Breast Cancer Research and Treatment (2006) 98: 63–70
DOI 10.1007/s10549-005-9132-5

Epidemiology

Presence of epithelial cells in nipple aspirate fluid is associated with subsequent breast cancer: a 25-year prospective study

Gertrude Case Buehring, Amy Letscher, Kathleen M. McGirr, Shruti Khandhar, Lisa H. Che, Christine T. Nguyen, and Adeline J. Hackett
Division of Infectious Diseases, School of Public Health, University of California, Berkeley, CA, USA

Key words: breast cancer risk, epithelial cells, nipple aspirate fluid

Summary

Fluid and epithelial cells obtained from the breasts of non-pregnant, non-lactating women by nipple aspiration, can be used for early diagnosis of breast neoplasms. However, since nipple aspirate fluid (NAF) with cells is obtainable from less than half of women sampled, the question arises: Is this method capable of targeting the women most likely to develop breast cancer? We approached this question with a 25-year prospective study to determine if subjects yielding NAF with or without epithelial cells were more likely to develop breast cancer during the follow-up period than subjects from whom no NAF or epithelial cells were obtained. Logistic regression analysis was used to determine relative risk (RR) with 95% confidence intervals (CI). The follow-up cohort of 972 was representative of the eligible cohort of 1605 for factors related to breast cancer risk and nipple aspiration outcome, and representative of the general population for breast cancer risk. After a mean follow-up period of 25 years, women with epithelial cells in NAF were significantly more likely to develop breast cancer (RR = 1.92; CI = 1.22–3.01; $p \leq 0.005$), especially invasive breast cancer (RR = 2.27; CI = 1.27–4.03; $p \leq 0.005$), than women with no NAF, or NAF without epithelial cells. These risks were higher for women < 55 years of age at the time of sampling (RR = 2.1 for any breast cancer, 2.5 for invasive breast cancer). We conclude that presence of NAF with epithelial cells is associated with subsequent breast cancer risk and may be a useful marker for women at higher risk.

Introduction

Breast fluids are usually produced in connection with childbearing, but occasional spontaneous fluid production in non-pregnant, non-lactating women has been recognized for centuries. It was thought to be abnormal and was labeled in textbooks as 'galactorrhea' [1–3]. Papanicolaou et al. [4] described the cells contained within these breast fluids and how they could be used diagnostically to identify breast neoplasms. The advent of the Sartorius aspirator [5], a simple suction device placed over the nipple, allowed painless and non-invasive breast fluid aspiration from about 50% of the female population. In the 1970s three studies evaluated the factors related to obtaining fluid and epithelial cells with this aspirator and the validity of atypical cells in nipple aspirate fluid (NAF) as an indicator of breast neoplasms confirmed by biopsy and mammography [5–7].

Wrensch et al. [8] followed the same cohort evaluated for NAF in 1975 [6] and found the following frequencies of breast cancer incidence during the 17 year period: 2.6% in women with no fluid; 4.8% in those with fluid but no epithelial cells; 4.6% in those with fluid and epithelial cells; and 10.3% in those with fluid containing abnormal epithelial cells. These results indicate that the women most likely to develop breast cancer are the ones most likely to have NAF and NAF cells and imply that ability to obtain NAF and/or epithelial cells may have prognostic significance.

These results are impressive, and it is of interest to determine whether similar results would occur with different cohorts. The three cohorts studied in the 1970s [5–7] varied, particularly in potential breast pathology of the subjects. The cohort evaluated by Sartorius et al. [5] consisted primarily (88%) of symptomatic women presenting to a specialized breast clinic for evaluation by cytology, mammography, and/or contrast ductography. The cohorts studied by the San Francisco investigators [6, 8–10] consisted partially of women from outpatient clinics and breast screening centers and partially of volunteers from the community. The Buehring study [7] used self-selected volunteers from the community; few subjects were referred by physicians or a clinic. This latter cohort may, therefore, consist of subjects with the least potential for existing breast pathology, and

provides an opportunity to investigate the prognostic applicability of NAF and NAF cells in a non-clinical population. We undertook a follow-up of this cohort to determine the incidence of breast cancer occurring during the 25 years since enrollment in the original study. This prospective design evaluates the relationship of the presence of NAF and NAF epithelial cells to the subsequent risk of developing breast cancer.

Materials and methods

Original study population and sample collection

Subjects were drawn from a cohort of 1744 women participating in a study on NAF and NAF cells during the years 1973–1976 in the Berkeley/Oakland, CA area [7]. This original cohort was self-selected in response to posted recruitment notices, media publicity, and oral presentations for womens' groups and medical units. A structured questionnaire recorded age, race, age at menarche and thelarche, reproductive and lactation history, menopausal status, and personal and familial history of breast neoplasms.

