Published in final edited form as:
Cancer Epidemiol Biomarkers Prev. 2009 October ; 18(10): 2751–2760. doi: 10.1158/1055-9965.EPI-09-0388.

Soy intake is associated with increased 2-hydroxylation and decreased 16α-hydroxylation of estrogens in Asian-American women

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Abstract

Introduction—In Asian women, soy consumption is associated with reduced breast cancer risk, perhaps due to effects on estrogen production or metabolism. In a sample of Asian-American women, we investigated associations of usual adult soy intake with urinary concentrations of 15 estrogens and estrogen metabolites (EM) measured using liquid chromatography-tandem mass spectrometry.

Methods—Participants included 430 Chinese-, Japanese-, and Filipino-American women, aged 20–55 years, and living in San Francisco-Oakland (CA), Los Angeles (CA) or Oahu (HI). They were postmenopausal (n=167) or premenopausal in luteal phase (n=263) when they collected 12-hour urines. Robust linear regression was used to assess soy tertiles as predictors of log-transformed EM measures. Individual and grouped EM were considered as concentrations (pmol/mg creatinine) and as percentages of total EM (%EM).

Results—Factor analysis confirmed that EM groups defined by metabolic pathways appropriately captured covariation in EM profiles. Total EM concentrations (pmol/mg creatinine) were not significantly associated with soy in pre- or postmenopausal women. Among all women, %2-hydroxylated EM and %4-hydroxylated EM were 16.3% higher (p_{trend}= 0.02) and 18.6% higher (p_{trend}= 0.03) in highest vs. lowest soy tertiles. In contrast, %16-hydroxylated EM were 10.6% lower (p_{trend}< 0.01). Results were consistent across ethnic and menopausal groups and after adjustment for Westernization measured by birthplace (Asia or U.S.).

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Discussion—Findings suggest that regular soy intake is associated with increased ratios of 2:16-pathway EM and with higher relative levels of 4-hydroxylated EM. Observed variations in estrogen metabolism may modify breast cancer risk.

Keywords

soy; isoflavones; estrogens; estrogen metabolite; urine

INTRODUCTION

Estrogens play important roles in the pathophysiology of breast tumors and are also recognized as causal factors in the etiology of this disease (1,2). It has long been known that estrogens exert mitogenic effects on cells in the breast via receptor-mediated signaling (3). More recently it has been recognized that some estrogen metabolites can be converted into reactive oxidative species which can damage DNA directly (4). Thus estrogens and estrogen metabolites (jointly referred to as EM) may contribute to cancer risk by acting as cancer-promoting growth factors, or as cancer-initiating mutagens. While prospective studies have demonstrated consistent associations between circulating estrogen levels and risk of subsequent breast cancer in postmenopausal women, the relative importance of proposed pathways in breast carcinogenesis is not yet understood.

Bradlow et al. suggested that variability among women in susceptibility to breast cancer may result from inter-individual variations in estrogen metabolism (5). Metabolism of estrogens occurs in the liver and kidneys as well as in breast and other target tissues, and includes oxidative metabolism (hydroxylation) and conjugative metabolism (glucuronidation, sulfation, glutathionation and/or O-methylation) (6). Hydroxylation of estrone and estradiol most commonly occurs at ring carbon positions 2, 4, or 16 to yield 2-hydroxylated EM, 4-hydroxylated EM, or 16-hydroxylated EM, respectively (7). EM may vary in binding affinity with estrogen receptors, susceptibility to oxidative conversion into reactive species, susceptibility to conjugative metabolism, and bioavailability in target tissues. Therefore EM are likely to vary with respect to specific roles in cancer etiology or cancer prevention.

Soy foods are a dietary staple in East Asian countries and may play a role in this region's historically low breast cancer incidence. Epidemiologic studies conducted to date among Asian women have found weak to moderate reductions in breast cancer risk associated with soy intake in adulthood (8,9). In three studies which included measures of childhood and/or adolescent soy intake, results suggest stronger protective effects of soy consumed early in life (10,11, 12). Isoflavones found in soy and soy-derived foods have structural similarities to estrogens and have been observed to have both estrogenic and anti-estrogenic effects in animal models and in humans (13,14). Health effects of isoflavones may be mediated through modulation of estrogen receptor signaling (15) but could also be mediated through their effects on enzymes with roles in production and/or metabolism of estrogens (16).

Various studies have tried to assess the effects of soy intake on endogenous levels of estrogens and/or estrogen metabolites, including observational studies (17,18,19), short-term feeding studies (20,21,22,23) and randomized controlled trials (24). However, the studies have produced conflicting results.

We therefore decided to study the association between usual adult soy intake and urinary concentrations of 15 EM among Asian American women. Study participants are of Chinese, Japanese and Filipino ancestry and were living in San Francisco-Oakland CA, Los Angeles CA, and Oahu HI when they were enrolled as controls in the population-based Asian American Breast Cancer Study. In that study we observed statistically significant inverse trends in breast

cancer risk across soy tertiles: risk was 58% lower and 29% lower, respectively, in women in the highest vs. lowest tertiles of childhood soy intake, and usual adult soy intake (12,25). In the present analysis, we test the hypothesis that adult intake of soy foods is associated with creatinine-adjusted urinary EM concentrations and/or relative proportions of urinary EM.

METHODS

Study population

Subjects for this cross-sectional analysis were drawn from a population-based breast cancer case-control study that has been described in detail previously (26). Briefly, cases in the parent study were Asian-American women of Chinese, Japanese or Filipino ethnicity, living in San Francisco-Oakland, California, Los Angeles, California, or Oahu, Hawaii who were diagnosed with breast cancer at 20–55 years of age during 1983–87. Controls were frequency matched to breast cancer cases by ethnicity, year of birth in 5-year age groups, and study center. These controls were the subjects for the current study. Controls completed in-home interviews which included questions about diet, anthropometry, menstrual and menopausal histories, and residential history. In addition, they were invited to provide overnight urine and/or fasting blood samples.

Of 966 controls in the parent study, 687 (71%) provided urine. Those who participated in the urine collection did not differ significantly from non-participants with respect to age, ethnicity, study center, or birthplace (Asia/West). Additionally, we excluded participants for whom urinary EM or urinary creatinine was not assayed (n=16); participants who reported recent (<6 months) exogenous hormone use (n=83), pregnancy (n=12) or lactation (n=5); and participants with missing data on adult soy intake (n=2).

