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Serum steroid hormones, sex hormone-binding globulin concentrations, and urinary hydroxylated estrogen metabolites in post-menopausal women in relation to daidzein-metabolizing phenotypes

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Abstract

Equol and *O*-desmethylangolensin (*O*-DMA) are products of bacterial metabolism of daidzein, an isoflavone in soybeans; thus, the presence or absence of equol and/or *O*-DMA in urine is a marker of particular intestinal bacteria profiles. Plasma hormone concentrations may be lower in pre-menopausal women who harbor the bacteria capable of producing equol (equol producers) compared to women who do not (equol non-producers). We evaluated concentrations of serum hormones, sex hormone-binding globulin (SHBG), and urinary 2-hydroxyestrone (2-OH E_1) and 16α-hydroxyestrone (16α-OH E_1) in relation to equol-producer and *O*-DMA-producer phenotypes in 89 post-menopausal women. Follicle stimulating hormone (FSH) was 23% greater in *O*-DMA-producers compared to non-producers ($P = 0.04$). No significant differences in serum estrogens, androgens, metabolic hormones, or SHBG were observed in relation to either daidzein-metabolizing phenotype. Compared with non-producers within each phenotype, age-adjusted 2-OH E_1:16α-OH E_1 was 27% greater ($P = 0.06$) in equol-producers and 9% greater ($P > 0.10$) in *O*-DMA-producers, and 2-OH E_1 concentrations were 24% greater in equol producers ($P = 0.07$) and 42% greater in *O*-DMA producers ($P = 0.02$). No significant differences in 16α-OH E_1 were observed in relation to either phenotype. These results suggest that interindividual variability in intestinal bacteria may be related to differences in products of hormone metabolism in post-menopausal women.

Keywords: Equol; *O*-desmethylangolensin; Steroid hormones; Postmenopause

1. Introduction

Circulating endogenous hormone concentrations, particularly sex hormones, are implicated in the etiology of certain cancers and other diseases arising in hormone-sensitive tissues [1,2]. For example, results from most prospective studies suggest an increased risk for post-menopausal breast cancer with increasing concentrations of plasma estradiol [3]. Post-menopausal breast cancer risk has been inversely associated with plasma sex hormone-binding globulin (SHBG) and leptin and positively associated with plasma estrone, androstenedione, dehydroepiandrosterone (DHEA), dehydroepiandrosterone-sulfate (DHEA-S), testosterone, and insulin-like growth factor binding protein 3 (IGFBP-3) [3–10].

Products of sex hormone metabolism may also be related to disease risk. Two hydroxylated metabolites of estrone are 2-hydroxyestrone (2-OH E_1) and 16α-hydroxyestrone (16α-OH E_1). 2-OH E_1 is believed to be less biologically active than 16α-OH E_1 [11]; thus; it has been hypothesized that preferential metabolism via the 2-hydroxylation pathway would be associated with lower risk of cancers in which sex-hormone exposure is believed to increase risk, such as breast cancer [12,13]. Two studies are known to have evaluated this association prospectively; greater

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2-OH E_1 compared to 16α-OH E_1 was associated with reduced risk of post-menopausal breast cancer in one study [14], but 2-OH E_1:16α-OH E_1 was not associated with post-menopausal breast cancer in another study [15].

Certain distinct intestinal bacteria are capable of metabolizing the soy isoflavone daidzein to equol and/or *O*-desmethylangolensin (*O*-DMA). The presence or absence of these daidzein metabolites in urine can serve as markers of the presence or absence of particular, although not-yet-identified, intestinal bacteria. Humans are considered equol-producers if they harbor bacteria capable of metabolizing daidzein to equol, and *O*-DMA producers if they harbor bacteria that can metabolize daidzein to *O*-DMA. The prevalence of equol producers ranges from about 30–50% of the population [16–21] and the prevalence of *O*-DMA producers is approximately 80–90% [20,22].

Intestinal bacteria are involved in the metabolism of steroid hormones. In vitro, hormone metabolic reactions appear to be species/strain-specific [23], suggesting that circulating hormones may be influenced by the presence of particular intestinal bacteria. In relation to the equol-producer phenotype, results from a study of pre-menopausal women observed that equol producers had lower circulating concentrations of several estrogens and androgens, including estrone and testosterone, compared to equol non-producers [24]. These daidzein-metabolizing phenotypes appear to remain stable within an individual over time [17], suggesting that physiologic effects of these phenotypes could have long-term impact to the human host. The objective of the present study was to evaluate selected serum sex hormone, metabolic hormone and SHBG concentrations and urinary excretion of 2-OH E_1, 16α-OH E_1 and their ratio by daidzein-metabolizing phenotypes in post-menopausal women.

