of mixed ethnicity; these differences, however, were not statistically significant. The mean urinary levels of  $E_1$  and  $E_2$  were also similar among ethnic groups (Table 1). However, statistically significant differences were observed in the mean urinary 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio, with the highest value for Caucasians and the lowest for Asians, reflecting a

**Table 1. Characteristics of the study population by ethnicity**

Variables	Asian ( <i>n</i> = 85)	Caucasian ( <i>n</i> = 83)	Mixed ( <i>n</i> = 52)	<i>P</i> *
Age (years)	43.5 (42.9-44.1)	42.9 (42.3-43.5)	42.4 (41.6-43.2)	0.11
BMI (kg/m <sup>2</sup> )	24.6 (23.5-25.6)	25.8 (24.7-26.9)	26.7 (25.3-28.2)	0.048
Family history of breast cancer (%)	11.0	17.1	15.7	0.52
Age at menarche before 13 (%)	60.0	51.8	67.3	0.19
Number of children (%)			17.3	
none	23.5	34.9	17.3	
one to two	57.7	44.6	51.9	0.10
three or more	18.8	20.5	30.9	
Estrogen levels in serum (means of multiple measurements over 2 years)				
$E_1$ (pg/mL)	84.7 (77.5-92.7)	86.6 (79.0-95.0)	91.5 (81.7-102.6)	0.56
$E_2$ (pg/mL)	129.0 (118.1-140.9)	135.1 (123.5-147.9)	141.4 (126.3-158.2)	0.58
Urinary estrogens and their metabolites (measured once at baseline)				
$E_1$ (ng/mg creatinine)	8.7 (7.2-10.4)	9.4 (7.8-1.2)	9.1 (7.2-11.4)	0.83
$E_2$ (ng/mg creatinine)	3.7 (3.2-4.3)	3.6 (3.1-4.2)	3.7 (3.1-4.4)	0.97
2-OHE <sub>1</sub> (ng/mg creatinine)	20.2 (17.2-23.7)	27.1 (23.2-31.7)	18.6 (15.3-22.6)	0.006
16 $\alpha$ -OHE <sub>1</sub> (ng/mg creatinine)	15.0 (13.0-17.4)	14.4 (12.5-16.5)	12.9 (10.8-15.4)	0.42
2-OHE <sub>1</sub> /16 $\alpha$ -OHE <sub>1</sub> ratio	1.35 (1.19-1.54)	1.89 (1.66-2.15)	1.51 (1.28-1.78)	0.008
Variant allele frequencies				
<i>COMT</i> <sup>158</sup> Met	0.25	0.57	0.22	<0.0001
<i>CYP1A1*2A</i>	0.44	0.13	0.41	<0.0001
<i>CYP1A1*2B</i>	0.30	0.07	0.23	<0.0001
<i>CYP1A2*1F</i>	0.27	0.22	0.27	0.07
<i>CYP1B1</i> <sup>432</sup> Leu	0.19	0.46	0.33	<0.0001
<i>CYP17</i> 27C	0.48	0.35	0.39	0.10

NOTE: The values are means for age and geometric means [95% confidence intervals (CI)] for other variables, except when indicated.

\**P* values from ANOVA for continuous variables and from  $\chi^2$  test for categorical variables.

significantly higher 2-OHE<sub>1</sub> excretion in Caucasian women. Adjustment for BMI, age, time of blood draw, intervention, and other variables under study did not change these findings.

**Correlations between serum and urinary estrogens and their metabolites.** We observed a positive moderate correlation between E<sub>1</sub> and E<sub>2</sub> in serum ( $r = 0.64$ ) and E<sub>1</sub> and E<sub>2</sub> in urine ( $r = 0.81$ ), and between urinary estrogens and 2-OHE<sub>1</sub> and 16 $\alpha$ -OHE<sub>1</sub> ( $r$  ranged from 0.48 to 0.65). Correlations between serum and urinary E<sub>1</sub> and E<sub>2</sub> were low ( $r = 0.26$  and 0.22, respectively). No correlations between serum estrogens and urinary 2-OHE<sub>1</sub> and 16 $\alpha$ -OHE<sub>1</sub> were found.