Because menopausal status is an important determinant of circulating and urinary endogenous estrogen levels and so many of the participants in this study were aged 45–55 years and potentially perimenopausal, we assessed menopausal status carefully using data from multiple sources, including self-reported histories of menopause and surgical procedures from the baseline interview, surgical procedures reported in interviews at the time of urine collection, a postcard mailed by participants to study investigators recording the day of the first menstrual period following urine collection, and levels of follicle stimulating hormone (FSH), progesterone, and estradiol in serum collected on the same day as urine. Decision rules (*detailed in the appendix*) were designed to identify women who were clearly premenopausal (having regular menstrual cycles or evidence of continuing ovulation) and women who were clearly post-menopausal (questionnaire data indicating cessation of menstrual cycles due to natural menopause at least 1 year prior to urine collection or circulating hormone levels indicating cessation of ovarian estrogen production). Postcard data and circulating progesterone levels were used to assess phase of the menstrual cycle. Participants who reported continuing periods were given urine collection appointments that coincided with the mid-luteal phase (days 19–26) of the menstrual cycle. Because Xu et al. found that estrogen metabolism changes over the course of the menstrual cycle (27) premenopausal women thought to be in other phases of the menstrual cycle at the time of urine collection have been excluded from most analyses.

Based on our criteria, of the 569 participants available for analysis 167 women were classified as postmenopausal, 263 were classified as premenopausal in luteal phase, 98 were classified as premenopausal but not in luteal phase, and 41 women were assigned unknown/perimenopausal status due to missing or ambiguous information. Thus we include 430 women, including both premenopausal women in luteal phase, and post-menopausal women in this study. Analyses were conducted separately for the two menopausal groups as well as combined. In sensitivity analyses we also considered whether inclusion of premenopausal women who were not in luteal phase would modify study findings.

Soy measures

During baseline interviews participants were queried about their usual intake of approximately 60 food items/food groups, of which 10–15 were specific to their ethnic group. Frequencies of intake were recorded as times per day, week, month, or year, according to the most convenient time frame for the food item and respondent. Estimates of weekly soy intake for Chinese and Filipino women were based on reported frequency of intake of "a tofu dish made with any fresh, dried or deep-fried tofu product" while estimates for Japanese women are based on frequencies of intake of tofu, natto and miso soup. Miso soup and natto were weighted as having 1/4 and 1/12, respectively, of the soy content of a tofu dish. Serving size was not queried. Participants were sorted into tertiles of soy intake using cut-points generated from the distribution in the entire study sample (n=430). Because soy intake was measured using more food items in Japanese compared to other ethnic groups, and because ethnic groups may differ both in EM profiles and in dietary patterns, all statistical models have been adjusted for ethnicity.

Urine Samples and Laboratory Methods

Participants were instructed to collect 12-hour overnight urine samples using half-gallon containers kept at 4°C on ice or in the refrigerator. Boric acid was added as a preservative (28). After delivery to the study center, urine was mixed and aliquoted into conical tubes and sent to a repository for long-term storage at −80°C.

Aliquots were sent to SAIC (Frederick, MD) where 15 urinary EM were measured, including estrone (E_1), estradiol (E_2), estriol (E_3), 2-hydroxyestrone (2-OHE_1), 2-methoxyestrone (2-$MeOE_1$), 2-hydroxyestradiol (2-OHE_2), 2-methoxyestradiol(2-$MeOE_2$), 2-hydroxyestrone-3-methyl ether (3-$MeOE_1$), 4-hydroxyestrone (4-OHE_1), 4-methoxyestrone (4-$MeOE_1$), 4-methoxyestradiol (4-$MeOE_2$), 16α-hydroxyestrone (16α-OHE_1), 17-epiestriol (17-epiE_3), 16-ketoestradiol (16-ketoE_2), and 16-epiestriol (16-epiE_3). The analytical method for measurement of urinary EM has been described previously (29). In brief, to each urine sample an internal standard solution containing four deuterium-labeled estrogens and estrogen metabolites (d-EM) was added. Urinary EM and d-EM were hydrolyzed using β-glucuronidase/sulfatase from *Helix pomatia*, and then, following a 20-hour incubation, extracted with dichloromethane. EM were quantitatively dansylated to improve their ionization efficiency. The LC-MS^2 analysis was performed using a ThermoFinnigan TSQ™ Quantum-AM triple quadrupole mass spectrometer equipped with an electrospray ionization source and coupled directly to a Surveyor HPLC system (ThermoFinnigan, San Jose, CA). Both the chromatography system and mass spectrometer were controlled using Xcalibur™ software (ThermoFinnigan).

The MS conditions used in this study were as follows: source, positive mode ESI; spray voltage, 4600 V; sheath and auxiliary gas, nitrogen; sheath gas pressure, 49 arbitrary units; auxiliary gas pressure, 23 arbitrary units; ion transfer capillary temperature, 350 °C; scan type, selected reaction monitoring (SRM); collision gas, argon; collision gas pressure, 1.5 mTorr. The SRM conditions for the protonated molecules [MH^+] of EM-Dansyl and d-EM-Dansyl were as follows: E_1 *m/z* 504 → 171 collision energy: 42 eV; E_2 *m/z* 506 → 171 collision energy: 43 eV; E_3, 16-epi E_3, and 17-epi E_3 *m/z* 522 → 171 collision energy: 43 eV; 16-ketoE_2, and 16α-OHE_1 *m/z* 520 → 171 collision energy: 43 eV; 2-$MeOE_1$, 4-$MeOE_1$, and 3-$MeOE_1$ *m/z* 534 → 171 collision energy: 42 eV; 2-$MeOE_2$ and 4-$MeOE_2$ *m/z* 536 → 171 collision energy: 43 eV; 2-OHE_1 and 4-OHE_1 *m/z* 753 → 170 collision energy: 44 eV; 2-OHE_2 *m/z* 755 → 170 collision energy: 43 eV; d_4-E_2 *m/z* 510 → 171 collision energy: 43 eV; d_3-E_3 and d_3-16-epiE_3 *m/z* 525 → 171 collision energy: 43 eV; d_5-2-$MeOE_2$ *m/z* 541 → 171 collision energy: 43 eV; d_5-2-OHE_2 *m/z* 760 → 170 collision energy: 43 eV. The following MS parameters were

used for all experiments: scan width, 0.7 u; scan time, 0.50 s; Q1 peak width, 0.70 u fwhm; Q3 peak width, 0.70 u fwhm.