2. Materials and methods

2.1. Subjects and experimental design

Participants were recruited for daidzein-metabolizing phenotyping from women who participated in the Physical Activity for Total Health Study (parent study), which is described in detail elsewhere [25]. This population was chosen to efficiently use previously collected outcome and co-variate data. Briefly, the parent study was a randomized trial designed to examine the effect of a moderate intensity exercise intervention on the sex hormone profile in overweight (BMI $\geq$ 25 or BMI between 24 and 25 if body fat >33%), sedentary (<60 min per week of moderate or strenuous exercise), post-menopausal (having no menstrual periods in prior year or follicle stimulating hormone (FSH) >30 mIU/ml for women who had a previous hysterectomy) women aged 50–75 years. Women were eligible for participation in the parent study if they were non-smokers, moderate or non-drinkers (two or fewer alcoholic drinks per day), physically able to undertake a moderate physical activity program, not using any exogenous hormone medications (currently or in the prior 6 months), free from diabetes mellitus or significant endocrine abnormalities, and free of prevalent cancers. Exclusion criteria for participation in the daidzein-metabolizing phenotyping were known allergy to soy and chronic antibiotic therapy. Reasons for not participating included: unable to locate or did not respond to calls ($n = 20$), consented but did not complete study ($n = 10$), indicated willingness to participate but did not return consent ($n = 8$), lack of interest ($n = 8$), no reason given ($n = 7$), dietary restrictions or food allergies ($n = 4$), and inconvenient ($n = 2$). Women who were receiving acute oral antibiotic therapy at the time of re-contact were scheduled for daidzein-metabolizing phenotyping at least 3 months after antibiotic therapy completion. The Institutional Review Board at the Fred Hutchinson Cancer Research Center (FHCRC) approved all procedures and written, informed consent was obtained from all participants.

2.2. Study procedures

Prior to randomization in the parent study, data regarding demographics and reproductive history were collected via self-administered questionnaire, and urine and blood samples were obtained. A food frequency questionnaire (FFQ) [26] was used to assess dietary intake in the 3 months prior to baseline.

From the parent study, 152 women, who previously indicated willingness to be re-contacted, were approached and 93 (61%) women completed a 3 day soy challenge and collected a 50–80 ml first-void urine sample on the morning of the fourth day to determine daidzein-metabolizing phenotypes. For daidzein-metabolizing phenotyping, women were mailed soy food items and a urine collection kit. For three consecutive days, each woman supplemented her usual diet each day with either a soy bar (Revival Soy, Physicians Laboratories, Winston-Salem, NC, ~83 mg daidzein per day) or one-third of a package of soy nuts (GeniSoy, Fairfield, CA, ~10 mg daidzein per day). Participants chose soy bars or soy nuts. On the morning of the fourth day, each woman collected a first-void urine sample (50–80 ml), and mailed the sample to the FHCRC. There was approximately a 2–3 year time interval between baseline data collection for the parent study and participation in this ancillary study.

2.3. Serum hormone and sex hormone-binding globulin concentrations

At the baseline data collection of the parent study, subjects provided a 50 ml sample of blood, after fasting for at least 12 h. Blood was processed into serum within 1 h of collection. Serum was aliquoted into 1.8 ml tubes and stored at −70 °C. Prior to analysis, a serum quality control (QC) pool was created from ineligible subjects (post-menopausal

and not taking hormone replacement therapy but ineligible for other reasons).

Serum estrone, estradiol, testosterone, androstenedione, and DHEA were quantified by sensitive and specific radioimmunoassays (RIA), following organic solvent extraction and Celite column partition chromatography [27,28]. Chromatographic separation of the steroids was achieved by use of different concentrations of toluene in isooctane and ethyl acetate in isooctane. SHBG, follicle stimulating hormone and DHEA-S were quantified via immunometric assays using the Immulite® Analyzer (Diagnostic Products Corporation, Los Angeles, CA). Concentrations of free estradiol and testosterone were calculated using the measured estradiol and testosterone, respectively, and SHBG concentrations and an assumed constant for albumin [29,30]. Insulin was quantified by a 48 h, PEG-accelerated, double-antibody radioimmunoassay; the primary antibody was guinea pig anti-human insulin and the secondary antibody was goat anti-guinea pig immunoglobulin. IGFBP-3 was quantified by competitive protein-binding radioimmunoassay using the IGFBP-3 100T Kit (Nichols Diagnostics Institute, San Juan Capistrano, CA). IGF-I was quantified via a two-site chemiluminescence immunoassay using the Nichols Advantage Specialty System (Nichols Diagnostic Institute). Leptin assays were performed in the lab of Dr. Weigle at University of Washington Harborview Medical Center using a commercially available radioimmunoassay kit (Linco Research, St. Charles, MO).

Two specimens of the pooled QC sample were placed in each batch. Laboratory personnel were blinded with regard to subject and QC sample identity. Intra-assay coefficients of variation (CV) were <10% for most measurements, except for estrone and estradiol (≤15%). Inter-assay CV were ≤10% for androstenedione, SHBG, FSH, IGF-I and insulin, ≤15% for testosterone, DHEA, DHEA-S, IGFBP-3, and leptin, and ≤20% CV for estrone and estradiol. Intra-individual CV were calculated using data from all the individuals with two baseline blood draws using a random-effects analysis of variance model and taking the square root of the sum of the residual and the individual covariance parameter. The intra-individual CV were 8.3% for leptin, ≤15% for IGF-I, IGFBP3 and testosterone, ≤25% for FSH, SHBG, estradiol, free estradiol and free testosterone, ≤30% for androstenedione and DHEA, and 46.6% for DHEA-S.

