Variation in Estradiol, Estradiol Precursors, and Estrogen-related Products in Nipple Aspirate Fluid from Normal Premenopausal Women

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Abstract

The purpose of the study was to measure the concentrations of estradiol, its primary precursors, and factors with which it interacts in the breast, and determine their sources of variation. Nipple aspirate fluid (NAF) was collected from premenopausal women during the midluteal phase of the menstrual cycle. The fluid was diluted and unconjugated steroids were extracted. Estradiol was further purified by a solvent partition into aqueous NaOH. Androgens were measured in the non-phenolic fraction. Water-soluble, conjugated steroids and proteins were measured in the aqueous residue. All analytes were measured by immunoassays. Permutation methods were used to determine the correlations over multiple periods of time. The average concentration of estradiol in NAF was 435 pmol/L after purification but was many times higher when assayed without purification. Estrone and dehydroepiandrosterone (DHEA) sulfates were present in 3.7 and 75 μmol/L concentrations, respectively, while unconjugated androstenedione and DHEA were present in nanomole per liter concentrations. Lack of the steroid sulfates in NAF in 19% of subjects had no effect on NAF estradiol levels but was associated with a 77% lower concentration of unconjugated DHEA. Progesterone was present in concentrations that were 3- to 4-fold higher than normal serum concentrations (mean: 291 nmol/L). Cathepsin D, epidermal growth factor, and interleukin 6 had average values of 3.4 μg/mL, 424 ng/mL, and 1.7 ng/mL, respectively. Correlations between breasts were between 0.57 and 0.84 for the several analytes; correlations over time ranged from 0.64 and 0.93 with estrone sulfate highest in both categories. The lower correlation between breasts than within breasts indicates that local factors play an important role in determining the levels of many of these analytes in the breast. The high stability of the concentrations of several analytes over time indicates that fluctuations in environmental factors have little immediate effect on levels in the breast, and portends their utility as surrogate breast cancer risk markers. (Cancer Epidemiol Biomarkers Prev 2004;13(6):928–35)

Introduction

Long-term estrogen exposure, in both endogenous and exogenous form, is now strongly established as an etiologic factor in breast cancer development. Our ability to exploit this knowledge in prevention research, however, has been limited by the need to measure estrogen and related compounds in the circulation, rather than in the target organ. In a limited number of studies, nipple aspirate fluid (NAF) has been obtained from women as a non-invasive means of assessing the concentrations of hormones and products of hormone action in the breast (1-6). The concentrations of estrogens seem to reflect the concentrations found in analyses of tissue extracts and breast cyst fluid (7-12). Estrogen administration results in increases in the estrogen inducible proteins pS2 and cathepsin D and decreases in estrogen-inhibited proteins apolipoprotein D and gross cystic disease fluid protein 15 (prolactin-inducible protein) in breast fluid, while estrogen deprivation results in the reverse effect on these proteins in NAF (4, 5). Thus, NAF seems to reflect the effects of estrogen on the breast. On the other hand, Ernster et al. (1) found no difference in the concentration of estradiol in NAF of pre- and postmenopausal women. This observation concurs with measurements of estrogens in breast tissue (11, 12). This unexpected result might be explained by formation of estradiol within the breast from precursors such as estrone sulfate and aromatizable androgens after menopause (13, 14).

Because hormone levels in NAF might provide a better reflection of estrogen concentrations within the breast than levels in serum do, the objective of the present study was to determine the following: the sensitivity and reproducibility of several newly developed assays, the stability of concentrations of estradiol and related compounds in NAF of premenopausal women who underwent repeat sampling over a period of 15 months, and the uniformity of measurements between breasts. In addition to measuring estradiol and its steroid precursors, we

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measured epidermal growth factor (EGF) and cathepsin D—two proteins up-regulated by estrogen activity—and interleukin 6 (IL-6)—a peptide involved in regulation of estradiol synthesis. This technique could provide novel intermediate biomarkers for breast cancer prevention trials or risk assessment procedures.

Methods

Subjects and Sample Collection. Women were recruited from the Chicago area by mass mailing, advertisements, and posters displayed near the Northwestern Medical Center area. All procedures with human subjects were approved by the Institutional Review Board of Northwestern University. Subjects were part of a study of hormonal responses to a high-fiber and low-fat diet, which included a high soy protein arm. This study has been described in detail elsewhere (15). Subjects reported having regular menstrual cycles of 25 to 35 days, had not taken any oral contraceptives for at least 6 months, were not taking any medications that would interfere with normal ovarian function, were not pregnant and did not plan a pregnancy, had not lactated for at least 6 months, and were between the ages of 20 and 40 years of age (mean 34.8 years). The racial makeup of the group was 83% White, 11% Black, 4% Hispanic, and 2% other. Subjects completed a life events questionnaire and recorded information on their diets and exercise throughout the study. Participants as a whole were physically more active than the average woman residing in Chicago, were self-selected, and may not reflect the general population. The risk of breast cancer in this group using the Gail model (16) was 0.24 ± 0.18% for 5 years and 12.0 ± 1.24% lifetime. Physical activity at baseline was assessed using a validated comprehensive questionnaire developed by Sidney et al. (17). The 1st, 4th, 12th, and 15th months were designated data collection months. In these months, the subjects collected urine specimens on days 10 to 15 for self-assessment of ovulation using a commercial test kit. They collected daily saliva samples and came to the clinic to have blood drawn and to have samples of NAF collected 5 to 8 days after the urinary luteinizing hormone peak was detected. For NAF collection, the breasts were warmed and massaged by the subjects themselves. Then a small volume of fluid was aspirated from each breast with a syringe connected to a bell-shaped applicator. When more than one duct in a breast provided fluid, the fluid from those ducts was combined. Overall, NAF was obtained from 48% of women enrolled in the study. Generally, if NAF was obtained from a subject at the first visit, it was obtained on all subsequent visits. The fluid was collected in calibrated capillary tubes, the volume was recorded, and the ends were sealed with clay. The median volume of NAF collected was 11 μL (range 0 to 151 μL). The samples were stored at −70°C until they were thawed for assay. Additional samples were collected using a ductal lavage procedure with instruments and reagents from Cytyc Corp., Boxborough, MA, described previously (18).

