HORM CANC (2015) 6:107–119
DOI 10.1007/s12672-015-0216-3

ORIGINAL PAPER

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

Gretchen L. Gierach · Deesha A. Patel · Roni T. Falk · Ruth M. Pfeiffer · Berta M. Geller · Pamela M. Vacek · Donald L. Weaver · Rachael E. Chicoine · John A. Shepherd · Amir Pasha Mahmoudzadeh · Jeff Wang · Bo Fan · Sally D. Herschorn · Xia Xu · Timothy Veenstra · Barbara Fuhrman · Mark E. Sherman · Louise A. Brinton

Received: 21 November 2014 / Accepted: 29 January 2015 / Published online: 11 March 2015

Abstract 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. 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. 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. 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 estrogen exposure to breast tissue composition.

Electronic supplementary material The online version of this article (doi:10.1007/s12672-015-0216-3) contains supplementary material, which is available to authorized users.

G. L. Gierach · D. A. Patel · R. T. Falk · L. A. Brinton
Hormonal and Reproductive Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA

D. A. Patel
e-mail: deesha.patel@nih.gov

R. T. Falk
e-mail: falkr@exchange.nih.gov

L. A. Brinton
e-mail: brintonl@exchange.nih.gov

R. M. Pfeiffer
Biostatistics Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
e-mail: pfeiffer@mail.nih.gov

B. M. Geller · P. M. Vacek · D. L. Weaver · R. E. Chicoine · S. D. Herschorn
University of Vermont, Burlington, VT, USA

B. M. Geller
e-mail: berta.geller@uvm.edu

P. M. Vacek
e-mail: pvacek@uvm.edu

D. L. Weaver
e-mail: Donald.Weaver@uvm.edu

R. E. Chicoine
e-mail: rchicoin@uvm.edu

S. D. Herschorn
e-mail: sally.herschorn@uvmhealth.org

Abbreviations

BMI	Body mass index
BREAST	Breast Radiology Evaluation and Study of Tissues
EM	Estrogens and estrogen metabolites
E_1	Estrone
E_2	Estradiol
E_3	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-OHE_1	2-Hydroxyestrone
2-$MeOE_1$	2-Methoxyestrone
4-$MeOE_1$	4-Methoxyestrone
16α-OHE_1	16α-Hydroxyestrone

Introduction

Mammographic density (MD), which reflects fibroglandular tissue content of the breast, is a strong breast cancer risk factor [1]. 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 [2]. In particular, menopausal hormone therapy is related to increased MD [3], while tamoxifen, a selective estrogen receptor modulator, decreases MD [4].

Endogenous estrogens are thought to increase breast cancer risk by stimulating cell proliferation [5], and this mechanism may partially explain the increased fibroglandular areas seen in high MD [6, 7]. Alternatively, estrogen may be metabolized to genotoxic and/or mutagenic metabolites [5], and enzymes involved in estrogen metabolism as measured in urine [8] and breast tissue [9] 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 [10, 11], data relating endogenous estrogens to MD are inconsistent [2, 12]. Furthermore, few studies have evaluated the effects of estrogen metabolites on MD [13–17], 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 [5] (Fig. 1 [18]). Specific patterns of estrogen metabolism have been associated with breast cancer risk in several recent studies using LC-MS/MS methods [18–21]. To date, five prior studies have examined estrogen metabolites in relation to MD, with mixed results [13–17]. All studies measured metabolites in urine, and the correspondence between urinary and circulating metabolites is not known. Two studies used immunoassays [16, 17], and three examined LC-MS/MS-measured metabolites [13–15]. In

J. A. Shepherd · A. P. Mahmoudzadeh · J. Wang · B. Fan
University of California, San Francisco,
San Francisco, CA, USA

J. A. Shepherd
e-mail: John.Shepherd@ucsf.edu

A. P. Mahmoudzadeh
e-mail: AmirPasha.Mahmoudzadeh@ucsf.edu

J. Wang
e-mail: jeff.wing.wang.walla.walla@gmail.com

B. Fan
e-mail: bo.fan@ucsf.edu

X. Xu · T. Veenstra
Laboratory of Proteomics and Analytical Technologies, Cancer Research Technology Program, Leidos Biomedical Research, Inc., Frederick National Laboratory for Cancer Research, Frederick, MD, USA

X. Xu
e-mail: xuxi@mail.nih.gov

T. Veenstra
e-mail: tveenstra@c2ndiagnostics.com

B. Fuhrman
Department of Epidemiology, Fay W. Boozman College of Public Health, University of Arkansas for Medical Sciences, Little Rock, AR, USA
e-mail: BJFuhrman@uams.edu

M. E. Sherman
Breast and Gynecologic Cancer Research Group, Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, Bethesda, MD, USA
e-mail: shermanm@exchange.nih.gov