2.4. Urinary estrogen metabolites

Clean-catch urine was collected at the same visit as the blood draw in the parent study, and was processed within 1 h of collection. Urine supplemented with Vitamin C (62.5 mg per 25 ml of urine) was used for analysis of 2-OH E_1 and 16α-OH E_1, and non-supplemented urine was used for creatinine analysis. Urinary 2-OH E_1 and 16α-OH E_1 were measured using the commercially available Estramet™ 2/16 enzyme immunoassay (EIA) kits (Immunacare Corporation, Bethlehem, PA), as described previously [31]. All kits were from the same lot number, and upon receipt, all components were stored prior to use as recommended by the manufacturer. The intra- and inter-assay CV were 7.2 and 12.7%, respectively, for 2-hydroxyestrone, and 8.4 and 12.9%, respectively, for 16-hydroxyestrone. All women had acceptable urinary creatinine concentrations (>1 mg/dl).

2.5. Equol-producer and O-DMA-producer phenotypes

Equol-producer and *O*-DMA-producer phenotypes were determined approximately 2–3 years post-intervention. Urine samples, frozen and stored at −70 °C until analysis, were ether-extracted using a variation of the method of Heinonen et al. [32] and analyzed for equol, *O*-DMA and daidzein by gas chromatography–mass spectrometry (GC–MS). 2.0 ml sample aliquots were centrifuged at 2000 rpm for 10 min. A deuterated internal standard was added and the pH was adjusted to 3.0 by adding 200 μl of 1/10 of 1.5 M acetic acid buffer, pH 3.0. Samples were mixed gently and applied to a conditioned SepPak C-18 cartridge. After rinsing each cartridge with 4.0 ml of 0.15 M acetic acid buffer (pH 3.0), the isoflavones were eluted in 3 ml of methanol. The methanol was evaporated to dryness under nitrogen flow, and samples were reconstituted in 5.0 ml of hydrolysis buffer (consisting of ascorbic acid, *Helix pomatia*, and 0.15 M acetic acid buffer, pH 4.1). Samples were then incubated at 37 °C for 16 h. Free aglycones were extracted with anhydrous diethyl ether (3 × 2 ml). The ether was then evaporated under nitrogen and the residues were dissolved in methanol and stored at −20 °C until derivatization. For derivatization, samples were brought to room temperature and the methanol was evaporated under nitrogen. The residues were dissolved in 15% MSTFA in acetonitrile (Pierce, Rockville, IL), and then incubated at room temperature in a vacuum dessicator for 30 min. The column used was a fused silica capillary, 12 m × 0.2 mm × 0.33 μm film thickness (Supelco, St. Louis, MO). The carrier gas was helium with a flow rate of 1 ml/min. The column temperature was as follows: 100 °C for 1 min, ramp at 20 °C/min to 290 °C and held for 5.5 min. The inlet temperature was held constant at 250 °C and the ion source and interface temperatures were 200 and 250 °C, respectively.

Two QC urine samples were included in each batch. The mean intra- and inter-assay CV for equol and *O*-DMA in the quality control urine samples, measured in duplicate in each batch, was <7 and <26%, respectively. Given the sensitivity of the assay, urinary equol concentrations less than 182 nmol/l (44 ng/ml in urine) and urinary *O*-DMA concentrations less than 170 nmol/l (44 ng/ml in urine) were considered below detectable limit. Equol/*O*-DMA-producers were defined as individuals with any detectable concentration of equol/*O*-DMA. All women had detectable concentrations (>170 nmol/l in urine) of daidzein (range: 350–51,100 nmol/l in urine), indicating compliance with soy consumption in all women.

2.6. Statistical analysis

Data from four women were excluded: one because of suspicion of exogenous estrogen use at baseline of the parent study based on circulating hormone concentrations and three because of high CV (>15%) for 2-OH E_1 or 16α-OH E_1 concentration; thus, data from 89 women were included in the statistical analysis. Prior to analysis, natural logarithmic transformations were performed for serum sex hormones (except FSH), SHBG, urinary estrogen metabolites, anthropometric variables, and caffeine and alcohol intake variables. Pearson correlations were used to create a correlation matrix for outcome variables. Age-adjusted linear regression was used to model differences in serum hormone and protein concentrations by individual daidzein-metabolizing phenotypes and combined phenotypes. Combined phenotypes was a four category variable for each combination of equol-producer and *O*-DMA-producer phenotypes. Further multivariate analyses were performed, adjusting for potential precision variables (variables associated with hormones or hormone metabolites), confounding variables such as dietary factors, and for the other phenotype. Percent differences and their standard errors were estimated as the beta-coefficients and standard errors from regression analyses on log-transformed outcomes. Analyses were performed using Stata 8.0 (Stata Corporation, College Station, TX).

