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Nutritional predictors for cellular nipple aspirate fluid: Nutrition and Breast Health Study

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Abstract

The presence of epithelial cells in breast nipple aspirate fluid (NAF), irrespective of abnormality, has been associated with increased risk of breast cancer in previous studies. We sought to investigate whether the presence of epithelial cells in NAF is associated with nutritional parameters among 71 healthy premenopausal women who participated in the Nutrition and Breast Health Study and provided any samples of NAF during the study. Total of 142 samples which were obtained over a 1-year period of intervention with low-fat and/or high vegetable-fruit diets were available for cytological evaluation. The odds ratios (ORs) and 95% confidence intervals (CIs) for the detection of epithelial cells in NAF were estimated by fitting generalized estimating equations models by quartile level of nutritional parameters. The probability of yielding epithelial cell-positive NAF progressively increased with increasing total fat intake ($p = 0.001$). The OR for the highest quartile level of fat intake, compared with lowest, was 7.22 (95% CI 1.14–45.82). On the other hand, there were a marginally significant inverse association with total fiber intake as well as an weak inverse association with the number of servings of fruit and vegetables. Furthermore, the probability of detecting epithelial cells in NAF decreased with increasing plasma levels of lutein and α-carotene (p-values for linear trend; 0.001 and 0.049, respectively). The ORs for the highest versus lowest quartile levels are 0.17 (95% CI 0.04–0.65) and 0.19 (95% CI 0.04–0.91), respectively. These results are generally in support of roles of nutritional factors in breast cancer and thus further studies are warranted.

Introduction

Breast fluids have received increasing interest as they provide potentially useful information for breast cancer risk in a minimally invasive fashion. Breast fluids can be obtained either by gentle suction with a simple syringe-adapted apparatus and massage (nipple aspiration fluid, NAF) or by microcatheter insertion and flushing (ductal lavage) [1]. Although fluids are not always cellular, particularly in case of NAF, certain cellular characteristics of breast fluids have been proven to predict risk of breast cancer. Cytological atypia found in NAF was associated with high risk patterns of mammograms [2], while cytological abnormality in NAF was more severe for women at elevated risk of breast cancer, i.e, with family history of breast cancer or with personal history of premalignant breast diseases, than women without such risk factors [3]. In a prospective cohort study by Wrensch et al. [4] women who yielded NAF containing epithelial cells with atypia or hyperplasia had a doubled risk of developing breast cancer, compared with women who did not yield any fluid. In addition, genetic and epigenetic changes associated with breast cancer, such as loss of heterozygosity [5] and promoter methylation [6], have been detected in cells found in ductal lavage. Interestingly, presence of cellularity in NAF itself, irrespective of abnormality, was associated with increased risk of breast cancer [4,7]. This may suggest that the presence of epithelial cells in NAF may also be a potential risk marker as it may reflect more proliferative state of breast ducts.

The role of dietary intakes in the etiology of breast cancer has long been studied, although it may be in part indirect effects through the endocrine system. While evidence is still inconclusive, meta-analyses have confirmed that diets high in fat [8] and low in vegetable [9] modestly increase the risk of breast cancer. Macro- and micronutrients in such diets have also been shown to modify surrogate biomarkers for breast cancer risk, such as circulating estrogen levels [10,11] and mammographic patterns [12]. In the present study, we sought to investigate whether the presence of epithelial cells in NAF is associated with nutritional parameters among healthy premenopausal women who participated in the Nutrition

and Breast Health Study, taking advantage of multiple NAF specimens collected under different diets.

Materials and methods

Study population

Details concerning the study design, characteristics of study participants and data and biological specimen collection of the Nutrition and Breast Health Study have been published elsewhere [13–15]. Briefly, it was designed as a two-by-two factorial randomized intervention trial of low fat and high vegetable/fruits diets over a 1-year period. With this study design, approximately one-fourth of the participants were assigned to the control arm (usual diet). Eligible participants were healthy premenopausal non-smoking women aged 21–50 years who had at least one first-degree relative with breast cancer. A total of 122 subjects were randomized. Witten informed consent was obtained from each subject before participation. Basic demographics and reproductive and medical history were obtained by structured questionnaires.