Calibration curves for the fifteen EM were constructed by plotting EM-dansyl/d-EM-dansyl peak area ratios obtained from calibration standards versus amounts of EM and fitting these data using linear regression with $1/X$ weighting. The amount of each EM in a urine sample was then interpolated using this linear function. Based on structural similarity and retention times, d_4-E_2 was used as the internal standard for E_2 and E_1; d_3-E_3 for E_3, 16-ketoE_2, and 16α-OHE_1; d_3-16-epiE_3 for 16-epiE_3 and 17-epiE_3; d_5-2-$MeOE_2$ for 2-$MeOE_2$, 4-$MeOE_2$, 2-$MeOE_1$, 4-$MeOE_1$, and 3-$MeOE_1$; d_5-2-OHE_2 for 2-OHE_2, 2-OHE_1, and 4-OHE_1.

Quality control samples at three concentrations (0.12, 0.96, and 6.4 ng of each EM/mL) were included in each batch of assays. In addition, urine samples from two premenopausal and two postmenopausal women were used as blinded quality control samples. Four quality control samples, including two from the same subject, were randomly included in every batch of ~40 samples. Total laboratory coefficients of variation (CV) were <10% for all EM except 4-methoxyestradiol (15%), and were ≤4% for estrone, estradiol, and estriol, and ~1% for total EM. In general, laboratory CV's decreased as mean urinary EM concentration increased.

Creatinine levels were obtained to adjust EM levels for differences in urine volume (30).

Statistical methods

Statistical analyses were conducted using SAS v. 9.1 (SAS Institute, Cary, NC). Absolute levels of each urinary EM were expressed in picomoles per mg creatinine. Relative EM levels (% EM) were expressed as a percentage of total EM. Each of these measures was log transformed to better approximate normal distributions.

To assess the impact of soy intake on EM measures, we first fit standard linear regression models and then robust linear regression models (PROC ROBUSTREG with option MM), which are less sensitive to outliers, to each EM measure as a dependent variable. We tested our hypotheses of linear trend across soy tertiles by assigning median frequency of soy intake to each tertile and treating the variable as a continuous covariate. We used regression coefficients associated with each of the second and highest soy tertiles to estimate percent differences based on the formula: $100\ \{\exp(\hat{\beta})-1\}$.

Models of EM and %EM measures were fit separately for pre- and postmenopausal strata and were adjusted for age and ethnicity. In additional models we adjusted, in turn, for body mass index (BMI) and birth place (Asia/West) to assess whether observed associations were independent of other aspects of acculturation. To assess effect modification, we fit models for EM grouped by metabolic pathways and expressed as a percent of total, to sample subgroups defined by menopausal status, ethnicity, birthplace (Asia/U.S.) and BMI. Statistical significance of interactions between soy intake and ethnicity, BMI, and birthplace were evaluated by comparing models with and without interaction terms using a likelihood ratio test.

We used Spearman correlations and factor analysis to evaluate covariation among urinary EM (pmol per mg creatinine), separately in premenopausal-luteal and postmenopausal women. Because factor analysis confirmed that EM cluster according to metabolic pathways described in the literature, EM groups were defined to include EM deriving from specific metabolic events: hydroxylation (at carbon 2, 4, or 16 of the estrogen ring) and methylation. %EM groups were calculated by summing absolute levels of related metabolites and then dividing by total EM; these measures represent relative proportions of total urinary EM in a metabolic pathway.

The composition of EM groups can be inferred from headings in table 2, and are also described in the appendix.

Finally, we calculated ratios of EM which may be associated with breast cancer risk according to various hypotheses of estrogen-mediated carcinogenesis, including the ratio of 2-hydroxylated EM to 16-hydroxylated EM, the ratio of 4-hydroxylated EM to 2-hydroxylated EM, and the ratio of unmethylated to methylated catechols in 2-hydroxylation and 4-hydroxylation pathways.

%EM groups and EM ratios were each log-transformed for use as dependent variables in regression analysis. Regression models to assess associations of soy intake with EM groups and ratios were fit to premenopausal and postmenopausal women combined and were adjusted for menopausal status, age, and ethnicity. In the combined population we evaluated effect modification by other variables including menopausal status, using the approach outlined above.

P-values ≤ 0.05 were considered statistically significant. All tests were two-sided.

RESULTS

Participant characteristics by tertiles of soy intake are presented in table 1 for premenopausal-luteal and postmenopausal women. Soy intake was non-significantly higher in postmenopausal compared to premenopausal-luteal women (p=0.09), with postmenopausal women making up 32.7% of the lowest tertile, but 44% of the highest soy tertile. Soy intake was associated with ethnicity in both pre- and postmenopausal women. Filipino women had the lowest frequency of soy intake (with median intakes of 0.2 and 0.5 servings per week in premenopausal-luteal and postmenopausal women, respectively) and so were over-represented in the lowest soy tertiles. In contrast, the median frequency of soy intake for each menopausal subgroup was approximately 1 serving per week among Chinese and Japanese American women. Soy intake was not significantly associated with age, study center, birthplace, or BMI in either menopausal group.

Median levels and interquartile ranges for absolute concentrations of each EM, in pmol/mg creatinine, and for EM expressed as a proportion of total EM, are presented by menopausal status in table 2. Even within menopausal groups urinary EM concentrations can vary by factors of 10–100. Median EM concentrations are 2–10 times higher in premenopausal-luteal women compared to postmenopausal women. In contrast, interquartile ranges for %EM in pre- and postmenopausal women overlap. Most median %EM differ by less than 2 percentage points between premenopausal-luteal and postmenopausal women. One exception is estriol, which is markedly lower as a percent of total EM in postmenopausal women compared to their premenopausal-luteal counterparts (23.6% vs. 32.2%, respectively).

We tested for associations of urinary concentrations of individual EM with soy intake by modeling each EM measure using robust linear regression and adjusting for age and ethnicity (table 3). No statistically significant associations were seen between soy intake tertiles and urinary concentrations of E_1, E_2, E_3, or total EM in either menopausal group. In postmenopausal women, urinary levels of 2-MeOE_1 were directly associated with soy intake (p_{trend}=0.02). Also among postmenopausal women, urinary levels of 16-ketoE_2 declined significantly across soy tertiles (p_{trend}=0.009). These associations remained statistically significant when birthplace or BMI was added to the model. Among premenopausal-luteal women, no significant associations were observed for absolute levels of any individual EM with soy intake.

In table 3 we also present results of robust linear regression models for individual EM expressed as a percentage of total EM, in a combined sample of premenopausal-luteal, and postmenopausal women. %4-OHE_1, %2-$MeOE_1$, and %2-$MeOE_2$ increased significantly, while %16-keto E_2 and %16-epiE_3 each had statistically significant inverse trends across tertiles of soy intake. Other %EM in these pathways had similar but non-significant associations with soy. This observation led us to examine covariation among EM.