Nipple aspirate fluid was obtained with a Sartorius aspirator [5] as previously described [7]. After cleansing the nipple with isopropanol to unclog ducts, the aspirator was placed over the nipple and negative pressure (90 mmHg) applied for 45 s. Before and during the procedure, the subject gently massaged her own breast from periphery toward the center of the breast. Aspiration was attempted on both breasts. If fluid appeared, a direct smear was made on a microscope slide, wet-fixed in 95% ethanol, and stained by the standard Papanicolaou method [11]. Smears were screened by a cytotechnologist and confirmed by a pathologist. The smear was graded on a scale of Classes I–V, with class I being 'No atypical cells identified' and Class V being 'Malignant cells are present.' Classes II–IV represented various stages of atypia, including alteration of cell size, nuclear/cytoplasmic ratio, nuclear chromatin, and cell aggregation patterns. When samples contained cells of several classifications, the most abnormal classification was recorded. A few subjects (3%) were sampled on subsequent occasions but all data used in these analyses were based on the first attempt.

Definition of the study population eligible for follow-up

Of the 1744 subjects originally enrolled, 139 were eliminated from follow-up for the following reasons: (1) 86 were pregnant, lactating, or weaning at the time of original sampling for NAF and therefore the ability to obtain NAF was physiologically biased; (2) for 41, information regarding pregnancy, lactation, and weaning was missing; (3) for 11, information on age or full name was missing or illegible, which interfered with attempts to locate subjects; (4) one subject had a bilateral prophylactic mastectomy shortly after the original enrollment. In both the original study and the follow-up, human subject use, which included informed consent, was approved by the University of California, Berkeley, Committee for the Protection of Human Subjects.

Methods for locating women in the eligible cohort

Locating the 1605 subjects eligible for follow-up was particularly difficult because at the time of the original study there was no intention of doing a long-term follow-up. Key information such as birth date and social security number had not been recorded, nor had subjects been periodically contacted to track change of address, and change of surname due to marriage or divorce. Approximately 15% of eligible subjects still had the same address and/or phone number recorded 20–25 years previously. Those who did not, were located through California Department of Motor Vehicles (DMV) records (after approval of the research project), Internet public databases for locating persons (www.ussearch.com and www.daplus.us), and phone directories. We found some subjects with licensed occupations through professional directories. Relatives, friends, and neighbors volunteered information about many subjects. The Social Security Death Index was used to determine which subjects were deceased. If they died in the local area, their death certificates were viewed to determine cause of death and next of kin, whom we then attempted to locate. DMV records supplied us with birth dates of many subjects, which enabled us to confirm identity of deceased subjects. Identity confirmation was made by matching race, their current age with expected current age, and querying their name, address, and employment at the time of original enrollment to see if it matched our recorded information.

Methods to establish breast cancer status in the follow-up cohort

From June 1999–April 2004, information about the located subject was obtained through a structured questionnaire requesting demographic information (age, race, and information about education, job/career title, and income that would provide an index of socioeconomic status); reproductive history; personal and familial history of breast disease/breast surgery; and frequency of mammograms, breast self-examination (BSE), and other breast diagnostic methods. Subjects signed a form to release pathology reports and other medical records to us. The development of breast cancer during the follow-up period was ascertained by self-report in the returned questionnaire and through the California Cancer Registry (CCR). Their data is based on mandatory reporting of all cancers statewide from 1988 on and links to another database (Northern California Cancer Center) covering the San Francisco Bay Area from 1973 on. Cases identified through the CCR were checked for correct match with our subjects based

on age and address. In a few cases, breast cancer status was determined because it was listed as the cause of death on the death certificate. For all cases where the questionnaire reported breast surgery for a tumor or suspicious lesion, attempts were made to obtain the pathology report to verify the diagnosis. Non-malignant pathologies were counted as associated with an increased risk of invasive breast cancer according to criteria established by the College of American Pathologists [12]. Examples of such pathology are atypical ductal and lobular hyperplasia, sclerosing adenosis, florid hyperplasia, papillomatosis associated with atypical hyperplasia, and ductal and lobular carcinomas *in situ*. Breast carcinomas were recorded as ductal or lobular and *in situ* versus invasive.

Data tracking and validation

A relational database (MS Access, 2000) was constructed to manage data from the initial (1973–1976) and the follow-up (1999–2004) cohorts. All data was double entered by different, trained, personnel. Accuracy was checked against the original questionnaire for all discordant entries, and systematically for 10 and 22.7% of the initial and follow-up datasets, respectively. Data entry error rates were 0.13% for the initial database and 0.24% for the follow-up database.

