Research Article

Cancer Prevention Research

Breast Hormone Concentrations in Random Fine-Needle Aspirates of Healthy Women Associate with Cytological Atypia and Gene Methylation

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Abstract

Sex steroid hormones contribute to breast cancer development, but data on concentrations of these within breast tissue are limited. We performed simultaneous multiparameter measurement of breast sex steroids, breast epithelial cytology, and DNA methylation in 119 healthy women (54 pre- and 65 postmenopausal) without a history of breast cancer. Random fine-needle aspiration (rFNA) of the breast was performed simultaneously with blood collection. Breast samples were analyzed by LC/MS-MS for estrone, estradiol, progesterone, androstenedione, and testosterone. Blood samples were assayed for estradiol and progesterone by immunoassay. Cytomorphology was classified using the Masood Score, and DNA methylation of eight genes was analyzed using quantitative multiplexed methylation-specific PCR, and expressed as the cumulative methylation index (CMI). Serum and breast concentrations of estradiol and progesterone showed significant correlation (Spearman $r = 0.34$, $P_{adj} = 0.001$ and $r = 0.69$, $P_{adj} < 0.0006$, respectively). Progesterone concentration was significantly higher in the premenopausal breast ($P_{adj} < 0.0008$), and showed a luteal surge. Breast estrone and estradiol concentrations did not differ significantly by menopause, but androstenedione concentration was higher in the breasts of postmenopausal women ($P = 0.026$ and $P_{adj} = 0.208$). Breast androgens were significantly correlated with breast density (Spearman $r = 0.27$, $P_{adj} = 0.02$ for testosterone) and CMI (Spearman $r = 0.3$, $P_{adj} = 0.038$ for androstenedione). Our data indicate that future larger studies of breast steroid hormones along with other parameters are feasible. Significant associations of breast androgen concentrations with breast density and gene methylation warrant future study. *Cancer Prev Res; 11(9); 557–68. ©2018 AACR.*

Introduction

Lifetime sex steroid exposure is a determinant of breast cancer risk, as evidenced by a wealth of epidemiologic data (1, 2). Direct measurement of systemic hormone exposure has been performed in multiple studies, which show that elevated concentration of serum estrogens and androgens are related to higher breast cancer risk among postmenopausal women (3–5). Local synthesis of estradiol has been directly demonstrated in the breast from circulating androgens and estrone sulfate (6–9), and is implicated as part of the mechanism for obesity-associated breast cancer (10, 11). Thus, hormone concentrations in breast tissue are

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hypothesized to be more closely related to breast cancer risk, but have been successfully analyzed in a few, relatively small studies, with variable methods, largely based on radioimmunoassay (12–15). Measurements of steroids in nipple aspirate fluid and breast ductal lavage provide some information suggesting differences in the local tissue compared with circulating concentrations of steroids (16–19), but these reflect the intraductal environment. The ability to measure sex hormones in breast tissue will allow the dissection of the importance of systemic exposure and the local endocrine environment, their relative contribution to risk, and may inform the development of specific preventive strategies for women with high hormone concentrations in the breast.

The major known risk factors that are observed locally in the breast include atypical hyperplasia and density of breast tissue. Women with atypical hyperplasia have 4-fold higher risk of developing breast carcinoma (20, 21), as do women with extremely dense breasts (22). Therefore, the contribution of local breast hormone concentrations to these breast-specific risk factors is of interest and may provide mechanistic insights. Also of significant interest is the influence of local hormone concentration on the DNA methylation status of cancer-related genes, a potentially powerful risk indicator for breast cancer (23–25), which we have previously shown to be related to cytologic atypia (26, 27).

We have therefore performed a prospective study to evaluate these breast-based risk parameters simultaneously. We enrolled healthy women, without regard for their breast cancer risk status, measured their breast density, and obtained breast samples through the minimally invasive technique of random fine-needle aspirate (rFNA) of breast (28–30). Our major goals were to demonstrate the feasibility of this multiparameter use of rFNA samples, and to evaluate variation of these promising molecular biomarkers with menstrual and menopausal states, a source of biological noise that is rarely defined in biomarker studies.

We enrolled 380 women with carefully documented menstrual and menopause status, and examined cumulative DNA methylation of eight genes in rFNA samples, and local breast steroid hormone concentration. We recently reported that cumulative DNA methylation of eight genes were not affected by menstrual or menopausal states, and showed significant positive associations with cytologic atypia (27). We now report on sex steroid hormone concentrations within breast tissue, its physiologic variation in a subset of 119 women, and explore relations of breast hormones with cytology, DNA methylation, and established clinical risk factors.

Materials and Methods

Participants

We have evaluated steroid hormones in rFNA and matching blood serum samples of 119 women whose rFNA samples were collected in PBS) with ascorbic acid from our recent published study (NU 08B2; ref. 27) where 380 pre- and postmenopausal women without a breast cancer history were recruited. Data on cellularity of the rFNA sample, cytomorphology, gene methylation (cumulative methylation index, CMI), along with clinical data including lifetime Gail risk and mammographic density of this study population have been described recently (27). Menopausal status and menstrual phase was determined by serum concentrations of follicular stimulating hormone (FSH), progesterone, and estradiol as described previously (18). The current report focuses on 119 women selected from the parent study to provide balanced groups of pre and postmenopausal women, with premenopausal women distributed roughly equally between follicular, mid-cycle, and luteal phases of the menstrual cycle (Table 1).