Factor analysis with rotation resulted in extraction of four independent factors in each menopausal group (figure 1). The composition of factors was observed to be similar in premenopausal luteal- and postmenopausal women, in spite of the large differences between these groups in absolute levels of total EM. The predominant factor in both pre- and postmenopausal women, termed 'catechols' and accounting for 46% and 30% of the variance in EM profiles, respectively, included all three catechol estrogens (2-OHE_1, 2-OHE_2, and 4-OHE_1) and some of their five methylated metabolites (2-$MeOE_1$ and 3-$MeOE_1$ in both groups, and 2-$MeOE_2$ in premenopausal women only). The second factor in both groups (termed '16-hydroxylated pathway EM') included all five EM that were initially hydroxylated at the 16-C position (16-keto E_2, 16a-OHE_1, 16-epiE_3, E_3, 17-epiE_3), and accounted for 18% and 16%, respectively, of the variance in EM profiles in the two menopausal groups.

The next factor, termed 'parent estrogens', was the third factor in premenopausal women, accounting for 7% of variation in EM profiles, and the fourth in postmenopausal women, accounting for 8% of variation in EM profiles. This factor included high loadings for E_1 and E_2 in both menopausal groups; in postmenopausal women only, two EM from the 16-pathway also contributed in a moderate fashion (E_3 and 16-EpiE_3). A fourth factor termed 'methylated catechols' (4-$MeOE_1$, and 4-$MeOE_2$ in both groups, and 2-$MeOE_2$ in postmenopausal women only) accounted for 6% and 14% of variation in pre- and postmenopausal women, respectively). The fact that methylated catechols in the 4-hydroxylation pathway did not cluster with those in the 2-hydroxylation pathway led us to define groups that examined these metabolites separately.

In table 4 we present results of robust linear regression models, fitted in each menopausal stratum and then in a combined sample, for EM grouped by metabolic pathway and expressed as a percentage of total EM. Patterns were similar in premenopausal-luteal and postmenopausal strata; accordingly there was no statistically significant effect modification of the associations by menopausal status. In the combined sample, %2-hydroxylation pathway EM and %4-pathway EM each significantly increased across increasing tertiles of soy intake; these pathways were 16% higher (p_{trend}= 0.02) and 19% higher (p_{trend}= 0.03), respectively, in the highest compared to the lowest tertile of soy intake. %16-hydroxylated pathway EM were 11% lower in the highest vs. the lowest tertile of soy intake (p_{trend}< 0.01). %Parent estrogens showed no significant trend across soy tertiles. %Methylated catechols, like %catechols, increased significantly across the range of soy intake (p_{trend}< 0.01). Although %catechol estrogens in the 2-hydroxylation and 4-hydroxylation pathways each increased significantly with soy intake, trends in %methylated catechols differed by pathway. Methylated catechols of the 2-pathway increased as a proportion of total EM across soy tertiles (p_{trend}<0.01) while % methylated catechols in the 4-hydroxylation pathway showed no linear trend (p_{trend}=0.80).

Results for ratios of EM were consistent with findings for EM groups. Ratios of 2-pathway EM and 4-pathway EM to 16α-pathway metabolites significantly increased across the range of soy intake (p_{trend}=0.01 for each). Among all women, the ratio of catechols: methylated catechols in the 4-pathway was 20% higher in the highest soy tertile compared to the lowest (p_{trend}=0.11); no trend was noted in the same ratio for the 2-pathway.

Finally we considered several potential confounders (data not shown) of the EM/soy relationships. Birthplace is considered a marker of acculturation and of breast cancer risk (26). BMI may play a causal role in breast cancer etiology and has sometimes been observed to be inversely associated with soy intake (31). Additional adjustment of all models for birthplace and for BMI did not modify the direction or magnitude of observed associations.

Figure 2 shows the percent difference between highest and lowest soy tertiles in relative levels of urinary EM for each stratum defined, in turn, by menopausal status, ethnicity, birthplace, and BMI. Results suggest that associations of %EM groups with soy intake were not significantly modified by these factors. One statistically significant interaction was noted: in women with BMI below the median the %2-hydroxylation pathway showed a significant increasing trend across soy tertiles and was 16.6% higher in the highest vs. lowest soy tertile; among heavier women the %2-hydroxylation pathway also increased with soy intake but the magnitude of the effect was smaller, with a 10% increase seen in the highest vs. lowest tertile (p for interaction = 0.04). This statistically significant finding may reflect the large number of statistical tests performed.

Inclusion of premenopausal women in other phases of the menstrual cycle also did not modify the direction or magnitude of observed associations; however it did result in wider confidence limits for many estimates.

DISCUSSION

In this study of pre- and postmenopausal Asian American women with varying diets, lifestyles and risks of breast cancer, we found no significant trends in absolute levels of estrone, estradiol, or total EM across tertiles of soy intake. This is consistent with the findings of a long-term intervention trial in which 220 premenopausal women were randomized to a soy intervention or control arm; in this study investigators found no differences between groups in circulating estrone, estradiol, or free estradiol at four time points up to 24 months (24). In contrast, in two observational studies, investigators found that high soy intake was associated with reduced serum estrone in postmenopausal Chinese women (18) and reduced serum estradiol in Japanese premenopausal women (17). Evidence from short-term feeding trials (<6 months) has been inconsistent (16,32,33).

While our findings do not support the hypothesis that soy intake influences endogenous production of estrogens, they do suggest that regular soy consumption modifies estrogen metabolism, perhaps though effects on enzymes involved in hydroxylation of estrogens. In our study, women who reported more soy intake had relatively higher levels of 2- and 4-hydroxylated estrogen metabolites and relatively lower levels of 16-hydroxylated estrogen metabolites.

There are more than 10 human cytochrome P450 isoforms capable of hydroxylating estrone and estradiol to produce a wide range of potential metabolites (34). These phase I enzymes vary in distribution across target tissues, catalytic activity, and regiospecificity. The activities of several specific hepatic enzymes likely influence EM profiles; for example, CYP1A2 preferentially hydroxylates estrogens at the second carbon, while CYP3A4 and CYP3A5 have relatively higher activity for production of 16-hydroxylated EM (35). The effects of polymorphic variants in extrahepatic enzymes CYP1A1, which preferentially produces catechol estrogens, and CYP1B1, which selectively produces 4-hydroxylated EM, can also be detected in women's profiles of circulating or excreted estrogen metabolites (36,37). Isoflavones can affect enzyme activity directly through competitive inhibition or indirectly, through interactions with receptors that induce enzyme expression (38). *In vitro* studies suggest that exposure to soy isoflavones can modulate both expression and activity of several of these

enzymes, however the net effects of soy exposure on estrogen metabolism *in vivo* remain unclear (39,40).

