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Circulating estrogens and estrogens within the breast among postmenopausal *BRCA1/2* mutation carriers

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Abstract

Accurately quantifying parent estrogens (PE) estrone (E_1) and estradiol (E_2) and their metabolites (EM) within breast tissue and serum may permit detailed investigations of their contributions to breast carcinogenesis among *BRCA1/2* mutation carriers. We conducted a study of PE/EM in serum, nipple aspirate fluid (NAF), and ductal lavage supernatant (DLS) among postmenopausal *BRCA1/2* mutation carriers. PE/EM (conjugated and unconjugated) were measured in paired serum/NAF ($n = 22$ women) and paired serum/DLS samples ($n = 24$ women) using quantitative liquid chromatography–tandem mass spectrometry (LC/MS/MS). The relationships between serum and tissue-specific PE/EM were measured using Pearson's correlation coefficients. Conjugated forms of PE/EM constituted the majority of estrogen in serum (88 %), NAF (59 %) and DLS (69 %). PE/EM in NAF and serum were highly correlated [E_1 ($r = 0.97$, $p < 0.0001$), E_2 ($r = 0.90$, $p < 0.0001$) and estriol (E_3) ($r = 0.74$, $p < 0.0001$)] as they were in DLS and serum [E_1 ($r = 0.92$, $p < 0.0001$; E_2 ($r = 0.70$, $p = 0.0001$; E_3 ($r = 0.67$, $p = 0.0004$)]. Analyses of paired total estrogen values for NAF and serum, and DLS and serum yielded ratios of 0.22 (95 % CI 0.19–0.25) and 0.28 (95 % CI 0.24–0.32), respectively. This report is the first to employ LC/MS/MS to quantify PE/EM in novel breast tissue-derived biospecimens (i.e., NAF and DLS). We demonstrate that circulating PE and EM are strongly and positively correlated with tissue-specific PE and EM measured in NAF and DLS among post-menopausal *BRCA1/2* mutation carriers. If confirmed, future etiologic studies could utilize the more readily obtainable serum hormone levels as a reliable surrogate measure of exposure at the tissue level.

Keywords

BRCA1/2; Estrogens; Parent estrogens; Estrogen metabolites; Nipple aspirate fluid; Ductal lavage supernatant; LC/MS/MS; Postmenopausal

Introduction

Epidemiological studies of endogenous sex steroid hormone levels [1–3] and randomized clinical trials of exogenous estrogen use [4–7] have demonstrated a role for estrogen in breast cancer carcinogenesis. In addition, evidence from animal models and cultured cell lines has supported the hypothesis that specific oxidative metabolites of estrogen have genotoxic, mutagenic, and carcinogenic potential [8–16] (Fig. 1). Estrogen metabolism entails hydroxylation of the parent estrogens (estrone and estradiol) at sites C2, C4, or C16, followed by conjugation (primarily via sulfation, glucuronidation, and methylation). Estrogen metabolites (EM) formed as a result of the 4- and 16-hydroxylation pathway have genotoxic properties, while EM produced by the 2-hydroxylation pathway are thought to inhibit tumorigenesis and are detectable in serum of postmenopausal women [7, 17–19]. Evidence also suggests that parent estrogens (PE [estrone (E_1) and estradiol (E_2)] and their EM are detectable in human breast tissues, both histologically normal and malignant, supporting the hypotheses that carcinogenic pathways exist within human breast tissue [20], and that twofold higher levels of both the 2- and 4-hydroxyestrogens can be detected within breast cancer tumors compared with normal breast tissue [21]. Growing evidence suggests that alterations in estrogen metabolism may lead to elevated serum and urine estrogen–DNA adduct levels, which are also associated with breast cancer in women at high risk (i.e., Gail Model score >1.66 %) and in women diagnosed with breast cancer compared with normal-risk women [16, 22].

In postmenopausal women, elevated levels of sex steroid hormones are linked to breast cancer risk, and accumulating evidence suggests that the contribution of intracrine estrogen production within the breast may be a critical factor in tumorigenesis [23]. In postmenopausal women, biologically active sex hormones are synthesized primarily in

target tissues, including the breast, although the regulation of enzymes involved in this process is complex and not well understood [24]. Further, the relationship between circulating estrogens and estrogens within the breast is not well-established. To date, research results across studies evaluating the relationship between E_1 and E_2 levels in breast tissue (normal, fatty, malignant) compared with concentrations of E_1 and E_2 in serum are inconsistent for both premenopausal and postmenopausal women [23, 24].

The role of estrogen metabolites in *BRCA1/2*-related breast carcinogenesis is complex and has not been well described [25, 26]. Estrogen receptor (ER)- α has been reported to activate *BRCA1* expression within the breast and to provide protection against DNA damage and genome instability in ER- α positive breast cells. In women who carry a *BRCA1* mutation, the loss of *BRCA1* function in precancerous cells could lead to an elevated risk of breast cancer by increasing genomic instability within the background of impaired DNA repair function.

In addition, an etiologic role for some genotoxic variants of estrogen has been suggested for *BRCA1*-related breast carcinogenesis, with estrogen receptor (ER)- α -negative breast cancers arising from the basal rather than luminal epithelium [8, 25–27]. In this model, *BRCA1* function is required for the differentiation of ER- α -negative stem/progenitor cells to ER- α -positive luminal cells. However, more recent work has identified an aberrant luminal progenitor population in *BRCA1* mutation carriers which may serve as a target for oncogenic events [28]. Similar work in postmenopausal women who are known *BRCA2* mutation carriers has not been reported, although *BRCA1* and *BRCA2* work in a common pathway of genome protection [29].