Table 1. Patient characteristics

Characteristics	Original study ($N = 380$)	Current study ($N = 119$)
Age, median (IQR)	50 (45–56)	51 (46–55)
Race	$N = 380$	$N = 119$
Caucasian	327 (86.1%)	100 (84%)
African American	40 (10.5%)	18 (15%)
Other	13 (3.4%)	1 (1%)
Hormonal menopausal status	$N = 366$	$N = 119$
Premenopausal	168 (45.9%)	54 (45%)
Postmenopausal	198 (54.1%)	65 (55%)
Menstrual phase	$N = 166$	$N = 54$
Follicular	53 (31.9%)	14 (26%)
Mid-cycle	67 (40.4%)	19 (35%)
Luteal	46 (27.7%)	21 (39%)
Lifetime Risk (Gail Model)	$N = 378$	$N = 119$
Median (IQR)	12.7 (9.6, 17.9)	11.1 (9.3, 16.2)
<10	112 (29.6%)	40 (34%)
10–19	206 (54.5%)	68 (57%)
≥20	60 (15.9%)	11 (9%)
BMI, kg/m^2	$N = 380$	$N = 119$
Median BMI	26.8 (23.3, 31.8)	28.1 (24.1, 33.2)
Low-Normal(≤25)	144 (38%)	39 (33%)
High(>25)	236 (62%)	80 (67%)
Breast percent density	$N = 379$	$N = 119$
Median (IQR)	18.1 (9.53, 29.1)	19.6 (9.17, 28.3)
<5%	15 (4%)	16 (13.4%)
5 to 24%	264 (70%)	63 (52.9%)
25 to 49%	92 (24%)	36 (30.3%)
50 to 75%	8 (2%)	4 (3.4%)
>75%	0 (0%)	0 (0%)
Cytology, standard	$N = 380$	$N = 119$
Insufficient (or no cells)	26 (6.8%)	12 (10%)
Benign	207 (54.5%)	61 (51%)
Borderline atypical	8 (2.1%)	1 (1%)
Atypical/Intermediate	136 (35.8%)	45 (38%)
Suspicious	3 (0.8%)	0 (0%)
Malignant	0 (0%)	0 (0%)
Masood Cytology score	$N = 354$	$N = 108$
Median (IQR)	13 (12, 15)	14 (13, 15.8)
Normal (6–10)	30 (8.5%)	5 (5%)
Hyperplasia, benign (11–14)	192 (54.2%)	59 (55%)
Hyperplasia, atypical (15–18)	132 (37.3%)	44 (40%)

rFNA samples

rFNA collection of breast tissue was conducted as described previously (28, 30). Briefly, rFNA from ten passes

of a 22-gauge needle was collected in two tubes of 10-mL PBS buffer containing 0.2 mg/mL EDTA and 0.1% (w/v) ascorbic acid. The two tubes were mixed, and centrifuged to separate cell pellets from supernatant and fat. The supernatant and fat were collected and stored at -80°C until analysis by LC/MS-MS.

Blood serum samples

The concentration of estradiol, progesterone, and FSH was measured by ELISA kits (R&D Systems, Inc.), and used to confirm menstrual and menopausal status of participants as described previously (18). The women with either no menstrual period for 12 months or with serum FSH >30 mIU/mL, estradiol <30 pg/mL, and progesterone <3 ng/mL were defined as postmenopausal women. The menstrual cycle was divided into three phases: follicular phase, serum estradiol <60 pg/mL and progesterone <3.0 ng/mL; mid-cycle, serum estradiol >60 pg/mL and progesterone <3.0 ng/mL; and luteal phase, estradiol >30 pg/mL and progesterone >3.0 ng/mL.

LC/MS-MS analysis for steroid hormone measurement in serum and rFNA

Initial attempts to measure hormones in rFNA samples were performed with methods we developed previously for nipple aspiration fluid (NAF) using high-performance liquid chromatography purification and immunoassay (18). However, we found that the recovery of androgens was incomplete in rFNA samples using these methods. These attempts were also hampered by discontinuation of some kits by manufacturers. We then decided to proceed using a new method with LC/MS-MS using 119 unprocessed original samples.