3. Results

The prevalence of equol producers was 25% ($n = 22$) and of *O*-DMA producers was 83% ($n = 72$). There appeared to be a relationship between equol-producer and *O*-DMA-producer phenotype classifications: 21 (24%) individuals were equol producers and *O*-DMA producers (equol+/*O*-DMA+), 14 (16%) individuals were equol−/*O*-DMA−, 1 (1%) individual was equol+/*O*-DMA−, and 53 (60%) individuals were equol−/*O*-DMA+. Ninety-five percent of equol producers were *O*-DMA producers, whereas 79% of equol non-producers were *O*-DMA producers.

Reproductive characteristics were similar between producers and non-producers for both daidzein-metabolizing phenotypes (Table 1). Anthropometric characteristics did not differ in relation to the equol-producer or *O*-DMA-producer phenotype (Table 2). Equol producers and non-producers were similar with respect to dietary intake. No differences in macronutrient composition or intakes of fiber, brassica vegetables servings, omega-3 fatty acids, or alcohol were observed, but equol producers consumed 45% more caffeine per day compared to equol non-producers. *O*-DMA producers consumed more energy from carbohydrate and less from fat compared to *O*-DMA non-producers. *O*-DMA producers also appeared to consume more fiber, caffeine and omega-3 fatty acids than non-producers, but these differences were not large. According to the baseline FFQ of the parent study, soy consumption was low in this population; one participant (1%) reported consuming soy milk and 31 women (35%) reported ever eating tofu in the past 3 months. However, only 10 (11%) reported eating tofu twice a week or more frequently. No appreciable difference in the consumption of two or more servings of tofu per week was observed in relation to equol-producer phenotype or *O*-DMA-producer phenotype.

Correlations among serum hormones, SHBG, and urinary estrogen metabolites are presented in Table 3. FSH concentrations were not highly correlated with concentrations of other serum hormones, but were modestly and positively correlated with SHBG and 2-OH E_1. Serum estrogens and androgens were highly and positively correlated with each other. SHBG concentrations were inversely correlated with

Table 1
Reproductive characteristics of 89 post-menopausal women by daidzein-metabolizing phenotypes

	Equol producers *n* (%) ($n = 22$)	Equol non-producers *n* (%) ($n = 67$)	*O*-DMA producers *n* (%) ($n = 74$)	*O*-DMA non-producers *n* (%) ($n = 15$)
Age at first menstrual period <14 years	16 (72)	53 (79)	56 (78)	12 (80)
Regular menstrual periods during most of reproductive life (yes vs. no or sometimes)	19 (86)	54 (86)	59 (82)	12 (80)
Nulligravid	5 (23)	7 (10)	10 (14)	2 (13)
Breastfeeding >1 month	14 (64)	40 (60)	44 (61)	10 (67)
Menopause occurred naturally	18 (82)	54 (81)	58 (81)	13 (87)
Had hysterectomy	3 (14)	13 (19)	14 (19)	1 (7)
Had ovariectomy	3 (14)	4 (6)	5 (7)	2 (13)
Ever used oral contraceptives	15 (68)	41 (61)	47 (65)	8 (53)
Ever used estrogen (non-contraceptive: estrogen or estrogen plus progestin)	12 (55)	28 (42)	32 (44)	7 (46)
Ever used herbal hormones	2 (9)	4 (6)	5 (7)	0 (0)
Family history of breast cancer in first or second degree female relative	6 (28)	18 (27)	21 (29)	3 (20)

No significant ($P < 0.05$) differences between producers and non-producers within each daidzein-metabolizing phenotype were observed.

Table 2
Anthropometric characteristics ($n = 89$) and dietary intake ($n = 87$) of selected nutrients in post-menopausal women by daidzein-metabolizing phenotypes in post-menopausal women[a]

	Equol-producers mean (S.E.) ($n = 22$)	Equol non-producers mean (S.E.) ($n = 65$)	*O*-DMA-producers mean (S.E.) ($n = 72$)	*O*-DMA non-producers mean (S.E.) ($n = 15$)
Anthropometric characteristics				
Height (m)	1.66 (0.06)	1.63 (0.08)	1.64 (0.08)	1.63 (0.05)
Weight (kg)[b]	80 (1.2)	80 (1.2)	80 (1.2)	78 (1.2)
Body mass index (kg/m^2)	30 (4.0)	30 (3.8)	30 (3.9)	31 (4.1)
Nutrient (intake per day)				
Energy (kcal)	1660 (111)	1610 (71)	1620 (66)	1620 (142)
Energy from carbohydrate (%)	48 (1.9)	48 (1.1)	49 (1.1)[d]	42 (1.7)
Energy from fat (%)	34 (2.0)	35 (1.0)	34 (0.9)[d]	41 (1.8)
Energy from protein (%)	18 (0.7)	17 (0.4)	17 (0.4)	17 (0.9)
Fiber (g)	16 (6.6)	16 (6.9)	17 (7.0)[d]	13 (3.5)
Brassica vegetables (servings)	0.4 (0.09)	0.5 (0.05)	0.5 (0.05)	0.4 (0.08)
Omega-3 fatty acids (g)	0.9 (0.06)	0.9 (0.03)	0.9 (0.03)[e]	1.0 (0.07)
Caffeine (mg)[b]	163 (0.2)[d]	89 (0.2)	121 (0.2)[e]	59 (0.5)
Alcohol (g)[b]	2.8 (0.2)	2.5 (0.1)	2.6 (0.1)	2.5 (0.2)

[a] Dietary data incomplete for two women.
[b] Geometric means.
[d] $P < 0.05$, producer relative to non-producer within the same daidzein-metabolizing phenotype.
[e] $P < 0.1$, producer relative to non-producer within the same daidzein-metabolizing phenotype.