G. L. Gierach (✉)
9609 Medical Center Drive, Rm. 7-E108,
Bethesda, MD 20892-9774, USA
e-mail: gierachg@mail.nih.gov

Present Address:
J. Wang
Graduate School of Medicine, Hokkaido University, Sapporo, Japan

Present Address:
T. Veenstra
C_2N Diagnostics, 4041 Forest Park Avenue, Saint Louis, MO, USA

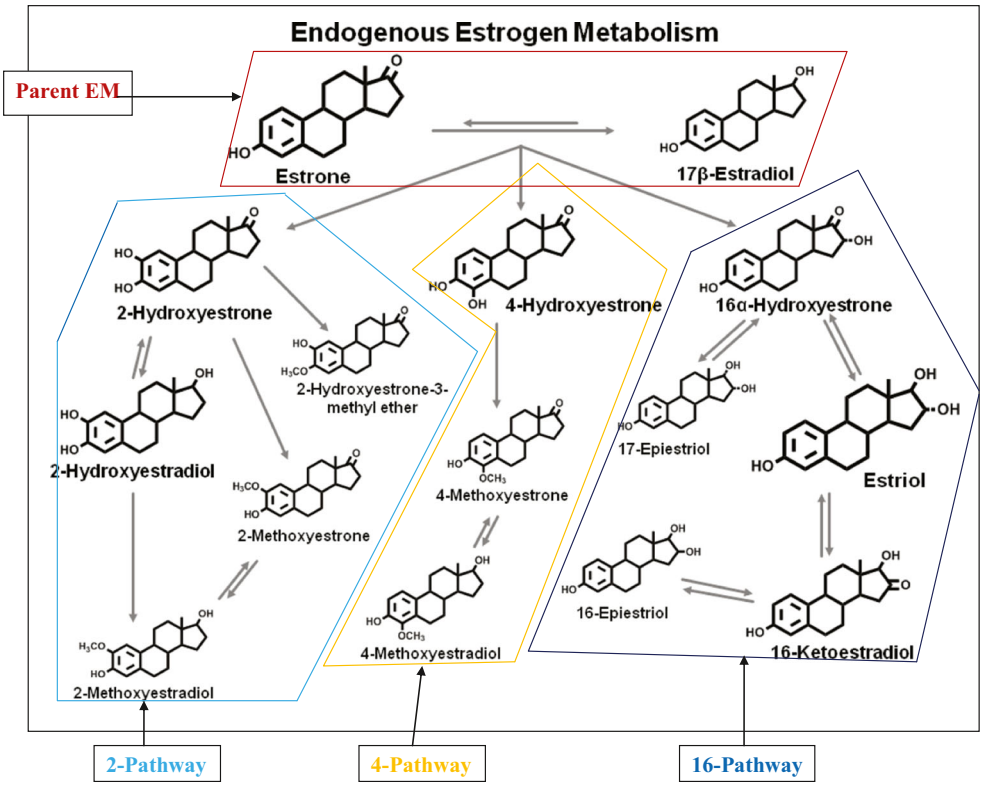

Fig. 1 Schematic of estrogen metabolic pathway [18]

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 [17] or quantitatively using computer-assisted methods [13–16]. Advanced methods now permit measurement of MD as a volume [22], 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 [23]. Briefly, 465 women who were referred for diagnostic image-guided breast biopsy were enrolled from 2007 to 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 1 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 min, and processed at the FAHC

General Clinical Research Center. Samples were centrifuged at 3000 rpm for 15 min, 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 [24], 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 (16α-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 glucoronidated 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) and 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 glucoronide groups from parent estrogens [24]. 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 prebiopsy craniocaudal 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 [25], using computer-assisted thresholding software comparable to other validated methods [26, 27]. One trained experienced reader [25, 27] 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 [28]. An SXA breast density phantom was affixed to the compression paddle and included in the X-ray field (Fig. 2). Mammographic grayscale values were compared to values of the SXA phantom with a known FGV composition and thickness [28]. 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 ±2 % accuracy for the entire thickness and density ranges [28].

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 premenopausal and 106 postmenopausal participants potentially eligible for this analysis. We further excluded four premenopausal and 11 postmenopausal participants with ≤3.2 mL of serum available, five premenopausal and five postmenopausal women who lacked prebiopsy MD results for the contralateral breast within 1 year of biopsy, and four premenopausal and three postmenopausal participants who underwent bilateral breast biopsies (preventing assessment of a contralateral breast). This resulted in a final analytic population of 100 premenopausal 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 [29], we square-root transformed all MD measures to better approximate normal distributions.