We measured estrone, estradiol, androstenedione, testosterone, and progesterone in rFNA samples. Sample aliquots were spiked with 10 μL of acetonitrile (ACN) containing the internal standards ISTD; E1-d4, E2-d3, androstenedione-d7, testosterone-d3 and progesterone-d9 (TRC) and extracted with twice its volume of methyl ter-butyl ether. After shaking for 5 minutes, the sample was centrifuged at 4°C for 10 minutes to separate the two phases and remove precipitated proteins and stored in a freezer at approximately −70°C for at least 2 hours; the top layer was then decanted into a preweighed tube and evaporated under nitrogen flow. After reweighing, the tube to record the total weight of lipids, the residue was dissolved in cold methanol, shaken for 0.5 minutes, centrifuged at 4°C for 10 minutes, and stored at −20°C for at least 1 hour to remove triglycerides; the top layer was then decanted into clean tube and evaporated again under nitrogen flow. The resulting purified lipid residue was derivatized by adding 0.1 mL of dansyl chloride solution (1 mg/mL in ACN) and 0.1 mL of sodium bicarbonate buffer (0.1 mol/L, pH 9.5), mixing well and incubation at 60°C for 20 minutes. The tube was then shaken for 0.5 minutes, centrifuged at 4°C for 10 minutes, and decanted into an autosampler vial for instrumental analysis. Freshly prepared calibration solutions were prepared for standard curves which were analyzed along with study rFNA specimens. Instrument calibrators and quality control (QC) samples were prepared by adding 10 μL of stock standard solutions in ACN to PBS buffer (calibrators) and rFNA matrix. Calibrator concentrations were prepared in the range of 5 to 200 pg (QC samples at 10, 40, and 100 pg) for E1 and E2; 50 to 2000 pg (QC samples at 100, 400, and 1,000 pg) for androstenedione, testosterone, and progesterone. Calibrator and QC samples were processed for analysis following the procedure described above. Concentrations of target analytes in the study specimens were determined from each sample's target peak to internal standard area ratios and the linear regression parameters derived for the calibration curves. Instrumental analysis was performed using a tandem mass spectrometer (4000 Q-TRAP; AB SCIEX) equipped with a high-performance liquid chromatograph (Agilent 1200; Agilent Technologies,). Separation of target analytes from matrix components was achieved with a Luna PFP (2), 3μ column, 100 × 2.1 mm (Phenomenex). The column temperature was maintained at 25 °C, and a flow rate of 0.3 mL/minute was used. The mobile phase consisted of solvent A: 0.1% formic acid in water (v/v) and solvent B: 0.1% formic acid in acetonitrile (v/v). The mobile phase gradient was as follows: after injection, initial conditions with solvent A at 40% were held for 0.5 min., decreased to 5% in 5.5 minutes, and held constant for 4 minutes, returning to initial conditions for another 4.5 minutes of reequilibration time. Total run time was 15 minutes. A heated nebulizer was used as the ion source for atmospheric pressure chemical ionization operating in positive mode. Acquisition was performed in multiple reaction monitoring mode using m/z 504.3 → 171.2, 508.3 → 171.2, 506.3 → 171.2, 509.3 → 171.2, 287.1→ 97.1 and 294.1→ 100.1, 289.2 → 97.1, 292.2 → 109.2, 315.2 → 97.1, and 324.2 → 100.1 at low resolution for E1, E1-d4, E2, E2-d3, androstenedione, androstenedione-d7, testosterone, testosterone-d3, progesterone, and progesterone-d9, respectively. Ion source temperature was 325°C, the declustering potential was 130 V, and the collision gas was nitrogen with collision energy of 50 V.

Statistical considerations

The primary objectives of this component of the study were to determine whether breast (i.e., rFNA) steroid hormone concentrations varied by age, menopausal status, and menstrual cycle as expected according to known physiology, how the breast hormone content compared with serum hormones, and whether breast hormone concentrations show associations with established risk factors (Gail risk estimates, mammographic density, and Masood cytology score) as well as novel risk markers such as CMI.

The clinical characteristics of our study followed a non-Gaussian distribution (27), therefore data were reported as medians and interquartile ranges (IQR). Univariate analyses were performed using Mann–Whitney *U* test for two group comparisons and Kruskal–Wallis tests were performed for comparison more than two groups. We used nonparametric Spearman correlation to evaluate dependency among hormone/risk factors as continuous variables. Results were reported as coefficient (r) with 95% confidence interval (CI) and considered significant at $P < 0.05$ for hormone concentrations, clinical factors, and CMI. P values were adjusted for multiple comparisons using the Bonferroni method. Linear regression analysis was performed for association between each hormone and breast density and body mass index (BMI). These analyses were adjusted for age. All statistical analyses were performed using GraphPad Prism version 5.04 (GraphPad Software) and SAS version 9.4 software.

Results

Participant characteristics

We evaluated rFNA samples of 119 women and their matching blood samples for endogenous hormone concentrations. Patient characteristics of the 119 women included in the current analysis were very similar to the parent study population, which included 380 women enrolled at two academic medical centers: (Northwestern University and Johns Hopkins University). As shown in Table 1, the median age of premenopausal women was 46 years, and of postmenopausal women was 55 years. Median lifetime risk of breast cancer using the Gail model was 11.1. Only 11 of 119 women (9%) had a lifetime risk of $\geq$20, 68 (57%) had a lifetime risk estimate of 10%–20%, and 40 (34%) had a lifetime Gail risk estimate of under 10%. Median BMI was 28.1, with 80 of 119 women (67%) being overweight or obese, with a BMI $\geq$25. Median percent area of mammographic breast density was 19.6; only 4 of 119 (3.4%) had 50%–75% dense area, whereas the rest had under 49% dense area. Cytomorphology analysis showed that of 119 rFNA samples 12 (10%) were insufficiently cellular for diagnosis, 61 (51%) had benign features, and 46 (38.7%) had atypical features without suspicion for malignancy (Masood score). Of the 108 samples, the median Masood score was 14; 64 of 108 samples (59.2%) showed normal to benign hyperplasia and 44 samples (40.7%) demonstrated atypical hyperplasia.

Because we used the lipid fraction of the rFNA sample for steroid hormone assays, and the cellular fraction for evaluation of cytology and DNA methylation, we examined the parameters that may be expected to influence these components (age, BMI, breast density). Correlations between these parameters are shown in Supplementary Table S1. After Bonferroni corrections, there was no significant association between age and any other parameter in this group of 119 women. As expected, BMI showed a significant inverse relation to breast density (Spearman $r = -0.54$, $P_{adj} < 0.0007$), and to total cell number ($r = 0.318$, $P_{adj} = 0.003$). The amount of lipid showed no significant correlations with other parameters after Bonferroni correction, but nominal P values were significant for age and cell number. Thus, the amount of lipid in rFNA samples reflects the fatty involution of aging rather than obesity. As expected, the total cell number in the rFNA samples was inversely related to BMI ($r = -0.318$, $P_{adj} = 0.003$), positively correlated to breast density ($r = 0.324$, $P_{adj} = 0.002$), to Masood score ($r = 0.656$, $P_{adj} < 0.0007$), and to CMI ($r = 0.505$, $P_{adj} < 0.0007$).