Several observational studies have identified dietary or lifestyle components associated with levels of 2-OHE_1, 16α-OHE_1, or with their ratio (41,42). The evidence regarding soy intake and levels of these estrogen metabolites is not conclusive. Of four intervention trials conducted in premenopausal women, two found an increased ratio of 2-hydroxyestrone to 16α-hydroxyestrone during periods of exposure to high levels of soy isoflavones (16,43) while two did not (44,45). A feeding trial conducted with postmenopausal participants (33) showed a decreased ratio of 2-hydroxyestrone: 16α-hydroxyestrone during a period of daily supplementation with 65 mg soy isoflavones in comparison to measures at baseline and during use of the control supplement; but found no significant effect of 135 mg soy isoflavones/day on the same parameter. This same study showed decreased levels of 4-OHE_1 during use of isoflavone supplements at both doses. A more recent trial showed no effects of supplementation with soy flour on the ratio of 2- hydroxyestrone to 16α-hydroxyestrone (46). Two studies found that soy interventions modified the ratio of 2-OHE_1 to 16α-OHE_1 only in a metabolic or ethnic subgroup (22,47).

Methylation is an important pathway for detoxification and excretion of catechol estrogens (48). The enzyme catechol-O-methyl transferase (COMT) is present in liver, kidney, and in breast tissue. Several *in vitro* studies have found that exposure of breast cells to soy isoflavones results in reduced expression and/or activity of *COMT* (49,50). Our findings suggest no significant difference in ratios of catechols: methylated catechols in the 2-hydroxylation pathway, but do show non-significant increases in this ratio for EM in the 4-pathway. Results of studies in human liver cells and in hamster kidney cells suggest that methylation by COMT is more efficient for 2-hydroxylated EM than for 4-hydroxylated EM (51). Methylated 2-hydroxylated EM may actually inhibit methylation of 4-hydroxylated catechols (52). Our findings suggest that soy isoflavones may directly or indirectly, reduce the rate at which 4-hydroxyestrone is methylated.

Our study has several strengths. It includes a large population-based sample of Asian-American women with varying levels of acculturation, corresponding to a wide range of dietary and lifestyle choices, and a 6-fold gradient in breast cancer risk (26). Measures of usual adult soy intake represent long-term exposures at doses that are relevant to levels and patterns of intake in free-living populations. Another major strength of this study is the use of a highly sensitive, specific and reliable assay to measure 15 of the most prevalent estrogens and estrogen metabolites in urine. Many studies on the role of estrogen metabolism in breast cancer risk have used genetic variants as surrogates because these are easy to measure on a large scale, however simulations suggest that genetic variants across metabolic pathways contribute in a complex fashion to levels of estrogen metabolites (53). The EM profile is a phenotypic measure and thus provides a direct way to test hypotheses about the effects of dietary and lifestyle factors on estrogen metabolism and to study the effects of estrogen metabolism on disease risk.

The primary weakness of this observational study is the potential for confounding. We assessed two potential confounders and found that observed associations were independent of birthplace (Asia/West), and of BMI, which are each associated with acculturation and breast cancer risk. Findings were also relatively consistent across ethnic subgroups, and are thus less likely to result from confounding by unmeasured dietary factors, since dietary patterns are somewhat different in each ethnic group. Another study weakness is that assessment of soy intake was based on a limited set of queried soy foods, and on frequency of intake without reference to portion size. Results of a study of Asian American women living in Los Angeles county (54), suggest that queried foods may account for most of the variation in soy intake among Japanese participants, but only half of the variability among Chinese and Filipino participants.

Pooled analyses of prospective studies in postmenopausal women have provided definitive evidence that high levels of circulating estrogens (estrone, estradiol, and free estradiol) are associated with increased breast cancer risk (2). Our data suggests that if soy decreases breast cancer risk, its effects are not mediated by effects on absolute levels of estrogens or total estrogens and estrogen metabolites. Increasing soy intake was associated with increased levels of 2-hydroxylated and 4-hydroxylated metabolites, and reduced levels of 16-hydroxylated metabolites. These variations in estrogen metabolism are consistent with a protective effect of soy on breast cancer risk based upon Bradlow's theory of estrogen-mediated carcinogenesis (5) However, the 'genotoxic metabolites' hypothesis, which posits oxidized products of catechol estrogens as primary causal agents in breast cancer (4) suggests that observed variations in estrogen metabolism could increase risk.

Methylated catechol estrogens have reduced estrogenicity, are readily cleared from circulation, and, unlike the catechol estrogens from which they are derived, are not readily oxidized to form DNA-damaging quinones (6). The metabolite 2-methoxyestradiol has anti-proliferative and anti-angiogenic effects and therefore may play a role as an endogenous anti-cancer agent (55). Thus, observed trends of increasing methylated catechols in the 2-pathway across tertiles of soy intake could be consistent with anti-cancer effects of soy.

Soy may also have effects on breast cancer risk mediated through other causal pathways; for example, through interactions of isoflavones with estrogen receptors. Further study of estrogen metabolism and EM phenotypes in relation to cancer risk will provide more information about the mechanisms by which soy can modify breast cancer risk.

Acknowledgments

This project has been funded in part by the Intramural Research Programs of the NIH, National Cancer Institute, Center for Cancer Research and Division of Cancer Epidemiology and Genetics and with federal funds from the National Cancer Institute, National Institutes of Health, under Contract N01-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organization imply endorsement by the United States Government. We thank David Check of the Biostatistics Branch, DCEG, NCI for assistance with figures.