Linear regression models were used to examine associations of serum EM concentrations with measures of MD

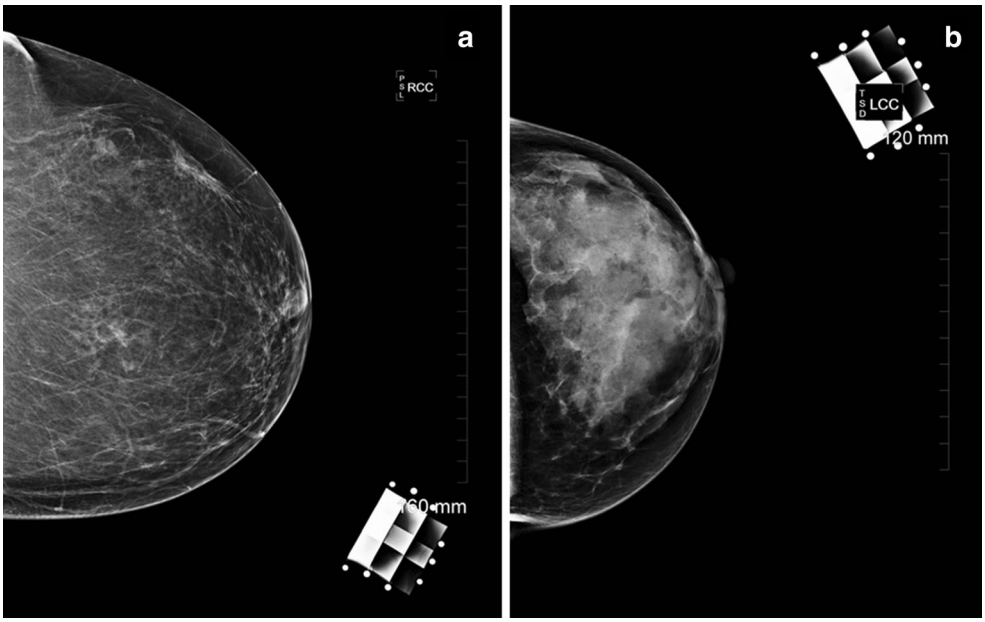

Fig. 2 Representative full-field digital mammograms from BREAST Stamp Project participants. The digital mammogram is acquired with the density phantom in the corner of the image to allow for automated computation of volume mammographic density (MD-V). In this example, **a** and **b** represent breasts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

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 premenopausal 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 premenopausal and 7 postmenopausal women). Residuals from linear models were assessed by a combination of visual inspection and the Anderson-Darling test 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 premenopausal versus postmenopausal women (25.9 (6.3) vs. 27.1 (6.3) kg/m^2). 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 premenopausal 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 premenopausal 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

Table 1 Baseline characteristics of the study population by menopausal status

	Premenopausal (n=100)		Postmenopausal (n=87)	
Continuous measures	Mean	SD	Mean	SD
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
Positive family history of breast cancer in a first 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
In situ**	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, 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[a]	Correlation with BMI[a]	Correlation with age[a]	Correlation with BMI[a]
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-OHE$_1$)	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**
16α-Hydroxyestrone (16α-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**

BMI body mass index (kg/m^2), *EM* estrogens and estrogen metabolites

[a] Spearman's correlation rho is presented. Correlation coefficients with p-values <0.05 are presented in bold font

were found between most EM and BMI (Table 2). The strongest correlation with BMI was observed for unconjugated E_1 (rho=0.57, p<0.0001).

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-OHE_1) relative to 16α-hydroxyestrone (16α-OHE_1) 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 E_3 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 E_1 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-OHE_1 to 16α-OHE_1 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 [23]. Nevertheless, we compared EM levels between participants diagnosed with breast cancer (n=7 premenopausal and 21 postmenopausal women) versus those diagnosed with benign breast disease (n=93 premenopausal 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 E_3. 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 E_3 and MD among luteal premenopausal women contrast with results from prior studies of premenopausal women, which measured urinary EM in the luteal phase [13, 15, 16]. Maskarinec et al. [15] reported an inverse association between EM in the 16-hydroxylation pathway, including E_3, and MD-A measures, whereas two other studies reported null associations [13, 16]. Maskarinec et al. also reported an elevated 2-OHE_1 to 16α-OHE_1 ratio among luteal phase women with higher MD [15], 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, intraindividual serum and urine EM have not been compared. Additionally, urinary EM are mostly found in conjugated form [30], whereas serum EM can be detected in both conjugated and unconjugated forms, the latter of which is thought to be more biologically active [24, 31].

