Endocrine Biomarkers in Ductal Lavage Samples from Women at High Risk for Breast Cancer

Deepa Bhandare,1 Ritu Nayar,2 Michele Bryk,1 Nanjiang Hou,3 Rachel Cohn,1 Nazar Golewale,1 Noah P. Parker,4 Robert T. Chatterton,4 Alfred Rademaker,3 and Seema A. Khan1

Departments of Surgery, Pathology, Preventive Medicine, and Obstetrics and Gynecology, Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, Illinois

Abstract

Background: Ductal lavage is a method of minimal epithelial sampling of the breast, with potential utility for repeat sampling and biomarker analysis in chemoprevention studies. We report here the baseline findings from a study designed to assess the utility of ducal lavage in this setting.

Methods: Tamoxifen-eligible, high-risk women underwent ducal lavage; epithelial cell number (ECN) and morphology were assessed on Papanicolaou-stained slides. Additional slides were immunostained for estrogen receptor (ER) α, Ki-67, and cyclooxygenase-2, and the labeling index (LI) was established by counting negative and positive cells. The ductal lavage supernatant (DLS) was assayed for estradiol, several of its precursors, progesterone, cathepsin D, interleukin-6, and epidermal growth factor (EGF).

Results: One hundred sixty-eight women have entered the study (mean age, 51 years; mean 5-year Gail score, 2.8).

Introduction

Studies of the normal human breast have, in the past, been hampered by the inability to access breast epithelial cells, although encouraging data have been published on cells acquired by aspiration of nipple fluid in healthy volunteers (1) and random fine-needle aspiration in high-risk healthy women (2). Ductal lavage is a minimally invasive procedure developed to analyze breast epithelial cells (3, 4). It has the theoretical advantage that the same duct may be sampled sequentially over time, thus facilitating the identification of biomarkers of breast cancer risk that are reversible with effective preventive agents (surrogate end point biomarkers). This issue has been identified as a high priority by a Task Force of the AACR (5), because the testing of new agents in II prevention trials. Our first study was designed to evaluate the utility of this technique in a cohort of high-risk women, particularly in assessing the reproducibility of cellular variables measured by lavage of the same duct over time. We have chosen to use tamoxifen as the preventive intervention because it is at present the gold standard in terms of pharmacologic breast cancer prevention, and its mechanisms of action are well understood, allowing the assessment of logical candidate biomarkers. Here, we report our findings regarding feasibility of cytologic and biomarker studies in the first 168 women who have entered this study. The accrual goal for this study is 262 women. The intent of this preliminary analysis is to assess the feasibility of biomarker measurement in these samples, the distribution of individual biomarkers in this population, and the interrelations between biomarkers. In addition, the analysis of two or more duct samples per subject per procedure adds significantly to the expense of the study and the complexity of analysis. We are therefore presenting the analysis both by duct and by woman (averaging the results across ducts) to assess whether separate analysis by duct provides a meaningful advantage.

Materials and Methods

Our study subjects were recruited from the Bluhm Family Program for Breast Cancer Early Detection and Prevention, at the Lynn Sage Breast Center of Northwestern Memorial Hospital, on an institutional review board–approved study protocol. Data on known breast cancer risk factors were used to calculate the 5-year Gail risk estimate (7). Eligible women included (a) unaffected healthy women with a 5-year Gail estimate of >1.6 and (b) those with unilateral invasive breast cancer, ≤1 cm in size (T1a or T1b, lesions of any histologic type), or duct carcinoma in situ. Eligible women with a history of
breast cancer were required to have one unaffected breast, and only the unaffected breast was lavaged.

After informed consent, ductal lavage was done in the office (baseline lavage); women were informed of the cytologic findings and allowed to choose tamoxifen therapy or observation. All subjects will undergo a repeat lavage 6 months after the first procedure or 6 months after initiation of tamoxifen (follow-up lavage). A final 12-month lavage will be done only on those women who have atypical cytology at the 6-month point. This report presents baseline data on the first 168 women entered into the study.

Ductal Lavage Procedure. Subjects applied an anaesthetic cream EMLA (2.5% lidocaine and 2.5% prilocaine; Astrazeneca, Wilmington, DE) 2 hours before the lavage procedure. Nitroglycerin cream (glyceryl trinitrate, E. Fougera & Co., Melville, NY) was applied to the nipple of each breast to be studied 20 minutes before the procedure along with a warm compress. The breast was massaged, and the Cytyc aspirator (Cytyc Corp., Boxborough, MA) was used to elicit nipple aspirate fluid. Lavage of fluid-yielding ducts and visualized non-fluid-yielding ducts was performed through a microcatheter (Cytyc), using Plasmalyte (Baxter Healthcare Corp., Deerfield, IL). The lavage effluent was collected in Cytolyte (Cytyc Corp., Boxborough, MA) was used to elicit nipple aspirate fluid. Lavage of fluid-yielding ducts and visualized non-fluid-yielding ducts was performed through a microcatheter (Cytyc), using Plasmalyte (Baxter Healthcare Corp., Deerfield, IL). Thelavage effluent was collected in Cytolyte (Cytyc). The location of the lavaged duct(s) was noted on an 8 × 8 nipple grid, and the duct orifice was marked with a prolene suture, the 12 o’clock axis of the areola marked with a skin pen, and the nipple was photographed to facilitate recannulation of the same duct in the future.