Table 3
Correlation matrix of serum hormones and SHBG, and urinary estrogen metabolites in 89 post-menopausal women[a]

	FSH	Estrogens			Androgens and pro-androgens					Urinary estrogen metabolites			SHBG	Metabolic hormones			
		E_1	E_2	Free E_2	T	Free T	A	DHEA	DHEA-S	2-OH E_1	16α-OH E_1	2:16α		Ins	Lep	IGF-I	IGFBP3
FSH	1.00																
E_1	−0.03	1.00															
E_2	0.00	0.80	1.00														
Free E_2	−0.10	0.74	0.92	1.00													
T	0.11	0.51	0.41	0.21	1.00												
Free T	−0.04	0.63	0.54	0.56	0.82	1.00											
A	−0.11	0.39	0.32	0.25	0.52	0.51	1.00										
DHEA	−0.19	0.36	0.21	0.21	0.36	0.41	0.69	1.00									
DHEA-S	−0.15	0.31	0.33	0.33	0.23	0.32	0.43	0.70	1.00								
2-OH E_1	0.33	0.02	−0.03	−0.08	0.09	0.00	−0.03	−0.07	−0.01	1.00							
16α-OH E_1	0.17	0.15	0.11	0.06	0.14	0.14	0.09	0.21	0.22	0.50	1.00						
2:16α	0.20	−0.12	−0.14	−0.15	−0.07	−0.13	−0.12	−0.27	−0.21	0.60	−0.40	1.00					
SHBG	0.34	−0.17	−0.20	−0.55	0.31	−0.24	0.04	−0.06	−0.14	0.19	0.14	0.08	1.00				
Ins	−0.19	0.39	0.42	0.56	−0.04	0.27	−0.03	−0.07	0.05	−0.16	−0.24	0.06	−0.61	1.00			
Lep	−0.07	0.39	0.39	0.51	0.08	0.35	0.16	0.05	0.12	0.02	−0.04	0.05	−0.49	0.57	1.00		
IGF-I	0.07	−0.06	−0.02	0.02	0.05	0.11	0.13	0.12	0.15	0.12	0.16	−0.02	−0.11	0.18	0.34	1.00	
IGFBP3	0.02	−0.08	−0.15	−0.05	−0.16	−0.06	0.14	0.08	−0.02	0.07	0.09	−0.01	−0.21	0.18	0.26	0.52	1.00

[a] FSH: follicle stimulating hormone; E_1: estrone, E_2: estradiol; T: testosterone; A: androstenedione; DHEA: dehydroepiandrosterone; DHEA-S: dehydroepiandrosterone-sulfate; SHBG: sex hormone-binding globulin; 2-OH E_1: 2-hydroxyestrone; 16a-OH E_1: 16α-hydroxyestrone; 2:16α: 2-OH E_1:16α-OH E_1; Ins: insulin; Lep: leptin; IGF-I: insulin-like growth factor-I; IGFBP3: insulin-like growth factor binding protein 3.

serum hormones. Urinary 2-OH E_1 and 16α-OH E_1 concentrations were positively correlated with each other, but not strongly correlated with concentrations of serum estrogens or androgens.

No statistically significant differences were observed by equol-producer phenotype for age-adjusted serum hormone or SHBG concentrations (Table 4). *O*-DMA-producers had 23% higher serum FSH concentrations than non-producers ($P = 0.04$). We assessed whether the differences we observed in some of the dietary factors between producers and non-producers explained the associated between *O*-DMA-producer phenotype and FSH, and adjusting for intakes of caffeine, omega-3 fatty acids, and fiber attenuated the differences between producers and non-producers to 15% ($P = 0.19$). Because sex hormones can exert feedback to FSH, we also assessed whether the association between

Table 4
Age-adjusted means (unadjusted range) of serum hormones and SHBG concentrations and urinary 2-OH E_1:16α-OH E_1 by daidzein-metabolizing phenotypes in 89 post-menopausal women