Table 3 Linear regression estimates for EM (nmol/L) and mammographic density measures, premenopausal luteal phase women (n=36)

Estrogens and estrogen metabolites (EM)[a]	% 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*
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	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-Pathway catechols	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_1)	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-3methly 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	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
4-Pathway catechol: hydroxyestrone (4-OHE_1)	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
4-Pathway methylated catechols	**75.20**	**0.02**	47.30	0.13	**58.10**	**0.04**	16.40	0.50	16.80	0.62	30.10	0.42	−59.90	0.31	−1.90	0.97
4-Methoxyestrone (4-$MeOE_1$)	**97.60**	**0.01**	65.00	0.09	**79.30**	**0.03**	31.40	0.29	23.50	0.58	39.00	0.40	−69.60	0.34	−1.20	0.99
4-Methoxyestradiol (4-$MeOE_2$)	174.40	0.14	64.40	0.57	100.30	0.36	−55.60	0.53	24.20	0.85	61.10	0.66	−209.50	0.34	−15.30	0.94
16-Hydroxylation pathway EM	0.50	0.16	0.30	0.38	0.30	0.33	0.00	0.90	0.00	0.90	0.10	0.78	−0.40	0.50	−0.10	0.92
16α-Hydroxyestrone (16α-OHE_1)	2.50	0.42	−0.20	0.95	1.70	0.55	−1.80	0.41	−1.20	0.72	−0.60	0.87	−5.70	0.32	−1.30	0.81
Estriol (E_3)	0.50	0.16	0.40	0.30	0.30	0.34	0.10	0.69	0.10	0.79	0.20	0.71	−0.30	0.64	0.00	0.97
Conjugated	0.50	0.18	0.30	0.33	0.30	0.37	0.10	0.74	0.10	0.86	0.10	0.77	−0.40	0.59	−0.10	0.91
Unconjugated	17.80	0.09	**18.90**	**0.04**	12.60	0.19	14.00	0.05	**26.30**	**0.01**	**26.10**	**0.02**	35.90	0.06	34.10	0.05
17-Epiestriol (17-epiE_3)	36.90	0.05	23.50	0.18	27.60	0.10	8.90	0.51	13.50	0.50	19.00	0.37	−7.60	0.82	19.80	0.54
16-Ketoestradiol (16-ketoE_2)	1.60	0.59	−0.90	0.75	1.00	0.71	−2.20	0.29	−1.70	0.58	−1.20	0.71	−6.10	0.24	−2.40	0.63
16-Epiestriol (16-epiE_3)	6.20	0.52	−0.40	0.97	3.40	0.70	−5.20	0.44	−2.70	0.79	−1.20	0.91	−11.20	0.52	−0.40	0.98
Pathway ratios																
Parent EM/total EM	6.31	0.06	5.59	0.06	4.16	0.18	3.22	0.17	4.69	0.19	4.91	0.18	4.27	0.49	5.57	0.33
2-Hydroxylation pathway EM/parent EM	−1.08	0.60	−1.45	0.43	−0.50	0.79	−0.96	0.50	−1.28	0.56	−1.19	0.59	−3.09	0.41	−2.50	0.46

Table 3 (continued)

Estrogens and estrogen metabolites (EM)[a]	% 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*
4-Hydroxylation pathway EM/parent EM	−19.09	0.20	−18.36	0.16	−15.34	0.25	−14.42	0.15	−14.25	0.36	−14.44	0.36	−18.27	0.50	−19.50	0.43
16-Hydroxylation pathway EM/parent EM	**−2.88**	**0.03**	**−2.52**	**0.03**	−2.19	0.07	−1.72	0.06	−2.24	0.11	−2.37	0.09	−1.85	0.45	−2.52	0.25
2-Hydroxylation pathway EM/16-hydroxylation pathway EM	2.68	0.27	1.20	0.59	3.14	0.15	1.27	0.46	0.97	0.71	1.42	0.60	−2.46	0.58	0.09	0.98
2-Hydroxyestrone/16α-hydroxyestrone	0.42	0.56	0.39	0.55	0.39	0.56	0.34	0.49	0.61	0.42	0.62	0.42	1.05	0.42	1.11	0.35
4-Hydroxylation pathway EM/2-hydroxylation pathway EM	**−48.02**	**0.003**	**−35.50**	**0.03**	**−48.72**	**0.0008**	**−31.26**	**0.01**	−8.99	0.62	−15.02	0.44	35.56	0.26	8.59	0.78
4-Pathway catechol/4-pathway methylated catechols	**−0.30**	**0.04**	−0.24	0.07	−0.20	0.13	−0.12	0.25	−0.15	0.34	−0.17	0.29	0.12	0.65	0.01	0.98
2-Pathway catechols/2-pathway methylated catechols	−0.37	0.17	−0.34	0.15	−0.44	0.06	**−0.41**	**0.02**	−0.09	0.75	−0.10	0.74	0.06	0.90	0.01	0.98

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

[a] 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

In postmenopausal women, prior studies have used immunoassays to measure circulating estrogens (including estrone, estrone sulfate, estradiol, and free estradiol) in relation to MD [32–43], generally showing that estrogens are not related to MD after BMI adjustment [32, 33, 37–43]. Our findings are in agreement with three studies that found positive associations between estrone [34] and estradiol with MD-A [35, 36] and only one prior report that utilized the LC-MS/MS assay to measure this panel of EM but did so in urine [14]. 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 [14]. 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 [14]. This suggests that less extensive hydroxylation of parent estrogens is associated with elevated MD-A [14]. 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 [18, 19, 21].