Cellular Analyses. The lavage effluent was spun at 1,125 x g for 10 minutes, and the supernatant was collected without disturbing the cell pellet and stored at −20°C for subsequent analyses. The cells were resuspended in 20 mL Preservecyt solution (Cytyc). The first one-fourth aliquot of this cell suspension was processed using a ThinPrep 2000 processor (Cytyc) to prepare a Papanicolaou-stained slide for cytologic diagnosis and estimation of cell yield. The diagnostic categories were insufficient cells for morphologic diagnosis (<10 cells per slide), benign, mild atypia, marked atypia, and malignant as described previously (ref. 8; see Fig. 1A). The fraction of the atypical cells (% atypia) was documented on every sample diagnosed with atypical cytology.

The second one-fourth aliquot was used for immunohistochemical evaluation of the fraction of cells expressing estrogen receptor (ER) α (see Fig. 1B) using heat antigen retrieval (DakoCytomation, Carpinteria, CA) and a 1:200 dilution of Clone SP1 (Lab Vision Corp., Fremont, CA). The third one-fourth aliquot was used for double immunohistochemical staining of nuclear Ki-67 and cytoplasmic cyclooxygenase-2 (COX-2; see Fig. 1C). Mouse monoclonal antibodies Ki-67, clone MIB-1 (DakoCytomation) diluted 1:200 and COX-2 (Cayman Chemical Co., Ann Arbor, MI) diluted 1:100 were used sequentially followed by color development (3,3’-diaminobenzidine for ERα and COX-2 and Vector Red for Ki-67). The labeling index (LI) for each marker was calculated by counting positively and negatively stained epithelial cells (average of 1,000 cells per slide). A colocalization index (colocalization LI) was calculated as cells expressing both COX-2 and Ki-67. The intraobserver variability was assessed by blinded repetition of counts on 20 random immunohistochemical slides each for ERα and Ki-67-COX-2.

The number of epithelial cells were counted on the Papanicolaou-stained, ERα and dual-labeled slides; the average number of cells per aliquot was calculated, and the fourth aliquot was assumed to contain the same number of cells. The cell number from all four aliquots was summed to generate the total epithelial cell number (ECN). Samples with total cell number of <100 were considered insufficient for analysis.

Analysis of DLS. The DLS was assayed for the following biomarkers: estradiol (E2), estrone sulfate, progesterone, cathepsin D, interleukin-6 (IL-6), epidermal growth factor (EGF), androstenedione, dehydroepiandrosterone (DHEA), and DHEA sulfate. Details of the procedure have been described elsewhere (6). Briefly, samples were put through a centrifugal evaporator, freeze-dried, and made up to an exact volume with water. Extraction with 1.0 mL ethyl acetate-hexane produced aqueous and nonaqueous fractions. Estrone sulfate, DHEA sulfate, cathepsin D, IL-6, and EGF were measured in the aqueous residue. The nonaqueous fraction was partitioned into phenolic and nonphenolic fractions. E2 was measured in the phenolic fraction and androstenedione, DHEA, and progesterone are measured in the nonphenolic fraction. RIA is used for measurement of progesterone, E2, and estrone sulfate. ELISA is used for measurements of cathepsin D, IL-6, EGF, androstenedione, DHEA, and DHEA sulfate. As seen with nipple aspirate fluid samples, the concentration of DHEA sulfate observed in direct assays was extremely high, suggesting interference of binding of the steroid to the antiserum. Therefore, the sulfate was hydrolyzed from DHEA sulfate and the resulting DHEA (DSH) assayed using ELISA (6). Quality-control preparations prepared from a pool of DLS are measured in each assay. Buffer blanks are carried through the procedure as well, and value of the blank is subtracted from each sample value. The hormones and proteins are

Figure 1. A. Papanicolaou-stained ThinPrep preparation of the first aliquot of the ducal lavage sample showing a cluster of ductal epithelial cells along with macrophages. B. Second aliquot showing ERα-positive ductal epithelial cells. C. Double immunostaining showing cytoplasmic COX-2 (brown) and nuclear Ki-67 (red) positivity in ductal epithelial cells.
expressed per milligram of total protein in the lavage supernatant.