Another determinant of the lipid component and cellularity of the rFNA sample may be menopausal status; the lipid amount of pre- and postmenopausal samples is shown in the first row of Table 2, and demonstrated a significant increase following menopause ($P_{adj} = 0.024$). The median lipid content of samples from the postmenopausal breast was 2.7-fold higher than that of premenopausal breast, supporting the notion that lipid fraction is related to the involution of the breast and increase in breast adiposity that occurs with menopause. The median total cell number was different by menopausal status (11,000 and 4,000 cells for premenopausal and postmenopausal women, respectively; $P = 0.02$, not significant after Bonferroni correction), but was not significantly different by menstrual phase (3,500, 14,000, 16,000 cells for follicular, mid-cycle, and luteal phase, respectively, Kruskal–Wallis rank sum test across the three groups; $P = 0.197$; Supplementary Table S3).

Hormone concentrations in rFNA and serum by menopausal status and menstrual phase

We measured endogenous hormone concentrations in the lipid fraction of rFNA samples; these included estrone, estradiol, progesterone, androstenedione, and testosterone. These results, and values for estradiol and progesterone in serum, are summarized in Table 2. Of note, the breast concentrations are expressed in ng/g total lipid, whereas the serum concentrations are shown in pg or ng/mL. The breast and serum concentrations are therefore not directly comparable but patterns can be discerned and compared.

Estrogens

Estrogen concentrations were not significantly greater in premenopausal breasts than in postmenopausal breasts (Table 2A). During the menstrual cycle, estrone concentration varied significantly, with follicular and mid-cycle values showing medians of 1.24 ng/g and 1.58 ng/g, whereas median luteal phase value was undetectable. Estradiol did not vary significantly in rFNA samples during the menstrual cycle, but the pattern of variation was similar to that in serum, with a mid-cycle peak, and

Table 2. Hormone concentrations in rFNA and serum samples and correlation to age

A. By menopausal status, median (IQR)

Hormones	Premenopausal ($n = 54$)	Postmenopausal ($n = 65$)	P^a
rFNA (per mg total lipid)			
Total lipid (mg)	1.57 (0.93–2.26)	4.31 (0.8–11.5)	0.003[b]
Estrone (ng/g)	1.03 (0–2.53)	0.48 (0–1.12)	0.26
Estradiol (ng/g)	1.66 (0.81–4.25)	1.12 (0.38–4.73)	0.24
Progesterone (ng/g)	37.4 (7.43–149)	0 (0–0)	<0.0001[b]
Androstenedione (ng/g)	32.3 (18.1–48.8)	39.6 (24.9–70.2)	0.026
Testosterone (ng/g)	15.4 (6.23–30.8)	15.4 (0–39.9)	0.38
Serum (per mL)			
Estradiol (pg/mL)	75.7 (48.7–141)	10.5 (0–29.7)	<0.0001[b]
Progesterone (ng/mL)	1.94 (1.37–5.30)	1.22 (1.05–1.39)	<0.0001[b]

B. By menstrual phase, median (IQR)

Hormones	Follicular ($n = 14$)	Midcycle ($n = 19$)	Luteal ($n = 21$)	P^c
rFNA (per mg total lipid)				
Total lipid (mg)	1.44 (0.93–2)	1.5 (0.9–2.56)	1.58 (0.96–2.26)	0.98
Estrone (ng/g)	1.24 (0–2.53)	1.58 (0–4.63)	0 (0–1.14)	0.021
Estradiol (ng/g)	0.91 (0.6–4.17)	3.09 (1.15–10.2)	1.44 (0.94–2.69)	0.12
Progesterone (ng/g)	11.0 (5.4–30.7)	7.43 (0–13.9)	151 (94–223)	<0.0001[b]
Androstenedione (ng/g)	29.6 (18.1–35.4)	33.4 (18.1–52)	29.7 (17.7–54)	0.76
Testosterone (ng/g)	20.4 (12.5–25.1)	6.21 (0–29.7)	15.2 (10.4–36.8)	0.047
Serum (per mL)				
Estradiol (pg/mL)	42.3 (37.8–51.5)	174 (84.2–259)	74.4 (49.6–85.7)	<0.0001[b]
Progesterone (ng/mL)	1.51 (1.35–1.85)	1.37 (1.22–1.72)	6.12 (4.97–9.72)	<0.0001[b]

C. Correlation between hormones and age

Hormones	*N*	Spearman *r* (95% CI)	*P*
rFNA			
Total lipid	119	0.22 (0.03–0.39)	0.019
Estrone	119	−0.11 (−0.29–0.07)	0.21
Estradiol	119	−0.18 (−0.35–0.01)	0.050
Progesterone	82	−0.49 (−0.35–0.01)	<0.0001[b]
Androstenedione	102	0.04 (−0.16–0.24)	0.68
Testosterone	116	−0.15 (−0.33–0.04)	0.10
Serum			
Estradiol	116	−0.57 (−0.68 to −0.43)	<0.0001[b]
Progesterone	117	−0.52 (−0.64 to −0.37)	<0.0001[b]

[a]Comparison between menopausal groups was calculated with Whitney *U* test.
[b]*P* values remain significant after adjustment for multiple comparisons with Bonferroni correction.
[c]Comparison among three menstrual phases was calculated with Kruskal–Wallis rank sum test.

a nadir in the follicular phase (Table 2B). We found a moderate but significant positive correlation between breast and circulating estradiol concentrations in both pre- and postmenopausal women (Fig. 1A; Supplementary Table S2A). The association was stronger in premenopausal women particularly during mid-cycle (Supplementary Table S2A). Despite the significant correlations between serum and breast estradiol, 26.4% (14/53) premenopausal women had breast estradiol values outside the 95% CI of the regression line relating serum to breast hormone values (13 below, one above, see Fig. 1). In contrast, among 64 postmenopausal women, only three (4.7%) had breast estradiol levels outside these confidence bounds (two above, one below).