Since BMI correlates strongly and positively with both the non-dense (i.e., fatty) and total breast areas and thus correlates inversely with percent MD [44], 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 [45] 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 [27]. 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

Table 4 Linear regression estimates for EM (nmol/L) and mammographic density measures, postmenopausal women (n=87)

Estrogens and estrogen metabolites (EM)[a]	% 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*
Total EM	−0.14	0.52	**0.40**	**0.03**	−0.17	0.26	0.15	0.29	0.09	0.69	**0.52**	**0.02**	**0.70**	**0.01**	0.42	0.19
Parent EM	−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
Estrone (E_1)	−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
Conjugated	−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
Unconjugated	**−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
Estradiol (E_2)	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
Conjugated	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
Unconjugated	−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	−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-Pathway catechols	−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-Hydroxyestrone (2-OHE_1)	−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-Hydroxyestradiol (2-OHE_2)	−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-Pathway methylated catechols	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
2-Methoxyestrone (2-$MeOE_1$)	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
Conjugated	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
Unconjugated	−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
2-Methoxyestradiol (2-$MeOe_2$)	−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
Conjugated	−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
Unconjugated	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
2-Hydroxyestrone-3methly ether (3-$MeOe_1$)	−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
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	−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
16α-Hydroxyestrone (16α-OHE_1)	−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
Estriol (E_3)	−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
Conjugated	−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
Unconjugated	−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
17-Epiestriol (17-epiE_3)	−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-Ketoestradiol (16-ketoE_2)	−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
16-Epiestriol (16-epiE_3)	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

Table 4 (continued)

Estrogens and estrogen metabolites (EM)[a]	% 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*
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/16α-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

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

[a] 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

by the inverse association between BMI and absolute MD-A in contrast to the positive association between BMI and absolute MD-V [23, 27, 46–49]. For SXA in particular, water contained in adipose tissue contributes to the absolute MD-V measure [27]. 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-OHE_1 to 16α-OHE_1 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 [50]. Whereas prior studies relating the 2-OHE_1 to 16α-OHE_1 ratio to MD-A among postmenopausal women have reported null [14] or positive [17] associations, our findings are consistent with the inverse associations reported in LC-MS/MS studies of breast cancer risk [19, 21].

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 [2, 51]. Strengths of our study include the utilization of a precise LC-MS/MS EM assay with demonstrated reliability in both premenopausal and postmenopausal women [24, 31] 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 [52], 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 premenopausal [31] and postmenopausal women [18, 19, 31].

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.

Acknowledgments The authors are indebted to the participants in the BREAST Stamp Project for their outstanding cooperation and to the physicians, pathologists, nurses, technologists, and interviewers for their efforts in the field. The authors thank Clair Bove, Patricia Lutton, Ellen Young, Aileen Burke, Laura Linville, and Daphne Papathomas for research assistance. We also thank Janet Lawler-Heaver and Kerry Grace Morrissey from Westat for study management support and Jane Demuth at Information Management Services for data support and analysis.

Funding This study was supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics of the National Cancer Institute and National Cancer Institute federal funds awarded under Contract No. HHSN261200800001E to SAIC-Frederick, Inc. Breast Cancer Research Stamp Funds and cooperative agreement U01CA70013 (B.M. Geller, P.M. Vacek, D.L. Weaver, R.E. Chicoine, S.D. Herschorn) and 1R21CA157254 (J.A. Shepherd, B. Fan, A.P. Mahmoudzadeh) from the National Cancer Institute funded some of the data collection and image analysis for this study. Grant number M01 RR000109 from the National Center for Research Resources funded the blood processing at the University of Vermont's General Clinical Research Center. 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 organizations imply endorsement by the US government.

Conflict of Interest S.D. Herschorn is a stockholder in Hologic.