Statistical Methods. For statistical analyses, the data for cellular and supernatant biomarkers were analyzed by duct, where each duct was an independent observation. We also generated weighted averages of the cellular and supernatant variables by women, averaging data across all the ducts examined in an individual woman. Here, the worst cytologic diagnosis per woman was used, so that if a single duct showed cytologic atypia among several that were lavaged the woman was classified as having atypical cytology.

Cytologic diagnosis was related to cellular and supernatant biomarkers using logistic regression analysis. Odds ratios (OR) were calculated as the change in odds of atypia for every SD unit increase in the marker, unless otherwise specified. ECN was related to biomarkers using Spearman correlation coefficients. Correlations among biomarkers were also assessed using Spearman correlation coefficients (R). Intraclass correlation coefficients were calculated using the ratio of the between-woman variance and the total variance as estimated from a one-way random-effects ANOVA (9). Intraclass variability was assessed using Lin’s concordance coefficient (10). A t test for zero correlation was used to determine whether the intraclass correlation was significantly different from zero.

A principal components factor analysis was done to obtain a composite score of the nine hormone and protein variables (androstenedione, DHEA, DSH, E₂, estrone sulfurate, progesterone, EGF, IL-6, and cathepsin D) and five other variables (total cell number, ER LI, COX-2 LI, Ki-67 LI, and colocalization LI). Two factors explained 52% of the variance in all 14 variables. The first factor representing the hormone and protein variables explained 36% of the total variance and that factor was subjected to further analysis. A high factor score means high values on some or all of these variables. For each of six variables (cytologic diagnosis of atypia, numerical measure of atypia, Ki-67 LI, COX-2 LI, colocalization LI, and total ECN), a regression model was run relating the ER LI and the factor score to the variable. Analyses were done two ways: one using duct as the unit of analysis and the other using woman as the unit of analysis.

Results

Subject Demographics. Thus far, 168 tamoxifen-eligible women have been enrolled in the study: The majority, 102 of 168 (60.7%) are of European ancestry; 126 of 168 (76.1%) women were asymptomatic and high-risk, 22 (13.1%) had ductal carcinoma in situ, and 18 (10.7%) had T₁a or T₁b, invasive cancer. Ninety-seven women (70 high-risk and 27 with cancer) were postmenopausal (mean age, 56 years; mean Gail score, 2.9). Four of the 97 postmenopausal women were on hormone replacement therapy within 6 months before lavage. The mean age of 71 premenopausal women was 45 years and their mean Gail score was 2.6. Of the 71 premenopausal women, 38 women had lavage done in the follicular phase, whereas 25 were lavaged in luteal phase. The menstrual phase could not be determined in 8 women. There were 14 participants with a BRCA mutation carrier probability of ≥10% as calculated using the BRCAPro program. There were 88 women of European ancestry, 13 of African ancestry, 12 women were Ashkenazi Jewish, 2 were of Asian Pacific origin, and 3 were of Hispanic origin.

Of the 168 women, 23 (13.7%) failed to produce nipple aspirate fluid and were not lavaged; 28 of 168 (16.6%) women yielded samples insufficient for analysis (i.e., with <100 total epithelial cells), leaving 117 of 168 (70%) women with samples adequate for analysis. The cytologic findings and number of cells recovered in the 117 women with sufficient cells for analysis are shown in Table 1. The concentration of hormones is standardized by protein; hence, we are only able to report the supernatant variables in women who had measurable protein in their DLS; 72 of 168 (43%) of women had measurable protein, of whom 4 had insufficient cells. This leaves 68 women in whom cellular and hormone variables can be related to each other. Figure 2 illustrates the breakdown of participants with measurable cellular and hormone variables.

Analysis of Cellular Biomarkers

By Duct. After exclusion of ducts with insufficient cells, the median ECN was 5,896 cells per duct lavaged (range, 100-58,293). There was no significant difference in median ECN per duct among women who were high-risk (5,737 cells) and those who had a history or contralateral malignancy (6,887 cells; Kruskal-Wallis P for difference = 0.22). Mild atypia was seen in 66 of 372 (17.7%) ducts and severe atypia was noted in 2 of 372 (0.5%) ducts (Table 1). The proportion of ducts with atypical cells was 27% in the high-risk group and 15% in women with contralateral malignancy (Fisher’s exact P = 0.12). When cytologic findings were benign, the mean ECN was 6,882 and when atypia was present, the mean ECN was 10,936. The odds of cytologic atypia as a categorical variable increased by 8% when the ECN increased by 1 SD or 1,134 cells (OR, 1.08; 95% confidence interval, 1,03-1.12; P = 0.0008; Table 2). In addition, the proportion of atypical cells in each sample was also positively related with epithelial cell yield (R = 0.24; P < 0.0001; Table 2). The other cellular biomarkers (ER, Ki-67, and COX-2) did not show any significant relationships with cytologic atypia. However, increasing cell yield per duct was significantly associated with increasing proportion of ER-positive cells (R = 0.28; P < 0.0001), increasing COX-2 LI (R = 0.23; P = 0.0003), increasing proliferation (R = 0.16; P = 0.01), and increasing colocalization of COX-2 and Ki-67 (R = 0.26; P < 0.0001).