**Relationships among covariates.** We did not find any significant associations between serum E<sub>1</sub> and E<sub>2</sub> and all covariates under study, except for an inverse association of serum E<sub>1</sub> levels with parity ( $P = 0.03$ ) and of E<sub>2</sub> levels with BMI ( $P = 0.049$ ). When stratified by ethnicity, E<sub>2</sub> levels in Asian subjects were inversely associated with age ( $P = 0.02$ ). Urinary 2-OHE<sub>1</sub> also showed a significant inverse association with parity ( $P = 0.03$ ) and BMI ( $P = 0.05$ ), and a direct association with ethanol consumption ( $P = 0.04$ ). CYP17 variant allele carriers had significantly younger age at menarche ( $P$  for trend = 0.02). This association remained significant after adjusting for BMI, but was no longer significant after adjusting for ethnicity ( $P = 0.056$ ). When examined by ethnic group, this association between CYP17 and age at menarche was statistically significant in Caucasian women only ( $P = 0.02$ ).

**Polymorphisms and serum E<sub>1</sub> and E<sub>2</sub>.** We did not find any significant associations between the genotypes under study and serum E<sub>1</sub> levels before (Table 2) and after (Table 3) adjusting for covariates. CYP1A2\*1F was the only polymorphism associated with serum E<sub>2</sub> levels (Table 2). This inverse association remained significant after adjusting for ethnicity and other covariates (Table 3). Further inclusion into the mixed model of the study group assignment, time of the blood collection, total daily calories, total daily calories from fat, daily consumption of

**Table 2. Unadjusted geometric mean (95% CI) serum E<sub>1</sub> and E<sub>2</sub> measured over a 2-year period by genotype**

Genotype	<i>n</i>	E <sub>1</sub> (pg/mL)		E <sub>2</sub> (pg/mL)	
		Mean (95% CI)	<i>P</i>	Mean (95% CI)	<i>P</i>
<i>COMT</i>					
Val/Val	84	80.4 (74-87.3)		123.7 (114.1-134.1)	
Val/Met	83	86.8 (79.9-94.3)	0.42*	131.3 (121.1-142.2)	0.59*
Met/Met	31	81.6 (71.3-93.5)	0.56 <sup>†</sup>	126.3 (110.7-144.1)	0.57 <sup>†</sup>
<i>CYP1A1*2A</i>					
m1/m1	100	81.1 (75.2-87.5)		124.7 (115.8-134.2)	
m1/m2	71	84.5 (77.2-92.4)	0.53*	128.5 (117.8-140.3)	0.74*
m2/m2	26	88.8 (76.5-103.1)	0.26 <sup>†</sup>	132.2 (114.3-153.0)	0.43 <sup>†</sup>
<i>CYP1A1*2B</i>					
AA	130	80.6 (75.4-86.1)		124.2 (116.5-132.5)	
AG	58	88.4 (80.1-97.6)	0.26*	133.4 (121.1-146.9)	0.46*
GG	10	89.3 (70.4-113.4)	0.12 <sup>†</sup>	131.6 (104.1-166.3)	0.26 <sup>†</sup>
<i>CYP1A2*1F</i>					
AA	110	82.1 (76.4-88.1)		126.4 (118.0-135.3)	
AC	76	86.9 (79.7-94.8)	0.25*	133.9 (123.3-145.4)	0.02*
CC	12	72.1 (58.0-89.7)	0.97 <sup>†</sup>	97.3 (79.0-119.8)	0.45 <sup>†</sup>
AA+AC	186	84.0 (67.5-104.5)	0.18 <sup>‡</sup>	129.4 (122.7-136.4)	0.01 <sup>‡</sup>
<i>CYP1B1</i>					
Val/Val	91	80.3 (74.2-86.9)		131.2 (121.5-141.7)	
Val/Leu	86	85.2 (78.5-92.4)	0.45*	125.4 (115.9-135.7)	0.44*
Leu/Leu	21	88.3 (75.0-104.10)	0.21 <sup>†</sup>	117.9 (100.8-137.9)	0.21 <sup>†</sup>
<i>CYP17</i>					
TT	70	87.2 (79.7-95.5)		128.3 (117.5-140.1)	
TC	96	81.9 (75.8-88.4)	0.41*	131.1 (121.7-141.2)	0.18*
CC	32	79.1 (69.3-90.3)	0.19 <sup>†</sup>	114.1 (100.3-129.8)	0.25 <sup>†</sup>

\**P* global test for comparison of means.