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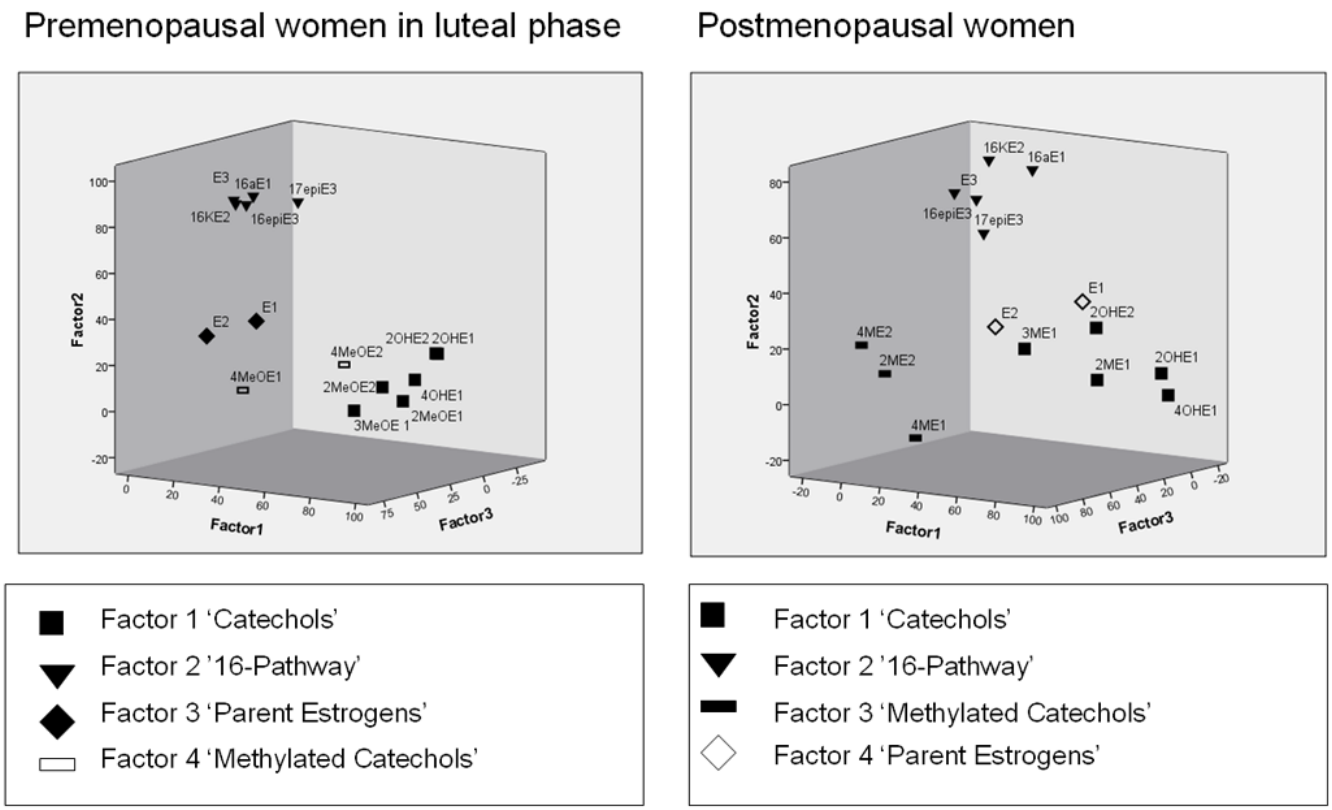

Figure 1. Covariation among urinary estrogens and estrogen metabolites (EM) in Asian American women by menopausal status

Factor analysis with rotation resulted in extraction of four independent factors with eigenvalues ≥ 1 in premenopausal-luteal and postmenopausal Asian American women. Factors were named according to predominant contributing EM. Each EM is graphed by loadings for the three factors accounting for the most variance in urinary EM concentrations. EM represented by solid shapes are statistically significant contributors to factors that correspond with axes shown in the graph, while EM contributing to the fourth extracted factor for each menopausal group (no axis shown) are represented by open shapes.

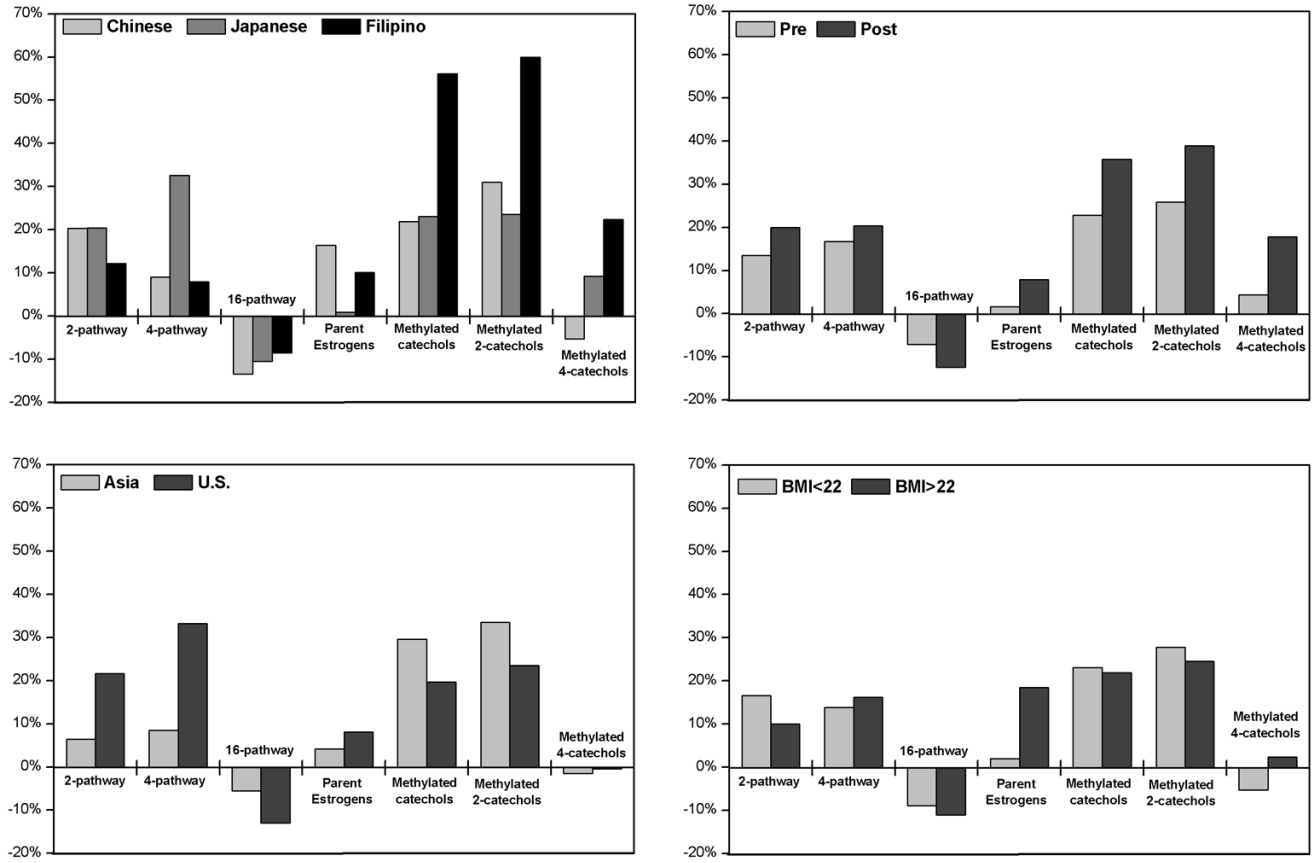

Figure 2. Percent difference between highest and lowest tertiles of soy intake in relative levels of urinary estrogens and estrogen metabolites (% of total) by menopausal status, ethnicity, and BMI