Information from the follow-up questionnaire was used to supply missing data or to correct erroneous data for the initial cohort in the following situations: (1) to supply missing information on age at enrollment, ethnicity, age at menarche, and age at menopause; (2) to update information for events occurring since the initial enrollment, e.g. age at first full-term pregnancy (FFTP), number of pregnancies, number of months of lactation, and family history of breast cancer. For validation of subject's recall ability, all initial and follow-up data were compared for the year of events that occurred before initial enrollment. When initial and follow-up responses differed for age at menarche, the responses in the initial study were used, as these were more likely remembered accurately.

Statistical analysis

Data was analyzed with Stata Statistical Software: Release 8.0 (Stat Corp., College Station, TX). Chi-square analysis was used to compare characteristics of the actual follow-up cohort to the eligible follow-up cohort (Table 1), and the frequencies of breast cancer detection practices in our follow-up cohort with those in other populations previously reported [13–16]. Continuous variables were first stratified into groups before chi-square analysis.

Differential logistic regression models were tested to determine which variables should be controlled when evaluating the risk of developing breast cancer (Table 2). The model that best fit the data controlled for subject's age at NAF sampling. To determine relative risks (RR) and 95% confidence intervals (CI) for developing breast neoplasms according to nipple aspiration categories, logistic regression analysis was refined using the method of Zou [17], a generalized linear model with Poisson distribution for the random part of the model and the log link to estimate the adjusted RRs and CIs. Data was adjusted simultaneously for age at nipple aspirate sampling, and length of time in study (interval between date of NAF sampling, and date of follow-up, breast cancer diagnosis, or death, whichever came first). Nipple aspiration outcomes were classified into three groups: (1) nipple aspiration attempted, no fluid obtained (No NAF) (reference group); (2) NAF obtained, no epithelial cells present (NAF/no epithelial cells); (3) NAF obtained, epithelial cells present (NAF/epithelial cells) (Table 4). Specimens with normal epithelial cells and those with atypical cells were combined in all analyses because only eight women in the original cohort and four women in the follow-up cohort had NAF with atypical cells, making this group too small for valid statistical analysis.

Results

Location of subjects and determination of their breast cancer status

Follow-up was completed on 61% (972 of 1605) of the women, for a total of 24,832 person-years of follow-up. The mean time between NAF sampling and follow-up data acquisition was 25.5 ($\pm$4.6 SD) years, with 92.0% having follow-up information gathered between 20 and 31.3 years after NAF sampling. The mean age at time of follow-up data acquisition was 67.2 ($\pm$11.2 SD) years, with a range of 41–100 years.

Overall, 120/972 (12.3%) of the subjects developed breast cancer during the follow-up period (1973–2003). There were two levels of certainty in determining the breast cancer status of the follow-up cohort. For 105/120 (87.5%) of the subjects, the occurrence of breast cancer was documented by records viz. a pathology report, listing in the CCR, or listing of breast cancer as cause of death on a death certificate. For 15/120 (12.5%) the occurrence of breast cancer was highly probable, but not certain because it was through self-report or next of kin report only. For 128/852 (15.0%) of subjects, the lack of breast cancer development during the follow-up period was virtually assured because the subject was not listed in the CCR and had lived at the same California address throughout the follow-up period, or because the subject was listed in the CCR for a type of cancer other than breast. Absence of breast cancer was highly probable but not certain for 724/852 (85.0%), because it was established only by self or next of kin report.

Of subjects diagnosed with breast cancer, 77/120 (64%) had invasive cancer, 13/120 (11%) had *in situ* carcinoma, and for 30/120 (25%) no detailed pathology information about the type of breast cancer was

Table 1. Characteristics of the actual follow-up cohort versus eligible follow-up cohort

Characteristic	Eligible cohort ($n=1605$)	Actual cohort ($n=972$)
Age at time of NAF sampling		
13–26 years	100(6.3%)	78 (8.0%)
27–39 years	507 (31.6%)	322 (33.1%)
40–52 years	541 (33.8%)	342 (35.2%)
53–66 years	361 (22.5%)	194 (20.0%)
>66 years	77 (4.8%)	34 (3.5%)
Missing	19 (1.0%)	2 (0.2%)
Ethnicity		
African–American	109 (6.8%)	52 (5.3%)
Asian–American	65 (4.0%)	34 (3.5%)
European–American	1307 (81.4%)	840 (86.4%)
Other	31 (1.9%)	23 (2.3%)
Missing	93 (5.8%)	23 (2.4%)
Parity		
Nulliparous	482 (30.0%)	270 (27.8%)
Parous	1106 (68.9%)	698 (71.8%)
Missing	17 (1.1%)	4 (0.4%)
Age at first full-term pregnancy		
≤ 24 years	591 (53.4%)	343 (49.1%)
≥25 years	445 (40.2%)	314 (45.0%)
Missing	70 (6.3%)	41 (5.9%)
Age at menarche		
≤ 12 years	699 (43.6%)	441 (45.4%)
>12 years	799 (49.8%)	498 (51.2%)
Missing	107 (6.7%)	33 (3.4%)
Family history of breast cancer		
No first-degree relative	1269 (79.1%)	776 (79.8%)
≥1 first-degree relative	119 (7.4%)	80 (8.2%)
Missing	217 (13.5%)	116 (11.9%)
NAF		
No NAF	850 (53.0%)	517 (53.2%)
NAF/no epithelial cells	510 (31.8%)	316 (32.5%)
NAF/normal epithelial cells	237 (14.8%)	135 (13.9%)
NAF/atypical epithelial cells	8 (0.4%)	4 (0.4%)