References

1. McCormack VA, dos Santos Silva I (2006) Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev 15(6):1159–1169. doi:10.1158/1055-9965.epi-06-0034
2. Martin LJ, Boyd NF (2008) Mammographic density—potential mechanisms of breast cancer risk associated with mammographic density: hypotheses based on epidemiological evidence. Breast Cancer Res 10(1):201
3. Greendale GA, Reboussin BA, Slone S, Wasilauskas C, Pike MC, Ursin G (2003) Postmenopausal hormone therapy and change in mammographic density. J Natl Cancer Inst 95:30–37
4. Cuzick J, Warwick J, Pinney E, Duffy SW, Cawthorn S, Howell A, Forbes JF, Warren RML (2011) Tamoxifen-induced reduction in mammographic density and breast cancer risk reduction: a nested case–control study. J Natl Cancer Inst 103:744–752. doi:10.1093/jnci/djr079
5. Yager JD, Davidson NE (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354(3):270–282
6. Boyd NF, Lockwood GA, Byng JW, Tritchler DL, Yaffe MJ (1998) Mammographic densities and breast cancer risk. Cancer Epidemiol Biomarkers Prev 7(12):1133–1144
7. Sun X, Gierach GL, Sandhu R, Williams T, Midkiff BR, Lissowska J, Wesolowska E et al (2013) Relationship of mammographic density and gene expression: analysis of normal breast tissue surrounding breast cancer. Clin Cancer Res 19(18):4972–4982. doi:10.1158/1078-0432.CCR-13-0029
8. Hong CC, Tang BK, Rao V, Agarwal S, Martin L, Tritchler D, Yaffe M, Boyd NF (2004) Cytochrome P450 1A2 (CYP1A2) activity, mammographic density, and oxidative stress: a cross-sectional study. Breast Cancer Res 6(4):R338–R351
9. Haakensen V, Biong M, Lingjaerde O, Holmen M, Frantzen J, Chen Y, Navjord D et al (2010) Expression levels of uridine 5′-diphosphoglucuronosyltransferase genes in breast tissue from healthy women are associated with mammographic density. Breast Cancer Res 12(4):R65
10. Key TJ, Appleby PN, Reeves GK, Travis RC, Alberg AJ, Barricarte A, Berrino F et al (2013) Sex hormones and risk of breast cancer in premenopausal women: a collaborative reanalysis of individual participant data from seven prospective studies. Lancet Oncol 14(10):1009–1019. doi:10.1016/s1470-2045(13)70301-2
11. Key T, Appleby P, Barnes I, Reeves G, Endogenous Hormones, and Breast Cancer Collaborative Group (2002) Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 94(8):606–616
12. Becker S, Kaaks R (2009) Exogenous and endogenous hormones, mammographic density and breast cancer risk: can mammographic density be considered an intermediate marker of risk? Recent Results Cancer Res 181:135–157
13. Bertrand KA, Eliassen AH, Hankinson SE, Gierach GL, Xu X, Rosner B, Ziegler RG, Tamimi RM (2012) Urinary estrogens and estrogen metabolites and mammographic density in premenopausal women. Breast Cancer Res Treat 136(1):277–287. doi:10.1007/s10549-012-2240-0
14. Fuhrman BJ, Brinton LA, Pfeiffer RM, Xu X, Veenstra TD, Teter BE, Byrne C et al (2012) Estrogen metabolism and mammographic density in postmenopausal women: a cross-sectional study. Cancer Epidemiol Biomarkers Prev 21(9):1582–1591. doi:10.1158/1055-9965.EPI-12-0247
15. Maskarinec G, Heak S, Morimoto Y, Custer L, Franke AA (2012) The relation of urinary estrogen metabolites with mammographic densities in premenopausal women. Cancer Epidemiol 36(5):e310–e316. doi:10.1016/j.canep.2012.03.014
16. Maskarinec G, Williams AE, Rinaldi S, Kaaks R (2005) Mammographic densities and urinary hormones in healthy women with different ethnic backgrounds. In: Li J, Li S, Llombart-Bosch A (eds) Hormonal carcinogenesis IV. New York, Springer, pp 277–286
17. Riza E, dos Santos Silva I, De Stavola B, Bradlow HL, Sepkovic DW, Linos D, Linos A (2001) Urinary estrogen metabolites and mammographic parenchymal patterns in postmenopausal women. Cancer Epidemiol Biomarkers Prev 10(6):627–634
18. Falk RT, Brinton LA, Dorgan JF, Fuhrman BJ, Veenstra TD, Xu X, Gierach GL (2013) Relationship of serum estrogens and estrogen metabolites to postmenopausal breast cancer risk: a nested case–control study. Breast Cancer Res 15(2):R34. doi:10.1186/bcr3416
19. Dallal CM, Tice JA, Buist DS, Bauer DC, Lacey JV Jr, Cauley JA, Hue TF et al (2014) Estrogen metabolism and breast cancer risk among postmenopausal women: a case-cohort study within B~FIT. Carcinogenesis 35(2):346–355. doi:10.1093/carcin/bgt367
20. Eliassen AH, Spiegelman D, Xu X, Keefer LK, Veenstra TD, Barbieri RL, Willett WC, Hankinson SE, Ziegler RG (2011) Urinary estrogens and estrogen metabolites and subsequent risk of breast cancer among premenopausal women. Cancer Res 72(3):696–706. doi:10.1158/0008-5472.can-11-2507
21. Fuhrman BJ, Schairer C, Gail MH, Boyd-Morin J, Xu X, Sue LY, Buys SS et al (2012) Estrogen metabolism and risk of breast cancer in postmenopausal women. J Natl Cancer Inst 104(4):326–339. doi:10.1093/jnci/djr531
22. Yaffe M (2008) Mammographic density. Measurement of mammographic density. Breast Cancer Res 10(3):209
23. Gierach GL, Geller BM, Shepherd JA, Patel DA, Vacek PM, Weaver DL, Chicoine RE et al (2014) Comparison of mammographic density assessed as volumes and areas among women undergoing diagnostic image-guided breast biopsy. Cancer Epidemiol Biomarkers Prev 23(11):2338–2348. doi:10.1158/1055-9965.EPI-14-0257
24. Xu X, Roman JM, Issaq HJ, Keefer LK, Veenstra TD, Ziegler RG (2007) Quantitative measurement of endogenous estrogens and estrogen metabolites in human serum by liquid chromatography-tandem mass spectrometry. Anal Chem 79(20):7813–7821