Sample Preparation. The volume of NAF was diluted 1:9 with PBS, pH 7.4 (PBS). Cellular components were not separated from the fluid. From the diluted sample, 50 μL were generally taken for extraction, although as little as 30 μL were used in some cases. This volume was further diluted to 700 μL with PBS. Ductal lavage fluid (DLF) samples that had been diluted in Cytolyt (Cytyc) were centrifuged to remove cells. The alcohol was evaporated with a centrifugal evaporator, the water was removed by freeze-drying, and the sample was made up to an exact volume with water. The samples were extracted 2× with 1.0 mL of ethyl acetate-hexane (3:2). The remaining aqueous fraction was kept for assay of water-soluble analytes and the organic solvent was evaporated and redissolved in 1.0 mL isooctane. Estradiol was extracted from the isooctane solution with 1.0 + 0.5 mL of 0.4 N aqueous NaOH, the alkaline solution was neutralized with HCl, and the estradiol was re-extracted into ethyl acetate. The ethyl acetate was washed with 0.5 mL of water and evaporated. The residue was dissolved in 1.0 mL PBS, pH 7.0, containing 0.1% gelatin for assay. Standards of estradiol for the assay were prepared in the same buffer. The isooctane solution containing the neutral steroids was diluted with 0.1 mL of ethyl acetate, washed with water, and evaporated. It was redissolved in 800 μL PBS, pH 7.4, containing 0.1% bovine serum albumin. Standards for dehydroepiandrosterone (DHEA), androstenedione, and progesterone assays were prepared in the same buffer. Water used in the procedures was deionized (Milli-Q water purification system, Millipore Corp., Bedford, MA) and then redistilled in an all glass still. The sodium phosphate salts used to prepare the buffers were recrystallized from aqueous methanol. Ethyl acetate, hexane, and isooctane were redistilled within 2 weeks of use. Pipet tips were washed in an ultrasonic cleaner with Liquinox (Alconox Corp., White Plains, NY) detergent before use and rinsed with deionized water.

The concentration of DHEA sulfate (DHEAS) that was observed in direct assays was extremely high, suggesting that something in the samples was interfering with binding of the steroid to the antiserum. Therefore, the solvolysis technique (19) was used to hydrolyze the sulfate from DHEAS, and the resulting DHEA was assayed in an aliquot of the free steroid.

Assays. All samples were assayed in duplicate. All samples from each subject were assayed within one assay. Each assay included two or more quality controls that were prepared from a pool of NAF that had been aliquoted in small volumes, and stored at −20°C before use. Buffer blanks were carried through the assay, and the values obtained were subtracted from the final results. When commercial kits were used, they were modified such that the standards for the assays were prepared in the same buffer that was used to redissolve the samples. Estradiol and progesterone were measured as described previously for salivary assays (20). DHEA and androstenedione were assayed using kits supplied by Diagnostic Systems Laboratories, Webster, TX. The intra-assay CVs for estradiol, progesterone, DHEA, and androstenedione were 13.7, 16.0, 11.0, and 11.1, respectively. Inter-assay CVs for estradiol, DHEA, and androstenedione were 16.8, 43.5, and 20.3, respectively. Values for progesterone in the pool were below the lowest standard and could not be used for this purpose. Samples from the same woman were all analyzed in a single batch, so inter-assay variation was not a factor in the comparisons between breasts or visits. However, variation between batches (kits) was high, especially for

DHEA, and this must be improved for between-subject comparisons. Using the sample preparation procedure described above, the buffer blanks for estradiol, progesterone, DHEA, and androstenedione were 1.37 ± 0.95 pmol/L, 0.11 ± 0.16 nmol/L, 0.0 ± 0.009 nmol/L, and 0.07 ± 0.18 nmol/L, respectively. The efficiency of the extraction and purification procedure for estradiol was evaluated using the tritiated hormone to determine procedural losses. The recovery of 22.9 and 45.9 pmol estradiol/L in buffer, after fractionation and measurement in the RIA, was 77 ± 4 and 83 ± 3%, respectively.