Percent difference was calculated based upon regression coefficients associated with the highest tertile of soy intake in robust regression models fitted to predict log-transformed EM measures, using the formula 100 {exp ($\hat{\beta}$)−1}. For menopause-specific strata, regression models were adjusted for continuous age and ethnicity (Chinese, Japanese, and Filipino); for ethnic-specific strata, regression models were adjusted for continuous age and menopausal status; for birthplace and BMI, models were adjusted for continuous age, ethnicity, and menopausal status. There was no statistically significant effect modification of the associations between soy and grouped %EM by menopausal status, by ethnicity or by birthplace. * p for interaction < 0.05.

Table 1

Participant Characteristics by Menopausal Status and Tertiles of Usual Adult Soy Intake

Characteristic	**Premenopausal women in luteal phase (n=263)**				**Postmenopausal women (n=167)**			
Soy Tertiles*	Low	Medium	High	P for trend/difference	Low	Medium	High	P for trend/difference
Weekly servings of soy	< 0.5	0.5 – 1.0	≥1.1		< 0.5	0.5 – 1.0	≥1.1	
n	109	75	79		53	50	64	
Age (mean ± s.d.)	41.4 ± 4.3	42.4 ± 4.8	42.2 ± 5.4	0.30	54.2 ± 4.2	55.3 ± 3.5	54.3 ± 3.8	0.27
BMI (mean ± s.d.)	20.8 ± 2.4	20.9 ± 2.6	21.2 ± 3.9	0.63	22.4 ± 3.7	22.5 ± 3.0	21.5 ± 2.6	0.15
Ethnicity (%)				<0.001				<0.001
Filipino	40.4	16.0	7.6		56.6	26.0	9.4	
Japanese	33.9	44.0	62.0		22.6	36.0	65.6	
Chinese	25.7	40.0	30.4		20.8	38.0	25.0	
Study Center (%)				0.72				0.66
Hawaii	41.3	48.0	38.0		39.6	42.0	46.9	
Los Angeles	28.4	22.7	26.6		32.1	30.0	20.3	
San Francisco	30.3	29.3	35.4		28.3	28.0	32.8	
Place of Birth (%)				0.19				0.46
In U.S.	55.6	45.3	59.5		54.7	54.0	50.0	
In Asia	44.4	54.7	40.5		45.3	46.0	50.0	

*Cut-points for soy tertiles were determined based upon the distribution of soy intake in the combined sample (n=430) of premenopausal-luteal and postmenopausal women.

**For continuous variables the p-value is for trend and comes from linear regression. For continuous covariates the p-value is for difference and comes from a chi-square test.

Table 2

Absolute and Relative Concentrations of Urinary Estrogens and Estrogen Metabolites (EM) in Premenopausal-luteal (n=263) and Postmenopausal (n=167) Asian-American women.

	EM (picomoles/mg creatinine)				%EM			
	Premenopausal-luteal		Postmenopausal		Premenopausal-luteal		Postmenopausal	
	median	IQR	median	IQR	median	IQR	median	IQR
Parent Estrogens								
Estrone (E_1)	23.9	16.6–35.7	2.8	1.7–4.0	12.7%	10.0–15.8%	11.3%	7.3–15.2%
Estradiol (E_2)	10.4	7.8–15.9	1.6	0.9–2.7	5.9%	4.3–8.1%	6.3%	4.2–10.0%
2-Hydroxylation Pathway EM								
Catechols								
2-hydroxyestrone (2-OHE_1)	26.5	16.2–40.9	3.2	1.8–5.0	14.3%	10.0–19.2%	12.5%	8.3–18.7%
2-hydroxyestradiol (2-OHE_2)	2.8	1.6–4.8	0.6	0.3–1.3	1.5%	0.9–2.3%	2.3%	1.3–4.9%
Methylated Catechols								
2-methoxyestrone (2-$MeOE_1$)	5.2	3.3–7.9	0.7	0.4–1.0	2.7%	1.8–4.0%	2.7%	1.6–3.9%
2-methoxyestradiol (2-$MeOE_2$)	0.5	0.3–0.8	0.1	0.1–0.2	0.3%	0.2–0.4%	0.5%	0.2–0.8%
2-hydroxyestrone-3-methyl ether (3-$MeOE_1$)	0.9	0.7–1.7	0.3	0.2–0.6	0.6%	0.3–0.9%	1.3%	0.7–2.0%
4-Hydroxylation Pathway EM								
Catechols								
4-hydroxyestrone (4-OHE_1)	3.5	2.1–5.3	0.7	0.4–1.0	1.8%	1.2–2.9%	2.8%	1.7–3.9%
Methylated Catechols								
4-methoxyestrone (4-$MeOE_1$)	0.2	0.1–0.4	0.1	0.1–0.2	0.1%	0.1–0.2%	0.5%	0.2–0.8%
4-methoxyestradiol (4-$MeOE_2$)	0.1	0.0–0.2	0.04	0.02–0.09	0.04%	0.02–0.08%	0.2%	0.1–0.4%
16-Hydroxylation Pathway EM								
16α-hydroxyestrone (16α-OHE_1)	11.7	7.3–17.3	1.3	0.8–2.1	5.9%	4.6–8.0%	5.3%	3.7–7.3%
Estriol (E_3)	62.3	36.2–90.7	5.6	3.6–9.4	32.2%	23.6–39.5%	23.6%	16.0–34.0%
17-epiestriol (17-epiE_3)	1.1	0.6–2.4	0.2	0.1–0.3	0.6%	0.3–1.2%	0.6%	0.3–1.3%
16-ketoestradiol (16-ketoE_2)	22.0	14.3–33.6	3.7	2.2–5.4	11.8%	9.1–15.0%	14.3%	10.5–20.3%
16-epiestriol (16-epiE_3)	9.0	5.5–12.3	0.9	0.6–1.3	4.6%	3.6–5.5%	3.6%	2.6–4.6%

	EM (picomoles/mg creatinine)				%EM			
	Premenopausal-luteal		Postmenopausal		Premenopausal-luteal		Postmenopausal	
	median	IQR	median	IQR	median	IQR	median	IQR
Total EM	194.9	138.5–267.9	25.3	17.7–34.8				

IQR interquartile range

Table 3

Percent difference[1] in concentrations of urinary estrogens and estrogen metabolites (EM) among Asian American women in highest vs. lowest tertiles of soy intake, by menopausal status.

