

Prostate-specific antigen and insulin-like growth factor binding protein-3 in nipple aspirate fluid are associated with breast cancer

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Received 3 January 2002; received in revised form 22 January 2002; accepted 1 March 2002

Abstract

Insulin-like growth factor-1 (IGF-1) is an important growth factor for breast cancer cells and insulin-like growth factor binding protein-3 (IGFBP-3) its most prevalent binding protein. Prostate-specific antigen (PSA) enzymatically cleaves IGFBP-3 into fragments (BP3-FR). Our purpose was to determine the association of these markers in nipple aspirate fluid (NAF) and serum with the presence of breast cancer. NAF from 175 and serum from 215 subjects were collected from women with or without breast cancer. In unadjusted analysis low NAF PSA ($P < 0.001$) and high NAF IGFBP-3 ($P = 0.023$) were associated with breast cancer. Low serum PSA was associated with postmenopausal breast cancer ($P = 0.034$). In separate multivariate analyses, controlling for age, menopausal status, and age at menarche, NAF PSA and IGFBP-3 were each associated with breast cancer. The association was significant for NAF IGFBP-3 in all women ($P = 0.031$), but for NAF PSA only in premenopausal women ($P < 0.001$). When considered jointly, only NAF PSA was significant. Therefore, NAF PSA, and to a lesser extent NAF IGFBP-3 and serum PSA, seem to be important predictors of breast cancer. © 2002 International Society for Preventive Oncology. Published by Elsevier Science Ltd. All rights reserved.

Keywords: Breast cancer screening; Risk factors for breast cancer; Nipple aspirate fluid; Prostate-specific antigen

1. Introduction

The breast is the leading site of cancer in American women [1]. Present efforts to evaluate the breast directly either through the analysis of tissue or individual cells are hindered because the assessment of these specimens generally requires an invasive procedure. The adult nonpregnant, nonlactating breast secretes fluid into the breast ductal system. This fluid can be obtained through aspiration of the nipple with a modified breast pump. Refinements in the ability to obtain this fluid, as well as epidemiologic studies to identify subjects most likely to yield NAF, have been ongoing for over 20 years. Nipple aspiration has the attractiveness of quickly, painlessly, and noninvasively obtaining both breast epithelial cells, the cells at risk for transforma-

tion to breast cancer, as well as secreted proteins, which are concentrated in the fluid. We have obtained nipple aspirate fluid (NAF) from over 500 women during the past 6 years, and demonstrated that secreted proteins in NAF, such as prostate-specific antigen (PSA) [2], can be analyzed and are highly associated with the presence of breast cancer. The purpose of this study is to reevaluate our previously reported findings in a larger cohort of subjects, determine in which subgroups of women PSA is most predictive of the presence of breast cancer, and evaluate proteins in the IGF-1 family with which PSA appears to interact and which may provide additional information about a subject's chance of having breast cancer.

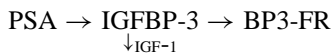
IGF-1 is a potent mitogen for human breast cancer cells. Patients who respond to tamoxifen treatment experience a 25–38% decrease in their plasma IGF-1 levels [3]. In the circulation, most IGF-1 is bound to IGFBP-3 [4]. IGFBP-3 is the major binding protein and serves as a carrier for the IGFs, prolonging their half-life in the circulation [5]. IGFBP-3 is secreted by a number of cell types, and can directly modulate IGF-1 actions in tissues [6]. Whether

Abbreviations: BP3-FR, fragmented insulin-like growth factor binding protein-3; DCIS, ductal carcinoma in situ; IC, invasive carcinoma; IGF-1, insulin-like growth factor-1; IGFBP-3, IGF binding protein-3; NAF, nipple aspirate fluid; PSA, prostate-specific antigen

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IGFBP-3 stimulates or inhibits breast epithelial growth is a matter of ongoing debate. In vitro studies indicate that IGFBP-3 inhibits the growth of human breast cancer cells in an IGF-1 independent manner [7] and facilitates apoptosis [8]. On the other hand, three studies have found that high levels of IGFBP-3 in breast cancer tissue were associated with unfavorable prognostic indicators of the disease, such as large tumor size, low levels of steroid hormone receptors, and elevated S-phase fraction [9–11].