Hormones and metabolites[a]	Equol producers ($n = 22$)		Equol non-producers ($n = 65$)		*O*-DMA producers ($n = 74$)		*O*-DMA non-producers ($n = 15$)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range
FSH (mIU/l)[b]	72	48–120	66	7–124	69[d]	36–81	56	7–124
E_1 (pg/ml)[c]	43	30–81	43	16–107	43	16–82	43	27–110
Total E_2 (pg/ml)[c]	17	10–31	17	10–50	17	10–34	18	13–50
Free E_2 (pg/ml)[c]	0.5	0.2–0.9	0.5	0.2–1.6	0.5	0.2–1.0	0.5	0.3–1.6
Total T (pg/ml)[c]	211	121–332	215	84–604	219	84–444	192	124–604
Free T (pg/ml)[c]	4.5	2.6–6.8	4.6	1.4–17	4.6	1.4–11	4.5	3.1–17
A (pg/ml)[c]	534	323–1040	567	215–1680	555	215–1680	572	256–1400
DHEA (ng/ml)[c]	2.1	0.8–5.7	2.5	0.9–9.6	2.3	0.8–6.1	2.7	0.9–9.6
DHEA-S (ng/ml)[c]	57	12–140	60	14–340	56	12–306	58	16–340
SHBG (nmol/l)[c]	36	15–108	35	12–98	36	12–110	30	13–58
2-OH E_1 (ng/mg Cr)[c]	8.1	0.7–13	6.6	0.3–34	7.4[d]	0.3–34	5.2	0.3–22
16α-OH E_1 (ng/mg Cr)[c]	5.7	0.6–14	5.9	0.4–15	6.1[e]	0.4–14	4.9	0.7–15
2-OH E_1:16-αOH E_1[c]	1.4[e]	0.5–2.3	1.1	0.2–3.8	1.2	0.3–2.9	1.1	0.2–3.8
Insulin[b]	20	7.7–45	19	5.2–44	19	5.2–45	21	9.9–44
Leptin[b]	27	8.8–46	28	9.1–51	28	8.8–47	28	9.1–51
IGF-I[b]	109	61–179	110	41–251	110	41–251	106	48–162
IGFBP3[b]	3.9	1.7–6.0	4.1	1.6–6.3	4.1	2.1–6.3	4.1	1.6–6.2

[a] FSH: follicle stimulating hormone; E_1: estrone; E_2: estradiol; T: testosterone; A: androstenedione; DHEA: dehydroepiandrosterone; DHEA-S: dehydroepiandrosterone-sulfate; SHBG: sex hormone-binding globulin; 2-OH E_1: 2-hydroxyestrone; 16α-OH E_1: 16α-hydroxyestrone; IGF-I: insulin-like growth factor-I; IGFBP3: insulin-like growth factor binding protein 3.
[b] Arithmetric means.
[c] Geometric means.
[d] $P < 0.05$, producers relative to non-producers within the same daidzein-metabolizing phenotype.
[e] $P < 0.1$, producers relative to non-producers within the same daidzein-metabolizing phenotype.

FSH and *O*-DMA-producer phenotype was explained by modest differences we observed in testosterone and DHEA concentrations. With adjustment for age, testosterone and DHEA, mean FSH for producers was 68.5 (standard deviation (S.D.)=2.5) mIU/l and for non-producers was 59.8 mIU/l (S.D. = 5.7) ($P = 0.17$). No other statistically significant differences for other serum reproductive hormones or SHBG concentrations were observed in relation to the *O*-DMA-producer phenotype.

Adjusting for ovariectomy status (had or not) and ever/never use of hormone replacement therapy did not appreciably alter the associations between daidzein-metabolizing phenotypes and any of the serum hormones or SHBG (data not shown). Adjusting for the other daidzein-metabolizing phenotype also did not appreciably alter the associations of individual phenotypes with serum hormones or SHBG (data not shown).

Geometric mean concentrations of age-adjusted urinary 2-OH E_1:16α-OH E_1 were 27% greater among equol producers relative to non-producers ($P = 0.06$) (Table 4). The difference in 2-OH E_1:16α-OH E_1 excretion appeared to be driven by a 24% difference in excretion of 2-OH E_1 ($P = 0.07$), given that mean urinary 16α-OH E_1 concentration of equol producers was only 3% lower than non-producers ($P = 0.88$). Geometric mean concentrations of urinary 2-OH E_1:16α-OH E_1 were not significantly different in *O*-DMA producers and non-producers (>16%, $P = 0.33$). However, *O*-DMA producers, compared to non-producers, had higher geometric mean concentrations of both 2-OH E_1 (>42%, $P = 0.02$) and 16α-OH E_1 (>24%, $P = 0.09$). Adjusting for consumption of caffeine, omega-3 fatty acids, and fiber did not attenuate the association between urinary 2-OH E_1 concentrations and the equol-producer phenotype (>23% difference, $P = 0.14$), but attenuated the association between 2-OH E_1 and the *O*-DMA-producer phenotype (>28%, $P = 0.12$).

Fig. 1 illustrates the relative (percent) differences by joint equol/*O*-DMA-producer phenotypes. Results for the equol+/*O*-DMA− phenotype are not presented due to insufficient frequency ($n = 1$). Considering the remaining three daidzein-metabolizing phenotypes, linear trends were observed across the categories of equol−/*O*-DMA−, equol−/*O*-DMA+, equol+/*O*-DMA+ for FSH ($P = 0.07$), DHEA ($P = 0.10$), 2-OH E_1:16α-OH E_1 ($P = 0.09$), and 2-OH E_1 ($P = 0.01$).

