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Relationship of Serum Estrogens and Metabolites with Area and Volume Mammographic Densities

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Abstract

Purpose—Elevated mammographic density is a breast cancer risk factor, which has a suggestive, but unproven, relationship with increased exposure to sex-steroid hormones. We examined associations of serum estrogens and estrogen metabolites with area and novel volume mammographic density measures among 187 women, ages 40–65, undergoing diagnostic breast biopsies at an academic facility in Vermont.

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Conflict of Interest Disclosure

S.D. Herschorn is a stockholder in Hologic.

Methods—Serum parent estrogens, estrone and estradiol, and their 2-, 4-, and 16-hydroxylated metabolites were measured using liquid chromatography-tandem mass spectrometry. Area mammographic density was measured in the breast contralateral to the biopsy using thresholding software; volume mammographic density was quantified using a density phantom. Linear regression was used to estimate associations of estrogens with mammographic densities, adjusted for age and body mass index, and stratified by menopausal status and menstrual cycle phase.

Results—Weak, positive associations between estrogens, estrogen metabolites and mammographic density were observed, primarily among postmenopausal women. Among premenopausal luteal phase women, the 16-pathway metabolite estriol was associated with percent area ($p=0.04$) and volume ($p=0.05$) mammographic densities and absolute area ($p=0.02$) and volume ($p=0.05$) densities. Among postmenopausal women, levels of total estrogens, the sum of parent estrogens, and 2-, 4- and 16-hydroxylation pathway metabolites were positively associated with area density measures (percent: $p=0.03$, $p=0.04$, $p=0.01$, $p=0.02$, $p=0.07$; absolute: $p=0.02$, $p=0.02$, $p=0.01$, $p=0.02$, $p=0.03$, respectively), but not volume density measures.

Conclusions—Our data suggest that serum estrogen profiles are weak determinants of mammographic density and that analysis of different density metrics may provide complementary information about relationships of exposures to breast tissue composition.

Keywords

Estrogens; Mammographic density; Breast Neoplasms/*etiology/*metabolism; Estrogens/blood/*metabolism

Introduction

Mammographic density (MD), which reflects fibroglandular tissue content of the breast, is a strong breast cancer risk factor [36]. However, the determinants of MD and mechanisms by which it increases breast cancer risk are poorly understood. Increased cumulative exposure to sex-steroid hormones is hypothesized to play a role, as several reproductive and hormonal factors associated with breast cancer are also associated with MD [31]. In particular, menopausal hormone therapy is related to increased MD [20], while tamoxifen, a selective estrogen receptor modulator, decreases MD [10].

Endogenous estrogens are thought to increase breast cancer risk by stimulating cell proliferation [52], and this mechanism may partially explain the increased fibroglandular areas seen in high MD [6, 43]. Alternatively, estrogen may be metabolized to genotoxic and/or mutagenic metabolites [52], and enzymes involved in estrogen metabolism as measured in urine [23] and breast tissue [22] have been linked to MD. Accordingly, hormonal mechanisms may partly mediate MD-related breast cancer risk. Although prospective studies have consistently demonstrated that breast cancer risk increases with circulating estrogens [27, 26], data relating endogenous estrogens to MD are inconsistent [31, 3]. Furthermore, few studies have evaluated the effects of estrogen metabolites on MD [4, 17, 32, 33, 38], in part because their comprehensive study has only recently become technically feasible with highly reproducible and sensitive liquid chromatography-tandem mass spectroscopy (LC-MS/MS) methods.

The parent estrogens, estrone and estradiol, can be metabolized along three pathways via irreversible hydroxylation at the C-2-, C-4-, or C-16-position of the steroid ring [52] (Figure 1 [13]). Specific patterns of estrogen metabolism have been associated with breast cancer risk in several recent studies using LC-MS/MS methods [11–13, 18]. To date, five prior studies have examined estrogen metabolites in relation to MD, with mixed results [4, 17, 32, 33, 38]. All studies measured metabolites in urine, and the correspondence between urinary and circulating metabolites is not known. Two studies used immunoassays [33, 38], and three examined LC-MS/MS-measured metabolites [4, 17, 32]. In addition to differences between populations, sample collections and hormone assays, varying approaches to measure MD may also contribute to inconsistent results. Prior studies have assessed MD subjectively using visual classification [38] or quantitatively using computer-assisted methods [4, 17, 32, 33]. Advanced methods now permit measurement of MD as a volume [51], which may better reflect the true amount of fibroglandular and adipose tissue in the breast.

In the current study, we aimed to clarify relationships between 15 circulating estrogens and estrogen metabolites (all jointly referred to as EM) and area and volume measures of MD in a cross-sectional study of women referred for image-guided breast biopsy. An association between EM and MD could provide clues as to the mechanisms that link high MD to increased breast cancer risk.

Materials and Methods

Study Population

The National Cancer Institute (NCI) Breast Radiology Evaluation and Study of Tissues (BREAST) Stamp Project is a molecular epidemiologic study of mammographic density undertaken at the University of Vermont College of Medicine and its affiliated academic hospital, Fletcher Allen Health Care (FAHC) as described previously [19]. Briefly, 465 women who were referred for diagnostic image-guided breast biopsy were enrolled from 2007–2010. Eligible women were aged 40–65 years, had no prior history of breast cancer or receiving cancer treatment, had not undergone breast surgery within one year, did not have breast implants, and were not taking breast cancer chemoprevention.

Study participants completed a standard health history questionnaire. A research coordinator administered an interview to collect additional health information and measured participants' height and weight. Compensation of \$50 was provided to participants who opted to donate blood. A woman was considered postmenopausal if menstrual periods had stopped more than 12 months prior to interview, she had undergone bilateral oophorectomy, or she had undergone a hysterectomy and was 55 years of age or older; otherwise, a woman was considered premenopausal. At the time of blood collection, premenopausal women were asked to report the date of last menstrual period and were provided with a postcard, to be returned with the date of the first day of their next menstrual period, in order to determine menstrual cycle length and phase. Final pathologic diagnoses were obtained from pathology reports. Participants provided written informed consent (in accordance with Institutional Review Boards at the University of Vermont and the NCI).