PSA is made by both normal and malignant human breast tissue [12]. We previously reported in a small cohort that PSA is detectable in NAF and low levels were associated with breast cancer [2]. On the other hand, we did not find that PSA was useful in predicting the presence of residual disease in women after breast biopsy demonstrated cancer with positive or indeterminate margins [13]. PSA is a chymotrypsin-like protease which is thought to cleave IGFBP-3 at a specific tyrosine residue (Tyr-159) [14], leading to fragmented IGFBP-3, or BP3-FR. BP3-FR has been found in a variety of body fluids from normal subjects, including lymph and serum [15] and seminal plasma [16]. BP3-FR inhibits the mitogenic effects of IGF-1 [17]. Multiple studies have confirmed that fragmented IGFBP-3 is biologically active [18]. Thus, the interactions of PSA, IGFBP-3 and BP3-FR appear to play an important role in IGF-1 regulation. We recently proposed a hypothetical model of IGF-1 regulation [19] which may help to explain how these molecules interact:



Because of the conflicting results regarding the association of IGFBP-3 in blood and tissue with breast cancer, we wondered if another source of material might be preferable. Breast NAF provides a source of material which is organ specific, not diluted by the contribution of other organs in the body. We demonstrate in this report that secreted proteins such as PSA are present in NAF at levels 100–1000-fold higher than in female serum. On the other hand, serum collection is currently more readily available than is the collection of NAF, so the identification of useful marker(s) in serum is highly desirable.

In this study, we set out to determine if NAF and serum levels of important growth factor regulators in the IGF-1 system were associated with breast cancer. We did this in a large cohort of subjects, some with and others without breast cancer. Each of the markers chosen for analysis has proven or suspected importance in breast cancer. If markers in NAF predictive of breast cancer are identified, they may prove useful as screening tools to detect new or recurrent disease.

2. Materials and methods

2.1. Subjects

NAF specimens from 175 subjects and serum specimens from 215 subjects aged 30–80 years were collected between

January 1995 and July 1999 after approval of the Institutional Review Board. These subjects were recruited either through local advertisement, by word of mouth, or, with the permission of the treating surgeons, by contacting subjects scheduled to undergo breast surgery. In some cases, more than one specimen was collected from an individual, with each specimen collected on a different day. If multiple results were available for an individual, only the median value was used for statistical analysis. Subjects included women with all stages of risk, from no risk factors for breast cancer (other than gender) to those with recently diagnosed carcinoma of the breast. Subjects were categorized as either (A) cancer: those with newly diagnosed, biopsy-proven ductal carcinoma in situ (DCIS) or invasive carcinoma (IC); or (B) non-cancer: no evidence of DCIS or IC. For subjects with cancer, NAF was collected from the breast with active disease.

2.2. Aspiration technique

Nipple fluid was aspirated by a trained physician or nurse clinician using a modified breast pump [20]. The breast nipple was cleansed with alcohol, the plunger of the aspiration device was withdrawn to the 7 ml level and held for 15 s. Fluid in the form of droplets was collected in capillary tubes. The quantity of fluid varied from 1 to 200 μ l.

2.3. PSA, IGFBP-3, BP3-FR

- A. *Specimen preparation (NAF)*: Every NAF sample collected was suitable for evaluation. In general, a sample was used to measure total protein and each one of the markers (PSA, IGFBP-3, or BP3-FR). Samples were collected in 50 μ l capillary tubes (generally 1–5 μ l per tube). For extraction, the portion of the capillary containing the sample was introduced into a 1.7 ml eppendorf tube and 100 μ l of a 0.1 mol/l solution of sodium bicarbonate (pH 7.8) was added. The capillary was then crushed by using a glass rod and the mixture was vortexed to disperse the sample. The mixture was centrifuged at 14,000 g for 5 min and the supernatant used without further dilution.
- B. *Specimen preparation (serum)*: A volume of 8 ml of blood was collected after informed consent was obtained and the serum separated from the cellular fraction.
- C. *Specimen analysis (NAF and serum)*: The NAF samples varied both in their total protein concentration and in the volume in the capillary used for marker analysis. For this reason, it has been our practice in NAF samples and the practice of others to control for total protein concentration and the degree to which the NAF was diluted prior to analysis. Total protein was measured using the bicinchoninic acid method (Pierce Chemical Co., Rockford, IL). PSA was analyzed using a highly sensitive technique [2]. Briefly, this procedure combines a time-resolved immunofluorometric assay (TRIFA)

with two monoclonal antibodies and has a detection limit of 1 ng/l. IGFBP-3 was measured according to the manufacturer's instructions with a commercially available enzyme-linked immunosorbent assay (ELISA) from Diagnostic Systems Laboratories, Webster, TX. This assay is based on a two-site immunoenzymatic principle with a polyclonal antibody used for capture and an enzyme-labeled polyclonal antibody used for detection. BP3-FR was measured with an immunoenzymatic assay based on a monoclonal capture antibody and another monoclonal detection antibody labeled with horseradish peroxidase. This assay has been previously described [21]. The assay was calibrated with intact recombinant IGFBP-3.