Nipple aspirate fluid (NAF) and ductal lavage offer means to directly evaluate the ductal microenvironment of the breast, where the majority of breast cancers arise. Data on the relationship between hormones in NAF, ductal lavage supernatant (DLS), and hormone concentrations in serum are inconsistent in both pre- and postmenopausal women [23, 30–32]. NAF estrogen concentrations have been reported as being higher than serum estrogen levels, and some studies have suggested a greater postmenopausal decline in serum estrogen levels than in NAF [31, 33, 34]. Most recently, correlations between serum and NAF estrogen levels in healthy postmenopausal women presenting for routine breast cancer surveillance ($n = 18$) were reported as low (E_1 , $r = -0.23$ [$P = 0.25$] E_2 , $r = 0.14$ [$P = 0.47$]) [31, 34]. The correlation between circulating and intra-breast levels (e.g., in NAF or DLS) has not been evaluated in *BRCA1/2* mutation-positive women.

To better understand the relationship between estrogens in serum and breast fluids, we conducted a cross-sectional feasibility study comparing PE and EM in serum, NAF, and DLS among postmenopausal, *BRCA1/2* mutation-positive women enrolled in the U.S. National Cancer Institute, Clinical Genetics Branch's Breast Imaging Study. Previous work has demonstrated the sensitivity, specificity, and reproducibility of LC/MS/MS methods in detecting TE, PE, and EM in urine and serum of postmenopausal women [35, 36]. The use of this sensitive and specific high-performance LC/MS/MS assay method provided the opportunity to quantify PE and an additional 13 EM in a single assay in serum, NAF, and DLS [36].

Materials and methods

Study population

The National Cancer Institute (NCI), Clinical Genetic Branch's Breast Imaging Study (BIS, NCI Protocol #01-C-0009; NCT-00012415) was a prospective assessment (2001–2008) of breast cancer screening modalities in women from families with known *BRCA1/2* mutations

[37, 38]. Participants were seen annually for 4 years, and underwent a physical examination, breast MRI, and a standard four-view mammogram at each visit. Nipple aspiration and ductal lavage (DL) were attempted at each visit. 200 women ages 25–56 years enrolled in this study, including 170 women with deleterious *BRCA1/2* mutations and 30 mutation-negative women from the same families. As previously reported [39], 171 participants underwent nipple aspiration and ductal lavage but only 45 yielded NAF (23 *BRCA* mutation carriers, 22 non-carriers) during the first visit and only 70 (57 *BRCA* mutation carriers, 13 non-carriers) had ductal lavage with adequate cell counts for cytological evaluation. 13 of the 30 mutation-negative women were postmenopausal at enrollment, but so few yielded NAF ($n = 3$) or were successfully cannulated for DL ($n = 8$), that the present analysis was restricted to *BRCA1/2* mutation carriers with adequate samples for the assay. This study was approved by the NCI Institutional Review Board (IRB), and written informed consent was obtained from all participants.

For the purposes of this study, all *BRCA1* or *BRCA2* mutation-positive, postmenopausal (defined as spontaneous amenorrhea for at least 12 months or documented bilateral oophorectomy) women with sufficient volumes of serum, NAF, and/or DLS specimens (> 0.5 mL), were eligible for inclusion. Serum, NAF, and/or DLS were collected at the same visit; however, both NAF and DLS could not always be collected from each participant.

Biological specimen collection and processing

Whole blood samples were collected at each visit using standard techniques, allowed to clot for 30 min, centrifuged at 3,000 rpm for 15 min, and the serum fraction aliquoted into 1 mL test tubes and frozen at -80°C . NAF was collected with a “clean” technique using sterile gloves to handle NAF instruments to avoid contamination of NAF specimens. After standard breast preparation [39], nipple aspiration was performed using a nipple aspirator (First-Cyte™; Cytoc Health Corporation) to identify all fluid-yielding ducts. Two methods of NAF collection were employed over the course of the study. Initially (2001–2007), visible NAF was collected with a 10- μL glass capillary tube; the tube was sealed and placed in a 10 cc test tube and frozen at -80°C ; 10 subjects’ samples in the current study were collected in this manner. Beginning in 2008, visible NAF was collected with a 10- μL glass capillary tube and up to 10- μL of NAF was immediately transferred to a 1 mL Nunc tube filled with 0.5 mL sterile normal saline; 12 subjects’ samples were collected in this way. An estimation of the quantity of NAF was made by recording the volume of NAF within the 10- μL glass capillary tube for both methods. When possible, each NAF-yielding duct was collected separately and placed into separate Nunc tubes. When NAF flowed freely from multiple ducts simultaneously, NAF was collected from the multiple ducts and processed in the same manner as NAF from a single duct.

A standardized ductal lavage procedure was attempted in all research participants [40]. Even when visible NAF was not produced by aspiration, an attempt was made to sample visually identifiable ducts with a microdilator, followed by cannulation (First-Cyte™; Cytoc Health Corporation). The locations of NAF-yielding ducts and non-NAF/cannulated ducts were recorded [39]. If catheter insertion was successful, 3–5 mL of 1 % lidocaine was infused into the duct, followed by approximately 20 mL of sterile normal saline, in incremental 5 mL aliquots. After each 5 mL aliquot was infused, breast massage was performed, and ductal fluid collected via the lavage catheter. Volumes of recovered lavage varied considerably across all participants. Recovered ductal lavage fluid volume was measured, and 20 % of the sample was placed in a vial for cell count and cytologic analysis. Cell count varied considerably across the total study population [39]. The remaining specimen was centrifuged at 2,000 rpm, and the DLS and pellet were separated. DLS was stored in 1 mL

test tubes and frozen for future research studies. Samples were placed on dry ice within 20–60 min after collection and stored at -80°C .