25. Prevrhal S, Shepherd JA, Smith-Bindman R, Cummings SR, Kerlikowske K (2002) Accuracy of mammographic breast density analysis: results of formal operator training. Cancer Epidemiol Biomarkers Prev 11(11):1389–1393
26. Byng JW, Boyd NF, Fishell E, Jong RA, Yaffe MJ (1994) The quantitative analysis of mammographic densities. Phys Med Biol 39(10): 1629–1638
27. Shepherd JA, Kerlikowske K, Ma L, Duewer F, Fan B, Wang J, Malkov S, Vittinghoff E, Cummings SR (2011) Volume of mammographic density and risk of breast cancer. Cancer Epidemiol Biomarkers Prev 20(7):1473–1482. doi:10.1158/1055-9965.EPI-10-1150
28. Malkov S, Wang J, Kerlikowske K, Cummings SR, Shepherd JA (2009) Single x-ray absorptiometry method for the quantitative mammographic measure of fibroglandular tissue volume. Med Phys 36(12):5525–5536
29. Box GEP, Cox DR (1964) An analysis of transformations. J R Stat Soc 26:211–522
30. Falk RT, Xia X, Keefer L, Veenstra TD, Ziegler RG (2008) A liquid chromatography-mass spectrometry method for the simultaneous measurement of 15 urinary estrogens and estrogen metabolites: assay reproducibility and interindividual variability. Cancer Epidemiol Biomarkers Prev 17(12):3411–3418. doi:10.1158/1055-9965.epi-08-0355
31. Fuhrman BJ, Xu X, Falk RT, Dallal CM, Veenstra TD, Keefer LK, Graubard BI, Brinton LA, Ziegler RG, Gierach GL (2014) Assay reproducibility and interindividual variation for 15 serum estrogens and estrogen metabolites measured by liquid chromatography-tandem mass spectrometry. Cancer Epidemiol Biomarkers Prev 23(12):2649–2657
32. Aiello EJ, Tworoger SS, Yasui Y, Stanczyk FZ, Potter J, Ulrich CM, Irwin M, McTiernan A (2005) Associations among circulating sex hormones, insulin-like growth factor, lipids, and mammographic density in postmenopausal women. Cancer Epidemiol Biomarkers Prev 14(6):1411–1417
33. Boyd NF, Stone J, Martin LJ, Jong R, Fishell E, Yaffe M (2002) The association of breast mitogens with mammographic densities. Br J Cancer 87:876–882
34. Bremnes Y, Ursin G, Bjurstam N, Rinaldi S, Kaaks R, Gram IT (2007) Endogenous sex hormones, prolactin and mammographic density in postmenopausal Norwegian women. Int J Cancer 121: 2506–2511
35. Greendale GA, Palla SL, Ursin G, Laughlin GA, Crandall C, Pike MC, Reboussin BA (2005) The association of endogenous sex steroids and sex steroid binding proteins with mammographic density: results from the postmenopausal estrogen/progestin interventions mammographic density study. Am J Epidemiol 162(9):826–834
36. Johansson H, Gandini S, Bonanni B, Mariette F, Guerrieri-Gonzaga A, Serrano D, Cassano E et al (2008) Relationships between circulating hormone levels, mammographic percent density and breast cancer risk factors in postmenopausal women. Breast Cancer Res Treat 108(1):57–67
37. McCormack VA, Dowsett M, Folkerd E, Johnson N, Palles C, Coupland B, Holly JM, Vinnicombe SJ, Perry NM, dos Santos Silva I (2009) Sex steroids, growth factors and mammographic density: a cross-sectional study of UK postmenopausal Caucasian and Afro-Caribbean women. Breast Cancer Res 11(3):R38. doi:10.1186/bcr2325
38. Schoemaker MJ, Folkerd EJ, Jones ME, Rae M, Allen S, Ashworth A, Dowsett M, Swerdlow AJ (2014) Combined effects of endogenous sex hormone levels and mammographic density on postmenopausal breast cancer risk: results from the Breakthrough Generations Study. Br J Cancer 110(7):1898–1907. doi:10.1038/bjc.2014.64