2.4. Serum IGF-1

- A. *Specimen collection*: A volume of 8 ml of blood was collected after informed consent was obtained and the serum separated from the cellular fraction.
- B. *Specimen analysis*: Serum IGF-1 concentration was measured with an immunoenzymatic assay commercially available from Diagnostic Systems Laboratories. The assay is based on a monoclonal capture antibody and a monoclonal detection antibody labeled with horseradish peroxidase. A non-extractive protocol was used, following the manufacturer's recommendations. The same method was used to measure IGF-1 in NAF but the concentration of the protein in the NAF samples was too low to detect.

2.5. Statistical analysis

Women were included in the cancer group if they had known DCIS or invasive cancer, and in the non-cancer group if they did not have either of these diagnoses. Because menopausal status has been shown to influence the expression of a variety of breast cancer markers, results were analyzed overall and by menopausal status (pre/peri- versus postmenopausal). The Wilcoxon two-sample test was performed to compare the levels of NAF and serum markers in the cancer and non-cancer groups. We used the exact version of the Wilcoxon test to verify all significant *P*-values. All tests of statistical significance were two sided.

Multivariate logistic regression analyses were then performed controlling for covariates potentially associated with breast cancer. We considered the following covariates: race, age, menopausal status, age at menarche, age when the first child was born, birth control usage and hormone replacement therapy. Age, menopausal status, and age at menarche were associated with the odds of cancer in some of the models, and generally improved the fit of all the models. We therefore analyzed the logistic regression models including these three factors, even if they were not statistically significant, and one or more of the markers. We used the Wald test to assess the significance of variables in the model and

the profile likelihood method to compute the confidence intervals for the odds ratios [22].

Although logistic regression models routinely estimate the increase in odds per one unit of increase in the covariate, because this increase was not biologically noteworthy for some of the covariates, we computed the odds ratios corresponding to 5 and 10 U increase in age and NAF IGFBP-3 as respectively the 5th and 10th power of the odds ratio per unit increase. NAF PSA was analyzed on the log scale, that is, the percent of increase in NAF PSA corresponded to the absolute increase in $\ln(\text{NAF PSA})$. The odds ratios corresponding to the 50 or 100% increase in NAF PSA were computed as the increase in odds corresponding to respectively $\ln(1.5)$ or $\ln(2)$ increase in the natural log transformed NAF PSA.

Logistic models combining NAF and serum markers were not feasible since only 21 women had both types of markers measured. Empirical logits were used to verify that the relationship between the log odds and each original continuous marker was approximately linear and to select the appropriate transformations for the marker variables, when necessary. Based on this analysis in the logistic models we used log-transformed NAF and serum PSA (adding 0.5 to the original value in order to accommodate multiple zero values) and squared serum IGFBP-3 and BP3-FR and the original values of NAF IGFBP-3 and BP3-FR. All markers were included as continuous variables in the logistic regression models. The following outlier data were excluded from the logistic analyses: serum PSA = 7335 and 219 ng/l, NAF PSA = 25016 and 17191 ng/g, and NAF IGFBP-3 = 583 $\mu\text{g}/\text{mg}$.

In order to define potential marker cut points associated with breast cancer, classification and regression trees (CART) analysis was performed on the data including significant covariates and markers from the corresponding logistic regression models. The data were analyzed using SAS 8.0 (SAS Institute Inc., Cary, NC) and classification and regression trees (CART, San Diego, CA: Salford Systems, 1997).

3. Results

We were able to obtain NAF from 98% of enrolled subjects. In Table 1, we report the results of the Wilcoxon two-sample tests which were performed to compare the levels of the markers in the cancer and non-cancer groups. Considering all subjects, median NAF PSA was significantly ($P < 0.001$) higher in non-cancer subjects (852 ng/g) than in subjects with cancer (46 ng/g), while median NAF IGFBP-3 was significantly lower ($P = 0.023$) in non-cancer subjects (4.7 ng/mg) than in subjects with cancer (9.8 ng/mg). NAF PSA and IGFBP-3 results were then analyzed divided by menopausal status. In premenopausal women, median NAF PSA was significantly higher ($P < 0.001$) in non-cancer subjects (1112 ng/g) than in subjects with cancer (49 ng/g) (Table 1, Fig. 1A). In postmenopausal women, median NAF PSA was significantly higher ($P = 0.010$) in non-cancer subjects (154 ng/g) than in cancer subjects (41 ng/g). Though

Table 1
Medians of PSA, IGF-1, IGFBP-3, BP3-FR levels in women with and without breast cancer^a