In the aqueous fraction, DHEAS was assayed directly by an ELISA from Diagnostic Systems Laboratories and, after solvolysis with 0.1% HCl in acetone (19), by an ELISA for DHEA (Diagnostic Systems Laboratories). Estrone sulfate was assayed directly by a RIA from Diagnostic Systems Laboratories. EGF and IL-6 were assayed by ELISAs from R&D Corp., Minneapolis, MN. Cathepsin D was measured by an ELISA from Calbiochem, San Diego, CA. The intra-assay CVs for DHEAS (after hydrolysis), estrone sulfate, cathepsin D, EGF, and IL-6 were 15.0, 7.8, 17.6, 7.1, and 16.2, respectively. Inter-assay CVs were 53.7, 20.0, 23.2, and 12.7, respectively. The concentration of IL-6 in the QC pool was not significantly above the blank and, therefore, the CV could not be calculated. The study uses only within-subject comparisons. The variability must be improved for between-subject comparisons, especially for DHEAS for which the hydrolysis procedure varied in efficiency between batches. The buffer blanks for DHEAS (after hydrolysis), estrone sulfate, cathepsin D, EGF, and IL-6 were 5.9 ± 11.8 μmol/L, 0.09 ± 0.13 nmol/L, 0.92 ± 1.0 ng/mL, −2.0 ± 3.0 ng/mL, and 10 ± 6.9 pmol/L, respectively. A pool of NAF was initially tested by comparison of direct assay with assay after the extraction procedure. After extraction, the average measured values for cathepsin D, EGF, and IL-6 were 96.8%, 94.4%, and 80.5%, respectively, of that in unextracted samples. The values presented have not been corrected for procedural losses. The sensitivity of the individual assays was sufficient for measurement of the mean value of all analytes in NAF when the sample size was 5.0 μL. Protein in NAF was assayed by the Bradford method (Bio-Rad, Hercules, CA).

Statistical Methods. Spearman correlation coefficients were calculated for each of 1000 bootstraps. Correlation coefficients and 95% confidence intervals were averaged across these bootstraps (21, 22). These results were used to examine reproducibility between breasts at a given time and over time within a single breast. This method was also employed to examine per-weight versus per-volume comparisons. Random effects models and the Statistical Analysis System (SAS) procedure PROC VARCOMP were used to obtain variance components for calculating between-subject intraclass correlation coefficients (ICC) using Type I estimation.

Results

In the present study, the median volume of NAF obtained was 11 μL (range 0 to 150 μL) with 49.3% of women producing sufficient volume for analysis on the first visit (i.e., ≥3 μL); 81.9% of those producing NAF on the first visit produced NAF on the second visit. Because of the small volume of NAF obtained from most women, we found it necessary to fractionate the analytes that we wished to measure to have sufficient amounts for assay. The dilution and extraction steps for separation of the unconjugated steroids did not adversely affect the immunoreactivity of the proteins that remained in the aqueous fraction. Furthermore, the conjugated steroids were retained by greater than 95% in the aqueous fraction when using the ethyl acetate-hexane (3:2) solvent for extraction. A further separation of the organic fraction was necessary for measurement of androgen precursors and estradiol. The partitioning of the phenolic estrogens into 0.4 N aqueous sodium hydroxide from isooctane gave excellent separation with almost no carryover to the opposite fraction. This system not only permitted analysis of nine different analytes in as little as 3 μL of NAF, but provided sufficient purification of estradiol for analysis.

Because the actual volume of breast fluid cannot be determined in the ductal lavage sample, the concentrations of analytes were expressed as units per milligram of protein. It was established in NAF that the relationships between analytes remains essentially the same when expressed per volume of fluid or per milligram of protein. Thus, the relationships before and after fractionation of the samples in DLF apply to NAF as well. On the other hand, there was no advantage, based on precision of sequential measurements, when data were expressed by total protein. A comparison of the results of direct assays and assays conducted after the fractionation procedure for all analytes in the ductal lavage samples is shown in Table 1. Only estradiol, cathepsin D, and EGF have been measured in NAF in previous studies. The concentration of estradiol was significantly less after purification (88% less, Table 1). It

Table 1. Comparison of direct assays with assays for the same substances after extraction and purification of the analytes in split DLF samples from 10 subjects

Analyte	DLF direct mean ± SE	DLF fractionated mean ± SE	Significance of difference *P*
Estradiol, pmol/g protein	90.7 ± 22.0	11.0 ± 4.4	0.002
Estrone sulfate, nmol/g	47.4 ± 11.4	52.6 ± 10.0	0.74
DHEA, nmol/g	2.7 ± 1.1	1.0 ± 0.6	0.20
DHEA sulfate, μmol/g	59.4 ± 13.6	55.6 ± 11.1	0.83
Androstenedione, nmol/g	4.5 ± 1.3	ND	—
Progesterone, nmol/g	4.1 ± 1.9	1.7 ± 0.6	0.24
Cathepsin D, ng/g	367 ± 132	199 ± 92	0.31
EGF, ng/g	6.7 ± 1.1	8.2 ± 1.2	0.64
IL-6, pg/g	7.9 ± 2.2	4.1 ± 2.3	0.25

Table 2. Correlations with 95% confidence intervals between analytes expressed per liter and per milligram of protein in NAF

Analyte	Correlation
Estradiol	0.94 (0.89-0.97)
Estrone sulfate	0.96 (0.92-0.98)
DHEAS	0.98 (0.96-0.99)
DHEA	0.97 (0.94-0.98)
Androstenedione	0.96 (0.93-0.98)
Progesterone	0.95 (0.90-0.97)
Cathepsin D	0.88 (0.79-0.93)
EGF	0.68 (0.49-0.81)
IL-6	0.95 (0.91-0.97)

