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14. ABSTRACT

The purpose of this research is to determine the extent to which exfoliated epithelial cells present in breast milk can be used to assess a woman's individual risk of developing breast cancer and to detect early signs of the disease. To accomplish this goal we collected breast milk samples from 250 lactating women who either have had a breast biopsy or were scheduled for a breast biopsy. We isolated the epithelial cells and completed DNA promoter methylation analysis of the proposed tumor suppressor genes. We presented results of our research at several scientific meetings and published a paper in "Epigenetics" demonstrating increased promoter methylation in cells from the breast milk of women at increased risk of developing breast cancer. While the study has concluded we continue with annual follow-up on all women who donated breast milk. A second manuscript describing the results for additional tumor suppressor genes is in preparation and additional studies are being conducted with the archived milk samples.

15. SUBJECT TERMS
Breast milk; recruitment; promoter hypermethylation; RASSF1, GSTP1, SFRP1

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Introduction

The purpose of this research is to determine the extent to which the promoter methylation pattern in exfoliated epithelial cells present in breast milk can serve as a reliable means of assessing an individual woman's risk of developing breast cancer. We have collected breast milk samples from 250 lactating women who were either scheduled for a breast biopsy or had a breast biopsy in the past. Women provided milk from both the biopsied and non-biopsied breast as well as a copy of their biopsy report. They also completed a health and reproductive history questionnaire. From each milk sample we isolated the epithelial cells from the total cell population and determined the methylation pattern of selected genes using pyrosequencing of bisulfite-modified DNA. We expected that roughly 10% or 25 women who had breast biopsies would be diagnosed with breast cancer. This design provides a unique opportunity to assess the development of cancer-associated methylation patterns in premenopausal women who either have breast cancer or a benign lesion and to compare the cells from the diseased breast with those from the healthy breast. We also will compare the methylation profiles we obtain from this At-Risk population (At-Risk because they have needed a breast biopsy) with the methylation profiles we obtained from women at Average-Risk (Wong *et al.*, 2010)

Body

This is the final report of a two-year project that required a one-year extension to complete. As noted in the first annual report, during the first year of the project we completed Tasks 1 – 4 and made significant progress on Tasks 5 and 6. During the second year of this study we completed subject recruitment and breast milk collection (Task 5). We have been extremely successful in subject recruitment and have exceeded our goal of collecting breast milk samples from 250 women.

During the third year we have completed all laboratory analyses. This involved processing each milk sample, isolating the cells, separating the cell populations, extracting the DNA from each cell population (epithelial-enriched and epithelial depleted which is primarily leucocytes) and finally assessing the gene-specific promoter methylation on bisulfite-modified DNA. In summary all of the proposed analyses have been completed and the milk has been archive for use in additional research as indicated in the original Informed Consent Document.

One aspect of the study continues and will continue for the foreseeable future: we contact women annually to inquire about their breast health and if a woman had a breast biopsy in the past year, we obtain a copy of her biopsy report. This annual follow-up is critical to determining whether the information we have collected on DNA methylation of tumor suppressor genes can be used to accurately assess individual breast cancer risk. We have obtained approval from the Institutional Review Board at the University of Massachusetts to conduct this long-term follow-up and each woman has signed a new Informed Consent Document. While we continue to collect these data we have published our first major paper on the results and are preparing our second manuscript.

In the first published report we focused our analysis on women whose biopsy report indicated that they had non-proliferative benign disease. We compared this group of 134 women with 102 women from our previous study in which having a breast biopsy was not an inclusion criteria. The details of the analyses are presented in the attached paper (Appendix 1: Increased promoter methylation in exfoliated epithelial cells in women with a previous breast biopsy; *Epigenetics* 2011). The overall conclusion from this first report indicates that the epigenetic marks on cells isolated from breast milk can be used to assess breast cancer risk as indicated by the findings presented below.