<sup>†</sup>*P* for gene dosage effect.

<sup>‡</sup>*P* for testing a recessive genetic model.

**Table 3. Geometric mean (95% CI) serum E<sub>1</sub> and E<sub>2</sub> measured over a 2-year period, adjusted in a mixed model for ethnicity, age, BMI, age at menarche, and parity by genotype**

Genotype	<i>n</i>	E <sub>1</sub> (pg/mL)		E <sub>2</sub> (pg/mL)	
		Mean (95% CI)	<i>P</i>	Mean (95% CI)	<i>P</i>
<i>COMT</i>					
Val/Val	84	80.1 (73.6-87.3)		123.9 (114.0-134.7)	
Val/Met	83	88.0 (80.9-95.9)	0.32*	133.2 (122.6-144.7)	0.49*
Met/Met	31	84.3 (72.7-97.8)	0.34 <sup>†</sup>	128.4 (111.1-148.4)	0.47 <sup>†</sup>
<i>CYP1A1*2A</i>					
m1/m1	100	81.7 (75.3-88.5)		124.7 (115.3-134.9)	
m1/m2	71	85.4 (77.6-93.8)	0.62*	131.4 (119.9-144.1)	0.66*
m2/m2	26	88.6 (76.1-103.2)	0.33 <sup>†</sup>	132.0 (113.8-153.1)	0.41 <sup>†</sup>
<i>CYP1A1*2B</i>					
AA	130	81.0 (75.6-86.8)		125.1 (116.9-133.8)	
AG	58	89.6 (81.0-99.2)	0.27*	134.9 (122.2-149.0)	0.47*
GG	10	88.0 (69.0-112.3)	0.15 <sup>†</sup>	130.6 (102.8-165.9)	0.30 <sup>†</sup>
<i>CYP1A2*1F</i>					
AA	110	82.6 (76.8-88.9)		128.3 (119.6-137.5)	
AC	76	88.6 (81.1-96.9)	0.14*	135.4 (124.3-147.5)	0.01*
CC	12	70.7 (56.9-87.8)	0.95 <sup>†</sup>	95.2 (77.3-117.2)	0.31 <sup>†</sup>
AA + AC	186	84.0 (79.5-88.7)	0.16 <sup>‡</sup>	129.2 (122.6-136.2)	0.01 <sup>‡</sup>
<i>CYP1B1</i>					
Val/Val	91	81.2 (74.4-88.2)		133.5 (123.1-144.8)	
Val/Leu	86	85.5 (78.7-92.8)	0.53*	124.4 (114.9-134.7)	0.42*
Leu/Leu	21	89.4 (75.3-106.3)	0.26 <sup>†</sup>	122.4 (103.5-144.8)	0.22 <sup>†</sup>
<i>CYP17</i>					
TT	70	87.2 (79.7-95.5)		128.3 (117.5-140.2)	
TC	96	84.0 (77.6-91.0)	0.31*	132.2 (122.3-142.8)	0.33*
CC	32	76.5 (66.7-87.9)	0.14 <sup>†</sup>	117.4 (102.6-134.3)	0.46 <sup>†</sup>

\**P* global test for comparison of means.

<sup>†</sup>*P* for gene dosage effect.

<sup>‡</sup>*P* for testing a recessive genetic model.

fat, saturated fat, alcohol, and caffeine did not affect these relationships. Subjects with the CYP1A2 CC genotype had mean serum E<sub>2</sub> levels that were 25.8% lower than subjects with the AA genotype and 29.7% lower than subjects with the AC genotype ( $P$  for pair-wise comparison = 0.01 and 0.003, respectively). Moreover, homozygous variant allele carriers had 26.3% lower mean serum E<sub>2</sub> levels than homo- and heterozygous common allele carriers combined ( $P$  for recessive model = 0.01). When stratified by ethnicity, lower mean E<sub>2</sub> levels were observed in subjects with the CC genotype in all three ethnic groups, reaching statistical significance in Caucasians (Table 4).