Table 4. ICCs for all analytes with 95% confidence intervals

Analyte	ICC
Estradiol	0.33 (0.27-0.39)
Estrone sulfate	0.87 (0.82-0.90)
DHEAS	0.72 (0.67-0.77)
DHEA	0.52 (0.46-0.58)
Androstenedione	0.71 (0.64-0.77)
Progesterone	0.53 (0.48-0.59)
Cathepsin D	0.62 (0.48-0.68)
EGF	0.62 (0.56-0.67)
IL-6	0.34 (0.29-0.40)

is evident that the purification procedure is necessary to eliminate cross-reacting substances from estradiol. The cross-reactivity of the estradiol antiserum for estrone sulfate was 0.0106% and that for DHEAS was 0.0015%. These cross-reactions can account for the entire difference between the measured concentrations of estradiol in the direct and extraction procedures. Some of the other analytes were also less after the extraction and purification procedure but not significantly so in this sample of 10 subjects. However, the levels of DHEAS measured in the direct assay may also be in excess of the actual amount of the sulfated steroid. After hydrolysis of the sulfate, the amount measured in the DHEA assay was only 3.42% (confidence interval: 2.39%-4.83%) of the direct assay concentration (not shown). However, the correlation between the measurement before and after the hydrolysis procedure was 0.89.

Shown in Table 2 are the correlations between data expressed per milliliter and per milligram of protein in DLF. It is clear that expressing the data per unit of protein did not change the relative amounts of the analytes. Thus, comparisons can be made between assays of DLF and NAF when both are expressed per milligram of protein. The average concentrations of the analytes for both breasts across all four samples of NAF are presented in Table 3. Correlations between breasts and across the 15-month period of the study are also shown in Table 3 for each of the analytes. The variability of the analytes was marginally greater for most analytes between breasts sampled at the same time than it was within breasts over 15 months. Table 4 lists the ICCs for each of the analytes. There is a wide range of values with estradiol and IL-6 being low, estrone sulfate and DHEAS having high values, and cathepsin D and EGF intermediate.

Seven of 47 subjects had undetectable concentrations of both estrone sulfate and DHEAS in both breasts at all time periods. These seven subjects had an average estradiol concentration of 439 ± 30 (SE) pmol/L compared with 435 ± 27 (SE) pmol/L for the remaining subjects. The difference was not significant. By comparison, unconjugated DHEA levels in the subjects with very low or absent steroid sulfates were significantly lower ($P < 0.001$) than the remaining subjects (10.2 ± 3.5 SE versus 56.2 ± 6.7 SE pmol/L, respectively).

Figure 1 displays the relationships between the first (baseline) and the fourth sample, approximately 15 months later, for all analytes.

Discussion

NAF represents fluid that accumulates in the ductal system of the non-pregnant breast. This report describes a new assay methodology for determining concentrations of estradiol—as well as its precursors and response proteins—in NAF. The results indicate that, in a single sample of NAF, concentrations of estradiol, estrone sulfate, DHEA, DHEAS, androstenedione, progesterone, cathepsin D, EGF, and IL-6 can be measured reproducibly,

Table 3. Mean concentration of analytes in NAF from premenopausal women with correlations between breasts and over time

Analyte	Number of subjects (samples)	Concentration (mean ± SE)	Right versus left breast correlations*	Correlations over 15 months per breast*
Protein	47 (271)	74.4 ± 2.1 mg/mL		
Estradiol	31 (188)	435 ± 27 pmol/L	0.61 (0.34-0.78)	0.65 (0.43-0.80)
Estrone sulfate	43 (284)	3.74 ± 0.26 μmol/L	0.84 (0.73-0.91)	0.93 (0.88-0.96)
DHEAS[c]	45 (292)	75.1 ± 6.7 μmol/L	0.67 (0.47-0.81)	0.88 (0.80-0.93)
DHEA	42 (269)	56.0 ± 6.1 nmol/L	0.65 (0.43-0.79)	0.71 (0.53-0.83)
Androstenedione	24 (147)	6.0 ± 0.6 nmol/L	0.71 (0.47-0.85)	0.77 (0.57-0.88)
Progesterone	43 (288)	291 ± 29 nmol/L	0.71 (0.53-0.83)	0.76 (0.61-0.86)
Cathepsin D	47 (292)	3.42 ± 0.38 μg/mL	0.68 (0.47-0.81)	0.74 (0.58-0.85)
EGF	47 (299)	424 ± 12 ng/mL	0.57 (0.33-0.74)	0.71 (0.54-0.83)
IL-6	45 (291)	1.74 ± 0.21 ng/mL	0.73 (0.53-0.85)	0.64 (0.42-0.79)

*Correlations by the permutation method with 95% confidence interval.
[c]Measured after hydrolysis as DHEA.