Differences in CpG Promoter Methylation in Epithelial Cells from the Breast Milk of Women in the Biopsy versus the Reference Group: We examined the methylation levels of 30 individual CpG sites within promoter regions of three tumor suppressor genes. As can be seen in Figure 1, the two populations differ in methylation scores. The most dramatic difference between the two populations is the greater number of outlier scores in the Biopsy Group, particularly for GSTP1. This might be expected given that the sample sizes in the Biopsy Group (individual breasts) were roughly twice that of the Reference Group (single sample per woman). Therefore we examined the ***percentage*** of population outliers by calculating the scores that were greater than the 75th percentile + 1.5*IQR of the combined Biopsy and Reference Groups. The percentage of outlier scores is significantly greater in the Biopsy Group than in the Reference Group for GSTP1 (16.3 *vs* 0%; Fisher's Exact p<0.00) but not for either RASSF1 (9.7 *vs* 5.9%) or SFRP1 (7.8 *vs* 5.9%). While less dramatic than the outliers, the overall means also are significantly higher in the Biopsy Group than in the Reference Group for both RASSF1 (7.00 *vs* 4.72; t = 2.93; p = 0.002) and GSTP1 (9.06 *vs* 3.64; t = 5.14; p =0.00), but not for SFRP1 (6.29 *vs* 5.80; t = 0.63; p = 0.27).

Next, to determine the extent to which the differences between the two study groups were related to demographic covariates, we conducted pooled OLS-ANOVA comparisons including study group, age, age at first birth, number of live births, baby's age (as a surrogate for length of nursing), BMI, and family history of breast cancer (first degree female relative with breast cancer and any family history of breast cancer were considered separately). Methylation