	All subjects	Overall median	Non-cancer		Cancer		P-value
			Subjects	Median	Subjects	Median	
<i>Both pre- and postmenopausal women^b</i>							
NAF							
PSA (ng/g)	171	133	58	852	113	46	<0.001
IGFBP-3 (ng/mg)	126	7.4	42	4.7	84	9.8	0.023
BP3-FR (ng/mg)	111	29	34	30	77	28	0.975
Serum							
PSA (ng/l)	89	1.1	40	1.5	49	0.9	0.071
IGF-1 (ng/ml)	151	166	89	173	62	157	0.125
IGFBP-3 (μg/ml)	198	3.2	82	3.2	116	3.2	0.838
BP3-FR (μg/ml)	198	1.2	82	1.1	116	1.2	0.071
<i>Premenopausal women only^b</i>							
NAF							
PSA (ng/g)	86	319	41	1112	45	49	<0.001
IGFBP-3 (ng/mg)	61	5.9	29	4.5	32	8.9	0.102
BP3-FR (ng/mg)	50	32	22	31	28	37.5	0.914
Serum							
PSA (ng/l)	35	1.4	21	1.0	14	2.7	0.172
IGF-1 (ng/ml)	73	182	51	187	22	173	0.564
IGFBP-3 (μg/ml)	92	3.2	49	3.2	43	2.9	0.214
BP3-FR (μg/ml)	92	1.1	49	1.1	43	1.1	0.837
<i>Postmenopausal women only^b</i>							
NAF							
PSA (ng/g)	85	55	17	154	68	41	0.010
IGFBP-3 (ng/mg)	65	9.6	13	5.7	52	10	0.251
BP3-FR (ng/mg)	61	28	12	29	49	28	0.986
Serum							
PSA (ng/l)	46	0.6	11	3.8	35	0.5	0.034
IGF-1 (ng/ml)	68	152	28	152	40	152	0.356
IGFBP-3 (μg/ml)	99	3.2	26	3.2	73	3.4	0.667
BP3-FR (μg/ml)	99	1.3	26	1.2	73	1.3	0.026

^a IGF-1 results are only available from serum. Some subjects provided only NAF or serum, but not both.

^b The number of pre- and postmenopausal women might not add up to the total number because of occasionally missing data on menopausal status.

both pre- and postmenopausal non-cancer subjects had lower median levels of IGFBP-3 than subjects in the cancer group (premenopausal: 4.5 versus 8.9 ng/mg; postmenopausal: 5.7 versus 10 ng/mg), these differences did not reach statistical significance (Table 1, Fig. 1B). NAF levels of BP3-FR were not different between the groups, whether considering all subjects or subjects divided by menopausal status.

Whether considering all subjects or premenopausal subjects only, no serum marker was significantly different in the cancer and non-cancer groups. On the other hand, in postmenopausal women median serum PSA was significantly higher ($P = 0.034$) in non-cancer subjects (3.8 ng/l) than in subjects with cancer (0.5 ng/l) (Table 1, Fig. 2A), and median serum BP3-FR was significantly lower ($P = 0.026$) in non-cancer subjects (1.2 μg/ml) than in subjects with cancer (1.3 μg/ml) (Table 1, Fig. 2B).

3.1. Logistic regression analyses of NAF and serum markers

Table 2 summarizes the results of the logistic regression models for NAF PSA (164 subjects) and NAF IGFBP-3

(119 subjects). NAF PSA was highly significant in predicting cancer in pre- ($P < 0.001$) but not in postmenopausal women ($P = 0.089$).

In premenopausal women for NAF PSA, the model implies that the odds of having cancer decrease 22.5% (95% CI: 14.3 and 31.7%) with each 50% rise in PSA. If NAF PSA is doubled (an increase of 100%), the odds of having cancer decrease 35.3% (95% CI: 23.2 and 47.8%). In postmenopausal women, the odds of having cancer decrease 8.2% (95% CI: -0.9 and 17.4%) with a 50% rise in NAF PSA and 13.7% for each 100% increase in PSA (CI: -1.6 and 27.9%).

NAF IGFBP-3 was a significant predictor of cancer in all women ($P = 0.031$), and results did not depend on menopausal status. Our model for NAF IGFBP-3 implies that the odds of having cancer increase 32.3% (95% CI: 6.5 and 76.7%) for every 5 ng/mg increase and 75% (95% CI: 13.4 and 212.2%) for every 10 ng/mg increase in this marker for women of all ages.

The model including both NAF PSA and NAF IGFBP-3 yielded a significant effect of NAF PSA, but a small and statistically insignificant effect of NAF IGFBP-3. Thus, if