Blood Collection and Laboratory Assays

Whole blood samples were collected using standard techniques, allowed to clot for 30 minutes, and processed at the FAHC General Clinical Research Center. Samples were centrifuged at 3,000 rpm for 15 minutes, and serum was aliquoted into 2.0 mL cryovials and frozen at -80°C until shipment to SeraCare Life Sciences (Gaithersburg, MD), where vials were stored in liquid nitrogen until transfer to the Laboratory of Proteomics and Analytical Technologies, Cancer Research Technology Program, Leidos Biomedical Research, Inc. (Frederick, MD) for testing.

Stable isotope dilution LC-MS/MS was used to quantitate 15 EM in serum as previously described [50], including: estrone, estradiol, 2-hydroxylation pathway metabolites (2-hydroxyestrone, 2-hydroxyestradiol, 2-methoxyestrone, 2-methoxyestradiol and 2-hydroxyestrone-3-methyl ether); 4-hydroxylation pathway metabolites (4-hydroxyestrone, 4-methoxyestrone and 4-methoxyestradiol); and 16-hydroxylation pathway metabolites (16 α -hydroxyestrone, estriol, 17-epiestriol, 16-ketoestradiol and 16-epiestriol). Six stable isotopically labeled standards were used including: deuterated 2-hydroxyestradiol, 2-methoxyestradiol and estriol (C/D/N Isotopes, Pointe-Claire, Quebec, Canada); deuterated 16-epiestriol (Medical Isotopes, Pelham, NH) and ^{13}C -labeled estrone and estradiol (Cambridge Isotope Laboratories, Andover, MA). In serum, this method detects 15 EM which circulate primarily as sulfated and/or glucuronidated conjugates; five EM (estrone, estradiol, estriol, 2-methoxyestrone and 2-methoxyestradiol) also circulate in unconjugated forms. The serum sample was split into two aliquots, one to measure total concentration of each of 15 metabolites (i.e., sum of conjugated plus unconjugated forms); the other, to measure unconjugated forms. To measure the sum of conjugated plus unconjugated forms, an enzyme with sulfatase and glucuronidase activity was added to samples to cleave any sulfate and glucuronide groups from parent estrogens [50]. To measure unconjugated forms only, addition of the enzyme was not included in sample preparation steps. For those metabolites with both total and unconjugated measurements, concentration of the conjugated form was calculated as the difference between total and unconjugated measurements. Assay reliability was monitored using 10% masked quality control samples. Coefficients of variation were $<3\%$ (median, 0.29%; range, 0.1–2.06%); intraclass correlation coefficients were $>99\%$ for each EM.

Mammographic Density Assessment

Digital raw mammographic images were transferred to the University of California at San Francisco for quantitative area and volume density assessment. This analysis was restricted to pre-biopsy cranio-caudal views of the contralateral breast. The mammogram taken closest in time prior to the breast biopsy date was selected. Area measures of density were estimated as described previously [37], using computer-assisted thresholding software comparable to other validated methods [9, 40]. One trained experienced reader [37, 40] measured absolute dense area (cm^2) by setting a pixel threshold for dense tissue. Percentage MD was calculated by dividing absolute dense breast area by total breast area and multiplying by 100. Breast density was quantified as an absolute fibroglandular tissue volume (cm^3) and percent fibroglandular tissue volume using Single X-ray Absorptiometry (SXA) as described previously [30]. An SXA breast density phantom was affixed to the compression paddle and

included in the X-ray field (Figure 2). Mammographic grayscale values were compared to values of the SXA phantom with a known FGV composition and thickness [30]. In this way, volumetric measures were achieved using a planar image. Previous estimates of reproducibility for SXA test phantoms demonstrated a repeatability standard deviation of 2%, with a $\pm 2\%$ accuracy for the entire thickness and density ranges [30].

Analytic Population

We restricted the study population to participants who donated blood, were not using exogenous hormones at blood collection, had information regarding menopausal status and, if premenopausal, menstrual cycle phase, and had non-missing MD measures. Of the 465 participants who consented, 12 were not subsequently biopsied and were excluded; at least one vial of serum was collected from 324 women (72%), of whom 29 were current hormone users and excluded. Of these, we excluded 57 premenopausal women whose menstrual cycle phase could not be classified and 19 women with indeterminate menopausal status, resulting in 113 pre- and 106 postmenopausal participants potentially eligible for this analysis. We further excluded four pre- and 11 postmenopausal participants with < 3.2 mL of serum available; five pre- and five postmenopausal women who lacked pre-biopsy MD results for the contralateral breast within one year of biopsy; and four pre- and three postmenopausal participants who underwent bilateral breast biopsies (preventing assessment of a contralateral breast). This resulted in a final analytic population of 100 pre- and 87 postmenopausal women.

Statistical Analysis

All analyses were stratified according to menopausal status at the time of blood draw. Analyses among premenopausal women were stratified by menstrual cycle phase. We computed Spearman's rank correlation coefficients to estimate the correlation of EM with age and body mass index (BMI), two factors which are strongly related to MD. Based on Box-Cox transformation analysis [5], we square-root transformed all MD measures to better approximate normal distributions.

Linear regression models were used to examine associations of serum EM concentrations (pmol/L) with measures of MD area (MD-A) and MD volume (MD-V) as the dependent variables. We examined individual EM, the sum of all 15 EM ("total EM"), the sum of parent EM, groups defined by metabolic pathway, and metabolic pathway ratios. To identify potential confounding factors, we examined the association of participant characteristics with MD measures in linear regression models and with serum EM (tertiles) in ordinal logistic regression models. Although serum EM concentrations were not significantly associated with age within menopausal strata, we chose to present age-adjusted models given the strong association between age and MD. Results from age-adjusted models were similar to unadjusted results, which are not shown here. The impact of BMI was assessed by comparing results from regression models with and without BMI. Additional adjustment for age at menarche, age at first birth, cigarette smoking, history of breast biopsy prior to enrollment and family history of breast cancer in a first degree relative had minimal effects (data not shown). In sensitivity analyses, we examined the relation between EM and MD after excluding a) women whose biopsy diagnosis included *in-situ* or invasive breast

carcinoma (n=7 pre- and 21 postmenopausal women) and b) women who reported 1 year since last use of menopausal hormone therapy or were missing time since last use (n=2 pre- and 7 postmenopausal women). Residuals from linear models were assessed by a combination of visual inspection and the Anderson-Darling test of and were not found to deviate substantially from normality.