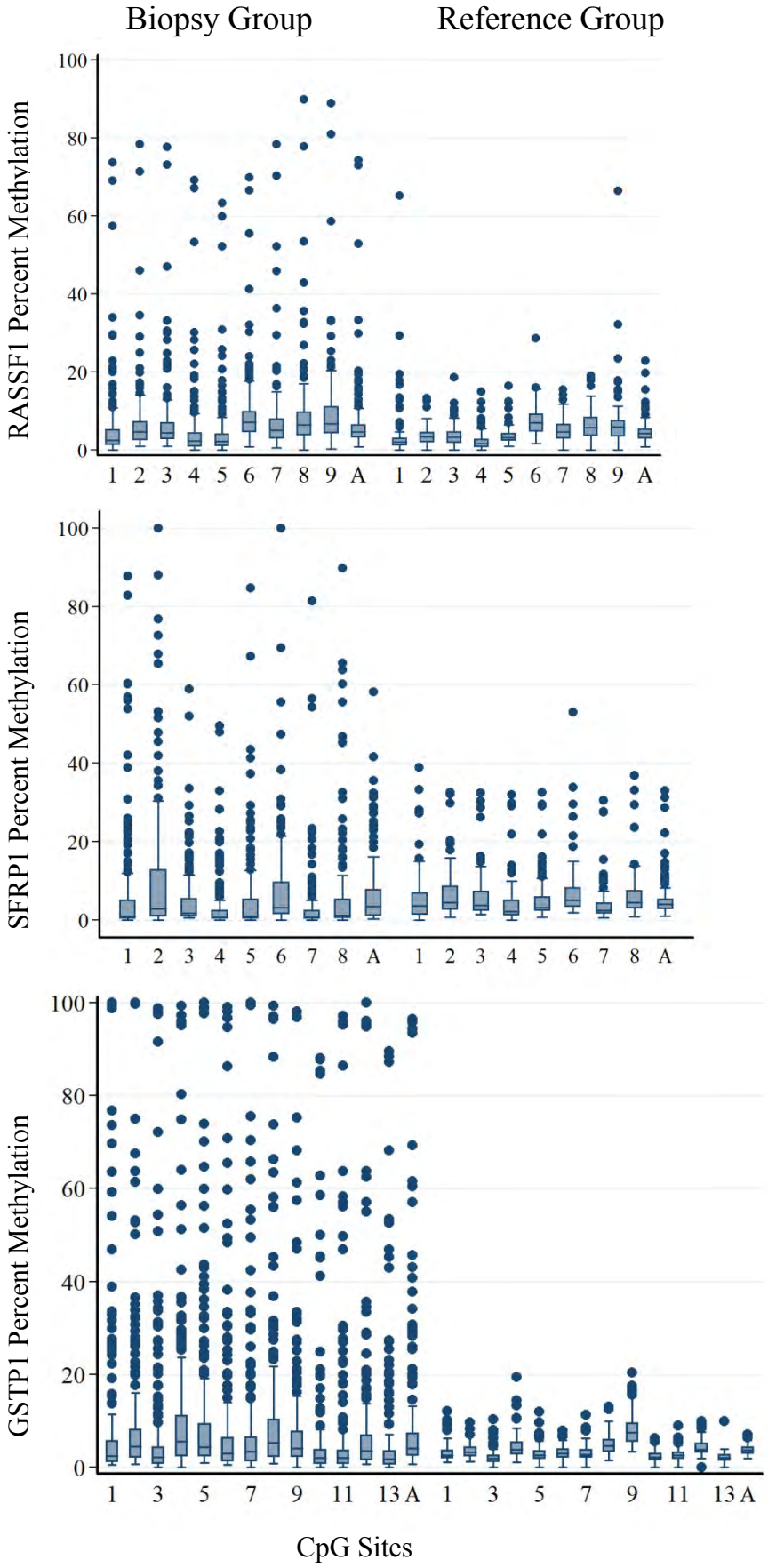

Figure 1. Percent methylation in the biopsy and reference groups.

scores were not associated with a woman's age, age at first birth, number of live births, baby's age, or her BMI for any of the three genes. However, a lack of any family history was associated with increased methylation for GSTP1 ($t = 2.83$; $p = 0.005$) but not for RASSF1 and SFRP1. Being Caucasian also was associated with slightly higher methylation scores for GSTP1 ($t = 2.12$; $p = 0.035$). By far the major contributor to the observed differences in methylation scores for RASSF1 and GSTP1 was study group ($t = 2.84$; $p = 0.005$ and $t = 4.05$; $p = 0.000$, respectively) suggesting that recruiting women who had breast biopsies resulted in a group with greater mean promoter methylation in exfoliated breast epithelial cells. However, despite the highly significant differences between study groups, only a small percentage of the variability in mean methylation scores is explained the regressions including study group (RASSF1; $R^2 = 0.052$; GSTP1: $R^2 = 0.075$). Given that little of the variation between the Groups was explained by established risk factors, we next focused on the Biopsy Group to determine whether the methylation signal was specific to the biopsied breast and the extent to which demographic factors explained the variation within the Biopsy Group.

Methylation Patterns in Biopsied and Non-Biopsied Breasts: Comparisons of the overall and individual CpG methylation means between the biopsied and non-biopsied breasts for all women in the Biopsy Group revealed differences among the three genes (see Table 4 in Appendix 1). For RASSF1 and SFRP1 the mean methylation scores of each of the individual CpG sites as well as the overall means were higher in the biopsied breast. However, for RASSF1 only one CpG site, CpG-6, had a significantly higher methylation score in the biopsied breast, while for SFRP1 the mean scores for CpG1 and CpG5, as well as the overall mean, were significantly higher in the biopsied breast. Likewise, the number of outliers was greater in the biopsied breast for all 9 of the CpG sites in RASSF1 and 7 of 8 CpG sites in SFRP1. In contrast, the individual mean CpG and overall mean methylation scores for GSTP1 were similar in the biopsied and non-biopsied breasts with the methylation scores for four of the 13 CpG sites slightly higher in the non-biopsied breast. Also, the number of outliers in the biopsied and non-biopsied breasts was similar between breasts, with three of the 13 GSTP1 CpG sites having a greater number of outliers in the non-biopsied breast. Neither family history of breast cancer nor time between biopsy and milk donation were significantly associated with mean methylation scores for any of the genes.

It is important to note that the 62 overall outlier scores, presented by gene and breast in Table 4 in the Appendix, come from a total of 51 different women or 38% of the 134 women. Only ten women had more than one outlier score (7.5%). Five women had outlier scores in two genes in their biopsied breast (3.7%), three women had outlier scores in two genes in their non-biopsied breast (2.2%), two women had an outlier score for a one gene in each breast (1.5%) and one woman with two outlier scores in her biopsied breast had an additional outlier score in her non-biopsied breast (0.75%).