Analysis of estrogens in serum, DLS, and NAF

Reagents and materials

Using methods previously described [36], parent estrogens (E_1), (E_2), and 13 estrogen metabolites (EM) [2-hydroxyestrone (2-OHE₁), 2-methoxyestrone (2-MeOE₁), 2-hydroxyestradiol (2-OHE₂), 2-methoxyestradiol (2-MeOE₂), 2-hydroxyestrone-3-methyl ether (3-MeOE₁), 4-hydroxyestrone (4-OHE₁), 4-methoxyestrone (4-MeOE₁), 4-methoxyestradiol (4-MeOE₂), 16 α -hydroxyestrone (16 α -OHE₁), 17-epiestriol (17-epiE₃), 16-ketoestradiol (16-ketoE₂), 16-epiestriol (16-epiE₃), and estriol (E_3)] were quantified. Standard samples for each estrogen metabolite were obtained from Steraloids, Inc. (Newport, RI, USA), while stable isotope-labeled estrogens (SI-EM), estrone-13,14,15,16,17,18- $^{13}\text{C}_6$ (estrone- $^{13}\text{C}_6$) and 17 β -estradiol-13,14,15,16,17,18- $^{13}\text{C}_6$ (17 β -estradiol- $^{13}\text{C}_6$) were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Estriol-2,4,17- d_3 (d_3 -E₃), 2-hydroxy-estradiol-1,4,16,16,17- d_5 (d_5 -2-OHE₂), and 2-methoxyestradiol-1,4,16,16,17- d_5 (d_5 -2-MeOE₂), were purchased from C/D/N Isotopes, Inc. (Pointe-Claire, Quebec, Canada). A sixth SI-EM, 16-epiestriol-2,4,16- d_3 (d_3 -16-epiE₃), was obtained from Medical Isotopes, Inc. (Pelham, NH, USA). All EM and SI-EM have reported chemical and isotopic purity 98 %, and were used without further purification.

Sample preparation procedure

Prior to the sample preparation, NAF specimens collected with capillary tubes were first sonicated in acetone/ethyl alcohol (1:1 v/v) for 30 min to quantitatively recover estrogens and estrogen metabolites from the glass capillaries followed by lyophilization to dryness, and were then reconstituted in 100 μL ethanol and 900 μL of 0.15 M sodium acetate buffer (pH 4.6). In addition, 0.5 mL normal saline was added to NAF samples collected in Nunc tubes to bring their volume to 1 mL as well. For serum, DLS, and NAF, the sample preparation procedures distinguished unconjugated and total estrogens (TE = conjugated + unconjugated) using techniques previously described [36]. To a single aliquot, 5 μL of SI-EM working internal standard solution was added. This sample was split into two separate 0.4 mL aliquots, one for total EM and the other for unconjugated EM. For measuring total EM, 0.5 mL of freshly prepared enzymatic hydrolysis buffer containing 2 mg of L-ascorbic acid, 5 μL of β -glucuronidase/sulfatase, and 0.5 mL of 0.15 M sodium acetate buffer (pH 4.6) were added. The sample was incubated 20 h at 37°C . For measuring unconjugated EM, a 0.5 mL aliquot of hydrolysis buffer, without β -glucuronidase/ sulfatase, was added to the second 0.4 mL aliquot. Both aliquots then underwent slow inverse extraction at 8 rpm (RKVSD^{TM}, ATR, Inc., Laurel, MD) with 5 mL dichloro-methane for 30 min. After extraction, the aqueous layer was discarded and the organic solvent portion was transferred into a clean glass tube and evaporated to dryness at 60°C under nitrogen gas (Reacti-Vap III^{TM}, Pierce, Rockford, IL, USA). To each NAF sample, 32 μL of 0.1 M sodium acetate solution (pH at 9.0) and 32 μL of dansyl chloride solution (1 mg/mL in acetone) were added. After vortexing, the sample was heated at 60°C (Reacti-Therm III^{TM} Heating Module, Pierce, Rockford, IL, USA) for 5 min to form the EM and SI-EM dansyl derivatives (EM-Dansyl and SI-EM-Dansyl, respectively). Calibration standards and quality control samples were hydrolyzed (or not hydrolyzed), extracted, and derivatized following the same procedure as that used for unknown samples. After derivatization, 8 μL (1/8 of final volume) of each sample was injected and analyzed by a capillary LC/MS/MS. Details of the LC/MS/MS method utilized for the analysis of serum, DLS, and NAF have been published previously [36].

Quantitation of estrogens and estrogen metabolites (EM)

Quantitation of estrogens and estrogen metabolites was carried out using Xcalibur™ Quan Browser (ThermoFisher). Calibration curves for each EM were constructed by plotting EM-Dansyl/SI-EM-Dansyl peak area ratios obtained from calibration standards versus amounts of EM and fitting these data using linear regression with 1/X weighting. The amount of EM in serum, DLS, or NAF samples was then interpolated using this linear function. Deuteriums at the α -position to the carbonyl group of the stable isotope-labeled ketolic estrogens were especially susceptible to exchange loss during sample preparation and analysis. To insure the quality of quantitative analyses, only stable isotope-labeled EM without exchange loss were employed in this study. Based on their similarity of structures and retention times, estrone- $^{13}\text{C}_6$ was used as the internal standard for estrone; 17β -estradiol- $^{13}\text{C}_6$ for E_2 ; d_3 - E_3 for E_3 , 16-keto E_2 , and 16 α -OHE $_1$; d_3 -16-epi E_3 for 16-epi E_3 and 17-epi E_3 ; d_5 -2-MeOE $_2$ for 2-MeOE $_2$, 4-MeOE $_2$, 2-MeOE $_1$, 4-MeOE $_1$, and 3-MeOE $_1$; d_5 -2-OHE $_2$ for 2-OHE $_2$, 2-OHE $_1$, and 4-OHE $_1$, respectively [36].