Abbreviations: NAF = nipple aspirate fluid.

obtainable either from medical sources or the CCR. Analysis of 54 surgical reports from women who had 'benign' lesions indicated that 21 had pathology associated with an increased risk of developing breast cancer. These latter cases were combined with the cancer cases for a total of 141, for some statistical analyses.

How representative of the eligible follow-up cohort is the actual follow-up cohort?

We terminated our attempts to locate subjects after reaching a level of 61% and obtaining a population that was representative of the eligible for follow-up cohort for ethnicity, factors that influence breast cancer risk (age, parity, age at FFTP, age at menarche, family history of breast cancer), and the factors under study (whether NAF and epithelial cells were obtained) (Table 1). There was no significant difference between eligible and actual follow-up cohorts for any subject characterization category examined (chi-square analysis, $p \geq 0.14$).

Did our follow-up cohort exhibit an increased incidence of breast cancer associated with known breast cancer risk factors?

Table 2 summarizes how our cohort's incidence and age-adjusted RR of breast cancer compares with known risk factors for breast cancer [18]. We found increased risks of breast cancer related to ethnicity, parity, age at FFTP, and age at menarche, but they were not statistically significant ($p \leq 0.71$, 0.74, 0.35, and 0.71 respectively). In contrast, age at the time of NAF sampling and family history of breast cancer were significant risk factors for developing breast cancer. Women aged 55 or greater at the time of NAF sampling had 3.9 times (p value ≤ 0.03) the risk relative to women aged 34 and under at the time of NAF sampling. Women with one or more first-degree relatives with breast cancer at the time of follow-up had a risk for developing breast cancer 2.5 times that of women reporting no first-degree relative with breast cancer at follow-up ($p \leq 0.0002$).

Were sufficient diagnostic tests performed to detect any breast cancer present?

Table 3 summarizes the overall frequency of BSE and mammograms in this cohort, and the frequencies stratified by ethnicity, believed to be important in influencing frequency of breast screening practices [13–16]. These are compared to frequencies reported in the published literature [13–16]. Our cohort utilized BSE somewhat less frequently and mammograms significantly more frequently than the other cohorts.

Is presence of nipple aspirate fluid and cells associated with the subsequent development of breast cancer?

Table 4 summarizes the age-adjusted RR of developing breast cancer for different nipple aspiration outcome categories. Part A combines invasive and *in situ* breast cancer into one group; part B includes only invasive breast cancer. There was no significant difference in the risk of developing breast cancer for women who yielded a NAF sample without epithelial cells versus women who did not yield a NAF sample (the referent group). The presence of epithelial cells in the NAF sample increased the RR of developing any type of breast cancer 1.92 times ($p \leq 0.005$), and the RR of developing invasive breast cancer 2.3 times ($p \leq 0.005$). Of the four women in the follow-up cohort who had atypical epithelial cells in NAF, two developed breast cancer. When precancerous breast pathologies ($n=21$) were grouped with malignant pathologies ($n=120$) for outcome analysis, the RR was somewhat less (RR = 1.70, $p \leq 0.013$) (data not shown).

Table 2. Breast cancer risk factors of the follow-up cohort

Risk factor	No. cases/total (%)	RR (95% CI)	*p* value*
Ethnicity			
Non-European American	13/111 (11.7%)	1.00 (referent)	
European American	117/851 (13.7%)	1.12 (0.61–2.08)	
Age at time of NAF sampling			
≤ 34 years	19/277 (6.8%)	1.00 (referent)	
35–54 years	63/505 (12.5%)	2.09 (0.98–4.43)	
≥55 years	38/190 (20.0%)	3.92 (1.13–13.66)	≤ 0.03
Parity, age at FFTP (determined at follow-up)			
Age at FFTP ≤ 24 years	45/366 (12.3%)	1.00 (referent)	
Age at FFTP ≥25 years	51/393 (13.0%)	1.06 (0.69–1.63)	
Nulliparous	23/182 (12.6%)	1.10 (0.64–1.88)	
Age at menarche			
≥15	10/81 (12.3%)	1.00 (referent)	
13–14	49/422 (11.6%)	1.00 (0.48–2.07)	
≤ 12	66/448 (14.7%)	1.40 (0.68–2.88)	
Number of first-degree relatives with breast cancer (determined at follow-up)			
None	34/557 (6.1%)	1.00 (referent)	
≥1	26/168 (15.5%)	2.54 (1.52–4.22)	≤ 0.0002