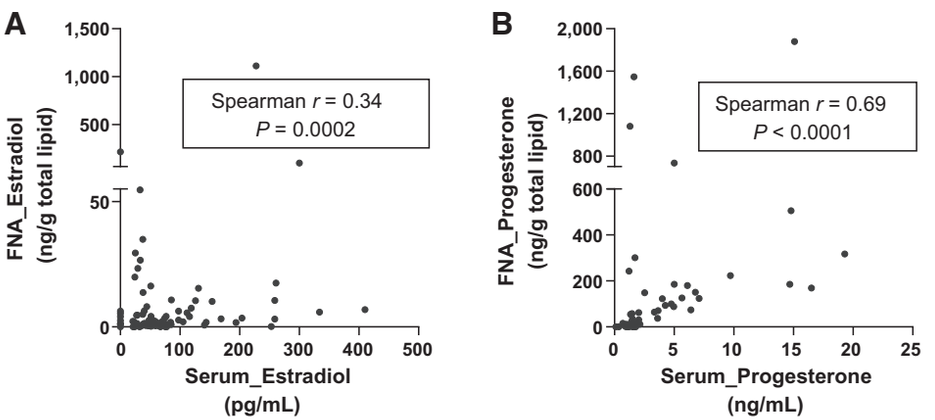

Figure 1.
Scatterplots with Spearman correlation between breast and serum hormone concentrations. Estradiol ($n = 117$; **A**) and progesterone concentrations ($n = 82$; **B**) showed significant positive correlation between breast and serum hormone concentrations.

Progesterone

Progesterone was detectable in 41 of 49 premenopausal breast samples (84%) but in only 6 of 33 postmenopausal samples (18%). The median progesterone concentration in the premenopausal breast was significantly higher than in postmenopausal breast ($P_{adj} < 0.0008$; Table 2A). Among premenopausal women, the breast progesterone concentration was 14-fold and 20-fold higher in the luteal phase than that in the follicular or mid-cycle phases, respectively ($P < 0.0008$ for both). The pattern of progesterone concentration in the breast was similar to that in serum (Table 2B). Breast and serum progesterone concentrations were strongly and positively correlated in premenopausal women particularly during mid-cycle and luteal phases, when progesterone concentrations were higher (Fig. 1B; Supplementary Table S2B). This correlation is not seen in the postmenopausal group (Supplementary Table S2B).

Breast androgens

We measured androgens in rFNA samples only; serum concentrations were not measured. Androstenedione was detectable in 53 of 54 (98%) and 49 of 65 (75%) of premenopausal and postmenopausal samples, respectively. The androstenedione concentration was significantly higher in postmenopausal samples than in premenopausal samples (39.6 ng/g vs. 32.3 ng/g, $P = 0.026$) but became not significant after Bonferroni correction (Table 2A). Testosterone was detectable in all premenopausal samples and in 62 of 65 (95%) of postmenopausal samples. The average testosterone concentration was equivalent in pre- and postmenopausal breasts (15.4 ng/g, $P = 0.381$; Table 2A). Among premenopausal women, the androstenedione concentrations in rFNA samples were relatively stable during the menstrual cycle while testosterone concentration varied, being highest during follicular phase, with a significant drop at mid-cycle (20.4 ng/g vs 6.1 ng/g, respectively, $P = 0.019$), and restoration during luteal phase (15.2 ng/g) similar to that of the follicular phase level, but this difference became nonsignificant after Bonferroni correction (Table 2B).

Effect of age on breast hormone content

When considering pre- and postmenopausal women together, the estradiol concentrations were inversely correlated with the subject age in both rFNA and serum samples, but the correlation was weak in the rFNA samples (Spearman $r = -0.18$, $P = 0.050$ for rFNA, and $r = -0.57$, $P < 0.0001$ for serum). The association between age and rFNA estradiol did not persist following Bonferroni correction (Table 2C). For progesterone, there was also a decrease with increasing age, which was similar between breast and serum samples ($r = -0.49$, $P_{adj} < 0.0008$ for rFNA and $r = -0.52$, $P_{adj} < 0.0008$ in serum; Table 2C). Other hormones measured in breast samples (estrone, androstenedione, testosterone) showed no correlation with age.

Other risk parameters in relation to breast and serum hormones

Because local breast hormone concentrations might affect other breast cancer risk parameters, we explored the relations of the five hormones we measured in the breast to two breast tissue–based risk factors, breast density, and cytologic atypia (Table 3). With a sample of 119 women, only 4 of whom had dense breasts, our evaluation of breast hormones with breast density is suboptimal; nevertheless, the only significant correlation was observed between breast density and breast testosterone concentration (Spearman $r = 0.26$, $P_{adj} = 0.02$; Table 3). This persisted when evaluated with linear regression analysis adjusted for age (regression coefficient = 0.09, $P = 0.011$). The Masood score was used to evaluate the epithelial cell cytology of rFNA samples. There was a significant positive correlation between breast progesterone concentration and Masood score (Spearman $r = 0.265$, $P = 0.024$), but this was no longer apparent following age adjustment and after Bonferroni correction. Other serum and breast hormones concentrations were unrelated to the Masood score. Masood scores were not affected by either menopausal status or menstrual phase (Supplementary Table S3A and S3B).