Key Research Accomplishments

- Established a unique breast milk bank by archiving milk samples from each breast of over 250 women who required a breast biopsy.
- Established a data base that includes information from a health and reproductive history questionnaire as well as the biopsy results for each of the women who donated breast milk.
- Processed milk samples from 252 women (from both right and left breasts); isolated DNA from all cell samples (both epithelial and non-epithelial fractions from both breasts); bisulfite-treated all DNA samples and completed DNA methylation analyses for all genes.

- Determined that mean levels of promoter methylation of selected tumor suppressor genes among women at increased risk is greater than among women at no elevated risk of breast cancer
- Determined that greater DNA methylation of selected tumor suppressor genes is observed in cell collected for the biopsied and compare to the non-biopsied breast
- Concluded that the cells in breast milk provide valuable information about breast cancer risk

Reportable Outcomes

Manuscripts supported by the present award

Browne EP, Punska EC, Lenington S, Otis CN, Anderton DL, **Arcaro KF**. **2011.** Increased promoter methylation in exfoliated breast epithelial cells in women with a previous breast biopsy. *Epigenetics* 6:12, published on-line December 1, 2011

Qin W, Zhang K, Kliethermes B, Ruhlen RL, Browne EP, **Arcaro KF** and Sauter ER. Differential expression of cancer associated proteins in breast milk based on age at first full term pregnancy and length of nursing. Submitted to: *Breast Cancer Research*

Arcaro KF, Browne EP, Qin W, Zhang K, Anderton DL and Sauter ER. Differential expression of cancer-related proteins in paired breast milk samples from women with breast cancer. Submitted to: *Breast Cancer Research*

Arcaro KF, Browne EP, Punska EC, Lenington S, Otis CN, Anderton DL and Schneider, SS. Promoter methylation in RASSF1, SFRP1 and GSTP1 in cells isolated from breast milk: a comparison between epithelial cells and leucocytes. In preparation.

Arcaro KF, Browne EP, Punska EC, Lenington S, Otis CN, Anderton DL and Schneider, SS. Detection of breast cancer and increased risk in epithelial cells isolated from breast milk. In preparation.

Presentations supported by the present award

Browne EP, Punska EC, Anderton DL, Lenington S, **Arcaro KF.** Methylation in exfoliated epithelial cells from lactating women at increased risk for developing breast cancer [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; **2011**. Abstract nr LB-443
Abstract accepted as a late-breaking and selected for AACR Press Release

Kristin E. Williams KE, Browne EP, Anderton DL, Poulin M, Yan L, **Arcaro KF**. Promoter methylation analysis of separate cell populations in human breast milk is required for assessing [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; **2011**. Abstract nr 1995

Pogo BGT, Moran H, Marin T, Lee A, Deligdisch L, **Arcaro KF**, Anderton D, Melana S, Holland JF. Detection of HMTV env sequences in hormonally dependent tissues [abstract]. In: Proceedings of the 102nd Annual Meeting of the American Association for Cancer Research; 2011 Apr 2-6; Orlando, Florida. Philadelphia (PA): AACR; **2011**. Abstract nr 2302

Arcaro KF. 2010 Analysis of promoter methylation in DNA obtained from breast milk to assess breast-cancer-risk. AACR 101st Annual Meeting, Washington D.C., April
Browne EP, Punska EC, Lenington S, Anderton DL, **Arcaro KF**. 2010. RASSF1A Promoter Methylation in Exfoliated Breast Cells Isolated from Breast Milk Donated by Women Who Have Had a Breast Biopsy. AACR 101st Annual Meeting, Washington D.C., April

Zimmers S, O'Keefe P, Reckhow D, Anderton D, **Arcaro KF**. Analysis of Bisphenol A (BPA) in Human Breast Milk: Are BPA Levels Related to DNA Promoter Methylation in Breast Epithelial Cells? Poster presented at the 13th Annual Vermont Cancer Center Breast Cancer Conference, October 15, **2010**. Burlington, VT

Received two awards based on the **breast milk samples** collected for the present study

Avon Foundation: *Impact of Environmental Estrogens on Epigenetics: Bisphenol A in Breast Milk and Promoter Methylation of Exfoliated Breast Epithelial Cells*
The goal of this study is to determine the relationship between levels of Bisphenol A (BPA) in breast milk and promoter methylation. We are examining BPA in the breast milk we collected from the participants in the present study. IRB protocol allows the use of the milk for other studies.