Probability values of <0.05 were considered statistically significant. All tests of statistical significance were two-tailed. Analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

Results

Participant Characteristics

The mean (standard deviation, SD) age of premenopausal and postmenopausal participants was 46 (4) and 57 (4) years, respectively (Table 1). Mean (SD) BMI was slightly lower among pre- versus postmenopausal women (25.9 (6.3) vs. 27.1 (6.3) kg/m²). Most participants were non-Hispanic white (premenopausal: 93%, postmenopausal: 92%), college graduates (premenopausal: 93.0%, postmenopausal 80.5%), and parous (premenopausal: 78.0%, postmenopausal: 80.5%). On average, percent and absolute measures of MD-A and MD-V were higher among pre- versus postmenopausal women, whereas postmenopausal women tended to have higher measures of total breast area and volume.

Distributions of Estrogens and Estrogen Metabolites

Median serum EM concentrations among pre- and postmenopausal women are presented in Supplementary Table 1. Among premenopausal women, the distribution by menstrual cycle phase was as follows: follicular (39%), periovulatory (25%), and luteal (36%). As expected, serum EM levels were lowest in the follicular phase and highest in the periovulatory phase. In general, we observed weak positive correlations between age and EM and weak inverse correlations between BMI and EM (Table 2).

Among postmenopausal women, we did not observe statistically significant correlations between any of the EM and age; however, significant positive correlations were found between most EM and BMI (Table 2). The strongest correlation with BMI was observed for unconjugated E₁ ($\rho = 0.57$, $p < .0001$).

Associations between Estrogens and Estrogen Metabolites and Measures of Mammographic Density

Premenopausal Women—Among premenopausal women in the follicular and periovulatory phases, individual EM were not significantly associated with percent or absolute MD-A or MD-V measures (Supplementary Tables 2 and 3). However, for periovulatory phase women, ratios of metabolic pathways showed that an increased ratio of 2-Hydroxyestrone (2-OHE₁) relative to 16 α -Hydroxyestrone (16 α -OHE₁) was associated with higher absolute MD-A and absolute MD-V (age- and BMI-adjusted p-value for MD-A=0.04 and MD-V=0.02, Supplementary Table 3). No other EM pathway ratios were associated with MD.

Among luteal phase women, total EM and parent EM were not significantly associated with percent or absolute measures of MD-A or MD-V after BMI adjustment (Table 3). Unconjugated E₃ was the only EM that tended to be positively associated with both percent and absolute measures of MD-A and MD-V after BMI adjustment (percent MD-A and MD-V: $p=0.04$ and $p=0.05$; absolute MD-A and MD-V: $p=0.02$ and 0.05). With respect to EM pathway ratios, the ratio of 16-hydroxylation pathway EM to parent EM was inversely associated with percent MD-A ($p=0.03$) and MD-V ($p=0.06$); likewise, the ratio of 4-hydroxylation pathway EM to 2-hydroxylation pathway EM was inversely associated with both percent MD-A ($p=0.03$) and MD-V ($p=0.01$). The ratio of 2-pathway catechols to 2-pathway methylated catechols was inversely associated with percent MD-A and MD-V, but findings were only statistically significant for percent MD-V ($p=0.02$). No statistically significant associations were observed between any EM measure and total breast area or volume (data not shown).

Postmenopausal Women—Among postmenopausal women, statistically significant positive associations between total EM and percent and absolute measures of MD-A were only apparent after BMI-adjustment ($p=0.03$ and 0.02 , respectively) (Table 4). In contrast, total EM was not associated with percent MD-V, and a positive association between total EM and absolute MD-V was attenuated and no longer statistically significant following BMI adjustment. Similar patterns were observed for MD relationships with individual EM, such that we only observed positive associations between E₁ and 2-, 4-, and 16-Hydroxylation pathway EM with percent and absolute MD-A after BMI adjustment, whereas positive associations for these individual EM with absolute MD-V were diminished with BMI adjustment. With respect to EM pathway ratios, the ratio of 2-OHE₁ to 16 α -OHE₁ was significantly and inversely associated with absolute MD-V both before and after BMI adjustment ($p=0.001$ and $p=0.01$, respectively). We did not observe any other statistically significant associations between EM pathway ratios and the various MD measures after BMI adjustment.

Sensitivity Analyses—As previously reported, MD measures are not associated with pathologic diagnosis in this population [19]. Nevertheless, we compared EM levels between participants diagnosed with breast cancer ($n=7$ pre- and 21 postmenopausal women) versus those diagnosed with benign breast disease ($n=93$ pre- and 66 postmenopausal women) and EM levels did not significantly differ. Additionally, in sensitivity analyses excluding a) breast cancer cases and b) women who reported 1 year since last use of menopausal hormone therapy or were missing time since last use, results were similar (data not shown).

Discussion

In this cross-sectional analysis of women referred for clinically indicated breast biopsies, circulating EM were not associated with MD among premenopausal women, with the exception of a suggestive positive association for E₃. In postmenopausal women, weak, positive associations were found between total EM as well as individual EM and area measures of MD after adjustment for BMI, but no associations were seen for absolute MD-V after accounting for BMI.