**Polymorphisms and Urinary E<sub>1</sub> and E<sub>2</sub>.** We did not observe any significant associations between single urinary E<sub>1</sub> and E<sub>2</sub> measurements and any of the polymorphisms under study (data not shown).

**Polymorphisms and urinary 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub>.** Tables 5 and 6 show the associations between the polymorphisms under study and urinary levels of 2-OHE<sub>1</sub> and 16 $\alpha$ -OHE<sub>1</sub>, as well as their ratio, before and after adjustment for covariates. A significantly lower mean ratio was observed in women with the CYP1A2 AC genotype compared with women with the AA or CC genotype ( $P$  for heterogeneity = 0.003), resulting from a higher 16 $\alpha$ -OHE<sub>1</sub> excretion ( $P$  for heterogeneity = 0.007). Moreover, prior to adjustment for covariates, the mean 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio was greater (1.87 versus 1.53) for homozygous COMT Met allele carriers compared with women with the Val/Val and Val/Met genotypes combined ( $P = 0.07$ ), due to significantly higher levels of 2-OHE<sub>1</sub> ( $P$  for trend = 0.008;  $P$  for a recessive model = 0.004). However, this association was no longer significant after adjustment other covariates ( $P = 0.11$ ).

## Discussion

In this study, we found an association between CYP1A2\*1F and both serum E<sub>2</sub> levels and the urinary 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub>

**Table 4. Geometric mean (95% CI) serum E<sub>2</sub> before and after adjustment in mixed models for age, BMI, age at menarche, and parity by ethnicity and CYP1A2\*1F genotype**

CYP1A2*1F	n	E <sub>2</sub> (pg/mL) unadjusted		E <sub>2</sub> (pg/mL) adjusted	
		Mean (95% CI)	P	Mean (95% CI)	P
<b>Asian</b>					
AA	38	132.8 (119.3-147.8)		132.6 (119.4-147.4)	
AC	37	123.1 (110.2-137.6)	0.01*	124.5 (11.6-139.0)	0.13*
CC	3	87.1 (59.4-127.6)	0.10†	88.5 (60.3-130.0)	0.11†
AA + AC	75	127.9 (118.4-138.1)	0.06‡	128.6 (119.3-138.6)	0.06‡
<b>Caucasian</b>					
AA	47	116.5 (106.0-128.1)		117.1b (106.4-128.9)	
AC	28	144.4 (127.2-163.9)	0.008*	143.6 (126.3-163.4)	0.009*
CC	3	87.7 (60.3-127.5)	0.025†	85.3 (58.2-124.9)	0.35†
AA + AC	75	137.7 (121.6-155.9)	0.04‡	136.7 (120.4-155.2)	0.06‡
<b>Mixed</b>					
AA	29	133.9 (115.1-155.8)		136.8 (117.2-159.7)	
AC	14	141.9 (115.5-174.3)	0.79*	140.9 (114.4-173.5)	0.64*
CC	6	125.1 (91.6-170.9)	0.95†	117.7 (85.8-161.4)	0.62†
AA + CC	43	137.5 (115.9-163.1)	0.80‡	134.5 (113.1-160.1)	0.92‡

\*P global test for comparison of means.

†P for gene dosage effect.

‡P for testing a recessive genetic model.

ratio, providing evidence for the role of this polymorphism in the regulation of circulating E<sub>2</sub> levels and its metabolism. No significant association was observed between CYP1A2\*1F and serum E<sub>1</sub> or urinary E<sub>1</sub> and E<sub>2</sub> levels. Our study also found no convincing evidence for an effect of the other polymorphisms on serum E<sub>1</sub> and E<sub>2</sub> levels, their excretion in urine, or the urinary 2-OHE<sub>1</sub>/16α-OHE<sub>1</sub> ratio. An effect of the COMT Val<sup>158</sup>Met polymorphism on urinary 2-OHE<sub>1</sub> excretion was observed, but this association was no longer present after adjustment for ethnicity.