*p Value listed only when significant at a ≤ 0.05 level.
Abbreviations: CI = confidence interval; FFTP = first full-term pregnancy; NAF = nipple aspirate fluid; RR = relative risk.

Table 3. Breast diagnosis practices in the follow-up cohort

	Study cohort *n* (%)	Other cohorts *n* (%)	References
Women >40 with mammogram during last 2 years			
European American	580/634 (91.5%)	5990/9.942 (60.2%)	[13, 14, 16]
African American	36/39 (92.3%)	3308/6,769 (48.9%)	[13, 14, 16]
Hispanic American	10/10 (100.0%)	NDA	
Asian American	25/31 (80.6%)	NDA	
Other	10/13 (76.9%)	NDA	
Total	661/727 (90.9%)	3041/4,907 (62.0%)	[14,15]
Women ≥40 who practiced breast self-examination in last month			
European American	176/633 (27.8%)	13,225/40,256 (32.9%)	[13, 14, 16]
African American	16/38 (42.1%)	4013/7839 (51.2%)	[13, 14, 16]
Hispanic American	6/10 (60.0%)	522/1826 (28.6%)	[16]
Asian American	7/31 (22.6%)	NDA	
Other	5/13 (38.5%)	NDA	
Total	210/724 (29.0%)	2012/4930 (40.8%)	[14,15]

Abbreviations: NDA = no data available.

Since both older age (≥55 years) at NAF sampling and number of first-degree relatives with breast cancer significantly increased a women's risk of developing breast cancer, the data were analyzed with adjustment for these variables. For women < 55 years of age at the time of NAF sampling, having NAF with epithelial cells was even more of a risk for developing breast cancer (RR = 2.1) ($p \leq 0.003$), especially invasive breast cancer (RR = 2.5) ($p \leq 0.003$), than for the study population as a whole (Table 4). Adjusting for number of first-degree relatives with breast cancer did not alter the RR (data not shown).

To determine if laterality made a difference in the effect of NAF status on subsequent breast cancer risk, only breast cancers in the same breast from which NAF was obtained were considered a positive outcome. Because laterality of the NAF specimen was not recorded in 27 cases, the number of breast cancer cases in this analysis was reduced to 93. When the outcome group was redefined in this way, the RR was less significant (RR = 1.6; CI = 0.99–2.51; $p \leq 0.057$) (data not shown).

Figure 1 illustrates the unadjusted cumulative percentages of subjects developing breast cancer during the years following NAF sampling. The three groups (no

Table 4. Relative risks of breast cancer by nipple aspiration outcome categories

	Invasive/*in situ* breast cancer cases/total (%)	RR (95% CI)	*p**
(A) Outcome			
Entire follow-up population			
No NAF	60/517 (11.6%)	1.0 (referent)	
NAF/no epithelial cells	35/318 (11.0%)	1.05 (0.71–1.57)	
NAF/epithelial cells	25/137 (18.3%)	1.92 (1.22–3.01)	≤0.005
Women aged 18–54 at time of NAF sampling			
No NAF	37/378 (9.8%)	1.0 (referent)	
NAF/no epithelial cells	21/274 (7.7%)	0.79 (0.47–1.32)	
NAF/epithelial cells	24/127 (18.9%)	2.06 (1.27–3.32)	≤0.003
	Invasive breast cancer cases only/total (%)	RR (95% CI)	*p**
(B) Outcome			
Entire follow-up population			
No NAF	37/502 (7.4%)	1.0 (referant)	
NAF/no epithelial cells	23/309 (7.4%)	1.17 (0.76–1.97)	
NAF/epithelial cells	17/130 (13.1%)	2.27 (1.27–4.03)	≤0.005
Women aged 18–54 at time of NAF sampling			
No NAF	23/369 (6.2%)	1.0 (referant)	
NAF/no epithelial cells	14/268 (5.2%)	0.90 (0.47–1.72)	
NAF/epithelial cells	17/121 (14.0%)	2.52 (1.37–4.64)	≤0.003

For all analyses there was adjustment for age and length of time in study as continuous variables.
*p Value listed only when significant at a ≤ 0.05 level.
Abbreviations: CI = confidence interval; NAF = nipple aspirate fluid; RR = relative risk.