The assay accuracy and intra- and inter-batch precision were evaluated by measuring PE and 13 EM in duplicate charcoal-stripped human sera at three levels, 0.5, 20, 250 pg/mL (Supplementary Table, available online). The limit of detection (LOD) for each estrogen and estrogen metabolite in this LC-MS assay was 10 fg on column. The reproducibility of the assay for serum ($n = 9$ individuals with replicate serum specimens) and DLS ($n = 5$), and NAF ($n = 3$) was evaluated in a masked fashion. The intra-class correlation coefficients (ICC) for the raw PE and EM values assessed in masked, paired serum/NAF, and serum/DLS specimens ranged from 0.98 to 0.99, indicating excellent reproducibility.

Assessment of breast cancer risk factors and covariates

Participants completed self-administered questionnaires that captured data on factors known to influence both estrogen levels and breast cancer risk, including: age, age at menarche, parity, age at menopause, surgical menopause, family and personal history of breast cancer, history of breast biopsy, and exogenous hormone use. Measured height and weight were used to calculate body mass index (BMI, kg/m^2).

Statistical Analysis

PE and EM measures were $\log_e$ transformed and geometric means were calculated. Pearson correlation coefficients were used to examine the strength of the association between $\log_e$ -transformed EM levels measured in serum with those measured in NAF or DLS. Each PE and EM was assessed separately. We examined whether relationships between circulating and intra-breast PE and EM differed between *BRCA1* and *BRCA2* mutation carriers; results were similar, and are therefore reported here for *BRCA1* and *BRCA2* mutation carriers combined. Nine women contributed both paired NAF-serum and DLS-serum samples in separate years. Analyses examining PE and EM serum concentrations were restricted to the earliest serum sample from each woman ($n = 37$). However, each NAF or DLS was paired with a serum sample from the same time point. We also examined whether associations differed by NAF collection method. The proportions of various EM were calculated for each woman, and the averages of these proportions were reported. Confidence intervals for the ratios of PE and EM levels in NAF were computed by analyzing the paired differences of $\log_e(\text{PE})$ minus $\log_e(\text{EM})$. The ratios and their confidence intervals were obtained by exponentiation of the mean difference of the logarithms and its confidence interval. Likewise, the ratio and its confidence interval of total estrogen (TE) in DLS compared with serum were obtained from the paired differences in $\log_e(\text{TE})$ values. Because these are exploratory analyses within a pilot study, the alpha level was not adjusted for multiple comparisons. Probability values of <0.05 were considered statistically significant. All tests

of significance were two-tailed. Analyses were performed using SAS software release 9.2 (SAS Institute Inc., Cary, NC, USA).

Results

The characteristics of the study participants are shown in Table 1. A total of 37 participants were included; 22 had paired NAF and serum measures ($n = 16$ *BRCA1*; $n = 6$ *BRCA2*) and 24 had paired DLS and serum measures ($n = 16$ *BRCA1*; $n = 8$ *BRCA2*). In general, characteristics of *BRCA1/2* mutation carriers contributing paired NAF-serum samples were comparable to those with paired DLS-serum samples. Both groups had an average BMI of ~ 26 kg/m² and an average age at menopause of ~ 42 years; the vast majority of study participants had undergone risk-reducing salpingo-oophorectomy (RRSO) and were in surgical menopause. Compared with those who contributed DLS, those from whom NAF was collected tended to be slightly older and less likely to be parous, to report ever breastfeeding, or to have had a breast biopsy. More detailed characteristics of subjects from whom either NAF or DLS samples were obtained have been reported previously [39].

The geometric mean [95 % confidence interval (CI)] for PE and EM concentrations in serum are shown in Table 2. Among the 37 postmenopausal *BRCA1/2* mutation carriers contributing serum, 88 % of TE was conjugated. Unconjugated forms of the PE, as well as three of the 13 EM [(2-methoxyestrone (2-MeOE₁), 2-methoxyestradiol (2-MeOE₂), and estriol (E₃)] could also be detected. On average, estrone (E₁), the 2-hydroxylation pathway EM catechols, and E₃ were detected in the highest concentrations.

Table 3 shows the geometric mean levels of PE and EM in NAF, as well as the correlation between the PE and EM levels in NAF and serum. The geometric means of total PE and EM levels in NAF were about four times lower than those observed in serum (Fig. 2). In NAF, unlike serum, concentrations of 2-OHE₁ and 2-OHE₂ were highest (Fig. 2). Analyses of the paired difference in mean PE and EM values in NAF and serum yielded a ratio of 0.22 (95 % CI 0.19–0.25). In NAF, approximately 59 % of TE were conjugated (Table 3), compared with 88 % for serum ($p < 0.001$). Both PE and five EM were also present as unconjugated forms, including 2-hydroxyestrone (2-OHE₁) and 2-hydroxyestradiol (2-OHE₂), whose unconjugated forms were not detected in serum. The 2-hydroxylation pathway EM catechols constituted 42 % of the total NAF EM compared with 19 % for serum ($p < 0.0001$). The PE constituted 26 % of the total NAF estrogens compared with 46 % for serum ($p < 0.0001$). NAF and serum estrogen levels were highly correlated for PE and many EMs (Table 3), with the strongest correlations for E₁ ($r = 0.97$, $p < 0.0001$), E₂ ($r = 0.90$, $p < 0.0001$), and 2-OHE₁ ($r = 0.89$, $p < 0.0001$). Geometric mean levels of estrogens in NAF and the correlation between estrogens in NAF and serum for the two NAF collection methods used in this study were similar (data not shown). As elaborated in the “Discussion” section, these high correlations suggest that serum measurements could serve as a good surrogate for NAF measurements. On the other hand, for some analytes, correlations were much lower.