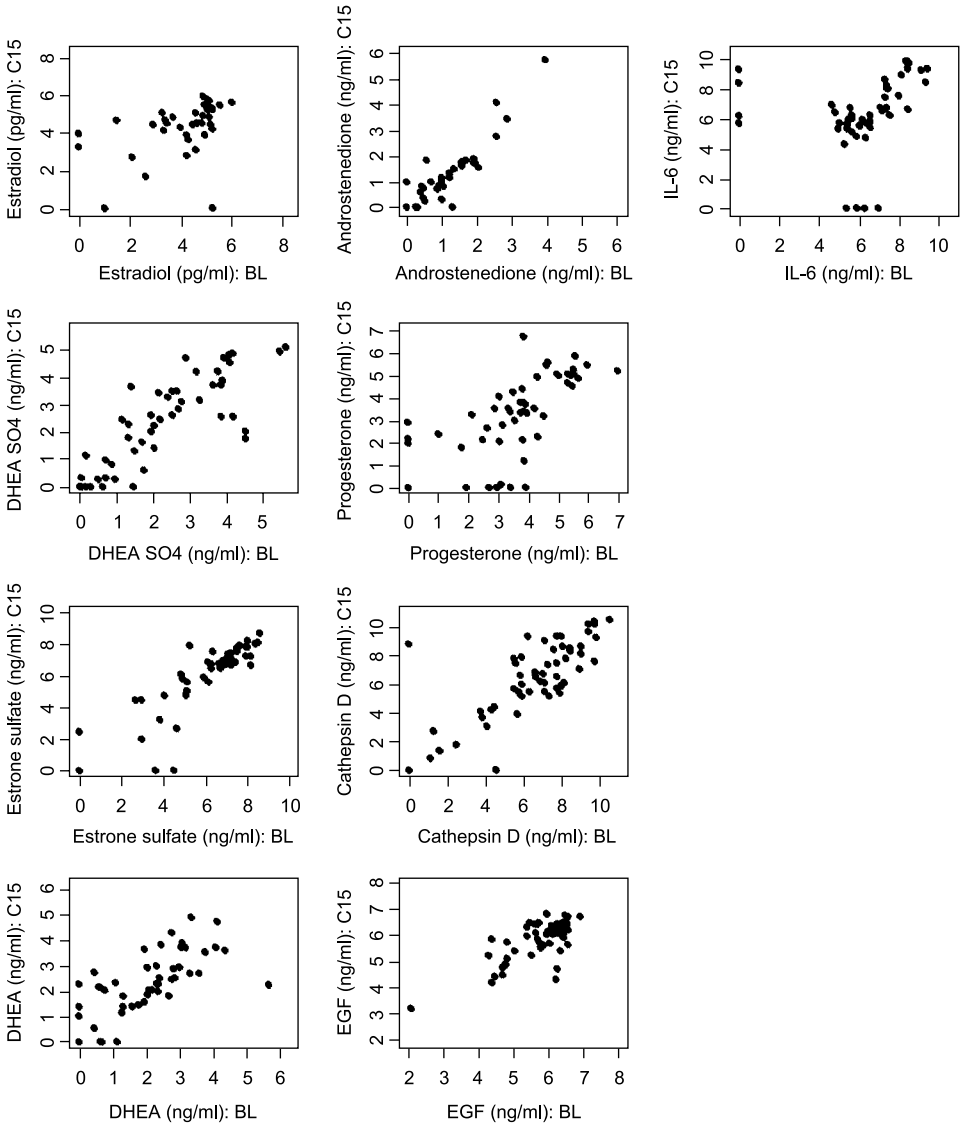

Figure 1. Distribution of log values for each analyte. Log values of the concentrations of each analyte in samples collected in the 15th month (*C15*) are plotted against the corresponding log values in the baseline (*BL*) samples. A correlation of 1.0 would be represented by a *diagonal line* from the lower left corner to the upper right of each plot. Plots of values from the 4th and 12th months versus the baseline samples are not shown for brevity, but the distributions were similar to those shown for 15th month. That is, there was little increase in dispersion between the 4th and 15th months.

with substantial correlations between breasts and between sampling visits in the same woman. The percentage of women who produce an adequate volume for analysis decreases progressively after menopause (23). Thus, the procedure cannot be used to ascertain the levels of hormones in the breast of all women but, among those capable of producing fluid, most can be studied longitudinally. It should be noted that women providing NAF might not be representative of the general population in that the capacity to produce fluid has been associated with elevated risk of breast cancer (24).

Estradiol concentrations in NAF were substantially lower than previously reported. After extraction and assay, Ernster et al. (1) obtained an average of 3,202 pmol/L estradiol in NAF obtained from 76 healthy, premenopausal women. In a previous study, using the same procedure described in this report with extraction and purification by solvent partition, we obtained an average value of 1,478 ± 324 pmol/L estradiol (14). The even lower mean level of estradiol in the present study, 435 pmol/L, might be attributable, in part, to the high level of physical activity among these women. The average physical activity score for this group of women was 605 ± 347 (SD), which is considerably higher than the average activity score (338) for white women over the age of 24 years with >12 years of education (the latter is the highest score for women obtained in the original study; ref. 17). The distributions of estradiol and estrone sulfate concentrations in NAF were skewed toward low values. All samples were collected from normally menstruating women in the mid-luteal phase of the menstrual cycle. Nevertheless, some women may not have had ovulatory cycles in the months that were sampled. It will remain for future analyses to examine the reproductive, anthropometric, and lifestyle characteristics associated with NAF hormone levels. The differences in estradiol among the studies were not due to losses of estradiol through the purification procedure. Assuming that the undiluted concentration of protein in NAF and in ductal lavage fluid was the same, the mean concentration of

estradiol, measured without extraction or further purification in our laboratory was 6,660 pmol/L. Clearly, effective extraction and purification are necessary to avoid overestimation of NAF estradiol levels.

Potential estradiol precursors, estrone sulfate, DHEA, and androstenedione, were present in the breast in concentrations that were manyfold greater than estradiol. Enzymes for conversion of these precursors to estradiol have all been identified in breast cells (25, 10). The estrone sulfate concentrations in NAF were surprisingly similar to those reported for human cyst fluid; the range in NAF was undetectable to 35.1 nmol/L compared with undetectable to 30.5 nmol/L in cyst fluid (26). DHEAS was also similar; the mean value in NAF was 75 μmol/L compared with 89 μmol/L (27) and 68 μmol/L (7) in cyst fluid. The potential for conversion of estrone sulfate to estradiol certainly exists (10, 14), although the extent to which potential precursors are converted in the breast of premenopausal women is not known.