By Woman. In the analyses by woman, data across all the ducts (and both breasts, in women who underwent bilateral lavage) were averaged. Mild atypia was seen in 43 of 145 (29.6%) women and severe atypia was noted in 2 of 145 (1.4%) women (Table 1). In the 117 women analyzed, the median ECN was 11,366 cells (range, 137-11,626). This did not differ between women who were high-risk and those with contralateral early breast cancer (11,278 versus 11,785; P = 0.84). The proportion of high-risk women with at least one duct showing cytologic atypia was 41.3% in the high-risk group and 20% in the women with contralateral breast cancer (P = 0.06). The presence of cytologic atypia in at least one duct was again significantly positively associated with total ECN across all ducts (see Table 2). In women with all ducts being cytologically benign, the mean ECN was 11,621, whereas when at least one duct was cytologically atypical, the mean ECN was 28,153. As in the analysis by duct, higher ECN was associated with increasing proportion of atypical cells (R = 0.35; P < 0.0001), increasing Ki-67 LI (R = 0.30; P = 0.001), and increasing colocalization of COX-2 and Ki-67 (R = 0.31; P = 0.0006). The analyses by women differed from the analyses by duct in that increasing ECN was no longer associated with ER LI and COX-2 LI. These data are shown in Table 2.

Table 1. Distribution of cytodiagnosis by woman and by duct

<table>
<thead>
<tr>
<th>Cytologic diagnosis</th>
<th>By woman (n = 145)</th>
<th>By duct (n = 372)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>Median ECN</td>
</tr>
<tr>
<td>ICMD*</td>
<td>29 (20)</td>
<td>0</td>
</tr>
<tr>
<td>Benign</td>
<td>71 (49)</td>
<td>4,875</td>
</tr>
<tr>
<td>Mild atypia</td>
<td>45 (32.6)</td>
<td>6,856</td>
</tr>
<tr>
<td>Severe atypia</td>
<td>2 (1.4)</td>
<td>4,898</td>
</tr>
</tbody>
</table>

*ICMD, insufficient cells for morphologic diagnosis.
related protein EGF, which showed significant correlations
the two groups (cellular and endocrine) was the estrogen-
supernatant biomarker with significant relationships between
exception of DSH levels when analyzed by woman). The

Intraobserver Variability. This was tested by blinded repeti-
tion of immunohistochemical counts on ERs and COX-2-Ki-67
labeled slides from 20 random ducts. We found high concordance coefficients for ER LI (0.87), COX-2 LI (0.87), and Ki-67 LI (0.71).

Analysis of Supernatant Biomarkers

By Duct. The correlation of the hormone and endocrine response proteins with cytologic atypia and ECN, found in the 72 women with measurable protein, is shown in Table 3. Of these, 31 were premenopausal, 34 were postmenopausal, and 2 were postmenopausal on hormone replacement therapy. Menopausal status could not be determined in 5 women.

Analysis of 151 such ducts showed that both cytologic atypia and ECN were significantly associated with increasing EGF concentrations in the lavage supernatant: for cytologic atypia, analyzed as a categorical variable, the OR was 1.87 ($P = 0.05$), and for ECN analyzed as a continuous variable, $R$ is 0.27 ($P = 0.002$).

By Woman. When similar analyses were done on 72 women who had measurable protein, increasing cytologic atypia was again positively and significantly associated with increasing levels of EGF, but in addition, a significant positive association was also seen with supernatant DSH levels (Table 3). Similarly, increasing ECN was associated with increasing DSH and estrone sulfate levels.

Analysis of Cellular Biomarkers with Supernatant Biomarkers. This analysis was done on samples where at least one duct had measurable data on cells and hormones (68 women and 141 ducts; see Table 4). In general, the cellular biomarkers (ER LI, COX-2 LI, and Ki-67 LI) were related to each other. The hormonal variables (estrone sulfate, DHEA, and DSH) showed significant interrelationships, although there was a remarkable paucity of correlation of $E_2$ with other variables (with the exception of DSH levels when analyzed by woman). The supernatant biomarker with significant relationships between the two groups (cellular and endocrine) was the estrogen-related protein EGF, which showed significant correlations with increasing ER LI and COX-2 LI as well as DHEA, DSH, and estrone sulfate, whether analyzed by woman or by duct.

Interclass Correlation Analysis. We examined between-subject variance (variance of subject) and within-subject variance (variance of duct). An intraclass correlation value of \( \leq 0.32 \), as determined by the \( t \) test, was used as evidence that the correlation was not significantly greater than zero, and \( \geq 0.33 \) indicated that the correlation was significantly different from zero. Results of variability in the levels of the biomarkers within women (duct to duct variability) and between women showed that measurements of $E_2$ (0.77), androstenedione (0.38), Ki-67 LI (0.41), COX-2 LI (0.34), and ER LI (0.33) had varying degrees of stability when measured across different ducts in the same woman.