Table 3. Correlation of breast hormone concentrations to breast density and epithelial cytology

Hormones	N	Spearman r (95% CI)	P	Regression coefficient[a]	P[b]
Correlation between breast density and breast hormone concentrations					
Estrone	119	0.14 (−0.05–0.32)	0.129	0.56	0.28
Estradiol	119	0.10 (−0.08–0.28)	0.259	0.003	0.80
Progesterone	82	0.10 (−0.13–0.31)	0.381	−0.003	0.58
Androstenedione	102	0.09 (−0.11–0.28)	0.379	0.02	0.53
Testosterone	116	0.26 (0.08–0.43)	0.004[a]	0.09	0.011
Correlation between cytology (Masood score) and breast hormone concentrations					
Estrone	108	−0.17 (−0.35–0.03)	0.079	−0.13	0.072
Estradiol	108	−0.01 (−0.20–0.19)	0.924	−0.002	0.33
Progesterone	73	0.27 (0.03–0.47)	0.024	0.000	0.64
Androstenedione	92	−0.14 (−0.34–0.07)	0.177	−0.004	0.51
Testosterone	105	0.05 (−0.15–0.25)	0.611	−0.003	0.56

[a]P value remains significant after adjustment for multiple comparisons with Bonferroni correction.
[b]Linear regression analysis was performed for each hormone. All the analyses were adjusted for age.

Table 4. Correlation between gene methylation and breast hormone concentration

Genes	Spearman correlation[a]	Estrone ($N = 119$)	Estradiol ($N = 119$)	Progesterone ($N = 72$)	Androstenedione ($N = 102$)	Testosterone ($N = 116$)
CMI	*R* (95% CI)	0.08 (−0.11–0.26)	0.03 (−0.16–0.21)	0.1 (−0.12–0.32)	0.3 (0.11–0.47)	0.18 (−0.005–0.36)
	P	0.384	0.762	0.355	0.002[b]	0.049
AKR1B1	*R* (95% CI)	0.01 (−0.17–0.20)	0.05 (−0.14–0.23)	0.13 (−0.10–0.34)	0.28 (0.09–0.46)	0.04 (−0.15–0.22)
	P	0.897	0.579	0.262	0.004[b]	0.706
HIN1	*R* (95% CI)	0.1 (−0.09–0.28)	0.07 (−0.12–0.26)	0.14 (−0.09–0.35)	0.32 (0.13–0.49)	0.11 (−0.08–0.29)
	P	0.293	0.422	0.215	0.001[b]	0.236
RARB	*R* (95% CI)	0.2 (0.01–0.37)	0.01 (−0.17–0.20)	−0.22 (−0.43–0.001)	0.25 (0.05–0.43)	0.13 (−0.07–0.31)
	P	0.033	0.884	0.045	0.011	0.183
CCND2	*R* (95% CI)	−0.05 (−0.23–0.14)	0.07 (−0.12–0.25)	0.24 (0.02–0.44)	0.16 (−0.04–0.35)	0.12 (−0.07–0.30)
	P	0.584	0.44	0.03	0.112	0.219
RASSF1	*R* (95% CI)	0.01 (−0.17–0.20)	0.06 (−0.13–0.24)	0.28 (0.06–0.47)	0.13 (−0.08–0.32)	0.21 (0.03–0.38)
	P	0.885	0.524	0.012	0.204	0.023
TWIST	*R* (95% CI)	0.07 (−0.12–0.25)	0.01 (−0.17–0.20)	0.09 (−0.13–0.31)	0.18 (−0.02–0.37)	0.22 (0.03–0.39)
	P	0.464	0.9	0.413	0.073	0.019
TMEFF2	*R* (95% CI)	0.13 (−0.06–0.31)	0.02 (−0.17–0.20)	−0.05 (−0.27–0.17)	0.18 (−0.03–0.36)	0.06 (−0.13–0.25)
	P	0.17	0.86	0.65	0.08	0.51

[a]Nonparametric Spearman correlation (*r*), 95% CI, and significance (*P*) are shown.
[b]*P* values remain significant after adjustment for multiple comparisons with Bonferroni correction.

Correlation between DNA methylation (CMI) and hormones

We recently published the associations between DNA methylation and cytologic atypia observed in the current study population (27). We now examined the relationships between CMI and hormones in breast and serum samples. We observed significant positive correlations between CMI and breast androgen concentrations (Spearman $r = 0.3$, $P = 0.002$ for androstenedione; $r = 0.183$, $P = 0.049$ for testosterone), and the correlation between CMI and androstenedione persisted after Bonferroni correction ($P_{adj} = 0.016$; Table 4). At the individual gene level, androstenedione correlated with methylation of AKR1B1 ($P = 0.004$), HIN1 ($P = 0.001$), and RARB ($P = 0.011$) while testosterone correlated with RASSF1 ($P = 0.023$), and TWIST methylation ($P = 0.019$; Table 4). The correlation of AKR1B1 and HIN1 with androstenedione remained significant after Bonferroni correction ($P_{adj} = 0.032$ for AKR1B1 and $P_{adj} = 0.008$ for HIN1). The estrogen and progesterone concentrations had no significant correlation with CMI (Table 4). At the individual gene level, however, breast estrone content was positively correlated with RARB methylation ($P = 0.033$), which did not persist after Bonferroni correction. On the other hand, an inverse correlation was present between breast progesterone concentrations and RARB gene methylation ($P = 0.045$). Interestingly, breast progesterone was positively correlated with methylation of CCND2 (nominal $P = 0.03$) and RASSF1 ($P = 0.012$; Table 4), which did not survive after Bonferroni correction. Two CMI genes, *TMEFF2* and *TM6SF1*, were not significantly associated with any hormone concentrations (data not shown).