Fasting venous blood specimens were obtained by venipuncture at 0, 3, 6 and 12 months, and NAF specimens with the self-expression method were obtained as described previously at 0, 6 and 12 months in order to assess plasma and NAF oxidative stress markers and antioxidant vitamin levels [14,15]. Approximately 60% of the participants were able to yield NAF. In addition, extra specimens of NAF were collected for cytological evaluation, which were placed in cytological preservative (Persev-Cyt: Cytyc Corp., Boxborough, MA). A total of 142 samples from 71 participants were available for cytological evaluation. Cytospin preparations of the sediment were made and stained with Papanicolaou's method. The presence of epithelial and other (including macrophages, foam cells, inflammatory cells and skin cells) cells, cell debris and epithelial cell atypia was recorded by the study pathologist. The number of epithelial cells was generally low or 'scanty' (e.g. less than 10 cells), although clumps of epithelial cells were noted in some of the samples.

Dietary assessment

Four day food records were collected at 0, 3, 6, 9 and 12 months as described previously [13]. The 4-day food records were kept by the participants in the week before the scheduled study visits. Registered dieticians taught the participants how to keep food records and estimate portion sizes and records were reviewed by the dieticians at each visit. Nutrient calculations were performed using the Minnesota Nutrition Data System (NDS) software (food database version 14A, nutrient database version 24, Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN). Registered dieticians enumerated servings of all vegetables and fruits recorded in the 4-day food records by hand using serving sizes defined for this study that were similar to American Dietetic Association exchange values with some consideration to usual serving sizes [13]. At each dietician visit, the women were weighted in clothing but without shoes using a professional beam scale (model 402KLS, Health-o-Meter, Bridgeview, IL) and percent body fat was measured using tetrapolar bioelectrical impedance (model BA101S, RJL Systems, Clinton Townshi, MI). Standing height was measured at baseline only and body mass index (BMI) was calculated as weight(kg)/height $(m)^2$.

Laboratory methods

Fasting blood samples were collected in heparinized tubes and processed immediately after drawing. All samples from a given individual were assayed in the same laboratory batch of 24–36 samples. Each blood sample was coded by number, and the diet arm assignment was blinded until the analyses were completed and the results calculated. Samples were spun to prepare plasma, and plasma aliquots were stored under argon at −70 °C.

For analysis of carotenoids and tocopherols in plasma, aliquots of plasma were mixed with ethanol, BHT and the internal standard Tocol, followed by extraction with hexane. Micronutrients were separated by HPLC using a YMC C-30 column. An ESA CoulArray electrochemical detector (Chelmsford, MA) was used for measurement of tocopherols and carotenoid using electrodes set up at 310, 390 and 470 mV. The analyses of carotenoids and tocopherols in NAF were performed by Craft technologies (Wilson, NC) also using a C-30 HPLC column. Levels of cholesterol in plasma and NAF were determined in a spectroscopic assay with Sigma (St. Louis, MO) Infinity Cholesterol Reagent using a standard curve constructed with plasma calibrators. For NAF, approximately 2 mg fluid was diluted with 10 μl dextrose solution before analysis. The volume of plasma or diluted NAF used in the assay was 2.5 μl and it was mixed with 250 μl of the cholesterol reagent in 96-well plates in triplicate.

Statistical analysis

First, the prevalence (%) of selected baseline demographic characteristics, mean ages at enrollment and mean fluid volume of samples were compared between epithelial cell positive and negative women or specimens by using the χ^2 test and 2 sample *t*-test, respectively. In this analysis subject were classified as 'epithelial cell positive' if epithelial cells were detected at any time in their NAF. The means of Spearman rank correlation was employed to examine correlations between baseline measurements of plasma and NAF micronutrient levels.

The odds ratios (ORs) and 95% confidence intervals (CIs) for the detection of epithelial cells in NAF specimens were estimated by fitting generalized estimating equations (GEE) models with SAS GENMOD procedure,

by quartile level of nutritional parameters based on the all data from of the entire study population, with adjustment for selected covariates. This analysis utilized all NAF cytology data from any time point and nutritional data of the corresponding visits, with an unstructured correlation matrix, which is deemed appropriate for the small number of repeated measurements [16], to model clustered observations within subjects. Tests for linear trend in the logit of risk associated with nutrition were performed using continuous nutritional variables. In these analyses, nutritional variables were log-transformed if the data better approximated a Gaussian or normal distribution. As a result, nutritional variables except for total calories, protein, carbohydrate, starch and fiber, percent energy from carbohydrate and fat and plasma cholesterol, were log-transformed.

Results

Twenty six of the 71 women (37%) yielded at least one NAF sample which contained epithelial cells at any time point during the study period. The positive rates at month 0, 6 and 12 were 25.4, 6.7 and 23.5%, respectively. Among the 27 positive samples, only one contained epithleial cells with atypia. There was no difference in fluid volume between samples positive to epithelial cells and those negative to epithelial cells (Table 1). As summarized in Table 1, women who yielded NAF positive to epithelial cells were not different from those who did not in terms of racial compositions, marital status, age at menarche and history of childbirth, breast feeding and breast biopsy. However, women diagnosed with epithelial cells were statistically significantly older at time of enrollment to the study than those who were not.