The positive associations we observed between serum unconjugated E₃ and MD among luteal premenopausal women contrast with results from prior studies of premenopausal women, which measured urinary EM in the luteal phase [4, 32, 33]. Maskarinec et al. [32] reported an inverse association between EM in the 16-hydroxylation pathway, including E₃, and MD-A measures, whereas two other studies reported null associations [4, 33]. Maskarinec et al. also reported an elevated 2-OHE₁ to 16α-OHE₁ ratio among luteal phase women with higher MD [32], whereas we observed a similar association only among women in the periovulatory phase. Unlike these prior studies, we found suggestive associations with percent MD-A and MD-V for several other EM pathway ratios among luteal phase women; however, our findings should be interpreted with caution due to small sample sizes. Reconciling analyses of EM and MD is complicated by differences in populations and unknowns about correlations between serum and urine EM levels. Notably, intra-individual serum and urine EM have not been compared. Additionally, urinary EM are mostly found in conjugated form [14], whereas serum EM can be detected in both conjugated and unconjugated forms, the latter of which is thought to be more biologically active [50, 16].

In postmenopausal women, prior studies have used immunoassays to measure circulating estrogens (including estrone, estrone sulfate, estradiol, and free estradiol) in relation to MD [7, 8, 45, 47, 48, 42, 34, 25, 21, 1, 49, 39], generally showing that estrogens are not related to MD after BMI adjustment [7, 45, 47, 48, 42, 34, 1, 49, 39]. Our findings are in agreement with three studies that found positive associations between estrone [8] and estradiol with MD-A [21, 25], and only one prior report that utilized the LC-MS/MS assay to measure this panel of EM, but did so in urine [17]. Whereas we identified positive associations between total, parent, and individual EM in each metabolic pathway with percent and absolute measures of MD-A, Fuhrman et al. did not find associations for individual urinary EM or each pathway [17]. However, consistent with our finding (albeit not statistically significant), Fuhrman et al. found inverse associations with percent and absolute measures of MD-A and ratios of each of the three metabolic pathways relative to parent EM [17]. This suggests that less extensive hydroxylation of parent estrogens is associated with elevated MD-A [17]. Reasons for discrepant results could be due to multiple differences between studies. Taken together, findings from the present study, along with those of Fuhrman et al., are consistent with prior LC-MS/MS studies of EM and postmenopausal breast cancer risk, which suggested that elevated parent estrogens and less extensive hydroxylation of parent estrogens were associated with increased risk [11, 13, 18].

Since BMI correlates strongly and positively with both the nondense (i.e., fatty) and total breast areas, and thus correlates inversely with percent MD [46], accounting for adiposity is important when studying factors, such as endogenous estrogens, that may be related to both percent MD and BMI. This issue is of particular concern among postmenopausal women where adipose tissue serves as a major source of estrogen production [41] and comprises a large proportion of the parenchyma. In light of this, we examined absolute MD-A, in addition to percent MD-A and found similar positive relationships with percent and absolute measures of MD-A among postmenopausal women after adjusting for BMI. This suggests that the observed associations may be mediated at least in part through an influence of EM on epithelial and stromal tissue content, independent of BMI.

We also had the opportunity to evaluate EM associations with percent and absolute SXA volumetric MD measures [40]. For postmenopausal women, we observed no associations for EM with percent MD-V; however, positive associations of total EM, parent EM and individual EM with absolute MD-V were observed. After BMI adjustment, EM levels were no longer associated with absolute MD-V, suggesting that the relationship between EM and absolute MD-V may be mediated by BMI. We and others have previously shown that absolute MD-V and MD-A likely capture different variations in breast tissue composition, an idea supported by the inverse association between BMI and absolute MD-A in contrast to the positive association between BMI and absolute MD-V [19, 40, 2, 24, 28, 35]. For SXA in particular, water contained in adipose tissue contributes to the absolute MD-V measure [40]. Because some degree of adiposity is already captured with absolute MD-V, it may be informative to consider EM associations with MD-V both before and after BMI-adjustment. Notably, we observed a significant inverse association between the 2-OHE₁ to 16 α -OHE₁ ratio and absolute MD-V that persisted after BMI adjustment; this particular ratio has been long hypothesized to be inversely associated with breast cancer risk [15]. Whereas prior studies relating the 2-OHE₁ to 16 α -OHE₁ ratio to MD-A among postmenopausal women have reported null [17] or positive [38] associations, our findings are consistent with the inverse associations reported in LC-MS/MS studies of breast cancer risk [11, 18].

The present study is the first comprehensive investigation of serum EM and MD assessed using area and volumetric measurements. Due to the exploratory nature of the study, results were not adjusted for multiple comparisons. The vast majority of our findings would not be considered statistically significant after a formal multiple testing adjustment, potentially due to limited sample size and/or the possible modest effects of estrogens on MD [31, 44]. Strengths of our study include the utilization of a precise LC-MS/MS EM assay with demonstrated reliability in both pre- and postmenopausal women [50, 16] and quantitative, reproducible area and volume MD measurements, improving the statistical power to detect associations.

While we have previously demonstrated strong, positive correlations between serum EM and EM in breast tissue-derived fluids [29], EM profiles may not be a valid proxy for breast tissue levels. In addition, our findings may reflect unique characteristics of our study population, which consisted primarily of white, highly educated women, who were referred for a breast biopsy from breast imaging. Despite potentially limited generalizability, interindividual variability in EM measures in the present study is similar to prior LC-MS/MS studies of serum EM in pre- [16] and postmenopausal women [13, 11, 16].

In summary, elevated serum estrogen profiles were weakly associated with higher MD among postmenopausal women undergoing breast biopsy. The positive associations between EM in the 2-, 4-, and 16-hydroxylation pathways with area, but not volume, measures of MD suggest a general role for EM in postmenopausal MD-A as opposed to a specific metabolic pathway. Our findings provide some support for the idea that hormonal mechanisms may mediate both MD and MD-related breast cancer risk. Future larger studies assessing relationships of EM and MD, using quantitative and reliable measures, may provide clues about factors influencing breast tissue composition and breast cancer etiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

BMI	body mass index
BREAST	Breast Radiology Evaluation and Study of Tissues
EM	estrogens and estrogen metabolites
E₁	estrone
E₂	estradiol
E₃	estriol
FAHC	Fletcher Allen Health Care
LC-MS/MS	liquid chromatography-tandem mass spectroscopy
MD	mammographic density
MD-A	area mammographic density
MD-V	volume mammographic density
NCI	National Cancer Institute
SD	standard deviation
SXA	Single X-ray Absorptiometry
2-OHE₁	2-Hydroxyestrone
2-MeOE₁	2-methoxyestrone
4-MeOE₁	4-methoxyestrone
16α-OHE₁	16 α -Hydroxyestrone

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Figure 1.
Schematic of estrogen metabolic pathway [13].