Factor Analysis. Because ER is a therapeutic target for tamoxifen, we analyzed the cellular variables by the score for the first factor (which included hormone and protein variables) and ER LI. This was again done in two ways: one using duct as the unit of analysis and one using woman as the unit of analysis (Table 5). When factor scores were low (below median), the proportion of atypical samples did not differ when ER LI was above or below median. In contrast, for high factor scores (above the median), the proportion of atypical samples was 53.9% when ER LI was below the median and 17.7% when ER LI was above the median, a difference of \(-36.2\%\). This interaction was significant ($P = 0.05$). When analyses were done by woman, the trend was similar, but the interaction was not significant ($P = 0.11$).

For both high and low factor scores, the mean ECN was higher when ER LI was high, with a statistically significant interaction ($P = 0.001$). Results by woman did not have a significant interaction ($P = 0.55$) but displayed a similar direction. The highest COX-2 LI values were seen when both factor and ER LI were high and the lowest when both were low in the analyses by duct. This interaction was significant ($P = 0.021$). When analyzed by woman, the trend was similar, but the interaction was not significant ($P = 0.35$). Ki-67 LI was...
higher when factor score was high. With low factor scores, Ki-67 indices were lower when factor score was high. This interaction was significant ($P = 0.021$). When analyzed by woman, the interaction was also significant, with the highest Ki-67 indices being found when both factor score and ER LI was above median ($P$ for interaction = 0.01).

### Discussion

Ductal lavage is a technique that allows serial sampling of breast epithelial cells from women at high risk for development of breast cancer. This ongoing study was designed to assess the feasibility and utility of ductal lavage for the monitoring of biomarkers in women undertaking a preventive intervention. We also hoped to obtain preliminary information regarding the distribution of endocrine biomarkers that are potential surrogate end points in a prevention setting. The process of sampling breast epithelial cells by ductal lavage has the theoretical advantage of repeat sampling of the same duct at different time points, but maintaining separate samples for each duct increases the cost of supplies and processing and complicates the statistical analysis. An alternative approach would be to pool samples across ducts, so that only one sample is analyzed per woman. We have therefore examined the relationships of biological variables with each other by duct and by woman to gain insight into whether the expected relationships are preserved when data from different ducts in the same woman are pooled.

Among the first 168 women entered, we were able to perform ductal lavage on 86% of women and obtain sufficient cells for cytologic evaluation in 81% of these (or 70% of the total number of women presenting for ductal lavage). The success rate of ductal lavage in this study compares favorably with those from the original multicenter study of ductal lavage, which showed that 299 of 500 (59.6%) women had cells sufficient for cytologic diagnosis (3). The number of ducts lavaged is also in a similar range (mean, 1.7 per breast in our hands versus 1.5 ducts per breast in the multicenter study). We used ThinPrep processing for cytologic slide preparation, whereas in the multicenter study Millipore filtration a technique that provides superior cell retrieval was used. In that study, 10 random clusters of epithelial cells were counted; the mean cells per cluster were multiplied with the number of clusters, and an estimate of single cells and small clusters was added, resulting in a median ECN of 13,500 per duct in the study (3). In another study where 19 ductal lavage samples were obtained in the operating room from women with a variety of breast findings (nipple discharge, breast cancer, and benign lumps), the median epithelial cell count was 15,680 (11). Our study population consisted of 145 asymptomatic, high-risk women, mean age was 51 years, 75 of whom were postmenopausal, all lavaged in an office setting, median cell count per duct was 5,896 cells (range, 100-58,293), and mean of 11,366 cells per woman (range 137-116, 626). This was based on the sum of direct counts of each of three slides. We estimate from experiments using cultured cells that the loss rate with ThinPrep processing is in the range of 20% to 40% (data not shown).

A cytologic finding of mild atypia was more frequent in our hands than in the multicenter trial (3), 29.6% versus 17%, whereas severe atypia was less frequent (0.3% versus 6%).