The geometric mean levels of the PE and EM in DLS and their correlations with serum estrogen are shown in Table 4. Total estrogen (TE) in DLS was about 11 times lower than that observed in serum, and about 2.5 times lower than those observed in NAF, most likely related to the dilution effect of ductal lavage. Analyses of the paired difference in log (TE) for DLS and serum yielded a ratio of TE in these two sources of 0.28 (95 % CI 0.24–0.32). In DLS, about 69 % of EM was conjugated. Similar to estrogens in NAF, both PE and five EM were present in their unconjugated form. The 2-hydroxylation pathway EM catechols comprised the overwhelming majority of DLS EM, constituting 66 % of the total DLS EM. Strong correlations were observed for E₁, E₂, and E₃ between DLS and serum ($r = 0.92$, $p < 0.0001$; $r = 0.70$, $p = 0.0001$; $r = 0.67$, $p = 0.0004$, respectively). In contrast, the levels of 2-

hydroxylation pathway EM catechols and the methylated catechols in DLS did not correlate with their serum counterparts. Correlations between serum and DLS EM (Table 4) tended to be weaker than those observed between serum and NAF EM (Table 3).

Discussion

This report is the first to utilize LC/MS/MS to compare concentrations of estrogens and their metabolites in intra-breast fluids and serum. Our data demonstrate that both PE and EM can be reliably measured in these breast tissue-derived biospecimens (i.e., NAF and DLS). Further, our data are the first to demonstrate that circulating PE and EM strongly and positively correlate with their paired breast tissue counterparts measured in NAF and DLS among postmenopausal *BRCA1/2* mutation carriers.

The circulating PE concentrations we observed were similar to those previously reported in postmenopausal women but the intra-breast hormone levels from both NAF and DLS samples were lower than prior reports. Two prior studies [24, 33] reported levels that were twofold to more than tenfold higher in the breast than in serum, using radioimmunoassays (RIA) to assess E_1 and E_2 levels in NAF among postmenopausal women. However, our population was younger (46 vs. 52 and 58 years [24]; [33], the majority were postmenopausal due to RRSO (not reported by prior groups), and all were *BRCA1/2* mutation carriers. It is possible that having undergone RRSO and not using menopausal hormone therapy may have contributed to the lower levels of EM we observed within the breast. The one prior study [33, 41] evaluating estrogen concentration in DLS included both pre- and postmenopausal women and women using hormone therapy, which limits our ability to make direct comparisons of estrogen concentrations in DLS between studies. However, for DLS, the lower levels observed in our study may also be related to the irrigation of the duct during the breast ductal lavage, leading to dilution of the sample. Differences in the mean concentrations of estrogens and their metabolites across studies may also reflect different measurement methods (i.e., RIA [24, 33] vs. LC/MS/MS). In prior publications [24, 33], it is possible that lower levels of estrogens in NAF and DLS compared with serum provided the opportunity for a higher degree of cross reactivity to the antibodies used in the RIA assays. Since LC/MS/MS provides a direct measurement, the potential for cross reactivity in our study is limited. In comparing urine EM measurement by RIA or ELISA and by LC/MS/MS, geometric mean concentration of E_1 , E_2 , and E_3 were 1.4–2.7 times higher ($p < 0.0001$) in postmenopausal women as detected by RIA compared with LC/MS/MS [42]. Geometric mean concentrations of 2-hydroxyestrone and 16 α -hydroxyestrone were 2.7–11.8 times higher ($p < 0.0001$) by ELISA than LC/MS/MS in postmenopausal women, suggesting that RIA and ELISA are problematic in detecting the low concentrations of TE, PE, and EM which are characteristic of postmenopausal women [42]. Our DLS results demonstrate that LC/MS/MS can detect exceedingly low concentrations of EM in intra-breast fluids obtained by ductal lavage. Future research will focus on standardizing the DL collection method to account for the dilution effect.

Our observation that intra-mammary estrogens are strongly and positively correlated with those in the circulation contrasts with one prior report in 16 postmenopausal women that observed no correlation between estradiol and estrone levels in NAF and serum [24]. Those participants were of comparable BMI and somewhat older than our population (i.e., 52 vs. 46 years). We also observed stronger correlations between serum and NAF than we did for serum and DLS. This result could be due to several factors, such as local tissue catabolism and conjugation enzyme activities, individual human variation and variable dilution of DLS samples due to ductal irrigation.

To our knowledge, no study to date has measured EM concentrations in NAF or DLS and only a few have evaluated levels in serum using LC/MS/MS. In those studies [18, 33, 41], levels of E_1 , E_3 , and 2-hydroxyestrone comprised more than 70 % of the estrogens in circulation. Similarly, in our study, these EM accounted for more than 60 % of the serum estrogens. However, both the absolute and relative concentration of serum E_3 was much lower in the *BRCA1/2* mutation carriers (accounting for 14.5 %, compared with literature values of 30–40 %), while 2-hydroxyestrone was higher. Five of the PE and 13 EM were present in both the conjugated and unconjugated forms in serum (E_1 , E_2 , 2-MeOE₁, 2-MeOE₂, and E_3), as previously reported [18]. In contrast to serum, the highest EM concentrations in NAF were observed for 2-hydroxyestrone and 2-hydroxyestradiol, followed by estrone and estriol. Unlike serum, both conjugated and unconjugated forms of these 2-hydroxyestrogen metabolites were found in NAF (in approximately equal concentrations). Interestingly, except for E_3 , the mitogenic 16-pathway EM were not detected in NAF samples, nor were 4-hydroxy catechols. Results for DLS were comparable to NAF. This finding of different patterns of EM in ductal fluids compared to serum supports accruing evidence that estrogen synthesis and catabolism within the breast is a significant contributor to the local hormonal milieu which is not reflected in serum hormone concentrations, particularly in postmenopausal women.