Figure 2. Representative full-field digital mammograms from BREAST Stamp Project participants. The digital mammogram is acquired with the density phantom in the corner of the image to allow for automated computation of volume mammographic density (MD-V). In this example, A and B represent breasts of low and high percent MD-V, respectively. Percent MD-V in panel A = 11.8% and in panel B = 83.6%.

Table 1

Baseline characteristics of the study population by menopausal status

	Premenopausal (n=100)		Postmenopausal (n=87)	
	mean	SD	mean	SD
Continuous measures				
Age at mammogram (years)	45.9	3.7	57.3	4.1
Body mass index, kg/m ²	25.9	6.3	27.1	6.3
Area mammographic density (MD-A):				
Percent dense area (%)	34.6	21.6	21.9	18.7
Absolute dense area (cm ²)	39.5	27.1	28.1	22.8
Total breast area (cm ²)	135.2	74.4	161.8	75.2
Volume mammographic density (MD-V):				
Percent fibroglandular volume (%)	46.3	22.3	32.4	17.7
Absolute fibroglandular volume (cm ³)	206.8	105.2	187.0	79.7
Total breast volume (cm ³)	582.6	429.0	723.7	429.8
Categorical measures				
	n	%	n	%
White, non-Hispanic race	93	93.0	80	92.0
College/Graduate school degree	93	93.0	70	80.5
Body mass index, kg/m ²				
<25	57	57.0	40	46.0
25–30	22	22.0	23	26.4
30+	21	21.0	24	27.6
Age at menarche (years)				
12	32	32.3	33	38.4
13	40	40.4	28	32.6
14	27	27.3	25	29.1
Parity				
Nulliparous	22	22.0	17	19.5
1	12	12.0	19	21.8
2	41	41.0	35	40.2
3+	25	25.0	16	18.4
Age at first birth (years)				
Nulliparous	22	22.0	17	19.5
<30	50	50.0	55	63.2
30	28	28.0	15	17.2
Former use of oral contraceptives	88	88.0	73	83.9
Former use of menopausal hormone therapy	10	10.2	30	34.5
Cigarette smoking, 100+ cigarettes/lifetime				
Never	59	62.1	31	38.3
Former	29	30.5	40	49.4
Current	7	7.4	10	12.3
Breast biopsy prior to enrollment	29	29.3	31	36.5

Continuous measures	Premenopausal (n=100)		Postmenopausal (n=87)	
	mean	SD	mean	SD
Positive family history of breast cancer in a 1st degree female relative	26	26.0	26	30.2
Pathologic diagnosis				
Benign	43	43.0	27	31.0
Proliferative	45	45.0	32	36.8
Proliferative with atypia *	5	5.0	7	8.0
<i>In situ</i> **	2	2.0	10	11.5
Invasive breast cancer	5	5.0	11	12.6

Missing values were excluded from percentage calculations.

* Includes n=5 atypical ductal hyperplasia diagnoses among premenopausal women, and n=3 atypical ductal hyperplasia and n=4 atypical lobular hyperplasia diagnoses among postmenopausal women

** Includes n=1 ductal and n=1 lobular carcinoma *in situ* diagnoses among premenopausal women and n=10 ductal carcinoma *in situ* diagnoses among postmenopausal women

Table 2

Correlations between serum estrogen metabolites with age and body mass index

Estrogens and estrogen metabolites (EM) [*]	Premenopausal women (n=100)		Postmenopausal women (n=87)	
	Correlation with age ^{**}	Correlation with BMI ^{**}	Correlation with age ^{**}	Correlation with BMI ^{**}
Total EM	0.07	-0.11	0.05	0.45
Parent EM	0.06	-0.11	-0.01	0.48
Estrone (E ₁)	0.05	-0.10	0.02	0.50
Conjugated	0.04	-0.10	0.02	0.47
Unconjugated	0.13	-0.09	-0.03	0.57
Estradiol (E ₂)	0.07	-0.15	-0.12	0.37
Conjugated	0.01	-0.11	-0.11	0.29
Unconjugated	0.10	-0.18	-0.15	0.43
2-Hydroxylation pathway EM	0.10	-0.16	0.12	0.37
2-Pathway catechols	0.08	-0.15	0.01	0.34
2-Hydroxyestrone (2-OHE ₁)	0.10	-0.15	0.06	0.40
2-Hydroxyestradiol (2-OHE ₂)	0.01	-0.10	-0.02	0.19
2-Pathway methylated catechols	0.07	-0.19	0.17	0.36
2-Methoxyestrone (2-MeOE ₁)	0.09	-0.17	0.17	0.30
Conjugated	0.11	-0.16	0.20	0.29
Unconjugated	0.07	-0.18	0.05	0.14
2-Methoxyestradiol (2-MeOE ₂)	-0.04	-0.24	0.04	0.22
Conjugated	-0.04	-0.21	0.05	0.27
Unconjugated	-0.05	-0.14	-0.08	-0.11
2-Hydroxyestrone-3methly ether (3-MeOE ₁)	0.06	-0.18	0.05	0.32
4-Hydroxylation pathway EM	0.13	-0.15	0.10	0.43
4-Pathway catechol: Hydroxyestrone (4-OHE₁)	0.10	-0.12	0.07	0.38
4-Pathway methylated catechols	0.10	-0.19	0.17	0.38
4-Methoxyestrone (4-MeOE ₁)	0.12	-0.18	0.18	0.37
4-Methoxyestradiol (4-MeOE ₂)	0.03	-0.14	0.05	0.32
16-Hydroxylation pathway EM	0.05	-0.09	0.04	0.43
16 α -Hydroxyestrone (16 α -OHE ₁)	0.08	-0.14	0.13	0.31
Estriol (E ₃)	0.03	-0.07	0.02	0.43
Conjugated	0.02	-0.08	-0.06	0.40
Unconjugated	0.01	0.18	0.20	0.003
17-Epiestriol (17-epiE ₃)	0.11	-0.16	0.01	0.45
16-Ketoestradiol (16-ketoE ₂)	0.05	-0.15	-0.01	0.45
16-Epiestriol (16-epiE ₃)	-0.05	-0.13	-0.03	0.30

* All hormones are presented in pmol/L.