Thus, in the following analyses the ORs based on 142 NAF specimens were calculated with adjustment for age at enrollment. Body mass index and percent body fat were not associated with the presence of epithelial cells in NAF (data not shown). When the ORs were calculated according quartile levels of nutritional intake from the 4-day food records at each visit (Table 2), the probability of detecting epithelial cells in NAF progressively increased with increasing total fat intake ($p = 0.001$), while total energy and protein intake did not show any associations. The OR for the highest quartile level of fat intake, compared with lowest, was 7.22 (95% CI 1.14–45.82). This association with total fat intake did not depend on specific types of fat, although the association appeared to be stronger for mono-unsaturated and saturated fats than poly-unsaturated fat. On the other hand, there were a marginally significant inverse association with total fiber intake as well as an weak inverse association with the number of servings of fruit and vegetables. Alcohol or caffeine intake was not associated with the presence of epithelial cells in NAF (data not shown).

When associations with plasma micronutrient levels were analyzed (Table 3), the probability of detecting epithelial cells in NAF progressively decreased with increasing plasma levels of lutein and α-carotene (p-values for linear trend; 0.001 and 0.049, respectively). The ORs for the highest versus lowest quartile levels are 0.17 (95% CI 0.04–0.65) and 0.19 (95% CI 0.04–0.91), respectively. β-Carotene and β-cryptoxanthin showed a similar but more modest inverse association with the probability of epithelial cell positive NAF, while there was marginally significant positive association with γ-tocopherol. On other hand, none of micronutrients in NAF, plasma cholesterol or NAF cholesterol were associated with the risk of yielding epithelial cell-positive NAF (data not shown). The Spearman correlation coefficients between baseline plasma and NAF micronutrient measurements were 0.025, 0.127, 0.139 and 0.167 for lutein, zeaxanthin, α-tocopherol and cholesterol, respectively ($p > 0.10$), and 0.222 ($p = 0.09$), 0.267 ($p = 0.04$) and 0.269 ($p = 0.04$) for γ-tocopherol, lycopene and β-cryptoxanthin, respectively, while 0.303 ($p = 0.02$) and 0.411 ($p = 0.001$) for β- and α-carotenes.

Discussion

The role of nutritional factors in the etiology of breast cancer has not yet been fully understood. A high fat diet

Table 1. Characteristics of women with nipple aspirate fluid positive or negative for presence of epithelial cells

Characteristics	Positive $N = 26$ (27)[a]	Negative $N = 45$ (115)	p-values
Mean fluid volume (ml)[b]	9.07	10.57	0.407
Mean age at enrollment (years)	40.8	37.6	0.048
Mean age at menarche (years)	12.6	12.4	0.428
Nonwhite (%)	26.9%	28.9%	0.859
Married (%)	69.2%	75.6%	0.615
Parous (%)	34.6%	26.7%	0.562
Breast feeding (%)	26.9%	20.0%	0.480
Breast biopsy (%)	50.0%	55.6%	0.501

[a]The numbers in the parentheses indicate the numbers of samples.
[b]Based on samples with known total fluid volume (25 positive and 106 negative samples).

Table 2. Age-adjusted OR and 95% CIs according to quartiles of macronutrient or food intakes per day

Nutritional Intake (quartiles)	No. of samples positive/negative	OR	95% CI	Linear trend[a]
Total energy (kcal)				
≤1478	5/21	1.00	–	$p=0.871$
1479–1742	6/28	0.91	0.23–3.55	
1743–1986	10/29	1.49	0.41–5.40	
≥1987	6/36	0.90	0.25–3.29	
Total protein (g)				
≤54.77	8/25	1.00	–	$p=0.304$
54.78–64.50	10/27	1.28	0.50–3.27	
64.51–76.19	3/31	0.29	0.08–1.03	
≥76.2	6/31	0.77	0.26–2.30	
Total fat (g)				
≤31.53	2/25	1.00	–	$p=0.009$
31.54–48.11	6/24	3.35	0.53–21.25	
48.12–66.29	8/36	4.90	0.89–26.94	
≥66.3	11/29	7.22	1.14–45.82	
Saturated fat (g)				
≤10.13	3/25	1.00	–	$p=0.008$
10.14–15.45	4/28	1.41	0.24–8.33	
15.46–22.33	8/27	3.27	0.73–14.63	
≥22.34	12/34	5.47	1.11–27.07	
Mono-unsaturated fat (g)				
≤11.07	1/26	1.00	–	$p=0.012$
11.08–17.14	7/22	5.79	0.62–54.09	
17.15–23.60	9/32	9.27	1.20–71.75	
≥23.61	10/34	8.93	0.93–85.52	
Poly-unsaturated fat (g)				
≤6.81	3/19	1.00	–	$p=0.052$
6.82–10.70	6/31	0.65	0.14–2.95	
10.71–15.2	7/30	1.94	0.60–6.21	
≥15.21	11/34	1.70	0.55–5.25	
Fiber (g)				
≤14.39	9/27	1.00	–	$p=0.065$
14.40–20.05	7/32	0.65	0.21–2.06	
20.06–26.38	8/31	0.70	0.24–2.04	
≥26.39	3/24	0.30	0.06–1.38	
Fruit/vegetables (servings)				
≤3.49	8/27	1.00	–	$p=0.112$
3.5–5.24	9/40	0.72	0.25–2.02	
5.35–9.73	7/25	0.78	0.28–2.23	
≥9.74	3/21	0.34	0.08–1.45	