Discussion

In this study, we utilized rFNA samples from clinically normal breasts of women, unselected by breast cancer risk estimates, to measure steroid hormones including estrogens (estradiol and estrone), progesterone, and androgens (androstenedione and testosterone) using LC/MS-MS. We show that analysis of hormone content in rFNA samples from the clinically normal breast is feasible, and the results are consistent with known physiology, as well as with prior smaller reports in the literature which utilized benign whole tissue samples

Table 5. Summary of studies examining sex steroid concentrations in benign breast samples[a]

Author, year, method	Summary value, unit, matrix	Menopause	N	Estradiol	Estrone	Androstenedione	Testosterone
Vermeulen, 1986, RIA	Mean ± SD, ng/g protein	Pre	—	—	—	—	—
		Post	14	5.7 ± 4.9	3.9 ± 5.2	198 ± 100	13.1 ± 12.4
Thijssen, 1989, RIA (Polish study)	Median, ng/g tissue	Pre	8	0.13	0.31	0.22	0.30
		Post	8	0.05	0.10	0.16	0.11
Lonning, 2009, RIA	Geometric mean (95% CI), ng/g tissue	Pre	13	0.11 (0.063–0.21)	0.31 (0.17–0.56)	—	—
		Post	30	0.007 (0.004–0.012)	0.12 (0.10–0.17)	—	—
Falk, 2012, RIA	Geometric mean, ng/g total lipid	Pre	—	—	—	—	—
		Post	94	0.052	0.311	5.250	0.249
Lee, 2017, LC/MS-MS	Median (IQR), ng/g total lipid	Pre	54	1.66 (0.81–4.25)	1.03 (0–2.53)	32.3 (18.1–48.8)	15.4 (6.23–30.8)
		Post	65	1.12 (0.38–4.73)	0.48 (0–1.12)	39.6 (24.9–70.2)	15.4 (0–39.9)

[a]We included the studies that (i) measured both estrogen and androgen, (ii) compared by menopausal status within a study. Lonning 2009 study was added as an exception which estrogen was only measured but compared by menopausal group. Progesterone concentration in the breast has not previously been reported.

from cancerous breast, and used a variety of methods of steroid extraction, purification, and detection (refs. 12, 14, 15, 31; Table 5). Our data enable larger scale investigations of local breast hormone exposure, which will enhance our understanding of the hormonal etiology of breast carcinogenesis, and the relative role of local synthesis versus systemic exposure. Such studies are not feasible with surgical breast samples, which must be acquired when fresh, and are subject to the biases associated with disease states. Minimally invasive methods therefore lend themselves well to these studies, given the feasibility we have demonstrated.

We report, for the first time, a profile of five hormones in the normal/benign breast (estradiol, estrone, progesterone, androstenedione, and testosterone) in a population of pre- and postmenopausal women where menopausal and menstrual phase status was thoroughly characterized. Also novel was our utilization of the lipid fraction of rFNA samples to measure sex steroid hormones in normal/benign breast tissue, with the epithelial component reserved for cytology, and DNA methylation; previously we have measured cytology and RNA expression (32). Thus, it is possible to develop a composite picture of the relationships between multiple parameters in the normal/benign breast, to develop and test etiologic hypotheses, and construct models that will aid efforts at improved risk stratification and cancer prevention.

Our data demonstrate that breast estradiol concentrations are nonsignificantly higher in the premenopausal compared with postmenopausal women, whereas progesterone concentrations are dramatically higher in premenopausal breasts. However, 18% of postmenopausal breast samples contained detectable amounts of progesterone; persistent exposure of the postmenopausal breast to progesterone could plausibly relate to higher breast cancer risk, a hypothesis to be pursued in future larger studies. Breast estradiol content was relatively stable across the menstrual cycle and menopause, despite marked changes in serum estradiol, but breast progesterone changed significantly across the menstrual cycle, resembling the fluctuations seen in serum progesterone.

We have previously reported steroid hormone concentration in nipple aspirate fluid (NAF) samples and compared them with serum concentrations (18). We see some similarities and some differences in our NAF and rFNA results. In both rFNA and NAF, we find strong correlations between breast and serum progesterone, with progesterone level in premenopausal breasts being vastly higher than postmenopausal breasts in both NAF and rFNA. Because NAF is a secretory product of the breast parenchyma, these data suggest an equilibrium of systemic progesterone with the breast adipose tissue, and breast parenchyma with secondary diffusion into ductal lumina and NAF. Despite the strong correlations observed for progesterone in the current study, there is clearly variation, with individual values that are discordant between serum and breast samples. For serum estradiol, the correlation is weaker and discordant values more numerous. This may be a function of the inherent fluctuations of hormonal exposure in premenopausal women, or may have biological and risk implications beyond that; the current data do not allow any conclusions in this regard. However, they do show that in the majority of women, the systemic progesterone fluctuation during the menstrual cycle is closely reflected in the breast.

Notably, while we found no correlation of estradiol content in NAF and serum (18), rFNA estradiol content is modestly but significantly related to serum estradiol in this study, with correlations of 0.3 or greater in all subgroups except luteal phase women (Supplementary Table S2). Thus, estradiol patterns between serum and breast adipose tissue are more similar than in NAF. Specifically, NAF estradiol was the lowest at midcycle (18) while rFNA estradiol peaks at midcycle, along with serum estradiol. This discordance between NAF and rFNA suggests either a slower diffusion of estradiol into the ducts compared with progesterone, or other local factors related to synthesis or degradation of estradiol. We also evaluated androgens in rFNA samples, and found that androstenedione content was significantly higher in the postmenopausal breast while testosterone concentrations did not change by menopausal status.