Labrie et al. (25) have pointed out that, unlike most other animals, humans use an intracrine system for production of much of the androgen and estrogen that is active in cells of target organs. DHEA and other precursors are produced in great quantity and are converted to active hormones within the target organs. Thus, the high concentrations of estrone sulfate and DHEAS found in NAF may serve as a reservoir for intracrine signaling. In such a system, the circulating concentrations of estradiol and testosterone are not necessarily indicative of the exposure of the responsive cells to these hormones.

Progesterone has been shown to promote EGF signaling in the breast (28) and has been implicated in increasing the risk of breast cancer in postmenopausal women taking combined hormone replacement therapy (29). Its correlation in NAF across time intervals was 0.76 with sampling occurring during the mid-luteal phase of the menstrual cycle. Thus, it had somewhat greater consistency over time than estradiol, and may represent an important potential marker of risk.

The finding that 7 of the 47 subjects had no detectable levels of estrone sulfate or DHEAS in NAF suggests that they either lack sulfotransferase activity or have very high sulfatase activity in the breast. How this may affect their risk of breast cancer is not known, but sulfotransferase/sulfatase activities have been suggested as a mechanism by which estrogens may be locally regulated (30-32). It is interesting that in this group of premenopausal women, the lack of estrone sulfate had no significant effect on the levels of estradiol in NAF. This suggests that if estrone sulfate is an important precursor of estradiol, compensating mechanisms exist to maintain estradiol concentrations. On the other hand, the lack of NAF DHEAS was associated with a significant 77% decline in NAF DHEA. This is taken as evidence that DHEAS is a source of DHEA in the breast and emphasizes the difference in regulation of the two unconjugated steroids.

Cathepsin D is the product of a gene that is promoted by estradiol (33, 34). Eskelinen et al. (35) and Harding et al. (4) have shown independently that the levels of cathepsin D in NAF increase in response to estrogens and decrease in response to estrogen deprivation. Cathepsin D is a lysosomal aspartyl protease that has been associated with metastasis but not by promotion of cellular escape. Rather, it facilitates growth of tumor cells at distant sites (36, 37) and is an indicator predictive of recurrence (38-40). Cathepsin D is present in high concentrations in NAF (>3 μg/mL), is normally distributed among subjects, and is relatively stable over time. EGF is known to up-regulate expression of the gene for cathepsin D (36). It is commonly elevated in breast cancer (41), and its elevation is associated with reduced survival (42). NAF EGF was shown previously by us to be correlated with serum levels of estradiol (21) within subjects and its production has been reported to be stimulated by progesterone (43). The concentration of EGF is high and easily measured in NAF. Stability over time was similar to that of cathepsin D, making it also a potentially useful marker of risk.

IL-6 promotes aromatization of androgen precursors (44) and stimulates 17β-hydroxysteroid dehydrogenase activity (45) in the breast. Therefore, it may mediate increases in estrogen levels in the breast that are caused by inflammatory processes (46). It has been considered a potential growth factor in the breast (47), and has been negatively associated with survival in patients with metastatic breast cancer (48). Its concentration in NAF is generally quite low, it is not easily measured in NAF, and, not surprisingly, it was the least stable over time among the analytes measured. Nevertheless, if its concentrations were persistently elevated, it could indicate a chronic inflammatory process or infection, which has been associated with breast cancer in mice and other cancers in humans (49).

Like estradiol, EGF levels tended to be skewed toward low values. The reason for this pattern is not known. The stability of the levels of all of the measured variables over the 15-month period of observation was quite high. Except for IL-6, the correlations calculated across the four periods by the permutation procedure for each breast were higher for all analytes than the correlations between breasts of the same woman. The greater correlations within breasts over time than between breasts suggests that local factors must be important in determining the level for each breast, and indicates that the level of surrogate markers in one breast must be applied cautiously in considering risk for the opposite breast. The sulfates of estrone and DHEA were remarkable in having correlations over time of 0.93 and 0.88. The sulfates have much longer half-lives than the unconjugated steroids, and this may be why they are more stable than the free steroids. Nevertheless, the correlation within breasts of 0.65 for estradiol is impressive, and indicates that processes that determine the level of estradiol in the breast are relatively invariant properties of the breast. The ICCs ranged widely among the several analytes. Estradiol was relatively low by comparison with serum (50) or saliva (51) but the androgens were similar to ICCs reported for serum (50). The ICC of estrone sulfate was much higher than that of estradiol, indicating that it may have some advantage in studies of estrogens in relation to cancer. Cathepsin D and EGF also had reasonably high ICCs; the value of 0.62 for EGF is significantly higher than that reported in a smaller earlier study (21).

Intermediate biomarkers of breast cancer risk that are sufficiently stable over time will provide a means of

predicting risk or measuring response to preventive interventions. At present, atypical cells from NAF, ductal lavage, or fine needle aspiration offer some measure of risk of breast cancer. Unfortunately, the findings of atypia are not very reproducible over time. Recent data on 25 women (67 ducts) from this laboratory show a reproducibility of 45% for benign or atypical cells from ductal lavage,[6] and, therefore, are not particularly useful for assessing risk. The hormonal variables described in this report, particularly estrone sulfate and cathepsin D, are remarkably stable within breasts over time, and may be useful surrogate markers of breast cancer risk. Future work will focus on the relationship of serum and NAF marker levels, the relationship of NAF biomarkers to each other, and the identification of factors that determine NAF hormone and biomarker levels.