** Spearman's correlation rho is presented. Correlation coefficients with p-values <0.05 are presented in bold font. BMI, body mass index (kg/m^2); EM, estrogens and estrogen metabolites

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Table 3

Linear regression estimates for EM (nmol/L) and mammographic density measures, premenopausal luteal phase women (n=36)

	% MD-A				% MD-V				Absolute MD-A (cm ²)				Absolute MD-V (cm ³)			
	Age-adjusted		Age- and BMI-adjusted		Age-adjusted		Age- and BMI-adjusted		Age-adjusted		Age- and BMI-adjusted		Age-adjusted		Age- and BMI-adjusted	
	β	p	β	p	β	p	β	p	β	p	β	p	β	p	β	p
Estrogens and estrogen metabolites (EM)*																
Total EM	0.10	0.05	0.10	0.14	0.10	0.17	0.00	0.56	0.00	0.59	0.10	0.48	-0.10	0.62	0.00	0.90
Parent EM	0.20	0.04	0.20	0.10	0.10	0.16	0.10	0.45	0.10	0.49	0.10	0.39	-0.10	0.73	0.00	0.81
Estrone (E ₁)	0.20	0.04	0.20	0.11	0.10	0.17	0.10	0.49	0.10	0.50	0.10	0.41	-0.10	0.77	0.10	0.76
Conjugated	0.30	0.04	0.20	0.12	0.20	0.17	0.10	0.52	0.10	0.50	0.10	0.40	-0.10	0.80	0.10	0.72
Unconjugated	2.60	0.06	2.00	0.10	1.80	0.16	1.00	0.29	0.80	0.59	1.00	0.52	-1.80	0.46	-0.90	0.71
Estradiol (E ₂)	1.30	0.03	1.00	0.06	0.90	0.12	0.40	0.32	0.50	0.43	0.60	0.35	-0.70	0.55	-0.10	0.93
Conjugated	2.70	0.06	2.20	0.09	1.60	0.22	1.00	0.34	0.80	0.61	0.90	0.56	-2.30	0.39	-1.40	0.56
Unconjugated	2.20	0.02	1.60	0.06	1.50	0.09	0.70	0.33	0.90	0.36	1.20	0.27	-0.70	0.69	0.40	0.80
2-Hydroxylation pathway EM																
2-Pathway catechols	1.60	0.10	0.80	0.41	1.30	0.14	0.20	0.83	0.00	0.98	0.30	0.80	-2.00	0.26	-0.50	0.77
2-Hydroxyestrone (2-OHE ₁)	1.20	0.38	0.10	0.96	0.90	0.48	-0.60	0.53	-0.50	0.74	-0.20	0.88	-2.90	0.25	-1.00	0.67
2-Hydroxyestrone (2-OHE ₂)	1.40	0.42	0.00	0.98	1.00	0.52	-0.80	0.49	-0.60	0.76	-0.20	0.90	-3.10	0.32	-0.80	0.78
2-Hydroxyestradiol (2-OHE ₂)	6.20	0.33	1.90	0.75	4.50	0.44	-1.20	0.78	-2.40	0.72	-1.40	0.84	-16.20	0.16	-9.30	0.39
2-Pathway methylated catechols	5.80	0.01	4.10	0.06	5.00	0.02	2.70	0.11	1.60	0.53	2.30	0.39	-3.40	0.43	0.00	1.00
2-Methoxyestrone (2-MeOE ₁)	6.10	0.01	4.50	0.05	5.20	0.02	3.00	0.10	1.80	0.50	2.50	0.38	-3.30	0.48	-0.10	0.98
Conjugated	6.90	0.01	5.30	0.03	5.60	0.02	3.40	0.07	2.60	0.38	3.20	0.28	-2.70	0.59	0.40	0.92
Unconjugated	1.70	0.88	-3.50	0.72	5.80	0.56	-0.70	0.93	-7.50	0.51	-6.40	0.59	-17.20	0.38	-9.00	0.62
2-Methoxyestradiol (2-MeOE ₂)	31.70	0.10	11.00	0.57	32.10	0.07	4.20	0.77	3.80	0.85	11.40	0.62	-21.80	0.54	19.10	0.59
Conjugated	24.70	0.23	5.10	0.80	25.00	0.18	-0.60	0.97	-1.00	0.97	4.70	0.84	-28.80	0.44	5.50	0.88
Unconjugated	72.90	0.17	40.90	0.41	73.50	0.13	32.10	0.40	35.50	0.53	46.40	0.43	30.70	0.76	92.10	0.31
2-Hydroxyestrone-3methyl ether (3-MeOE ₁)	72.00	0.10	42.70	0.30	64.10	0.11	25.20	0.43	-6.90	0.88	1.20	0.98	-100.00	0.21	-49.90	0.51
4-Hydroxylation pathway EM																
4-Pathway catechol: Hydroxyestrone (4-OHE ₁)	7.00	0.45	-0.50	0.95	4.90	0.56	-4.90	0.46	-2.50	0.80	-0.80	0.94	-15.20	0.36	-3.20	0.84
	1.30	0.91	-5.70	0.58	0.30	0.98	-8.60	0.27	-5.70	0.63	-4.20	0.73	-15.20	0.45	-4.30	0.82