Although NAF and serum estrogen levels were highly correlated, they were not perfectly so. If local estrogens more accurately reflect breast cancer risk when compared with serum estrogens, then we can use the observed correlations between NAF and serum PE and EM to inform our thinking about the design and interpretation of epidemiologic studies of circulating estrogens and breast cancer risk. If the NAF values come to be regarded as the gold standard exposure metric for the association between estrogen and breast cancer risk, and if the $\log_e$ odds ratio of association $\log_e(EM)$ in NAF with breast cancer is β , then using serum $\log_e(EM)$ measurements instead of NAF measurements will attenuate the $\log_e$ odds ratio β by a factor equal to the square of the correlation. This attenuation factor would be about $0.90^2 = 0.81$ for E_2 (Table 3) and about $0.30^2 = 0.090$ for conjugated 2-MeOE₂ (Table 3). The reciprocal of this attenuation factor is the factor by which the sample size for a study of serum EM would need to be increased to have the same power as a study of the “gold standard” NAF EM measurement. More data and larger studies are needed to obtain precise estimates of these correlations, which may give an important indication of the usefulness of serum measurements if the true biologic effect is mediated by EM levels in NAF and if $\log_e$ odds ratios are linear in the logarithm of EM levels in NAF. It would be fair to say that the increase in sample size that would be required if serum rather than NAF measures of exposure were used would not be prohibitive for many of the estrogens in Table 3, because epidemiologic studies are typically large, and serum measurements are much easier and less expensive to obtain. Similar comments apply to DLS, but the correlations with serum levels tended to be smaller (Table 4).

Due to the small size of this pilot study, the results observed for specific EM need to be interpreted with caution and require replication in a larger sample of women. Despite our small sample size, this study is the first to have evaluated the association between circulating and intra-breast levels of estrogen among postmenopausal *BRCA1/2* mutation carriers. Other estrogen metabolites (16 α -OHE and 4 hydroxylation pathway EM), thought to be carcinogenic through their strong estrogenic or genotoxic properties, were detected in serum but neither in NAF (Fig. 2) nor in DLS. Furthermore, we were able to detect unconjugated forms of estrogen in the 2-hydroxylation pathway within NAF and DLS but not in serum, suggesting that this biologically active form of estrogen may be locally produced and may serve an important biological function that remains to be defined.

Conclusion

The ability to accurately detect and quantify estrogen metabolites would greatly enhance the use of these measures as biomarkers of breast cancer risk and to monitor the success of breast cancer prevention strategies. These pilot data provide evidence that accurate, reproducible measurement of exceedingly low EM concentrations within novel breast tissue fluids is achievable, and provide support for undertaking larger studies designed to determine whether serum levels of PE and EM are truly reflective of intra-breast levels, as our results suggest. Larger prospective studies with repeated samples will be required to confirm that circulating and/or intra-breast PE and EM comprise useful surrogate biomarkers of breast cancer risk. Future work should seek to confirm the relationships that have been demonstrated here, and to determine whether serum PE and EM levels could serve as a surrogate for intra-breast PE and EM levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BRCA1/2	Breast Cancer genes 1 and 2
PE	Parent estrogen
E1	Estrone
E2	Estradiol
E3	Estriol
EM	Estrogen metabolites
TE	Total estrogens, conjugated + unconjugated
NAF	Nipple aspirate fluid
DLS	Ductal lavage supernatant
LC/MS/MS	Liquid chromatography–tandem mass spectrometry
DNA	Deoxyribonucleic acid
ER	Estrogen receptor
NCI	National Cancer Institute
BIS	Breast Imaging Study

MRI	Magnetic resonance imaging
IRB	Institutional Review Board
2-OHE1	2-Hydroxyestrone
2-MeOE1	2-Methoxyestrone
2-OHE2	2-Hydroxyestradiol
2-MeOE2	2-Methoxyestradiol
3-MeOE1	2-Hydroxyestrone-3-methyl ether
4-OHE1	4-Hydroxyestrone
4-MeOE1	4-Methoxyestrone
4-MeOE2	4-Methoxyestradiol
16α-OHE1	16 α -Hydroxyestrone
17-epiE3	17-Epiestriol
16-ketoE2	16-Ketoestradiol
16-epiE3	16-Epiestriol
SI-EM	Stable isotope-labeled estrogens
Estrone-13C6	Estrone-13,14,15,16,17,18-13C6
17β-estradiol-13C6	17 β -Estradiol-13,14,15,16,17, 18-13C6
d3-E3	Estriol-2,4,17-d3
d5-2-OHE2	2-Hydroxyestradiol-1,4,16,16,17-d5
d5-2-MeOE2	2-Methoxyestradiol-1,4,16,16,17-d5
d3-16-epiE3	16-Epiestriol-2,4,16-d3
ICC	Intra-class correlation coefficients
BMI	Body mass index
RRSO	Risk reducing salpingo-oophorectomy
SD	Standard deviation
RIA	Radioimmunoassay

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Potential Carcinogenic Pathways of Estrogen Metabolites (EM)

Fig. 1.
Carcinogenic pathways of estrogen metabolism

Fig. 2.
Geometric means of, and EM in serum and NAF (error bars = Standard deviation of the geometric means)

Table 1

Breast imaging study participants

Patient characteristics ^a	Nipple aspirate fluid (NAF)		Duct lavage supernatant (DLS)	
	BRCA1/2 (n = 22)		BRCA1/2 (n = 24)	
	Mean	SD	Mean	SD
Age (years)	46	6.9	43.7	5.8
Range	33–57		32–55	
Body mass index ^b	26.3	5.7	26.8	5.8
Range	20.0–39.9		20.1–43.7	
Age at menopause (years)	42.9	4.8	42.1	5.4
Range	32.6–50.0		32–54	
Years since menopause	3.1	3.1	1.8	2.7
Range	0.1–11.1		0.2–13.0	

	n	% ^b	n	%
Age at menarche (years)				
< 12	2	9.5	0	0
12–13	15	71.4	21	87.5
14	4	19.1	3	12.5
Missing	1			
Parous				
No	8	36.4	3	12.5
Yes	14	63.6	21	87.5
Breastfeeding				
Never	12	54.6	6	25
Ever	10	45.5	18	75
Menopausal status				
Postmenopausal, natural	5	22.7	2	8.33
Postmenopausal, surgical	17	77.3	22	91.67
Current oral contraceptives use	0	–	0	–
Current hormone therapy use	1	4.6	0	–
Current tamoxifen use	0	0	2	8.33
Breast biopsy prior to enrollment				
0	14	63.6	13	54.17
1+	8	36.4	11	45.83
Personal history of breast cancer				
No	20	90.9	21	87.5
Yes	2	9.1	3	12.5