[a]Based on continuous values of nutrient/food intake.

has been one hypothesis to account for considerable variation in breast cancer risk among populations. Dietary fat may increase levels of systemic oxidative stress through lipid peroxidation [17]. More specific to breast cancer, high fat diet has been linked to elevated levels of circulating estrogens in women [10]. In addition, high fat diet is often associated with an increase in the amount of adipose tissue, which is now recognized as an endocrine organ capable of producing a variety of bioactive peptides that play roles in inflammation, lipid and hormone metabolisms and cell growth and differentiation [18,19]. The mammary gland is indeed embedded in adipose tissue and adipocyte-secreted factors have been shown to promote experimental mammary carcinogenesis [20].

Despite relatively weak associations between dietary fat and breast cancer risk in observational epidemiological studies, the results from intervention trials suggest that low fat diet may reduce the prevalence of established risk factors for breast cancer, such as increased mammographic density and circulating estrogens. In addition, hyperplasia, atypia and carcinoma *in situ* are more commonly found in mammographically dense breasts [2,21], which consist of more epithelial cells and connective tissue relative to fat. Higher mammographic density has also been associated with

Table 3. Age-adjusted ORs and 95% CIs according to quartiles of plasma vitamin levels (μg/ml)

Vitamin levels (quartiles)	No. of samples positive/negative	OR	95% CI	Linear trend[a]
Lutein				
≤0.056	12/28	1.00	–	$p = 0.001$
0.057–0.114	7/29	0.40	0.14–1.12	
0.115–0.236	3/31	0.28	0.09–0.91	
≥0.237	4/27	0.17	0.04–0.65	
Zeaxanthin				
≤0.009	7/29	1.00	–	$p = 0.671$
0.01–0.020	8/36	0.62	0.24–2.72	
0.021–0.040	8/29	0.54	0.37–3.06	
≥0.041	3/21	0.63	0.19–2.23	
β-Cryptoxanthin				
≤0.062	9/29	1.00	–	$p = 0.216$
0.063–0.092	6/31	0.41	0.12–1.44	
0.093–0.145	5/27	0.49	0.15–1.64	
≥0.146	6/28	0.42	0.13–1.38	
α-Carotene				
≤0.035	9/23	1.00	–	$p = 0.049$
0.036–0.80	6/31	0.37	0.11–1.23	
0.081–0.172	8/35	0.40	0.14–1.21	
≥0.173	3/26	0.19	0.04–0.91	
β-Carotene				
≤0.159	5/29	1.00	–	$p = 0.401$
0.160–0.303	8/33	0.89	0.26–3.00	
0.304–0.526	7/23	1.35	0.41–4.39	
≥0.527	6/30	0.59	0.16–2.18	
Lycopene				
≤0.331	5/23	1.00	–	$p = 0.442$
0.332–0.450	8/30	1.33	0.38–4.66	
0.451–0.678	6/37	0.59	0.15–2.28	
≥0.679	7/25	1.05	0.32–3.46	
α-Tocopherol				
≤7.94	4/25	1.00	–	$p = 0.715$
7.95–10.59	10/28	2.07	0.55–7.73	
10.6 14.00	6/33	1.07	0.29–4.02	
≥14.01	6/29	0.92	0.24–3.49	
γ-Tocopherol				
≤1.049	3/24	1.00	–	$p = 0.064$
1.05–1.695	6/38	1.41	0.35–5.61	
1.70–2.433	8/31	1.83	0.47–7.40	
≥2.434	9/22	3.40	0.98–11.75	

[a]Based on continuous values of nutrient measurements.