References

1. Ernster VL, Wrensch MR, Petrakis NL, et al. Benign and malignant breast disease: initial study results of serum and breast fluid analyses of endogenous estrogens. J Natl Cancer Inst 1987;79:949-60.
2. Rose DP. Hormones and growth factors in nipple aspirates from normal women and benign breast disease patients. Cancer Detect Prev 1992;16:43-51.
3. Rose DP, Lahti H, Laakso K, Kettunen K, Wynder EL. Serum and breast duct fluid prolactin and estrogen levels in healthy Finnish and American women and patients with fibrocystic disease. Cancer 1986; 57:1550-4.
4. Harding C, Osundeko O, Tetlow L, Faragher EB, Howell A, Bundred NJ. Hormonally-regulated proteins in breast secretions are markers of target organ sensitivity. Br J Cancer 2000;82:354-60.
5. Hargreaves DF, Potten CS, Harding C, et al. Two-week dietary soy supplementation has an estrogenic effect on normal premenopausal breast. J Clin Endocrinol & Metab 1999;84:4017-24.
6. Sauter ER, Babb J, Daly M, et al. Prostate-specific antigen production in the female breast: association with progesterone. Cancer Epidemiol, Biomarkers & Prev 1998;7:315-20.
7. Inaudi P, DeLeo V, Baldini S, D'Antona N. Adrenal steroids and tumor antigens in plasma and cyst fluid of women with gross cystic breast disease. Ann NY Acad Sci 1990;586:117-20.
8. van Landeghem J, Poortmen J, Nabuurs M. Endogenous concentrations and subcellular distribution of oestrogens in normal and malignant human breast tissue. Cancer Res 1985;45:2900-6.
9. Geisler J, Berntsen H, Lonning PE. A novel HPLC-RIA method for the simultaneous detection of estrone, estradiol and estrone sulphate levels in breast cancer tissue. J Steroid Biochem Mol Biol 2000;72: 259-64.
10. Pasqualini JR, Cortes-Prieto J, Chetrite G, Talbi M, Ruiz A. Concentrations of estrone, estradiol and their sulfates, and evaluation of sulfatase and aromatase activities in patients with breast fibroadenoma. Int J Cancer 1997;70:639-43.
11. Thijssen JHH, Blankenstein MA. Uptake and metabolism of estradiol by normal and abnormal breast tissues. Ann NY Acad Sci 1990;586: 252-8.
12. Yue W, Santner SJ, Masamura S, et al. Determinants of tissue estradiol levels and biologic responsiveness in breast tumors. Breast Cancer Res Treat 1998;49 Suppl 1:S1-S7.
13. Pasqualini JR, Schatz B, Varin C, Nguyen BL. Recent data on estrogen sulfatases and sulfotransferases activities in human breast cancer. J Steroid Biochem Mol Biol 1992;41:323-9.
14. Chatterton RT Jr, Geiger AS, Gann PH, Khan SA. Formation of estrone and estradiol from estrone sulfate by normal breast parenchymal tissue. J Steroid Biochem Mol Biol 2003;86:159-66.
15. Gann PH, Chatterton RT Jr, Gapstur SM, et al. The effects of a low fat high fiber diet on sex hormone levels and menstrual cycling in premenopausal women: a 12-month randomized trial. Cancer 2003; 98:1870-9.
16. Gail MH, Greene MH. Gail model and breast cancer. Lancet 2000; 355:1017-2000.
17. Sidney S, Jacobs DR Jr, Haskell WL, et al. Comparison of two methods of assessing physical activity in the Coronary Artery Risk Development in Young Adults (CARDIA) Study. Am J Epidemiol 1991;133:1231-45.
18. Dooley WC, Ljung B-M, Veronesi U, et al. Ductal lavage for detection of cellular atypia in women at high risk for breast cancer. J Natl Cancer Inst 2001;93:1624-32.
19. Jacobsohn GM, Lieberman S. Studies on the chemical cleavage of the urine glucuronosides of the 17-ketosteroids. J Biol Chem 1962; 237:1469-75.
20. Lu Y-C, Bentley GR, Gann PH, Hodges KR, Chatterton RT Jr. Salivary estradiol and progesterone levels in conception and nonconception cycles in women. Evaluation of a new assay for salivary estradiol. Fertil Steril 1999;71:863-8.
21. Gann P, Chatterton R, Vogelsong K, Dupuis J, Ellman A. Mitogenic growth factors in breast fluid obtained from healthy women: evaluation of biological and extraneous sources of variability. Cancer Epidemiol, Biomarkers & Prev 1997;6:421-8.
22. Good P. Permutation test: a practical guide to resampling methods for testing hypotheses. 2nd ed. New York: Springer-Verlag; 2001.
23. Petrakis NL. Physiologic, biochemical, and cytologic aspects of nipple aspirate fluid. Breast Cancer Res Treat 1986;8:7-19.
24. Wrensch MR, Petrakis NL, Miike R, et al. Breast cancer risk in women with abnormal cytology in nipple aspirates of breast fluid. J Natl Cancer Inst 2002;93:1791-8.
25. Labrie F, Luu-The V, Labrie C, et al. Endocrine and intracrine sources of androgens in women: inhibition of breast cancer and other roles of androgens and their precursor dehydroepiandrosterone. Endocr Rev 2003;24:152-82.
26. Castagnetta L, Granata OM, Brignone G, et al. Steroid patterns of benign breast disease. Ann NY Acad Sci 1990;586:121-36.
27. Maeda Y, Tanaka E, Fujiwara M, et al. Accumulation of 4- and 5-ene steroid sulfates in human breast cyst fluids. J Steroid Biochem Mol Biol 2002;81:249-53.
28. Lange CA, Richer JK, Shen T, Horwitz KB. Convergence of progesterone and epidermal growth factor signaling in breast cancer. Potentiation of mitogen-activated protein kinase pathways. J Biol Chem 1998;273:31308-16.
29. Rossouw JE, Anderson GL, Prentice RL, et al. Risks and benefits of estrogen plus progestin in healthy postmenopausal women: principal results from the Women's Health Initiative randomized controlled trial. J Am Med Assoc 2002;288:321-33.
30. Falany JL, Falany CN. Expression of cytosolic sulfotransferases in normal mammary epithelial cells and breast cancer cell lines. Cancer Res 1996;56:1551-5.
31. Purohit A, Reed MJ. Oestrogen sulphatase activity in hormone-dependent and hormone-independent breast-cancer cells: modulation by steroidal and non-steroidal therapeutic agents. Int J Cancer 1992;50:901-5.
32. Pasqualini JR, Chetrite G, Nestour EL. Control and expression of oestrone sulphatase activities in human breast cancer. J Endocrinol 1996;150 Suppl:S99-105.
33. Cavailles V, Augereau P, Rochefort H. Cathepsin D gene is controlled by a mixed promoter, and estrogens stimulate only TATA-dependent transcription in breast cancer. Proc Natl Acad Sci USA 1993;90:203-7.
34. Wang F, Porter W, Xing W, Archer TK, Safe S. Identification of a functional imperfect estrogen-responsive element in the 5′-promoter region of the human cathepsin D gene. Biochemistry 1997;36:7793-801.
35. Eskelinen M, Kataja V, Hamalainen E, Kosma VM. Receiver-operating characteristic (ROC) curve analysis of cathepsin D and pS2 in breast disease; results from a prospective study. Anticancer Res 1999;19:3463-7.
36. Rochefort H, Garcia M, Glondu M, et al. Cathepsin D in breast cancer: mechanisms and clinical applications, a 1999 overview. Clin Chim Acta 2000;291:157-70.
37. Garcia M, Platet N, Liaudet E, et al. Biological and clinical significance of cathepsin D in breast cancer metastasis. Stem Cells 1996;14:642-50.
38. Gillesby BE, Zacharewski TR. pS2 (TFF1) levels in human breast cancer tumor samples: correlation with clinical and histological prognostic markers. Breast Cancer Res Treat 1999;56:253-65.
39. Fulco RA, Petix M, Salimbeni V, Torre EA. Prognostic significance of the estrogen-regulated proteins, cathepsin-D and pS2, in breast cancer. Minerva Med 1998;89:5-10.
40. Bankowska A, Gacko M, Chyczewska E, Worowska A. Biological and diagnostic role of cathepsin D. Rocz Akad Med Bialymst 1997;42 Suppl 1:79-85.