^aNo statistically significant differences were observed between women from whom NAF or DLS was collected

^bMissing values were excluded from percentage calculations; Weight (kg)/height² (m²)

Table 2

Serum parent estrogens and estrogen metabolite measures

Serum PE and EM	<i>BRCA1</i> (n = 27) + <i>BRCA2</i> (n = 10)	
	Serum	
	Geometric mean concentrations (pmol/L) ^a	95 % CI
Parent estrogens (PE)		
Estrone (E ₁)	331.75	48.28–2,279.66
Conjugated	278.19	37.06–2,088.45
Unconjugated	44.92	7.91–255.01
Estradiol (E ₂)	42.52	2.94–615.30
Conjugated	30.15	1.72–527.04
Unconjugated	8.02	0.55–117.66
2-Hydroxylation pathway catechols		
2-Hydroxyestrone (2-OHE ₁)	116.92	18.68–731.62
2-Hydroxyestradiol (2-OHE ₂)	51.29	12.28–214.18
2-Hydroxylation pathway methylated catechols		
2-Methoxyestrone (2-MeOE ₁)	30.59	5.32–175.91
Conjugated	19.75	2.54–153.61
Unconjugated	5.53	0.41–75.72
2-Methoxyestradiol (2-MeOE ₂)	17.04	2.23–130.28
Conjugated	11	0.9–134.70
Unconjugated	3.98	0.77–20.57
2-Hydroxyestrone-3methly ether (3-MeOE ₁)	7.4	1.35–40.52
4-Hydroxylation pathway catechols		
4-Hydroxyestrone (4-OHE ₁)	22.6	4.38–116.60
4-Hydroxylation pathway methylated catechols		
4-Methoxyestrone (4-MeOE ₁)	6.93	1.5–32.06
4-Methoxyestradiol (4-MeOE ₂)	3.78	0.48–29.75
16-Hydroxylation pathway		
16 α -Hydroxyestrone (16 α -OHE ₁)	22.36	5.62–88.94
Estriol (E ₃)	137.37	35.37–533.55
Conjugated	101.31	19.99–513.38
Unconjugated	22.67	3.06–168.21
17-Epiestriol (17-epiE ₃)	4.61	0.65–32.77
16-Ketoestradiol (16-ketoE ₂)	21.03	5.47–80.82
Total estrogen (TE) ^b	948.45	211.9–4,245.08
Conjugated	833.06	185.83–3,734.44
Unconjugated	102.8	18.7–565.03

^aThe unconjugated and conjugated EM levels do not sum to the total because they are geometric means^bTotal estrogens (TE) = PE + EM

Table 3

Association between estrogens in nipple aspirate fluid and serum

Estrogens in NAF and serum	<i>BRCA1/2 combined (n = 22); BRCA1 (n = 16); BRCA2 (n = 6)</i>				
	Serum		NAF		
	Geometric mean (pmol/L) ^a	95 % CI	Geometric mean (pmol/L) ^a	95 % CI	Pearson's <i>r</i> with log _e (serum EM)
Parent estrogens (PE)					
Estrone (E ₁)	388.11	39.09–3,853.75	36.01	1.01–1,284.54	0.97 <0.0001
Conjugated	333.77	30.89–3,606.02	24.61	0.79–766.22	0.96 <0.0001
Unconjugated	47.63	6.66–340.74	9.98	0.2–490.43	0.96 <0.0001
Estradiol (E ₂)	51.30	2.61–1,009.28	10.91	1.21–98.07	0.90 <0.0001
Conjugated	38.04	1.89–764.81	5.4	0.73–40.22	0.82 <0.0001
Unconjugated	7.24	0.185–284.23	4.21	0.27–65.80	0.77 <0.0001
2-Hydroxylation pathway catechols					
2-Hydroxyestrone (2-OHE ₁)	124.31	21.77–709.90	46.76	6.52–335.36	0.89 <0.0001
Conjugated	<i>c</i>		24.33	1.63–362.30	
Unconjugated	<i>c</i>		18.15	5.08–64.89	
2-Hydroxyestradiol (2-OHE ₂)	57.73	11.51–289.70	40.18	9.01–179.25	0.75 <0.0001
Conjugated	<i>c</i>		14.11	1.17–170.62	
Unconjugated	<i>c</i>		22.09	8.63–56.55	
2-Hydroxylation pathway methylated catechols					
2-Methoxyestrone (2-MeOE ₁)	38.57	6.46–230.38	18.94	3.75–95.77	0.78 <0.0001
Conjugated	23.71	3.97–141.45	9.4	0.76–116.83	0.63 0.002
Unconjugated	8.00	0.33–196.54	7.03	2.94–16.79	0.48 0.03
2-Methoxyestradiol (2-MeOE ₂)	17.92	2.22–144.76	7.23	2.36–22.14	0.70 0.0003
Conjugated	11.11	2.22–149.42	2.85	0.57–14.25	0.30 0.17
Unconjugated	3.66	2.22–40.56	3.74	1.11–12.55	0.45 0.04
2-Hydroxyestrone-3methyl ether (3-MeOE ₁)	6.86	2.22–50.38			
4-Hydroxylation pathway catechols					
4-Hydroxyestrone (4-OHE ₁)	24.06	5.08–114.03	<i>c</i>		
4-Methoxyestrone (4-MeOE ₁)	5.71	1.62–20.15	<i>c</i>		