CYP1A2 is thought to be the most important enzyme in the 2-hydroxylation of E<sub>1</sub> and E<sub>2</sub> (49). This enzyme is known to be

inducible, and previous studies have shown variation in activity level by sex, age, race, smoking status, coffee and alcohol consumption, and exposure to various combustion products and contaminants [e.g., dioxin; reviewed in ref. 50]. In our study, we did not have an opportunity to investigate the effect of smoking on the relationship between CYP1A2\*1F and serum E<sub>2</sub> as few women were smokers; however, alcohol and coffee consumption did not influence this relationship. Our finding of lower estradiol levels in carriers of CYP1A2\*1F polymorphism is consistent with the lower mammographic density in subjects with this genotype, reported in recent study by our group (36). We are not aware of any other studies of this polymorphism in relation to circulating estrogen levels or the urinary 2-OHE<sub>1</sub>/16α-OHE<sub>1</sub> ratio.

COMT catalyzes the conjugation of catecholestrogens, resulting in their conversion into monomethylethers. Studies have shown that individuals with the Met/Met genotype have 2- to 3-fold decreased activity of this enzyme (51), and that this phenotype is inherited as an autosomal recessive trait (52). Increased mean levels of 2-OHE<sub>1</sub> in homozygous COMT Met allele carriers in our study are in agreement with both of these findings and, also, confirm the results obtained by Tworoger et al. (32) in postmenopausal women. However, in our study, the association was not significant after adjustment for ethnicity.

Higher serum levels of E<sub>2</sub> measured during the luteal phase of the menstrual cycle were found in CYP17 A27C allele carriers by Feigelson et al. (26). Other studies of premenopausal women failed to produce evidence to support this finding (29). Consistent with these data, we found no association between the CYP17 27C allele and either serum or urine estrogens or the urinary 2-OHE<sub>1</sub>/16α-OHE<sub>1</sub> ratio. The only previous study that investigated estrogens and polymorphisms in premenopausal women other than CYP17 observed an association between the CYP1B1 <sup>432</sup>Leu allele and increased serum luteal E<sub>2</sub> levels (29). In contrast, we found no association with this genetic

**Table 5. Unadjusted geometric mean (95% CI) urinary estrogen metabolites (measured at baseline by genotype)**

Genotype	n	2-OHE/16-OHE Ratio		2-OHE <sub>1</sub> (ng/mg creatinine)		16α-OHE <sub>1</sub> (ng/mg creatinine)	
		Mean (95% CI)	P	Mean (95% CI)	P	Mean (95% CI)	P
<b>COMT</b>							
Val/Val	75	1.50 (1.32-1.71)		20.0 (13.8-29.0)		13.7 (11.9-15.7)	
Val/Met	74	1.55 (1.36-1.76)	0.18*	21.5 (14.8-31.2)	0.01*	14.0 (12.2-16.0)	0.36*
Met/Met	31	1.87 (1.53-2.29)	0.10†	30.6 (21.1-44.4)	0.008†	16.4 (13.2-20.2)	0.22†
Val/Val + Val/Met	149	1.53 (1.39-1.67)	0.07‡	20.7 (16.3-26.3)	0.004‡	13.8 (12.6-15.2)	0.16‡
<b>CYP1A1*2A</b>							
m1/m1	88	1.60 (1.42-1.80)		23.4 (20.3-27.0)		14.6 (13.4-16.0)	
m1/m2	67	1.63 (1.42-1.87)	0.53*	21.8 (18.5-25.7)	0.35*	13.5 (12.4-14.8)	0.71*
m2/m2	24	1.40 (1.12-1.76)	0.47†	18.7 (14.3-24.6)	0.16†	14.5 (13.3-15.9)	0.71†
<b>CYP1A1*2B</b>							
AA	117	1.61 (1.45-1.78)		22.3 (19.7-25.2)		13.8 (12.4-15.4)	
AG	54	1.56 (1.34-1.81)	0.69*	22.2 (18.5-26.6)	0.91*	14.4 (12.3-16.9)	0.35*
GG	9	1.36 (0.94-1.98)	0.44†	20.1 (13.1-30.9)	0.75†	18.7 (12.6-27.7)	0.24†
<b>CYP1A2*1F</b>							
AA	100	1.79 (1.60-1.99)		22.6 (19.8-25.8)		13.0 (11.6-14.6)	
AC	70	1.33 (1.17-1.51)	0.003*	22.4 (19.1-26.2)	0.39*	16.8 (14.7-19.3)	0.004*
CC	10	1.61 (1.14-2.27)	0.008†	16.5 (10.8-25.4)	0.37†	10.3 (7.1-14.8)	0.25†
AC + CC	80	1.36 (1.21-1.53)	0.001§	21.6 (18.6-25.0)	0.65§	15.9 (13.9-18.0)	0.03§
<b>CYP1B1</b>							
Val/Val	79	1.49 (1.32-1.69)		27.7 (19.5-26.4)		15.4 (13.5-17.5)	
Val/Leu	83	1.62 (1.44-1.84)	0.41*	21.3 (18.4-24.7)	0.78*	13.5 (11.8-15.3)	0.31*
Leu/Leu	18	1.78 (1.38-2.30)	0.18†	23.4 (17.1-31.9)	0.37†	13.1 (10.0-17.2)	0.15†
<b>CYP17</b>							
TT	66	1.55 (1.35-1.77)		21.1 (17.9-24.9)		14.1 (12.2-16.3)	
TC	89	1.67 (1.48-1.87)	0.89*	23.3 (20.2-26.9)	0.59*	14.1 (12.5-16.0)	0.33*
CC	25	1.39 (1.12-1.73)	0.70†	20.8 (16.0-27.2)	0.79†	15.0 (11.9-18.9)	0.71†