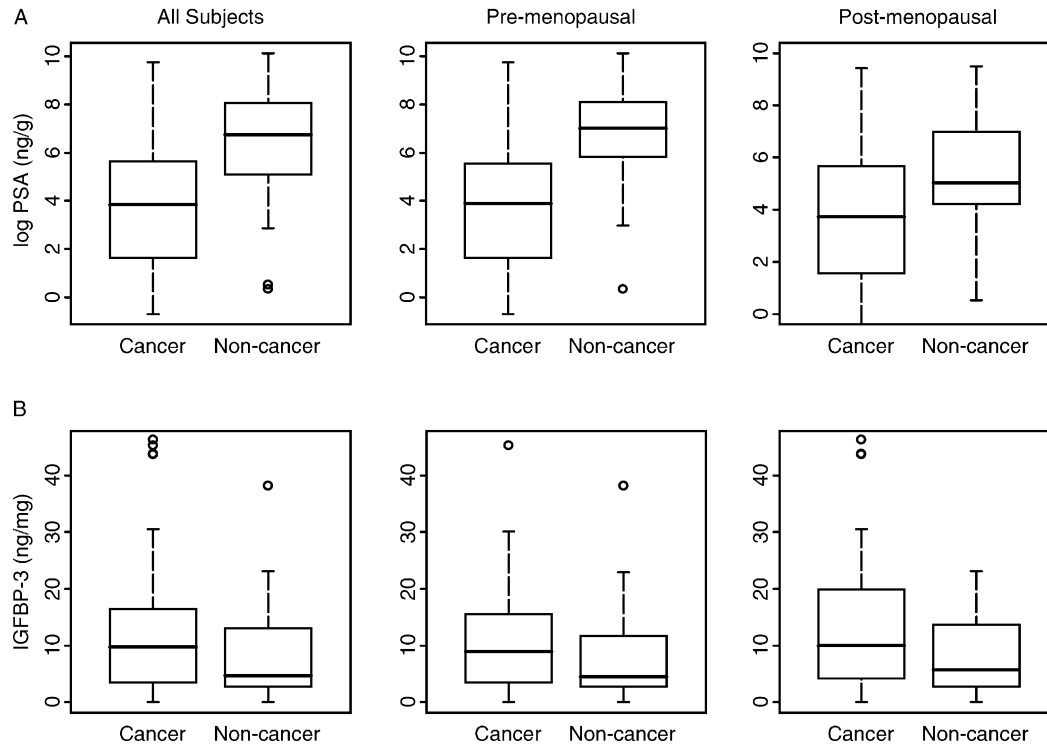


Fig. 1. NAF box plots in cancer patients (cancer) and in subjects free of cancer (non-cancer) overall and by menopausal status: (A) PSA in log scale (for each data point 0.5 has been added to avoid log0), (B) IGFBP-3. The median (middle line), 25 and 75th percentiles (lower and upper boundaries of the box, respectively), and lowest and highest data within 1.5 times the 25–75th percentiles (lower and upper hatch lines) are illustrated. Points more extreme are shown by individual plot symbols. IGFBP-3 = 340.7 and 205.3 are out of bounds and not plotted.

age, menopausal status, age at menarche, and NAF PSA are known, NAF IGFBP-3 contributed little additional information about the chances that a subject had breast cancer.

Models for the serum markers indicated that after controlling for age, menopausal status and age at menarche, no serum marker was significantly associated with the odds of a subject having cancer.

Table 2
Odds ratios and confidence intervals from the logistic regression models

Variable	Unit of increase	Odds ratio	Lower 95% CI	Upper 95% CI	P-value
<i>Model with NAF PSA</i>					
Age	5 years	1.564	1.164	2.160	0.004
	10 years	2.445	1.355	4.665	
Menopausal status	Post vs. pre	0.099	0.008	1.093	0.064
Age at menarche	1 year	1.050	0.966	1.129	0.245
NAF PSA in premenopausal women	50%	0.775	0.683	0.857	<0.001
	100%	0.647	0.522	0.768	
NAF PSA in postmenopausal women	50%	0.918	0.826	1.009	0.089
	100%	0.863	0.721	1.016	
<i>Model with NAF IGFBP-3</i>					
Age	5 years	1.511	1.116	2.137	0.012
	10 years	2.284	1.245	4.566	
Menopausal status	Post vs. pre	1.024	0.283	3.604	0.970
Age at menarche	1 year	1.068	0.878	1.121	0.099
NAF IGFBP-3	5 ng/mg	1.323	1.065	1.767	0.031
	10 ng/mg	1.750	1.134	3.122	

NAF IGFBP-3: the effect of NAF IGFBP-3 was not different by menopausal status.