### Table 3. Correlation of supernatant biomarkers in 72 women and 151 ducts with cytologic atypia and ECN

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>By duct</th>
<th>By woman</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>n</td>
<td>OR*</td>
</tr>
<tr>
<td>Androstenedione (ng/mg)</td>
<td>110</td>
<td>1.02</td>
</tr>
<tr>
<td>DHEA (ng/mg)</td>
<td>133</td>
<td>1.80</td>
</tr>
<tr>
<td>DSH (ng/mg)</td>
<td>64</td>
<td>4.77</td>
</tr>
<tr>
<td>E2 (pg/mg)</td>
<td>128</td>
<td>1.14</td>
</tr>
<tr>
<td>Estrone sulfate (ng/mg)</td>
<td>136</td>
<td>0.12</td>
</tr>
<tr>
<td>Progesterone (ng/mg)</td>
<td>86</td>
<td>2.60</td>
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<tr>
<td>EGF (ng/mg)</td>
<td>126</td>
<td>1.87</td>
</tr>
<tr>
<td>IL-6 (pg/mg)</td>
<td>77</td>
<td>0.81</td>
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<tr>
<td>Cathepsin D (ng/mg)</td>
<td>138</td>
<td>0.84</td>
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### Table 4. Spearman correlation coefficients of supernatant biomarkers with cell indices and with each other

<table>
<thead>
<tr>
<th>Biomarker</th>
<th>By duct</th>
<th>% Atypia</th>
<th>ERLI</th>
<th>COX-2</th>
<th>Ki-67</th>
<th>L1</th>
<th>Androstenedione</th>
<th>DHEA</th>
<th>DSH</th>
<th>E2</th>
<th>Estrone sulfate</th>
<th>Progesterone</th>
<th>EGF</th>
<th>IL-6</th>
<th>Cathepsin D</th>
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<tr>
<td>By woman</td>
<td>% Atypia</td>
<td>0.02</td>
<td>0.11</td>
<td>0.03</td>
<td>0.06</td>
<td>0.11</td>
<td>0.13</td>
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<td>0.03</td>
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<td>0.07</td>
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<tr>
<td>COX-2 L1</td>
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<td>Ki-67 L1</td>
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<td>0.14</td>
<td>0.004</td>
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<tr>
<td>Estrone sulfate</td>
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<td>0.13</td>
<td>0.27</td>
<td>0.26</td>
<td>0.25</td>
<td>0.53</td>
<td>0.67</td>
<td>0.07</td>
<td>0.05</td>
<td>0.43</td>
<td>0.02</td>
<td>0.15</td>
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</tr>
<tr>
<td>Progesterone</td>
<td>0.31</td>
<td>0.15</td>
<td>0.01</td>
<td>0.07</td>
<td>0.28</td>
<td>0.33</td>
<td>0.27</td>
<td>0.15</td>
<td>0.17</td>
<td>0.01</td>
<td>0.44</td>
<td>0.21</td>
<td></td>
<td></td>
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<tr>
<td>EGF</td>
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<td>0.26</td>
<td>0.29</td>
<td>0.25</td>
<td>0.24</td>
<td>0.26</td>
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<td>0.17</td>
<td>0.54</td>
<td>0.09</td>
<td>0.06</td>
<td>0.37</td>
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<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>0.32</td>
<td>0.05</td>
<td>0.08</td>
<td>-0.10</td>
<td>0.07</td>
<td>-0.02</td>
<td>0.12</td>
<td>0.21</td>
<td>0.04</td>
<td>-0.35</td>
<td>0.12</td>
<td>0.27</td>
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<tr>
<td>Cathepsin D</td>
<td>0.15</td>
<td>0.19</td>
<td>0.14</td>
<td>0.21</td>
<td>0.08</td>
<td>0.02</td>
<td>0.25</td>
<td>0.13</td>
<td>0.27</td>
<td>-0.20</td>
<td>0.48</td>
<td>0.35</td>
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<td></td>
</tr>
</tbody>
</table>

NOTE: Correlations in the top right are by duct; correlations in the bottom left are by woman. Sample sizes are 68 women and 141 ducts. The values in boldfaced have $P < 0.05$. 

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When we analyzed data by ducts, the rate of mild atypia was 17.7% (66 of 372) and severe atypia was 0.5% (2 of 372). In a study of 480 healthy high-risk women undergoing random fine-needle aspiration, hyperplasia with atypia was reported in 21% of the subjects (2). Ductal lavage samples with atypical morphology contained higher mean number of epithelial cells, consistent with the notion that proliferative epithelium is more cellular and exfoliates readily, yielding a larger number of cells on lavage. This is similar to an observed association of high cell yield and cytologic atypia using random fine-needle aspiration (12, 13).