NAF, NAF/no epithelial cells, and NAF/epithelial cells) have a similar percent morbidity from time of nipple aspiration up until about 23 years after NAF sampling, with no time point having greater than a 4% difference. After 23 years, the 'NAF/epithelial cells' group climbs steeply, resulting in an increase to 21.4% cumulative morbidity by the 30 year time point, while both the 'no NAF' and the 'NAF/no epithelial cells' groups remain <12.5% during this time.

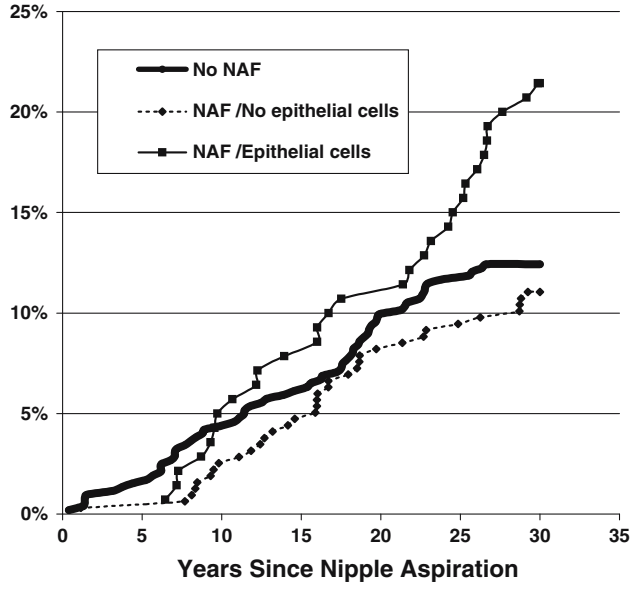

Figure 1. Unadjusted cumulative percentages of subjects that developed breast cancer during the years of follow-up since nipple aspiration.

Discussion

Despite the relatively low follow-up rate of 61%, the cohort obtained is representative of the eligible original cohort for all variables crucial for analysis (Table 1) and is of sufficient size for valid statistical analyses. Wrensch et al. reported follow-up rates of 87% [8], 80% [9], and 90.0% [10] in analogous studies. The most obvious explanation for their higher rate is that they planned to do long-term prospective studies at the time of original enrollment, and contacted subjects periodically, whereas we did not have such plans until 24 years after the original study began. Another explanation for our lower rate is that we did not exclude any group of subjects because of poor follow-up rate, whereas Wrensch et al. [9] excluded those under 30 years at original enrollment because of a 66% follow-up rate, as opposed to >80% rate for those over 30.

How representative is this self-selected follow-up cohort of the general population in terms of risk factors for developing breast cancer? This is an important question if these data are to represent a non-clinical population as opposed to a population with breast symptomology. Our follow-up cohort appears to be quite representative. Known strong risk factors such as aging and one or more first-degree relatives with breast cancer [18] confer the expected significantly increased risk in our cohort. Other known risk factors such as European ancestry, nulliparity, older age at FFTP, and younger age at menarche, show the expected trends in this cohort, but fall short of statistical significance. This is not surprising, since these

risk factors are weaker and often not significant unless the study population is very large [18]. Also, the overall breast cancer incidence of 12.3% during the ≈25 year follow-up period is remarkably similar to the reported average of one in eight (12.5%) of California women developing breast cancer during their lifetimes [19]. The Group I cohort of Wrensch et al. [10], which was most similar to our cohort in terms of recruitment from a non-clinical population, had a somewhat lower overall incidence of breast cancer (7.8%). Since frequencies of breast cancer risk factors were similar in both cohorts, the most likely explanation for the difference in incidence is that our longer mean duration of follow-up (25 years versus 21 years) allowed more time for breast cancer to develop.

The follow-up questionnaire included items about breast cancer detection practices, because we wanted to know to what extent breast cancer would be detected in our population if it were present. The results indicate a far higher rate of mammograms than reported for other cohorts, which may be due to the relatively high educational level of our cohort (70% with a college education or higher). This would make it more likely that subjects would know the value of mammograms and have health insurance which would cover regular mammograms. Volunteer study populations have been found to have a higher average educational level [20]. Breast self-examination, was practiced at a somewhat lower rate than reported for some ethnic groups by other studies [13–16]. We were unable to find published mammogram data for Hispanic and Asian American women and BSE data for Asian Americans reported for the same age group and time since last examination, to allow us to make direct comparisons.