4. Discussion

The objective of this study was to evaluate serum hormones and SHBG and urinary estrogen metabolites in relation to daidzein-metabolizing phenotypes in post-menopausal women. We observed no appreciable differences in serum hormone concentrations in relation

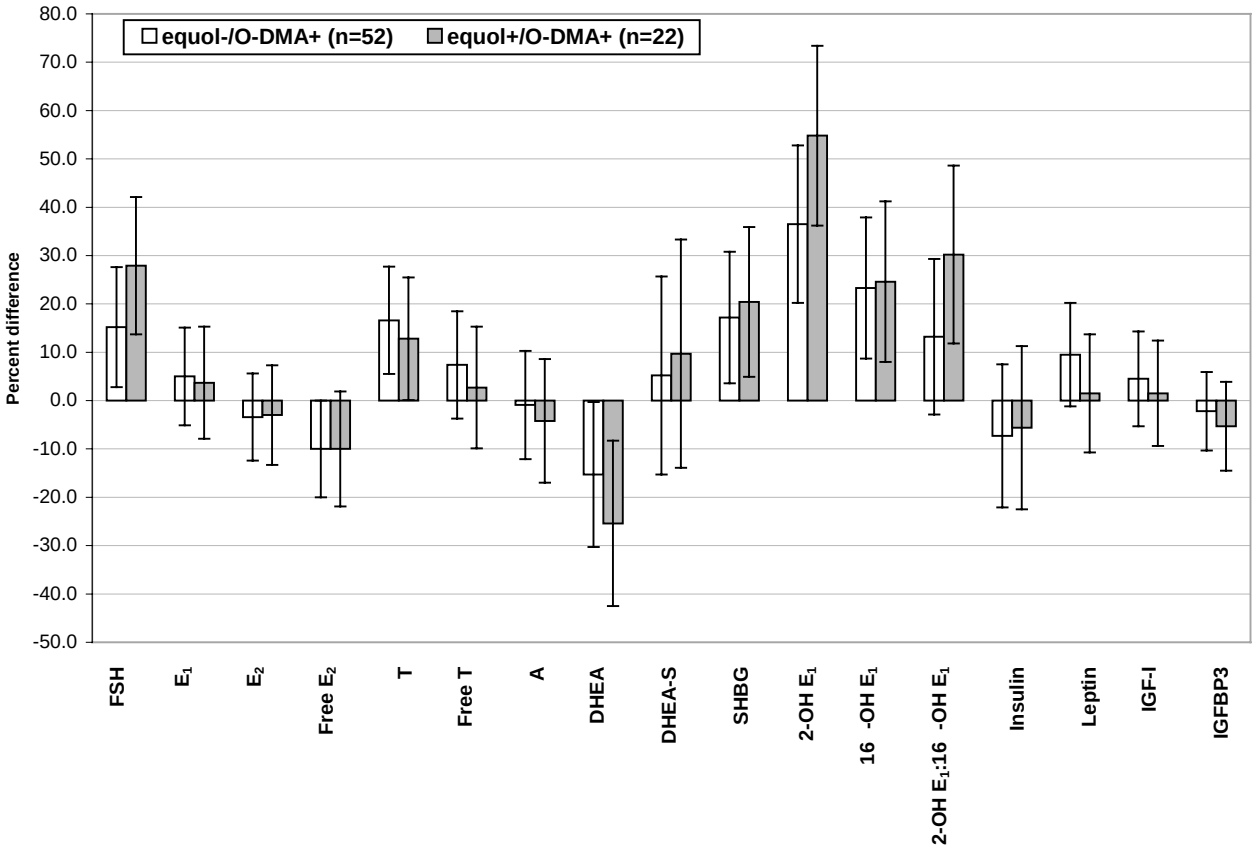

Fig. 1. Age-adjusted percent difference (with standard error bars) in circulating sex hormone, SHBG, and urinary estrogen metabolite concentrations by combined daidzein-metabolizing phenotypes in 89 post-menopausal women. Percent differences reflect equol−/*O*-DMA+ and equol+/*O*-DMA+ phenotypes compared to equol–/*O*-DMA− phenotype (equol–/*O*-DMA−) ($n = 14$). FSH: follicle stimulating hormone; E_1: estrone; E_2: estradiol; T: testosterone; A: androstenedione; DHEA: dehydroepiandrosterone; DHEA-S: dehydroepiandrosterone-sulfate; SHBG: sex hormone-binding globulin; 2-OH E_1: 2-hydroxyestrone; 16a-OH E_1: 16α-hydroxyestrone; IGF–I: insulin–like growth factor–I; IGFBP3: insulin–like growth factor binding protein 3.