estrogen replacement therapy in postmenopausal women [22,23] and with elevated levels of urinary malondialdehyde (MDA), a marker for oxidized lipids, in premenopausal women [24,25]. Preliminary results from the Women's Intervention Nutrition Study indicate that a low-fat diet can reduce risk of breast cancer recurrence [26]. Thus, our result pointing to an association of high fat intake with epithelial cells in NAF accords with these earlier observations. Furthermore, it is interesting to note that Lee et al. [27] have reported that the odds for women to yield NAF increase linearly with increasing dietary fat intake, which may be mediated through the stimulation of prolactin secretion. Such hormonal stimulation of the breast may also affect mammary epithelial cells, because the supplementation of foods with weak estrogenic properties, such as soy protein, has been demonstrated to increase not only breast fluid secretion but also the appearance of hyperplastic epithelial cells in NAF, as well as plasma estradiol levels [28].

There was a weak indication in the present study that dietary fiber intake may reduce the probability of yielding epithelial cells-positive NAF. Likewise, an inverse association between dietary fiber intake and breast cancer risk has been observed in some cohort and case–control studies [29,30]. Dietary fiber has been postulated

to inhibit intestinal reabsorption of biliary excreted estrogens [31]. Interestingly, constipation, which may result from low fiber intake, has been associated with increased risks of breast cancer [32] as well as of cytological abnormality in NAF [33].

The protective effects of vegetables and fruits against cancer have often been ascribed to antioxidant vitamins abundant in these foods, although it has been difficult to separate effects of individual vitamins in observational studies. Several prospective cohort studies have reported an inverse association between breast cancer risk and serum/plasma carotenoids, including α- and β-carotenes and lutein [34,35]. These observations are consistent with our results concerning plasma antioxidant vitamin levels. Despite being a relatively minor component among total carotenoids, it is intriguing that lutein showed a pronounced inverse association. It has been speculated the chemical structures of lutein enable it to act as a more effective membrane antioxidant than β-carotene and lycopene [36]. However, it is also possible that other micronutrients, such as isothiocyanate and folate, which are abundantly present in dark-green vegetables rich in lutein, may account for the protective effects.

In the present study, the associations with antioxidant vitamin levels was only observed in plasma but not in NAF. This may undermine their potential effects on breast epithelial cells. However, it is worth noting that levels of a variety of endogenous and exogenous biochemical components in NAF, such as cholesterol, estrogens, and isoflavone, are not correlated with their blood levels [37–39]. In fact, when the correlations between plasma and NAF levels were examined for cholesterol and antioxidant vitamins at baseline, significant correlations were only observed for α- and β-carotenes and for lycopene and β-cryptoxanthin to the lesser extent. Carotenoids can also be oxidized in the lipid-rich environment of the breast. Lipid oxidation products can be very high in the breast. We found previously that the oxidation product of lycopene, 5,6-cyclolycopene-1,5-diol, was much higher in NAF than in plasma and in some NAF samples all the lycopene detected was oxidized [40]. Also, we have reported that levels of these antioxidant vitamin levels in NAF are affected by lactation history, i.e., longer duration, higher concentrations [15], whereas history of lactation itself is a major predictor for yielding NAF [41]. These suggest that factors other than circulating concentrations may be important determinants of biochemical compositions of NAF, regulating the uptake and excretion of specific biochemical molecules by mammary ductal epithelial cells. Despite the fact that ductal epithelial cells are in direct contact with ductal fluid, it is now clear that not all ducts within the same individuals produce fluid and that the frequency of atypia is actually higher in non-fluid-yielding than fluid-yielding ducts [42]. Therefore, it is possible that exposures through systemic circulation entail more sustained effects on ductal epithelial cells.

Strengths of the present study include the quality of dietary data that was obtained under direct supervision of registered dieticians and relatively wide range of dietary intake due to intervention diets. However, we acknowledge several weakness of the study. First, our study did not have the ability to address the effect of nutritional variables on abnormality of epithelial cells as there was only one atypia in the whole study population. Second, only approximately 60% of the study population were able to obtain NAF samples and specimens for cytological evaluation which were not always available, limited the number of study subjects and specimens, leading to rather wide confidence intervals of the ORs. However the proportion of women who were able to obtain NAF was comparable to the rates reported in the literature [15]. Third, some of significant associations observed in the present study may be a chance finding due to multiple comparisons. Finally, caution should be exercised in generalizing our results because the study subjects were a selected population who agreed to participate in an intervention trail and had family history of breast cancer. Nevertheless, the result of the present study are generally in support of roles of nutritional factors in breast cancer, some of the associations were highly statistically significant and thus further studies with an expanded sample size are warranted.

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