0Estrogens in NAF and serum	<i>BRCA1/2</i> combined (<i>n</i> = 22) <i>BRCA1</i> (<i>n</i> = 16); <i>BRCA2</i> (<i>n</i> = 6)					
	Serum			NAF		
	Geometric mean (pmol/L) ^a	95 % CI		Geometric mean (pmol/L) ^a	95 % CI	Pearson's <i>r</i> with log _e (serum EM)
4-Methoxyestradiol (4-MeOE ₂)	2.41	0.489–11.86	<i>c</i>			
16-Hydroxylation pathway						
16α-Hydroxyestrone (16α-OHE ₁)	24.14	7.62–76.45	<i>c</i>			
Estradiol (E ₂)	155.56	32.43–746.15	34.71		10.00–120.46	0.74
Conjugated	117.98	17.03–817.54	28.01		5.40–145.41	0.68
Unconjugated	117.98	1.6–190.95	4.79		1.52–15.08	0.08
17-Epiestradiol (17-epiE ₂)	117.98	0.85–40.12	<i>c</i>			0.72
16-Ketoestradiol (16-ketoE ₂)	117.98	7.55–65.73	<i>c</i>			
16-Epiestradiol (16-epiE ₂)	117.98	0.43–58.54	<i>c</i>			
Total estrogen (TE) ^b	1,029.41	151.63–6,988.58	224.09		21.53–2,332.72	0.98
Conjugated	900.46	130.86–6,196.11	127.49		9.34–1,741.28	0.96
Unconjugated	111.57	14.35–867.37	86.02		9.50–779.26	0.92

^aThe unconjugated and conjugated EM levels do not sum to the total because they are geometric means

^bTotal estrogens (TE) = PE + EM

^cNot detected

Table 4

Association between estrogens in ductal lavage supernatant and serum

Estrogens in DLS and serum	BRCA1/2 combined (<i>n</i> = 24) BRCA1 (<i>n</i> = 16); BRCA2 (<i>n</i> = 8)			
	Serum		DLS	
	Geometric mean concentrations (pmol/L) ^a	95 % CI	Geometric mean concentrations (pmol/L) ^a	Pearson's <i>r</i> with log _e (serum EM)
Parent estrogens (PE)				
Estrone (E ₁)	276.04	79.06–963.79	12.08	0.92
Conjugated	228.39	59.63–874.68	8.98	0.90
Unconjugated	38.80	11.38–132.31	2.83	0.93
Estradiol (E ₂)	32.98	5.02–216.48	5.29	0.70
Conjugated	22.13	2.27–215.54	3.07	0.40
Unconjugated	7.07	1.26–39.86	1.75	0.56
2-Hydroxylation pathway catechols				
2-Hydroxyestrone (2-OHE ₁)	110.61	19.13–639.58	40.96	–0.03
Conjugated	<i>c</i>		27.53	5.70–133.17
Unconjugated	<i>c</i>		11.07	3.12–39.26
2-Hydroxyestradiol (2-OHE ₂)	45.59	14.83–140.17	16.06	–0.05
Conjugated	<i>c</i>		12.03	2.93–49.40
Unconjugated	<i>c</i>		3.53	1.99–6.27
2-Hydroxylation pathway methylated catechols				
2-Methoxyestrone (2-MeOE ₁)	23.32	5.62–96.82	2.95	0.10
Conjugated	16.08	2.19–118.14	2.12	–0.01
Unconjugated	3.98	0.87–18.28	1.27	0.01
2-Methoxyestradiol (2-MeOE ₂)	15.28	2.62–89.01	3.39	0.36
Conjugated	9.59	1–91.89	1.06	0.35
Unconjugated	3.99	0.94–16.83	1.97	0.50
2-Hydroxyestrone-3methyl ether (3MeOE ₁)	7.87	1.68–36.85	<i>c</i>	0.01
4-Hydroxylation pathway catechols				
4-Hydroxyestrone (4-OHE ₁)	20.92	3.95–110.74	<i>c</i>	
4-Hydroxylation pathway methylated catechols				

Estrogens in DLS and serum	<i>BRCA1/2</i> combined (<i>n</i> = 24) <i>BRCA1</i> (<i>n</i> = 16); <i>BRCA2</i> (<i>n</i> = 8)					
	Serum			DLS		
	Geometric mean concentrations (pmol/L) ^a	95 % CI		Geometric mean concentrations (pmol/L) ^a	95 % CI	Pearson's <i>r</i> with log ₁₀ (serum EM)
4-Methoxyestrone (4-MeOE ₁)	8.31	1.60–43.12	<i>c</i>			
4-Methoxyestradiol (4-MeOE ₂)	5.27	0.76–36.41	<i>c</i>			
16-hydroxylation pathway			<i>c</i>			
16 α -Hydroxyestrone (16 α -OHE ₁)	20.97	4.86–90.57				
Estriol (E ₃)	121.54	49.04–301.18	4.07		1.93–8.57	0.67
Conjugated	86.22	28.21–263.51	1.32		0.29–5.96	0.47
Unconjugated	27.11	6.00–122.46	2.08		0.33–13.08	0.09
17-Epiestriol (17-epiE ₃)	4.46	0.60–33.29	<i>c</i>			
16-Ketoestradiol (16-ketoE ₂)	20.42	4.68–89.00	<i>c</i>			
16-Epiestriol (16-epiE ₃)	2.25	0.27–18.97	<i>c</i>			
Total estrogen (TE) ^b	833.58	387.60–1,792.72	86.9		28.43–265.57	0.83
Conjugated	734.05	337.13–1,598.32	59.34		16.49–213.61	0.79
Unconjugated	89.59	30.58–262.49	24.79		7.97–77.10	0.83

^aThe unconjugated and conjugated EM levels do not sum to the total because they are geometric means

^bTotal estrogens (TE) = PE + EM

^cNot detected