	% MD-A						% MD-V						Absolute MD-A (cm ²)						Absolute MD-V (cm ³)					
	Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age- and BMI-adjusted		
	β	p		β	p		β	p		β	p		β	p		β	p		β	p		β	p	
Estrogens and estrogen metabolites (EM)*																								
16-Hydroxylation pathway EM																								
4-Pathway methylated catechols																								
4-Methoxyestrone (4-MeOE ₁)																								
4-Methoxyestradiol (4-MeOE ₂)																								
16-Hydroxylation pathway EM																								
16 α -Hydroxyestrone (16 α -OHE ₁)																								
Estriol (E ₃)																								
Conjugated																								
Unconjugated																								
17-Epiestriol (17-epiE ₃)																								
16-Ketoestradiol (16-ketoE ₂)																								
16-Epiestriol (16-epiE ₃)																								
Pathway ratios																								
Parent EM / Total EM																								
2-Hydroxylation pathway EM / Parent EM																								
4-Hydroxylation pathway EM / Parent EM																								
16-Hydroxylation pathway EM / Parent EM																								
2-Hydroxylation pathway EM / 16-Hydroxylation pathway EM																								
2-Hydroxyestrone / 16 α -Hydroxyestrone																								
4-Hydroxylation pathway EM / 2-Hydroxylation pathway EM																								
4-Pathway catechol / 4-Pathway methylated catechols																								
2-Pathway catechols / 2-Pathway methylated catechols																								

All mammographic density measures were square-root transformed.

Statistically significant estimates with p-values <0.05 are presented in bold font.

EM, estrogens and estrogen metabolites; MD-A, mammographic density-area measure; MD-V, mammographic density-volume measure

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* For ease of presentation, β values for individual EM are presented in nmol/L; β values and standard errors in pmol/L may be found in Supplementary Table 4.

Table 4

Linear regression estimates for EM (nmol/L) and mammographic density measures, postmenopausal women (n=87)

	% MD-A			Age- and BMI-adjusted			Age-adjusted			% MD-V			Age- and BMI-adjusted			Age-adjusted			Absolute MD-A (cm ²)			Absolute MD-V (cm ³)		
	Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age-adjusted			Age- and BMI-adjusted			Age- and BMI-adjusted		
	β	p		β	p		β	p		β	p		β	p		β	p		β	p		β	p	
Estrogens and estrogen metabolites (EM)*																								
Total EM	-0.14	0.52		0.40	0.03		-0.17	0.26		0.15	0.12		0.09	0.69		0.52	0.02		0.70	0.01		0.42	0.19	
Parent EM																								
Estrone (E ₁)	-0.36	0.45		0.86	0.04		-0.51	0.12		0.18	0.55		0.15	0.75		1.12	0.02		1.60	0.01		0.88	0.20	
Conjugated	-0.48	0.36		0.90	0.05		-0.63	0.09		0.16	0.65		0.14	0.80		1.23	0.02		1.80	0.01		1.03	0.18	
Unconjugated	-0.43	0.44		0.91	0.06		-0.63	0.10		0.13	0.72		0.21	0.71		1.27	0.02		1.90	0.01		1.08	0.17	
Estradiol (E ₂)	-15.82	0.02		12.37	0.08		-9.58	0.05		8.14	0.12		-9.96	0.16		11.40	0.16		22.60	0.02		5.12	0.66	
Conjugated	1.10	0.70		4.14	0.07		-0.06	0.98		1.81	0.30		1.52	0.61		3.75	0.17		3.90	0.34		1.59	0.68	
Unconjugated	2.13	0.54		4.60	0.10		0.25	0.92		1.77	0.40		2.42	0.50		4.23	0.19		3.40	0.49		1.45	0.76	
	-7.06	0.57		19.23	0.07		-4.41	0.62		11.96	0.13		-2.75	0.83		16.64	0.18		30.30	0.09		11.98	0.50	
2-Hydroxylation pathway EM																								
2-P-Pathway catechols	-0.21	0.90		3.60	0.01		-0.76	0.53		1.52	0.16		1.14	0.52		4.09	0.01		5.10	0.03		2.68	0.27	
2-Hydroxysterone (2-OHE ₁)	-0.31	0.87		3.20	0.05		-0.88	0.53		1.24	0.31		1.29	0.52		3.98	0.04		5.30	0.06		2.89	0.29	
2-Hydroxysterone (2-OHE ₂)	-0.08	0.97		4.22	0.03		-1.15	0.49		1.41	0.34		1.87	0.44		5.17	0.02		6.00	0.07		3.09	0.35	
2-Pathway methylated catechols	-7.37	0.48		6.82	0.43		-2.02	0.79		6.96	0.28		-0.52	0.96		10.06	0.32		30.40	0.04		20.18	0.16	
2-Methoxysterone (2-MeOE ₁)	0.24	0.97		12.99	0.01		-1.17	0.78		6.60	0.08		1.91	0.76		11.38	0.05		13.50	0.11		4.77	0.57	
Conjugated	6.32	0.42		18.82	0.003		1.31	0.81		8.90	0.07		8.00	0.32		17.31	0.02		12.70	0.25		3.67	0.74	
Unconjugated	10.72	0.26		26.16	0.0006		1.82	0.79		11.09	0.06		11.80	0.23		23.30	0.01		10.30	0.45		-1.04	0.94	
2-Methoxysterone (2-MeOE ₂)	-3.84	0.81		3.75	0.76		0.26	0.98		5.01	0.59		-0.15	0.99		5.38	0.71		22.20	0.31		16.15	0.44	
Conjugated	-19.49	0.26		0.96	0.95		-10.35	0.40		2.46	0.82		-13.81	0.44		1.04	0.95		38.60	0.12		22.82	0.34	
Unconjugated	-24.20	0.20		2.02	0.90		-12.18	0.36		4.33	0.71		-18.19	0.35		0.78	0.97		42.20	0.11		22.14	0.40	
2-Hydroxysterone-3methyl ether (3-MeOE ₁)	21.38	0.78		-13.48	0.83		0.47	0.99		-21.33	0.64		32.26	0.68		7.09	0.92		50.20	0.64		79.37	0.44	
	-43.15	0.24		56.89	0.08		-27.08	0.30		35.16	0.15		-42.05	0.27		27.98	0.46		61.60	0.24		-14.52	0.79	
4-Hydroxylation pathway EM																								
	-3.18	0.77		21.40	0.02		-6.53	0.40		8.12	0.25		6.17	0.59		25.24	0.02		31.50	0.04		15.54	0.33	