It was difficult to accurately assess the relationship between cytologic diagnosis of atypias at the time of original NAF sampling and subsequent development of breast cancer because in the original cohort there were only 8/1744 (0.5%) who had samples containing atypical cells and only 4 of these subjects were located for follow-up. In the Group I cohort of Wrensch et al. [10] 87/3633 (2.4%) of the follow-up cohort had produced NAF with atypical cells, allowing for valid statistical analysis. One explanation for the greater frequency of atypical cells in their population is that a larger proportion of their population was recruited from breast cancer clinics, and may have been more likely to have existing pathologies at the time of sampling. In addition, there could have been slight differences in cytology interpretation by the respective pathologists [21].

Also different from the San Francisco group [8,9], we did not find a significantly increased RR for women with NAF without epithelial cells. However, for women with normal NAF epithelial cells, both studies indicate a statistically significant RR for developing breast cancer. The RR we obtained (1.9 for any type of breast cancer) was similar to that obtained by Wrensch et al. [10] for their Group I cohort (RR = 1.6–2.4, depending on NAF cytologic classification). Refining our analysis to include only invasive cancers as the breast cancer outcome, increased the RR to 2.3. Similar to the findings of the San Francisco investigators [8,9], we found this effect increased in women <55 years of age at the time of sampling (RR = 2.1, and 2.5 for invasive breast cancers). In fact, none of the cases of invasive breast cancer developing in women with epithelial cells in NAF were in women aged >54 years at the time of sampling. This might relate to the fact that older women are less likely to yield NAF (6,7) and therefore, their epithelial cells might be missed. Alternatively, breast cancer in older women might have a different etiology and pathology from the disease in younger women.

Why should the presence of NAF epithelial cells be predictive of breast cancer diagnosis 20–25 years later? The most obvious explanation is the slow nature of breast cancer development, taking place over 20–30 years [22,23] and involving a gradual progression from normal epithelium through hyperplasia, atypical hyperplasia, and carcinoma *in situ* to invasive carcinoma [24]. Some of the most common non-malignant breast pathologies associated with an increased risk of invasive breast cancer, e.g. atypical and florid hyperplasia, involve excessive proliferation of epithelial cells. This could result in increased exfoliation of mammary epithelial cells into breast fluids and recovery in NAF. Our data did not allow determination of whether the breast cancers that developed were located in the same section of the mammary tree from which NAF was obtained, but for 78% of the breast cancer cases, we had information on the laterality of the NAF and the subsequent breast cancer. Interestingly, when these cases were analyzed separately, the RR was not increased. This fits with the idea that changes occurring in one area of the breast are not localized to that area, but rather, could reflect generalized influences in all areas of both breasts (field effect). Histologic studies have shown that women with hyperplasia and atypical hyperplasia in a single biopsied breast lesion have a 1.5–4× risk of subsequently developing breast cancer in *either or both* breasts, indicating that the local manifestation represents a generalized condition [25].

Our data and those of Wrensch et al. [8–10] suggest the potential usefulness of NAF epithelial cells as a biomarker for breast cancer risk, particularly in women <55 years of age. A possible clinical application of this, is routine nipple aspiration and NAF cytology during general physicals and gynecologic examinations of premenopausal women. A Sartorius-like aspirator is currently available commercially (Cytyc Corp., Boxborough, MA). Women with epithelial cells obtained by NAF could be more closely followed over the years, and those with atypical epithelial cells could be immediately evaluated with other diagnostic modalities. Although not a highly effective screening method, nipple aspiration is less invasive, less expensive, and requires less time and skill to perform than other cellular modalities such as ductal lavage and fine needle aspiration [26]. Breast cancer is curable when detected at an early stage. The

fact that it remains the second most frequent cause of cancer death in women indicates that detection is not occurring early enough. NAF cytology may be an important adjunct to the battery of early detection methods currently available.

Acknowledgements

We are grateful to the women who volunteered as subjects for this study and without whom it would not have been possible. We thank the following at the University of California, Berkeley: Maureen Lahiff, Biostatistics Division for consultation on biostatistics; Katherine Miller, Janice Casamina, Rhonda Kropp, Jessica Kirschbraun, Kelly Fujikawa, and Hua Min Shen for locating subjects and/or data entry and processing. We thank David Harris and Robert Schlag of the California Cancer Registry, Sacramento, CA for help with the Registry database. This research was supported by a grant from the Dr Susan Love Research Foundation.