**Cellular Indices.** The median ER LI in this cohort of high-risk women was 24.8%. This is higher than what we have observed in the past (14) and is probably explained by newer, more sensitive antibodies, sampling of purely luminal cells that are more likely to be ER positive, and the fact that this is a high-risk population. In another case-control study using histologic material, ER LI in normal breast lobules was found to be 20% in controls and 28.2% in cases (15). The ER LI in these ducetal lavage samples is in a similar range. For analyses by duct, increasing epithelial cell yield was significantly associated with the fraction of cells expressing ER, COX-2, and Ki-67, with additional significant association seen between these markers (Table 2). These associations between ECN and the cellular markers, all of which would be expected to increase with increasing abnormality of epithelial cells, suggest that ECN may in itself be a useful marker of neoplastic progression of epithelial cells and lead to the speculation that ECN may be an indicator of epithelial cell mass. In recently published data on random fine-needle aspiration done in a cohort of 147 high-risk women, the median Ki-67 index was higher in women displaying hyperplasia with atypia when compared with women with only hyperplasia (median Ki-67, 2.8% versus 1.1%). Increasing Ki-67 was also noted with increasing total cell number as assessed by Masood scoring system (12). Our findings are similar, and increasing epithelial cell yield is significantly associated with higher cell proliferation. However, we did not find any correlation of cytologic atypia with Ki-67 LI (Table 2). One of the reasons for the dissimilar findings could be that, in the above-mentioned study, Ki-67 assessment was attempted only in those samples with cell numbers greater than 500 and hyperplastic clusters were preferentially assessed. We have, instead, counted all epithelial cells in five blindly chosen areas of the slide, covering ~75% of the area of the cytologic spread, and included single negatively stained epithelial cells in the denominator of the LI. An additional explanation may involve the assessment of mild atypia in ductal lavage samples, which is not quite on par with the category of hyperplasia with atypia used for random fine-needle aspiration evaluation.

**Superfetated Biomarkers.** The entire range of biomarkers was analyzed to evaluate the effect of local endocrine microenvironment on breast epithelium in high-risk women. The fluid component of lavage sample represents a diluted combination of these factors. We have examined differences in the proliferation rates in the epithelial cells from larger ducts versus the terminal duct lobular unit in Ki-67-labeled samples from 20 normal breast tissue samples. We found that the median Ki-67 LI was 0.75% in the ducts and 2.3% in the lobules. Finally, it is possible that exfoliated cells captured in ducetal lavage samples are largely a senescent population and therefore truly has a lower proliferative index. We did a trypan blue exclusion test to assess the percentage of viable cells in lavage samples from 10 ducts and found that >30% cells recovered were nonviable (data not shown). The low proliferative rate seen in these samples may therefore result from a combination of these factors.

**Table 5. Results of factor analyses by duct and by woman with interactions between factor score and ER LI indicated by Ps**

<table>
<thead>
<tr>
<th>Factor score</th>
<th>ER LI</th>
<th>P</th>
<th>Factor score</th>
<th>ER LI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤Median (%)</td>
<td>&gt;Median (%)</td>
<td>1/13 (7.7)</td>
<td>1/15 (6.7)</td>
<td>0.05</td>
<td>2/7 (28.6)</td>
</tr>
<tr>
<td>&gt;Median (%)</td>
<td>7/13 (53.9)</td>
<td>3/7 (17.7)</td>
<td>0.001</td>
<td>7/280 (7)</td>
<td>18,417 (6)</td>
</tr>
<tr>
<td>Mean total ECN Factor score</td>
<td>≤Median (r)</td>
<td>&gt;Median (r)</td>
<td>4,418 (13)</td>
<td>6,565 (15)</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean COX-2 LI Factor score</td>
<td>≤Median (r)</td>
<td>&gt;Median (r)</td>
<td>35.2 (13)</td>
<td>47.6 (15)</td>
<td>0.02</td>
</tr>
<tr>
<td>Mean Ki-67 LI Factor score</td>
<td>≤Median (r)</td>
<td>&gt;Median (r)</td>
<td>0.29 (13)</td>
<td>0.48 (15)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

NOTE: Complete data for factor analysis are on 58 ducts and 27 women.

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5 S.A. Khan et al., unpublished data.
steroids are freely diffusible across cell membranes; hence, their concentration in DLS is likely to be representative of that in the tissue as a whole. During the ductal lavage procedure, the fluid within the ducts is diluted to an undetermined degree by the physiologic solution (Plasmalyte) used to lavage the ducts. We assumed that protein content in the lavage fluid has a constant ratio to the analytes of interest (hormones and hormone response proteins) that we have expressed as a concentration per milligram of total protein. This assumption is based on the relatively constant protein concentration in colostrum (26). Cathepsin D, EGF, and IL-6 are secreted proteins that are affected by or affect estrogen activity and hence were chosen in our study. Similar to results from studies using nipple aspirate fluid, estrone sulfate and the unconjugated androgen precursors (DHEA and DSH), to a lesser extent, are more abundant in DLS than E2 (6), consistent with the idea that estrogen precursors represent a reservoir from which E2 is synthesized locally. It is worth noting that E2 is present in very small quantities in DLS, and variability in measurement related to this may be an explanation for the absence of significant correlations of other biomarkers with E2.