Our findings are not directly comparable with prior studies of breast hormones, as those were performed using radioimmunoassay on benign tissue samples from tumor-bearing breasts, and the steroids were expressed per unit of protein, tissue, or oil (total lipid). Two small studies that had compared the hormone content of pre- and postmenopausal breasts have also found higher estradiol and estrone concentrations in premenopausal breasts (14, 31), although the units of quantity are not directly comparable (Table 5). In a study of 8 premenopausal and 8 postmenopausal breast using radioimmunoassay, Thijssen and colleagues found that both androstenedione and testosterone concentrations were nonsignificantly higher in premenopausal breasts than in postmenopausal breasts (31). In our substantially larger study using LC/MS-MS, we find that androstenedione content was significantly higher in postmenopausal compared with premenopausal breasts, but testosterone concentrations were similar across menopausal status. Although we did not measure serum androgens in our current study, in our previous NAF study (18) serum androstenedione level was significantly lower in postmenopausal than in premenopausal women. Together, these findings of lower serum androstenedione (NAF study) and higher breast androstenedione (current study) after menopause suggest that postmenopausal women have locally abundant androstenedione in the breast tissue despite lower circulating concentrations in the serum. These data support the notion that androstenedione is an abundant resource for local estrogen production by aromatase activity in the postmenopausal breast, as

does a small study by Falk and colleagues, who measured estrogen and androgen concentrations in breast adipocytes from reduction mammoplasty and benign tissue of cancer-bearing breast (33). They also found that androstenedione was abundant in the breast fat regardless of menopausal status.

Menstrual cycle variations in androgens have not been previously examined. We find that androstenedione concentrations in the breasts were relatively stable while testosterone concentrations varied during menstrual cycle. However, serum and NAF androstenedione and testosterone concentrations were not affected by menstrual cycle phase (18).

Turning to DNA measurements, we recently reported significant associations between cumulative DNA methylation and cytologic atypia measured as the Masood Score in 380 women (27); that association is also observed in the current subset study of 119 women (Spearman $r = 0.374$; $P_{adj} < 0.0007$; Supplementary Table S1). In exploratory analyses of associations between promoter methylation of specific genes, we see nominally significant positive relationships between breast progesterone content and CCND2 and RASSF1A methylation; and between DNA methylation and breast androgens (androstenedione and testosterone). Elevated concentrations of androstenedione in breast samples was associated with methylation of three genes (*AKR1B1*, *HIN1*, and *RARB*), while elevated testosterone concentrations in breast samples were associated with methylation of two genes (*RASSF1* and *TWIST*). These do not survive correction for multiple comparisons, except for the association of androstenedione with methylation of two genes (*AKR1B1* and *HIN1*); nevertheless these suggestive findings are worthy of pursuit in future studies, particularly as Bonferroni correction can increase type II error when used in small datasets. Surprisingly, there was no significant association of breast estradiol concentration with DNA methylation. These findings raise the possibility of a direct action of androgens on DNA methylation, and potentially, the utility of androgens as risk markers for breast cancer. This is consistent with our NAF case–control study, where we found that NAF androgen concentrations in the contralateral breasts of incident breast cancer cases were significantly higher than in age, race, and menopause-matched controls (18). The lack of correlations with estradiol, however, suggests that these correlations are not driven by androgens serving as a precursor pool to drive estradiol synthesis in the breast, but by direct androgenic signaling. It is also possible that estradiol concentrations are determined by both the rate of synthesis and the rate of degradation, and therefore the quantity of estradiol by itself does not provide the full picture.

Finally, in exploratory analyses in this group of 119 women, we did not observe significant associations of breast hormones with cytologic atypia following age-adjustment. However, a significant association between breast testosterone and breast density did persist following adjustment for age. These potential relationships remain of interest and should be pursued in future larger studies.

Limitations of this report are that it includes roughly one-third of the parent population of 380 women. This was related to initial difficulties in defining a sample handling protocol which addressed the need for cytology and hormone measurements, and to the fact that samples were expended earlier in the study in attempts at HPLC purification and detection by immunoassay. However it became clear that LC/MS-MS would be more accurate and reproducible. Another limitation is the fact that we did not measure all five hormones in the serum; this was partially related to budgetary considerations, and partially to the fact that serum concentrations have been extensively studies and are well known. Finally, despite the fact that this is the largest study to date of breast tissue hormones in healthy women, the results pertaining to correlations with other risk parameters are hypothesis-generating.

Conclusions

We used LC/MS-MS to evaluate the breast hormone concentrations of 119 healthy pre- and postmenopausal women who were well characterized for menopausal and menstrual status and were not a selected high-risk population. We found that breast progesterone fluctuations reflect those in the circulation, whereas estradiol concentrations are less closely related. Postmenopausal breasts remain rich in androgens. In parallel assessments of breast epithelial cytology and DNA methylation, breast androstenedione was positively associated with methylation of AKR1B1 and HIN1 after adjustment for multiple comparisons. These observations demonstrate that multiparameter evaluation of minimal breast samples obtained by fine-needle aspiration is feasible, and can yield data relevant to the characterization of breast cancer risk, in addition to providing mechanistic insights that can be pursued in future hypothesis-testing investigations of breast cancer risk.

Disclosure of Potential Conflicts of Interest

M.J. Fackler reports receiving a commercial research grant, has ownership interest (including stock, patents, etc.), and is a consultant/advisory board member for Cepheid. V. Stearns is a consultant/advisory board member for Iridium Therapeutics, Inc. S. Sukumar reports receiving a commercial research grant and is a consultant/advisory board member for Cepheid. No potential conflicts of interest were disclosed by the other authors.

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