References

1. Koss LG: Diagnostic Cytology. JB Lippincott, Philadelphia, 1968 541
2. Haagensen CD: Diseases of the Breast. 2nd ed. WB Saunders, Philadelphia, 1971
3. Vorherr H: The Breast. Academic Press, New York, 1974 218–246
4. Papanicolaou GN, Holmquist DG, Bader FM, Falk EA: Exfoliative cytology of the human mammary gland and its value in the diagnosis of cancer and other diseases of the breast. Cancer 1: 377–409, 1958
5. Sartorius OW, Smith HS, Morris P, Benedict D, Friesen L: Cytological evaluation of breast fluid in the detection of breast diseases. J Natl Cancer Inst 59: 1073–1108, 1977
6. Petrakis NL, Mason L, Lee R, Sugimoto B, Pawson S, Catchpool F: Association of race, age, menopausal status, and cerumen type with breast fluid secretion in nonlactating women, as determined by nipple aspiration. J Natl Cancer Inst 54: 829–834, 1975
7. Buehring GC: Screening for breast atypias using exfoliative cytology. Cancer 43: 1788–1799, 1979
8. Wrensch MR, Petrakis NL, King EB, Miike R, Mason L, Chew K, Lee MM, Ernster VL, Hilton JF, Schweitzer R, Goodson WH III, Hunt TK: Breast cancer incidence in women with abnormal cytology in nipple aspirates of breast fluid. Am J Epidem 135: 130–141, 1992
9. Wrensch MR, Petrakis NL, King EB, Lee MM, Miike R: Breast cancer risk associated with abnormal cytology in nipple aspirates of breast fluid and prior history of breast biopsy. Am J Epidem 137: 829–833, 1993
10. Wrensch MR, Petrakis NL, Miike R, King EB, Chew K, Neuhaus J, Lee MM, Rhys M: Breast cancer risk in women with abnormal cytology in nipple aspirates of breast fluid. J Natl Cancer Inst 93: 1791–1798, 2001
11. Papanicolaou GN: New procedure for staining vaginal smears. Science 95: 438–439, 1942
12. Fitzgibbons PL, Henson DE, Hutter RVP, for the Cancer Committee of the College of American Pathologists. Benign breast changes and the risk for subsequent breast cancer: an update of the 1985 consensus statement. Arch Pathol Lab Med 122: 1053–1055, 1998
13. Coleman EA, O'Sullivan PO: Racial differences in breast cancer screening among women from 65–74 years of age: trends from 1987–1993 and barriers to screening. J Women Aging 13: 23–39, 2001
14. Harris DM, Miller JE, Davis DM: Racial differences in breast cancer screening, knowledge and compliance. J Natl Med Asscoc 95: 693–701, 2003
15. Katapodi MC, Faclone NC, Miakowski C, Dodd MJ, Waters C: The influence of social support on breast cancer screening in a multicultural community sample. Oncol Nurs Forum 29: 845–852, 2002
16. Vernon SW, Vogel VG, Halabi A, Jackson GL, Lundy RO, Peters GN: Breast cancer screening behaviors and attitudes in three racial/ethnic groups. Cancer 69: 165–174, 1992
17. Zou G: A modified Poisson regression approach to prospective studies with binary data. Am J Epidemiol 159: 702–706, 2004
18. MacMahon B, Cole P, Brown J: Etiology of human breast caner: a review. J Natl Cancer Inst 50: 21–42, 1973
19. California Cancer Registry: Breast Cancer in California, 2003
20. Ganguli M, Lytle ME, Reynolds MD, Dodge HH: Random vs. volunteer selection for a community-based study. J Gerontol: Series A, Biol Sci Med Sci 53: M39–M46, 1998
21. King E, Barrett D, Petrakis NL: Cellular composition of the nipple aspirate specimen of breast fluid. II. Abnormal findings. Am J Natl Clin Pathol 64: 739–748, 1975
22. Fournier DV, Weber E, Hoeffken W, Bauer M, Kubli F, Barth V: Growth rate of 147 mammary carcinomas. Cancer 45: 2198–2207, 1980
23. Lundgren B: Observations on growth rate of breast carcinomas and its possible implications for lead time. Cancer 40: 1722–1725, 1977
24. Wellings SR, Jensen HM, Marcum RG: An atlas of subgross pathology of the human breast with special reference to possible precancerous lesions. J Natl Cancer Inst 55: 231–273, 1975
25. Page DL: The woman at high risk for breast cancer: importance of hyperplasia. Surg Clin N Am 76: 221–230, 1996
26. Fabian CJ, Kimler BF, Mayo MS, Khan SA: Breast-tissue sampling for risk assessment and prevention. Endocr-relat Cancer 12: 185–213, 2005

Address for offprints and correspondence: Gertrude Case Buehring, Division of Infectious Diseases, School of Public Health, University of California, 140 Warren Hall, Berkeley, CA, 94720, USA; *Tel.:* +1-510-642-3870 ; *Fax:* +1-510-642-6350 ; *E-mail:*buehring@berkeley.edu