Among the estrogen-related proteins, EGF was the variable with the most significant and consistent associations with hormone and cell-based variables in analyses both by duct and by woman. Higher EGF levels were significantly and positively associated with E2 precursors, such as DSH and estrone sulfate, in the lavage supernatant (but not with E2), raising the possibility that these abundant precursors have sufficient estrogenic activity to induce EGF production. Increasing levels of EGF were seen with increasing ECN in the analyses by duct, suggesting that EGF induced as a result of local estrogen influences plays a role in producing a larger epithelial cell mass. However, there was no significant association of EGF with Ki-67 LI; it is possible that this is a statistical artifact related to the very low fraction of Ki-67-positive cells in these samples. However, we did not measure cellular EGF levels, and it is also possible that the levels of cellular EGF may show stronger relationships. We will address this in future studies by measuring EGF gene expression in epithelial cells. Increasing levels of EGF were seen with increasing ER LI and probably reflect the known ER-dependent induction of EGF by estrogenic hormones (27). Increasing COX-2 LI was also positively and significantly associated with EGF levels in the superant fluid in the analyses both by duct and by woman. The significance of this is not clear but may reflect induction of COX-2 expression by EGF, a phenomenon that has been observed in breast stromal cells (28).

In previous studies of breast epithelial ER expression in surgical biopsy material from breast cancer cases and benign disease controls, we have seen a statistically significant interaction between serum E2 and breast epithelial ER LI in standard-risk and high-risk women. Whereas breast epithelial ER expression decreased with increasing serum E2 in standard-risk women, this down-regulation is lost in high-risk cases. In the same study, we also saw a significant interaction between Ki-67 LI and ER LI, so that Ki-67 LI was inversely related to ER LI in standard-risk women but not in high-risk cases. We postulated from these and other data (14, 29) that the ER expression of breast epithelium is dysregulated in high-risk women, normal down-regulation of ER with high ambient E2 levels is lost, and this loss of regulation is pivotal in determining the extent of cell proliferation. In addition, the existing paradigm of hormonal promotion of breast cancer predicts that cell proliferation, and therefore cell number and cytologic atypia, should all be associated with an abundance of estrogenic hormones, and our previous data suggest that ER functions as an amplifier of this response in high-risk women. We looked for interactions between ER expression and local breast hormones in the present study by performing a factor analysis, where the hormones and hormone response proteins in the supernatant fluid from the ductal lavage samples were expressed as a factor score. This factor score is a composite measure that correlates highly with most of the hormones and proteins. The sulfated E2 precursors DSH and estrone sulfate and EGF were most highly correlated with the composite score, with correlations exceeding 0.75.

We examined a series of cell-based variables (cytologic atypia, Ki-67 LI, COX-2 LI, and total ECN) to identify interactions between factor score and ER LI. Whether analyzed by duct or by woman, cytologic atypia was more frequent when the factor score was high and the ER LI was lower than the median. This was surprising but is in fact not inconsistent with earlier data on ER expression and breast cancer risk, where it seems that the threshold of ER expression required for a significant association with risk is very low (14) and may be lower than the median level of ER expression in this group of uniformly high-risk women. From the present data, it seems that an abundance of estrogenic hormones is more strongly related to cytologic atypia than ER LI of ≥26.1%.

For total ECN, which is strongly related to cytologic atypia, we saw the highest values by duct when both factor and ER LI were high and the lowest when both were low; this interaction was significant (P = 0.001). By woman, the trend was similar, but the interaction was not statistically significant. These results are consistent with the notion that an abundance of ER-positive cells drives proliferative processes resulting in a larger epithelial cell mass. It also suggests that ECN may be a more robust indicator of this than either cytologic atypia (see above) or Ki-67 labeling (see below). For Ki-67 LI, we saw a significant interaction between ER LI and factor score, with the highest values being seen in the analyses by duct and by woman when both factor score and ER expression were high. However, by duct, the significance of the interaction was based on the difference in Ki-67 when factor score was low; by woman, the significance of the interaction was based on the difference in Ki-67 when the factor score was high. Thus, there seems to be a trend here in support of the concept of high hormone levels and a high proportion of ER-expressing cells driving increased proliferation, but measurement instability in the Ki-67 LI may prevent us from seeing this consistently in the analyses presented.

Thus far, 35 women have accepted tamoxifen intervention and will be following up for repeat lavages. This would allow serial observation of the breast epithelium and the local hormonal levels. We will be able to evaluate the effect of tamoxifen as well as comment on the stability of these candidate biomarkers.

**Conclusion**

The measurement of estrogen-related biomarkers in ductal lavage samples is feasible. High epithelial cell yield is associated with the presence of cytologic atypia, which is mild in the overwhelming majority of samples. ECN increases with increasing fraction of cells expressing the cellular markers ER LI, COX-2 LI, and Ki-67 LI. EGF shows significant associations with estrogenic precursors, ER LI and with ECN, suggesting a role in increasing epithelial cell mass. The proportion of Ki-67-positive cells are very low in duct lavage samples and may not be a useful marker in this setting. The associations between biomarkers are stronger in general when analyses are done by duct rather than by woman, but analysis by duct introduces additional complexity and expense.

**References**


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