EM Measure	Percent Difference[1]					
	EM (pmol/mg creatinine)				EM (% of total)	
	Premenopausal-luteal women (n=263)		Postmenopausal women (n=167)		Combined Premenopausal-luteal and Postmenopausal women (n=430)	
	highest v. lowest tertile	p trend[2]	highest v. lowest tertile	p trend[2]	highest v. lowest tertile	p trend[2]
Parent EM						
Estrone (E_1)	−1.8	0.80	7.5	0.70	7.7	0.21
Estradiol (E_2)	−7.7	0.45	5.9	0.68	0.6	0.94
2-Hydroxylation Pathway EM						
Catechols						
2-hydroxyestrone (2-OHE_1)	1.7	0.93	7.8	0.85	14.7	0.06
2-hydroxyestradiol (2-OHE_2)	−4.1	0.79	14.5	0.51	15.9	0.15
Methylated Catechols						
2-methoxyestrone (2-$MeOE_1$)	14.7	0.31	51.8*	0.03	34.7*	<0.01
2-methoxyestradiol (2-$MeOE_2$)	7.9	0.56	17.3	0.35	27.8*	0.02
2-hydroxyestrone-3-methyl ether (3-$MeOE_1$)	17.6	0.33	14.2	0.53	12.1	0.22
4-Hydroxylation Pathway EM						
Catechols						
4-hydroxyestrone (4-OHE_1)	5.9	0.68	25.2	0.19	22.1*	0.02
Methylated Catechols						
4-methoxyestrone (4-$MeOE_1$)	6.6	0.94	22.0	0.24	10.6	0.37
4-methoxyestradiol (4-$MeOE_2$)	−7.6	0.75	15.6	0.72	6.9	0.64
16-Hydroxylation Pathway EM						
16α-hydroxyestrone (16α-OHE_1)	−7.5	0.33	−19.6	0.17	−6.6	0.28

EM Measure	Percent Difference[1] EM (pmol/mg creatinine)				EM (% of total)	
	Premenopausal-luteal women (n=263)		Postmenopausal women (n=167)		Combined Premenopausal-luteal and Postmenopausal women (n=430)	
	highest v. lowest tertile	p trend[2]	highest v. lowest tertile	p trend[2]	highest v. lowest tertile	p trend[2]
Estriol (E_3)	−7.5	0.30	−2.8	0.98	−5.2	0.36
17-epiestriol (17-epiE_3)	2.6	0.90	−25.0	0.30	−8.4	0.51
16-ketoestradiol (16-ketoE_2)	−10.0	0.26	−29.7*	0.01	−12.1*	0.02
16-epiestriol (16-epiE_3)	−13.0	0.12	−16.6	0.25	−8.4*	0.04
Total EM	−2.3	0.62	−2.3	0.62		

[1] Percent difference = 100 {exp ($\hat{\beta}$)−1} where β is the coefficient from robust linear regression corresponding to women in the highest compared to the lowest tertile of soy on log-transformed EM measures (pmol/mg creatinine or mol% of total EM). For menopause-specific strata, regression models were adjusted for continuous age and ethnicity (Chinese, Japanese, and Filipino); for the combined sample, regression models were also adjusted for menopausal status.

[2] p for linear trend was tested by assigning median frequency of soy intake to each tertile and treating the variable as a continuous covariate.

* p associated with tertile estimate <0.05.

Table 4

Percent difference[1] in urinary EM grouped by metabolic pathway in highest vs. lowest tertiles of soy intake among premenopausal (luteal) and postmenopausal Asian American women.

	Percent Difference[1]					
	Premenopausal-Luteal N=263		Postmenopausal N=167		Combined N=430	
	highest v. lowest tertile	p-trend[2]	highest v. lowest tertile	p-trend[2]	highest v. lowest tertile	p-trend[2]
Grouped EM by Pathway (% of total EM)						
2-Hydroxylation Pathway EM	13.5	0.06	20.0	0.16	16.3*	0.02
4-Hydroxylation Pathway EM	16.7	0.11	20.3	0.15	18.6*	0.03
16-Hydroxylation Pathway EM	−7.1	0.05	−12.5	0.08	−10.6*	<0.01
Parent Estrogens	1.6	0.70	7.9	0.50	6.1	0.27
Catechol Estrogens	10.9	0.10	15.3	0.35	15.0*	0.05
Methylated Catechols	22.8*	0.06	35.8*	0.02	23.9*	0.01
Methylated Catechols of the 2-Hydroxylation Pathway	25.8*	0.04	38.9*	0.02	27.4*	<0.01
Methylated Catechols of the 4-Hydroxylation Pathway	4.3	0.90	17.8	0.41	2.6	0.81
EM Ratios						
4-Pathway/2-Pathway	−1.7	0.95	−1.3	0.98	−1.5	0.86
2-Pathway/16-Hydroxylation Pathway	21.9	0.05	42.0	0.10	31.4*	0.01
4-Pathway/16-Hydroxylation Pathway	24.5	0.08	39.6	0.09	32.7*	0.01
2-Pathway Catechols/Methylated 2-Pathway Catechols	−8.74	0.49	−5.1	0.68	−2.3	0.80
4-Pathway Catechols/Methylated 4-Pathway Catechols	13.89	0.30	16.4	0.52	20.5	0.11

[1] Percent difference = 100 {exp ($\hat{\beta}$)−1} where β is the coefficient from robust linear regression corresponding to women in the highest compared to the lowest tertile of soy on log-transformed EM measures (pmol/mg creatinine or mol% of total EM). For menopause-specific strata, regression models were adjusted for continuous age and ethnicity (Chinese, Japanese, and Filipino); for the combined sample, regression models were also adjusted for menopausal status.

[2] p for linear trend was tested by assigning median frequency of soy intake to each tertile and treating the variable as a continuous covariate.

* p for tertile estimate ≤0.05.