Avon Foundation: *Presence, Prevalence, Impact of Intracellular Chlamydia in Cells of Breast Milk*
The goal of this study is to determine the relationship between infection with *Chlamydia* and promoter methylation in breast cells. Using RT-qPCR we are determining the levels of two species of Chalmydia DNA obtained from the cells in breast milk collected from the participants in the present study. IRB protocol allows the use of the milk for other studies.

Received one award based on results from the present study

Avon Foundation: *Epigenetics and Breast Cancer Risk in African American Women*
The goal of this proposal is to examine the relationship between DNA methylation and Breast cancer risk in African American women.

Submitted four additional proposals that build on the results from the present study

CDMRP Idea Expansion: *Epigenetic and Protein Markers in Exfoliated Epithelial Cells from Breast Milk and Breast Cancer Risk*

CDMRP Idea: *Dietary Intervention to Reduce Breast Cancer Risk: Monitoring Epigenetic Changes in Exfoliated Epithelial Cells Isolated from Breast Milk*

NIH R21: *Diet Intervention and Epigenetics of Breast Cells in African American Women*

Keep a Breast Foundation: *Diet Intervention to Reduce Breast Cancer Risk in Hispanic/Latina Women in Western Massachusetts*

Conclusion

During the final year of this award we have completed the methylation analyses on all genes and published our first paper. Analysis of DNA methylation in the exfoliated epithelial cells isolated from the milk of 134 women with a breast biopsy diagnosis of non-proliferative disease revealed three major findings: 1) the group of women with a non-proliferative breast biopsy diagnosis had a slightly increased probability of CpG promoter methylation in two of three genes examined as compared to a group for whom a breast biopsy was not a requirement, 2) the methylation patterns differed between the biopsied and non-biopsied breasts for two of the three genes, and 3) while the mean methylation scores were low for most women, a subset of women had significantly higher CpG promoter methylation.

We are presently analyzing the data for all eight genes for all 250 women and expect to complete a manuscript in February 2012. These data together as well as long-term follow-up of participants and a greatly expanded panel of genes are needed to determine the specificity and precision with which we can use promoter methylation of exfoliated cells in breast milk to predict which women will develop breast cancer. However, this study provides evidence that analyzing promoter methylation of key genes known to be methylated early in the etiology of breast cancer could provide a more individualized measure of risk. While we are using previous biopsy to obtain a high risk population, analysis of promoter methylation would not need to be restricted to a high risk population; indeed, it would be most useful in detecting increased risk among women who will develop sporadic breast cancer. Young women of child bearing age are an excellent population for assessing individual risk. These women have already experienced changes associated with critical windows of exposure, yet there is still time to stop and potentially reverse the epigenetic effects associated with many deleterious exposures. Furthermore, young mothers who learn that they are at increased risk of developing breast cancer, and that diet and lifestyle changes could reduce their risk, will be highly motivated to make the needed changes.

References

Wong CM, Anderton DL, Smith-Schneider S, Wing MA, Greven MC and ***Arcaro KF*.** 2010 Quantitative analysis of promoter methylation in exfoliated epithelial cells isolated from breast milk of healthy women *Epigenetics* 5(7): 1-11.

Publication: (available on line)

1. Browne EP, Punska EC, Lenington S, Otis CN, Anderton DL, ***Arcaro KF***. **2011.** Increased promoter methylation in exfoliated breast epithelial cells in women with a previous breast biopsy. *Epigenetics* 6:12, published on-line December 1, 2011