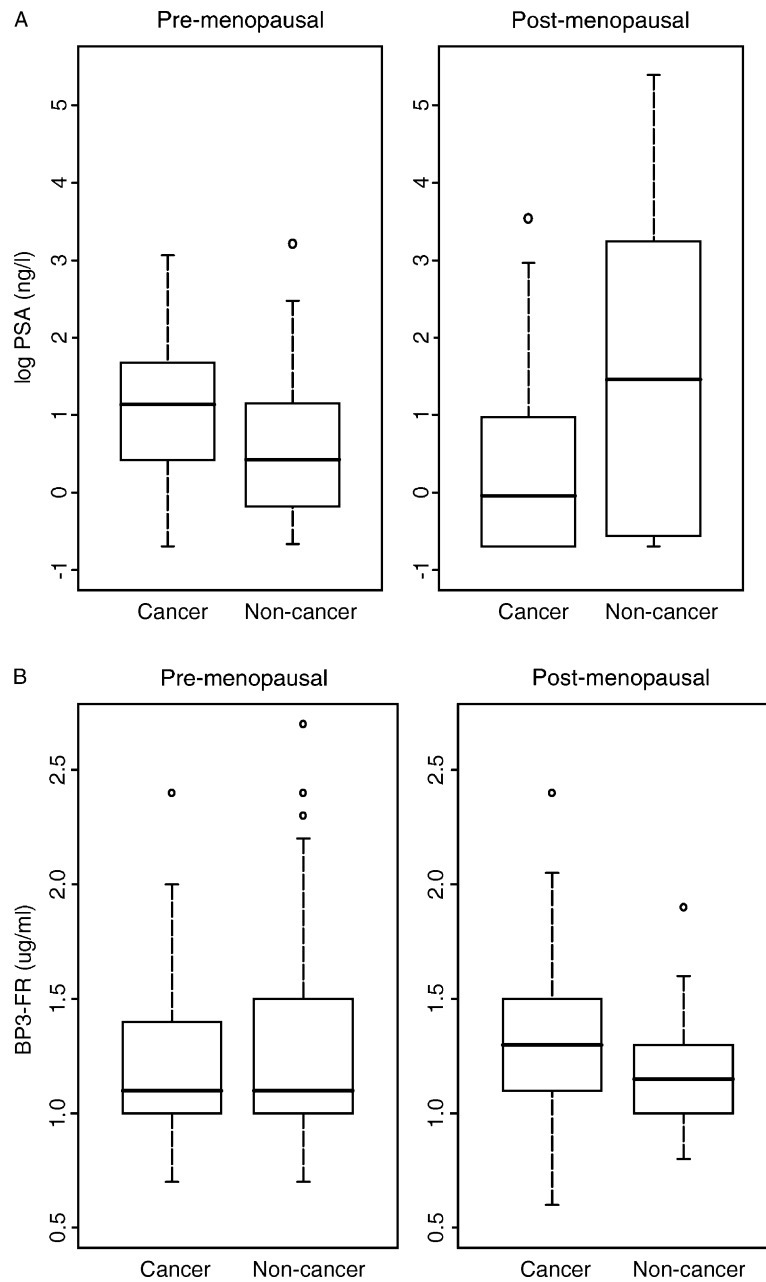


Fig. 2. Serum box plots in cancer patients (cancer) and in subjects free of cancer (non-cancer) overall and by menopausal status: (A) PSA in log scale (for each data point 0.5 has been added to avoid log 0). (B) BP3-FR. The median (middle line), 25 and 75th percentiles (lower and upper boundaries of the box, respectively), and lowest and highest data within 1.5 times the 25–75th percentiles (lower and upper hatch lines) are illustrated. Points more extreme are shown by individual plot symbols. BP3-FR = 6.1 is out of bounds and not plotted.

The inverse association of NAF PSA with the odds of having breast cancer in pre- and postmenopausal women is illustrated in Fig. 3A. The odds are shown relative to a woman with a median NAF PSA value from the non-cancer premenopausal group (1112 ng/g). The direct association of NAF IGFBP-3 with the odds of breast cancer for both pre- and postmenopausal women is illustrated in Fig. 3B. Odds are shown relative to a woman with a median NAF IGFBP-3 value from the non-cancer group (4.72 ng/mg).

In response to the reviewer's request we considered the ratios in NAF of IGFBP-3 to PSA and IGFBP-FR to PSA, as well as IGF-1 to IGFBP-3, IGF-1 to PSA, IGFBP-3 to PSA, and IGFBP-FR to PSA in serum. In separate logistic regression models, controlling for age, menopausal status, and age at menarche, only the ratio of the IGFBP-FR to PSA in NAF from premenopausal women was significantly associated with breast cancer ($P = 0.004$), although the ratio of the IGFBP-3 to PSA in NAF was of marginal statistical