\*P global test for comparison of means.

†P for gene dosage effect.

‡P for testing a recessive genetic model.

§P for testing a dominant genetic model.

**Table 6. Geometric mean (95% CI) urinary estrogen metabolites (measured at baseline, adjusted in a mixed models for age, ethnicity, BMI, age at menarche, and parity by genotype)**

Genotype	n	2-OHE/16-OHE Ratio		2-OHE <sub>1</sub> (ng/mg creatinine)		16 $\alpha$ -OHE <sub>1</sub> (ng/mg creatinine)	
		Mean (95% CI)	P	Mean (95% CI)	P	Mean (95% CI)	P
<i>COMT</i>							
Val/Val	75	1.58 (1.39-1.80)		20.8 (17.8-24.2)		13.5 (11.7-15.5)	
Val/Met	74	1.55 (1.36-1.76)	0.89*	21.2 (18.2-24.8)	0.27*	13.9 (12.0-16.0)	0.38*
Met/Met	31	1.63 (1.32-2.02)	0.91 <sup>†</sup>	26.5 (20.5-34.3)	0.19 <sup>†</sup>	16.4 (13.0-20.7)	0.23 <sup>†</sup>
Val/Val + Val/Met	149	1.56 (1.43-1.71)	0.69 <sup>‡</sup>	21.0 (18.9-23.4)	0.11 <sup>‡</sup>	13.7 (12.4-15.1)	0.17 <sup>‡</sup>
<i>CYP1A1*2A</i>							
m1/m1	88	1.50 (1.33-1.69)		19.7 (16.4-23.6)		14.6 (12.7-16.7)	
m1/m2	67	1.72 (1.50-1.97)	0.32*	21.1 (17.8-25.0)	0.75*	13.3 (11.4-15.4)	0.65*
m2/m2	24	1.54 (1.23-1.93)	0.47 <sup>†</sup>	19.0 (14.4-25.3)	0.95 <sup>†</sup>	14.4 (11.2-18.6)	0.71 <sup>†</sup>
<i>CYP1A1*2B</i>							
AA	117	1.55 (1.40-1.72)		21.1 (18.6-23.9)		13.6 (12.2-15.3)	
AG	54	1.65 (1.42-1.92)	0.73*	23.3 (19.5-27.9)	0.68*	14.3 (12.1-16.9)	0.33*
GG	9	1.47 (1.01-2.13)	0.78 <sup>†</sup>	21.6 (14.1-33.1)	0.53 <sup>†</sup>	18.9 (12.5-28.7)	0.23 <sup>†</sup>
<i>CYP1A2*1F</i>							
AA	100	1.76 (1.59-1.96)		22.1 (19.4-25.2)		12.9 (11.5-14.5)	
AC	70	1.32 (1.17-1.50)	0.003*	22.0 (18.7-25.8)	0.63*	16.7 (14.5-19.3)	0.007*
CC	10	1.71 (1.23-2.39)	0.02 <sup>†</sup>	17.8 (11.8-27.1)	0.52 <sup>†</sup>	10.3 (7.1-15.0)	0.25 <sup>†</sup>
AC + CC	80	1.37 (1.22-1.54)	0.002 <sup>§</sup>	21.4 (18.4-24.8)	0.73 <sup>§</sup>	15.7 (13.7-17.9)	0.03 <sup>§</sup>
<i>CYP1B1</i>							
Val/Val	79	1.54 (1.36-1.74)		23.0 (19.8-26.8)		15.1 (13.1-17.3)	
Val/Leu	83	1.60 (1.42-1.80)	0.87*	20.9 (18.1-24.2)	0.64*	13.4 (11.8-15.3)	0.39*
Leu/Leu	18	1.64 (1.26-2.13)	0.61 <sup>†</sup>	20.5 (14.9-28.3)	0.37 <sup>†</sup>	12.7 (9.5-17.0)	0.18 <sup>†</sup>
<i>CYP17</i>							
TT	66	1.51 (1.32-1.72)		20.5 (17.4-24.0)		14.0 (12.1-16.3)	
TC	89	1.76 (1.49-1.88)	0.37*	22.9 (19.8-26.4)	0.59*	13.8 (12.1-15.7)	0.80*
CC	25	1.45 (1.17-1.79)	0.88 <sup>†</sup>	21.8 (16.8-28.3)	0.48 <sup>†</sup>	15.1 (11.9-19.2)	0.73 <sup>†</sup>