to equol-producer phenotype. In contrast, Duncan et al. [24] observed that, among 14 pre-menopausal women, equol producers, compared to non-producers, had statistically significantly lower serum concentrations of estrone, estrone-sulfate, testosterone, androstenedione, DHEA, DHEA-S, and cortisol during certain menstrual cycle phases, and had higher concentrations of sex hormone-binding globulin and mid-luteal phase progesterone. No other studies are known to have evaluated circulating hormones and daidzein-metabolizing phenotypes in post-menopausal women. Our results may differ from those of Duncan et al. [24] because post-menopausal women, compared to pre-menopausal women, have lower circulating concentrations of several sex hormones and SHBG [33], which, along with the women in our study being overweight, may have reduced the variation in serum hormones. Less variability could possibly lower the power to detection an association if there is an association. Additionally, post-menopausal concentrations of these hormones may not be reflective of pre-menopausal concentrations [34,35]; thus, it would not necessarily be expected for associations between equol-producer phenotype and serum hormones to be similar in pre-menopausal and post-menopausal women.

We observed significantly greater FSH concentrations in *O*-DMA-producers compared to *O*-DMA non-producers. Dietary factors appeared to mediate or confound some of this association. The association between FSH and *O*-DMA-producer phenotype was also attenuated when adjusted for total testosterone and DHEA, suggesting that some of these hormone differences may be partly related, possibly through metabolic feedback, to FSH concentration. To our knowledge, no other studies are known to have evaluated serum sex hormone concentrations by the *O*-DMA-producer phenotype.

We observed that equol-producing women, relative to non-producing women, had higher mean 2-OH E_1:16α-OH E_1 ratio, which appeared to be driven by higher mean 2-OH E_1. No other studies are known to have evaluated urinary estrogen metabolite concentrations by these daidzein-metabolizing phenotypes. One study looked at continuous equol excretion and urinary estrogen metabolites in a US population [36]. However, because some foods, including cow's milk, contain low levels of equol

[37], continuous equol excretion in a low soy-consuming populations, such as populations in the US [38,39], does not provide an adequate assessment of the equol-producer phenotype as a marker of the presence of particular intestinal bacteria. Given that results from a few prospective studies suggest that greater 2-OH E_1:16α-OH E_1 may be associated with lower risk for breast cancer [14,15], we hypothesize that equol-producing women may be at lower risk for breast cancer.

O-DMA producers had greater concentrations of mean 2-OH E_1 than non-producers, and adjusting for caffeine, fiber and omega-3 fatty acid intake attenuated this association, suggesting that dietary factors may be confounding factors, i.e. dietary factors influence the capacity to produce *O*-DMA and dietary factors are associated with hormones and metabolites. Little is known about estrogen metabolites and caffeine or omega-3 fatty acids. However, fiber has been associated with estrogen metabolites; luteal phase 2-OH E_1 and 16α-OH E_1 were lower after dietary intervention with prunes, but not wheat bran, in pre-menopausal women [40,41].

Little is known about associations between the equol-producer phenotype and *O*-DMA-producer phenotype. It was suggested in our study that the two phenotypes were positively associated. However, each phenotype did not appear to confound or modify the association between the other phenotype and hormonal outcomes.

Our study has several limitations. One, given the small sample of women, we may have failed to identify some true associations. Two, because we evaluated a number of hormones and metabolites, significant findings may have occurred by chance. Three, a single serum sample was used and there was high variability for some of the sex hormones. It is not expected that this variability would be related to either daidzein-metabolizing phenotype; thus, the use of a single hormone measurement would be expected to attenuate associations towards the null. Four, the parent study was a randomized trial, and the women were selected for the parent study based on particular selection criteria, which may have also reduced variability in our outcomes and decreased the power to detect associations in this ancillary study. There are also some strengths of our study, including a comprehensive panel of hormones and metabolites and eliminating menstrual cycle variation in circulating reproductive hormones.

Setchell et al. [42] proposed a mechanism by which the equol-producer phenotype influences hormone-related outcomes; equol, produced in response to soy consumption by equol producers, acts on hormone-sensitive tissues. Modification of the effect of soy by equol-producer phenotype may be important in populations regularly consuming soy or for soy intervention studies, but would have little influence in low soy-consuming populations, such as post-menopausal women in the United States [38,43]. A number of women in this study reported tofu consumption, but few were regular consumers, suggesting that the associations between daidzein-metabolizing phenotypes and hormones and metabolites we observed are more likely to be reflective of effects associated with the presence of a particular intestinal bacterial composition rather than effects of circulating equol or *O*-DMA concentrations.

While a prior study observed that the equol-producer phenotype was related to hormone concentrations in pre-menopausal women, we observed few appreciable differences in serum hormones in post-menopausal women. Daidzein-metabolizing phenotypes were associated with products of estrogen metabolism. The results of this study should be confirmed in a larger, more diverse sample. Overall, our findings suggest that, if the associations we observed between daidzein-metabolizing phenotypes and hydroxylated estrogen metabolites can be confirmed in other studies, these phenotypes may be relevant with regards to hormone-associated diseases, and further study of daidzein-metabolizing phenotype associations with health, and of mechanisms of daidzein-metabolizing phenotype actions, would be warranted.

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