[6] SA Khan, unpublished data.

41. Bolufer P, Lluch A, Molina R, et al. Epidermal growth factor in human breast cancer, endometrial carcinoma and lung cancer. Its relationship to epidermal growth factor receptor, estradiol receptor and tumor TNM. Clin Chim Acta 1993;215:51-61.
42. Rosfjord EC, Dickson RB. Growth factors, apoptosis, and survival of mammary epithelial cells. J Mammary Gland Biol Neoplasia 1999; 4:229-37.
43. Murphy LC, Murphy LJ, Dubik D, Bell GI, Shiu RP. Epidermal growth factor gene expression in human breast cancer cells: regulation of expression by progestins. Cancer Res 1988;48:4555-60.
44. Reed MJ, Purohit A. Breast cancer and the role of cytokines in regulating estrogen synthesis: an emerging hypothesis. Endocrine Rev 1997;18:701-15.
45. Purohit A, Ghilchik MW, Dincan LJ, et al. Aromatase activity and interleukin-6 production by normal and malignant breast tissues. J Clin Endocrinol & Metab 1995;80:3052-8.
46. Morrow LE, McClellan JL, Conn CA, Kluger MJ. Glucocorticoids alter fever and IL-6 responses to psychological stress and to lipopolysaccharide. Am J Physiol Regul Integr Comp Physiol 1993;264: R1010-16.
47. Chiu JJ, Sgagias MK, Cowan KH. Interleukin 6 acts as a paracrine growth factor in human mammary carcinoma cell lines. Clin Cancer Res 1996;2:215-21.
48. Salgado R, Junius S, Benoy I, et al. Circulating interleukin-6 predicts survival in patients with metastatic breast cancer. Int J Cancer 2003; 103:642-6.
49. zur Hausen H. Viruses in human cancers. Eur J Cancer 1999;35: 1878-85.
50. Hankinson SE, Manson JE, Spiegelman D, Willett WC, Longcope C, Speizer FE. Reproducibility of plasma hormone levels in postmenopausal women over a 2-3 year period. Cancer Epidemiol, Biomarkers & Prev 1995;4:649-54.
51. Gann PH, Giovanazzi S, Van Horn L, Branning A, Chatterton RT Jr. Saliva as a medium for investigating intra- and interindividual differences in sex hormone levels in premenopausal women. Cancer Epidemiol, Biomarkers & Prev 2001;10:59-64.