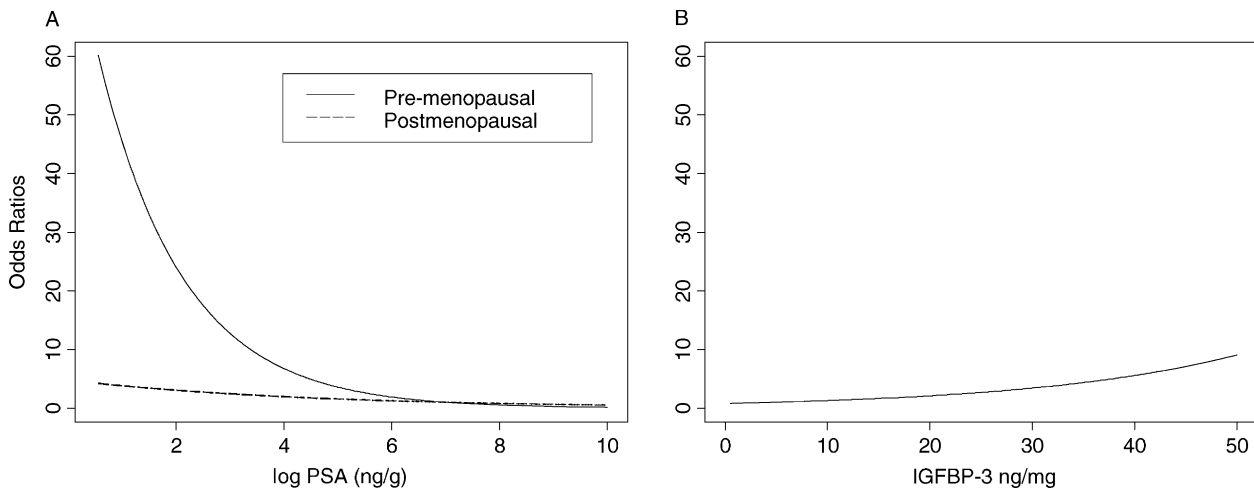


Fig. 3. NAF plots demonstrating the odds of breast cancer for (A) PSA and (B) IGFBP-3 (reference values are the medians in the non-cancer group).

significance ($P = 0.083$). There were 92 observations available for these models. None of the ratios in the serum was significantly associated with breast cancer, although due to missing data there were only 56 observations available for these models.

3.2. Categorizing risk using classification and regression trees (CART)

Since only age, PSA and the interaction between menopausal status and PSA were significant in the logistic regression model for NAF PSA, we sought cut points for PSA in the CART model which included age and menopausal status. PSA was more discriminatory than age and menopausal status regarding risk. In our sample, 92% (65/71) of women with a PSA value (≤ 65 ng/g) had breast cancer, whereas analysis of women with PSA > 65 was inconclusive (44/93, or 47% had cancer).

In the logistic regression model for NAF IGFBP-3, only age and IGFBP-3 were significant. We sought cut points for IGFBP-3 including only these variables in the CART model. In contrast to PSA, IGFBP-3 was less discriminatory than age regarding risk, and was not informative for women > 60 years old. The 100% (8/8) of women younger than 60 with IGFBP-3 > 26 ng/mg had breast cancer. Analysis of women younger than 60 with IGFBP-3 ≤ 26 was inconclusive (39/80 or 49% had cancer).

4. Discussion

We initiated our nipple aspiration studies to identify biomarkers that were associated with breast cancer. The breast ducts of adult nonpregnant women secrete small amounts of fluid [23]. This fluid does not escape because the nipple ducts are occluded by smooth muscle contraction, dried secretions, and keratinized epithelium. Breast

fluid can be obtained by nipple aspiration in women without spontaneous nipple discharge with the use of a modified breast pump [24]. This fluid contains several types of cells, including exfoliated breast epithelial cells [25]. Because breast cancer develops from ductal and lobular epithelium, NAF is a potentially useful epidemiologic and clinical research tool.

IGF-1 is a known mitogen for breast cancer growth. We attempted to measure IGF-1 in NAF, but for most samples the levels of the growth factor were too low to detect. We did not find an association between IGF-1 serum concentration and breast cancer, whether considering the entire group or divided by menopausal status. A prior report [26] also did not find an association between plasma IGF-1 and breast cancer risk in premenopausal women, postmenopausal women or among all women. A pilot study of postmenopausal women identified an association between IGF-1 plasma levels and postmenopausal breast cancer [20]. Larger studies are needed to determine if IGF-1 will become useful in the identification of women with breast cancer or in the development of risk reduction strategies.

We found a significant association between PSA levels in NAF and the odds of breast cancer, both for the group as a whole, and when divided by menopausal status. The observation that the odds of both pre- and postmenopausal women having breast cancer is low if their PSA is high extends our earlier findings [2]. Moreover, it is noteworthy that the median PSA value was more than 22-fold greater in premenopausal women without breast cancer than in those with cancer, but only 3.76-fold higher in postmenopausal women. To some, our observations are counterintuitive, for it is well known that circulating PSA levels are higher in men with prostate cancer than in those without the disease. Nonetheless, it has been demonstrated that PSA levels are higher in prostate tissue from normal subjects than from subjects with prostate cancer [27]. Moreover, PSA levels in

prostate cancer tissue are inversely associated with T stage and grade [28].