\*P global test for comparison of means.

<sup>†</sup>P for gene dosage effect.<sup>‡</sup>P for testing a recessive genetic model.<sup>§</sup>P for testing a dominant genetic model.

variant. This inconsistency might be explained by differences in study populations: our subjects were significantly older and more ethnically diverse.

Most importantly, our study used repeated measures of E<sub>1</sub> and E<sub>2</sub> levels over a 2-year period, in contrast to the one-time assessment universally used in past studies. Multiple measurements are likely to greatly increase the reliability of the hormone measurements, and, thus, the statistical power, by decreasing the intraindividual variation in serum estrogen levels, which is known to be substantial (38). The fact that ovulation was confirmed by progesterone measurements and that sampling was narrowly timed in the luteal phase of the menstrual cycle also adds confidence to the standardization of the hormonal assessment. Although the urinary 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio was only measured once in our study, the within-person variability of this ratio over a 2-month period in Caucasian women has been shown to be moderate, so that a single urine sample is an adequate predictor of long-term levels (39). The data by Westerlind et al. (53) also suggested that a first-morning void (and thus, an overnight sample as in our study) is representative of a 24-hour collection and that the 2-OHE<sub>1</sub>/16 $\alpha$ -OHE<sub>1</sub> ratio is relatively constant throughout a 24-hour period.

Another strength of the present study is that we only included women who had regular menstrual cycles and who were not taking hormonal contraceptives. We also carefully collected data on reproductive history and nutrition to adjust estrogen levels for potential confounders. Moreover, the precision of the hormonal measurements was monitored using internal standards and the laboratory reproducibility of these measurements was satisfactory. Finally, the genotype distributions in our study were in agreement with previous reports (54), suggesting that our sample is representative of the general population. The main limitation of our study was that the sample size was not large enough to test for gene-gene and gene-environment interactions.

A life-long exposure to modestly increased estrogen levels could produce a substantial cumulative effect on disease risk. For example, a model proposed by Pike et al. (55) showed that a 20% difference in circulating estrogen may result in a >2-fold increase in lifetime breast cancer risk. We found that carriers of the *CYP1A2* CC genotype had a consistently 26.3% lower estradiol levels. If these levels measured during a 2-year period were representative of lifetime patterns, these women may be at 50% lower risk of breast cancer. The present study suggests that the *CYP1A2\*1F* allele should be studied in relation to breast cancer susceptibility.

## Acknowledgments

The authors are grateful for the cooperation and enthusiasm of the participants in the Breast, Estrogens and Nutrition (BEAN) study. We thank Elizabeth Gentzschlein and Sabina Rinaldi for performing the hormone assays, and Yvonne Tatsumura for help with genotyping; and Drs. Lynne Wilkens and Ian Pagano for their advice on the statistical analysis.

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