	% MD-A						% MD-V						Absolute MD-A (cm ²)						Absolute MD-V (cm ³)					
	Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age- and BMI-adjusted			Age-adjusted			Age- and BMI-adjusted		
	β	<i>p</i>	β	β	<i>p</i>	β	β	<i>p</i>	β	β	<i>p</i>	β	β	<i>p</i>	β	β	<i>p</i>	β	β	<i>p</i>	β	β	<i>p</i>	
Estrogens and estrogen metabolites (EM) [*]																								
4-Pathway catechol: Hydroxyestrone (4-OHE ₁)	-1.38	0.91	20.16	0.04		-4.97	0.55	8.04	0.28	6.63	0.59	23.01	0.05	29.50	0.08	14.57	0.38							
4-Pathway methylated catechols	-38.87	0.43	61.84	0.14		-43.49	0.21	16.61	0.59	8.37	0.87	86.35	0.07	117.50	0.09	46.17	0.51							
4-Methoxyestrone (4-MeOE ₁)	-45.18	0.53	121.10	0.05		-66.91	0.18	30.23	0.51	8.03	0.91	135.39	0.06	101.40	0.32	-24.69	0.81							
4-Methoxyestradiol (4-MeOE ₂)	-59.16	0.51	20.21	0.78		-40.74	0.52	8.57	0.87	15.38	0.87	74.47	0.38	233.40	0.06	172.98	0.15							
16-Hydroxylation pathway EM																								
16 α -Hydroxyestrone (16 α -OHE ₁)	-0.30	0.54	0.76	0.07		-0.25	0.48	0.41	0.19	0.19	0.70	1.03	0.03	1.60	0.02	0.90	0.20							
Estriol (E ₃)	-0.27	0.94	5.76	0.04		-1.71	0.47	1.89	0.37	2.55	0.46	7.18	0.03	9.40	0.05	5.37	0.25							
Conjugated	-0.52	0.43	0.96	0.09		-0.34	0.47	0.58	0.18	0.15	0.83	1.30	0.05	2.20	0.02	1.20	0.21							
Unconjugated	-0.48	0.47	0.97	0.09		-0.33	0.49	0.57	0.18	0.21	0.76	1.34	0.04	2.10	0.02	1.17	0.22							
17-Epiestriol (17-epiE ₃)	-2.40	0.66	-2.10	0.63		-0.63	0.87	-0.44	0.89	-4.45	0.42	-4.23	0.40	-0.30	0.97	-0.52	0.94							
16-Ketoestradiol (16-ketoE ₂)	-8.52	0.88	80.72	0.07		-34.33	0.37	18.86	0.58	56.23	0.31	126.06	0.02	177.70	0.02	118.23	0.12							
16-Epiestriol (16-epiE ₃)	-1.08	0.77	5.85	0.06		-1.18	0.65	3.08	0.19	2.58	0.50	7.96	0.03	11.50	0.03	6.90	0.19							
	11.44	0.67	30.09	0.16		-9.30	0.62	2.06	0.90	29.55	0.28	43.40	0.08	42.30	0.27	27.72	0.44							
Pathway ratios																								
Parent EM / Total EM	-3.37	0.12	1.15	0.54		-2.99	0.05	-0.31	0.83	-1.48	0.51	1.94	0.37	7.09	0.02	4.02	0.20							
2-Hydroxylation pathway EM / Parent EM	0.69	0.10	-0.27	0.47		0.69	0.02	0.13	0.63	0.19	0.66	-0.56	0.19	-1.52	0.01	-0.89	0.15							
4-Hydroxylation pathway EM / Parent EM	5.16	0.16	-3.40	0.29		5.33	0.04	0.39	0.87	1.10	0.77	-5.59	0.13	-13.40	0.01	-8.04	0.13							
16-Hydroxylation pathway EM / Parent EM	0.08	0.61	-0.19	0.15		0.25	0.03	0.09	0.36	-0.02	0.89	-0.23	0.14	-0.34	0.13	-0.14	0.54							
2-Hydroxylation pathway EM / 16-Hydroxylation pathway EM	2.57	0.07	0.24	0.84		1.81	0.07	0.38	0.68	0.82	0.58	-0.96	0.49	-4.52	0.03	-2.83	0.16							
2-Hydroxyestrone / 16 α -Hydroxyestrone	0.40	0.56	-0.66	0.24		0.28	0.56	-0.38	0.37	-0.32	0.65	-1.14	0.08	-3.10	0.001	-2.43	0.01							
4-Hydroxylation pathway EM / 2-Hydroxylation pathway EM	-13.43	0.17	-7.68	0.34		-6.75	0.33	-3.15	0.60	-12.95	0.20	-8.81	0.34	1.16	0.93	-3.69	0.78							
4-Pathway catechol / 4-Pathway methylated catechols	0.09	0.40	0.09	0.31		0.10	0.23	0.09	0.17	0.10	0.40	0.09	0.37	0.15	0.35	0.15	0.31							
2-Pathway catechols / 2-Pathway methylated catechols	-0.03	0.89	0.02	0.92		-0.08	0.59	-0.05	0.69	0.08	0.70	0.12	0.55	0.17	0.57	0.13	0.64							

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All mammographic density measures were square-root transformed.

Statistically significant estimates with p-values <0.05 are presented in bold font.

EM, estrogens and estrogen metabolites; MD-A, mammographic density-area measure; MD-V, mammographic density-volume measure

* For ease of presentation, β values for individual EM are presented in nmol/L; β values and standard errors in pmol/L may be found in Supplementary Table 5.