The odds of a woman having breast cancer changed most dramatically at the lowest PSA levels, especially for premenopausal women. For example, for a premenopausal woman with NAF PSA = 200 ng/g, a 100 ng/g decrease to 100 ng/g increased her odds of cancer by 55%, a 150 ng/g decrease to 50 ng/g increased her odds of cancer by 139%, and a 195 ng/g decrease to 5 ng/g increased her odds of cancer by 916%. On the other hand, for a premenopausal woman with NAF PSA = 1000 ng/g, a 100 ng/g decrease to 900 ng/g increased her odds of cancer only by 7% and a 200 ng/g decrease to 800 ng/g increased her odds of cancer only by 15%.

In an effort to better understand the association of members of the IGF-1 family with breast cancer, we also analyzed IGFBP-3 and BP3-FR. The median NAF level of IGFBP-3 was twice as high in women with as in women without breast cancer (Table 1, Fig. 1B), a statistically significant difference. This degree of difference was similar in both pre- and postmenopausal women yet significance was lost when the data were separated, which may have been due to sample size. Consistently, in the logistic regression model the effect of IGFBP-3 was not different by the menopausal status. We were surprised not to observe an association between BP3-FR and breast cancer, given prior reports of its ability to inhibit the mitogenic function of IGF-1 [17]. We are currently investigating possible reasons for this.

Although serum PSA results were univariately associated with postmenopausal breast cancer, when we controlled for age, menopausal status, and age at menarche, no serum marker was significantly associated with the odds of a subject having cancer. We believe that serum results are less reliable than NAF because they reflect the contribution of a variety of bodily organs.

In our logistic regression models which considered a number of clinical covariates, higher levels of NAF PSA in premenopausal women improved our ability to predict whether or not they had breast cancer, whereas it did not in postmenopausal women. PSA was a powerful predictor in premenopausal women, more powerful than age, which has been shown to be highly associated with a woman's odds of having breast cancer [29]. The association of PSA with postmenopausal breast cancer was not significant in the logistic regression model. While we are not sure of the reason for this, our sample size may lack sufficient power to detect a difference, as there were only nine postmenopausal subjects without breast cancer in the model. When no information is available on NAF PSA, IGFBP-3 is also helpful in predicting a woman's odds of having breast cancer using logistic regression. In the presence of PSA, IGFBP-3 is no longer significantly associated with a woman's odds of having breast cancer.

In secondary statistical analysis we found that controlling for age, menopausal status, and age at menarche, the ratio of BP3-FR to PSA in NAF from premenopausal women was

significantly associated with breast cancer ($P = 0.004$) and the ratio of IGFBP-3 to PSA in NAF was of marginal statistical significance ($P = 0.083$). Both these results appear to reflect the significant effect of PSA and do not seem to provide additional insight above and beyond the effect of PSA.

We used CART analysis to determine cut points below or above which we could identify women likely to have or be free of breast cancer, and found that 92% of women in our sample with a PSA value (<65 ng/g) had breast cancer, while 100% of women younger than 60 with IGFBP-3 > 26 ng/mg had breast cancer. While we realize the limitations of our sample, the cut points provide a starting point from which to evaluate the association of these markers with breast cancer in a larger population.

Two recent reports evaluated the association of PSA in NAF with breast cancer. In both reports, the fraction of subjects with invasive breast cancer was 16% or less. In the first [30], the authors found that the mean concentration of PSA in NAF from women without breast cancer was significantly higher ($P < 0.001$) than in women with breast cancer. On the other hand, the second report [31] failed to observe a difference in NAF PSA in tumor-free breasts compared to specimens from subjects with recently diagnosed breast cancer. The second study was limited by the fact that only 29% of the subjects enrolled provided evaluable samples, introducing the potential for selection bias. Our results, collected in a large cohort of women with or without breast cancer and in which NAF was obtained in 98% of subjects enrolled, suggest that levels of PSA in NAF are inversely associated with breast cancer, confirming both our earlier findings and those of the first report above [30]. Further studies will help to determine the usefulness of NAF PSA to predict which women have or will develop breast cancer.

In summary, we extended our earlier findings that PSA levels in NAF are inversely associated with the presence of breast cancer, especially in premenopausal women. We found that NAF IGFBP-3, an important binding protein of IGF-1, is significantly higher in women with breast cancer. Serum PSA and BP3-FR were associated with postmenopausal breast cancer. Using logistic regression we determined that both NAF PSA and IGFBP-3 were helpful in identifying women with breast cancer, even controlling for clinical variables known to be associated with the disease. We also determined cut points which identified subjects at very high risk of having breast cancer, suggesting that NAF biomarkers can be evaluated and criteria established to identify women who have the disease. NAF PSA and IGFBP-3 may prove useful for breast cancer screening.

Acknowledgements

We wish to thank Dr. Constantine Daskalakis, member of the Biostatistics Section of the Kimmel Cancer Center at Thomas Jefferson University, for his helpful review of this manuscript.

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