39. Sprague BL, Trentham-Dietz A, Gangnon RE, Buist DS, Burnside ES, Bowles EJ, Stanczyk FZ, Sisney GS (2011) Circulating sex hormones and mammographic breast density among postmenopausal women. Horm Cancer 2(1):62–72. doi:10.1007/s12672-010-0056-0
40. Tamimi RM, Hankinson SE, Colditz GA, Byrne C (2005) Endogenous sex hormone levels and mammographic density among postmenopausal women. Cancer Epidemiol Biomarkers Prev 14(11): 2641–2647
41. Verheus M, Peeters PH, van Noord PA, van der Schouw YT, Grobbee DE, van Gils CH (2007) No relationship between circulating levels of sex steroids and mammographic breast density: the Prospect-EPIC cohort. Breast Cancer Res 9(4):R53
42. Warren R, Skinner J, Sala E, Denton E, Dowsett M, Folkerd E, Healey CS et al (2006) Associations among mammographic density, circulating sex hormones, and polymorphisms in sex hormone metabolism genes in postmenopausal women. Cancer Epidemiol Biomarkers Prev 15(8):1502–1508. doi:10.1158/1055-9965.epi-05-0828
43. Woolcott CG, Courneya KS, Boyd NF, Yaffe MJ, McTiernan A, Brant R, Jones CA et al (2013) Association between sex hormones, glucose homeostasis, adipokines, and inflammatory markers and mammographic density among postmenopausal women. Breast Cancer Res Treat 1–11. doi:10.1007/s10549-013-2534-x
44. Vachon C, van Gils C, Sellers T, Ghosh K, Pruthi S, Brandt K, Shane Pankratz V (2007) Mammographic density, breast cancer risk and risk prediction. Breast Cancer Res 9(6):217
45. Siiteri PK (1987) Adipose tissue as a source of hormones. Am J Clin Nutr 45(1):277–282
46. Aitken Z, McCormack VA, Highnam RP, Martin L, Gunasekara A, Melnichouk O, Mawdsley G et al (2010) Screen-film mammographic density and breast cancer risk: a comparison of the volumetric standard mammogram form and the interactive threshold measurement methods. Cancer Epidemiol Biomark Prev 19(2):418–428. doi:10.1158/1055-9965.epi-09-1059
47. Jeffreys M, Warren R, Highnam R, Davey Smith G (2008) Breast cancer risk factors and a novel measure of volumetric breast density: cross-sectional study. Br J Cancer 98(1):210–216. doi:10.1038/sj.bjc.6604122
48. Lokate M, Kallenberg MG, Karssemeijer N, Van den Bosch MA, Peeters PH, Van Gils CH (2010) Volumetric breast density from full-field digital mammograms and its association with breast cancer risk factors: a comparison with a threshold method. Cancer Epidemiol Biomarkers Prev 19(12):3096–3105. doi:10.1158/1055-9965.EPI-10-0703
49. McCormack VA, Highnam R, Perry N, dos Santos Silva I (2007) Comparison of a new and existing method of mammographic density measurement: intramethod reliability and associations with known risk factors. Cancer Epidemiol Biomarkers Prev 16(6):1148–1154. doi:10.1158/1055-9965.EPI-07-0085
50. Fishman J, Schneider J, Hershcope RJ, Bradlow HL (1984) Increased estrogen-16 alpha-hydroxylase activity in women with breast and endometrial cancer. J Steroid Biochem 20(4B):1077–1081
51. Tamimi RM, Byrne C, Colditz GA, Hankinson SE (2007) Endogenous hormone levels, mammographic density, and subsequent risk of breast cancer in postmenopausal women. JNCI 99(15):1178–1187. doi:10.1093/jnci/djm062
52. Loud JT, Gierach GL, Veenstra TD, Falk RT, Nichols K, Guttmann A, Xu X, Greene MH, Gail MH (2014) Circulating estrogens and estrogens within the breast among postmenopausal BRCA1/2 mutation carriers. Breast Cancer Res Treat 143(3):517–529. doi:10.1007/s10549-013-2821-6