Nipple Aspirate Fluid Proteome of Healthy Females and Patients with Breast Cancer

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BACKGROUND: The ductal/alveolar system of the female breast constantly secretes and reabsorbs fluid in non-pregnant/nonlactating women. This fluid, referred to as nipple aspirate fluid (NAF), can be obtained by a noninvasive procedure and it is part of the microenvironment where more than 95% of breast cancers arise.

METHODS: Using an Orbitrap® mass analyzer coupled to a linear ion trap, we performed an in-depth proteomic analysis of NAF samples obtained from 3 healthy individuals and 3 patients with breast cancer. Multiple fractionation methods such as size-exclusion and anion-exchange chromatography were applied for protein separation before mass spectrometric analysis.

RESULTS: We identified more than 800 unique proteins in total, generating the most extensive NAF proteome thus far. Using gene ontology, we classified the identified proteins by their subcellular localization and found that more than 50% were extracellular or plasma membrane proteins. By searching against the Plasma Proteome Database, we confirmed that 40% of the proteins were also found in the plasma. Unigene database searching for transcripts of the proteins not found in the plasma revealed that the vast majority were expressed in the mammary gland.

CONCLUSIONS: Our extensive proteome database for NAF may be helpful in the identification of novel cancer biomarkers.

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The female breast is a gland that after puberty, and throughout life, exhibits a constant secreting and absorptive activity (1). This activity is apparent in pregnant and lactating women but it usually remains unnoticed during the rest of a woman's life because the volume of the secreted fluid is small and the nipple is usually occluded by keratin plugs, which do not allow fluid release. In the mid 1950s, Papanicolaou and Fleming first cytologically studied breast secretions (2, 3); since then, the literature on nipple aspirate fluid (NAF)[6] and breast secretions has expanded considerably (4).

The ductal tree is embedded in a dense fibrous stroma of the breast and consists of 5–20 duct systems that originate on orifices of the nipple. They branch into smaller and smaller ducts, eventually giving rise to the lobule. The entire ductal/alveolar system is lined by 2 cell layers; an inner epithelium and an outer myoepithelium. The myoepithelial layer exhibits contractile-like properties and plays a role in both the support of the ductal/alveolar system and milk ejection, whereas the epithelial cells have secretory and absorptive functions.

The breast differs from other secretory glands in that the components of the nipple aspirate fluid are continuously secreted, metabolized, concentrated, and absorbed (5, 6). These components are synthesized by the epithelium and secreted into the duct or taken from plasma by breast cells and transported intracellularly to the ducts (7, 8). In view of the aforementioned characteristics of NAF and the fact that the vast majority of breast cancers (>95%) arise from the epithelial cells lining the ductal/alveolar system, proteomic analysis of NAF may reveal breast-specific proteins that are useful for understanding the physiology of breast cancer and may serve as potential breast cancer biomarkers.

Nipple aspiration is a noninvasive and low-cost procedure. During NAF collection a hand-held suction cup is placed over the nipple and with the help of microcapillary tubes is used to quickly obtain concentrated fluid droplets at nipple openings. The success of the technique varies from 39% to 66% (9, 10) (although it may be higher in specialized centers (4)), and the volume of the sample is always in the microliter

range. The variable success rate for sample collection and the low volume of sample obtained have been the main drawbacks for the clinical use of NAF. Certain factors may increase the likelihood of obtaining NAF, such as age range 35–50 years, earlier age of menarche, previous lactation history, and non-Asian ethnicity (10). Recently, Suijkerbuijk et al. increased the success of nipple aspiration to 94% with use of nasal oxytocin (11), a technique that opens new horizons for the use of NAF in diagnostics.

In the present study, we used an Orbitrap mass analyzer coupled to a linear ion trap to perform an in-depth proteomic analysis of NAF samples from healthy individuals and women with breast cancer. The Orbitrap is a mass analyzer that delivers high resolution and low parts-per-million mass accuracy. It is a modified ion trap in which the ions are trapped in an electrostatic flask. Accurate reading of mass-to-charge ratios of ions with different masses can be determined by the oscillation frequencies after a Fourier transformation.

By using tandem mass spectrometry (MS/MS) in combination with multidimensional chromatographic fractionation, we identified more than 800 unique proteins in NAF, the largest list of proteins reported for this fluid thus far.

Experimental Procedures

STUDY PARTICIPANTS

All women enrolled in the study provided written informed consent. Pregnant and lactating women were excluded. All women enrolled underwent biopsy for suspected breast cancer. Study participants were classified into 1 of 2 categories: "cancer" if there was biopsy-proven ductal carcinoma in situ or invasive breast cancer; or "normal" if cancer was not present on the biopsied breast tissue. NAF was collected and analyzed from only 1 breast (the pathological one for 2 patients and the normal one for 1 cancer patient). NAF samples were collected before diagnostic biopsy at the same clinic.

SPECIMEN COLLECTION

NAF samples were collected by use of a modified breast pump (12) into capillary tubes and stored at −80 °C until use. In total, we collected samples from 3 cancer and 3 normal patients. The samples were coded as NAF-C1, NAF-C2, NAF-C3 (cancer) and NAF-N1, NAF-N2, NAF-N3 (normal). The clinicopathological characteristics of the donors are summarized in Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol56/issue5.

SAMPLE PREPARATION

We extracted samples from the capillary tubes using sulfate/phosphate buffer (100 mmol/L NaH_2PO_4, and 100 mmol/L Na_2SO_4, pH 6.8) (10-fold dilution). All additional procedures were performed with these diluted NAF solutions. The total protein of diluted NAF varied between 2.9 and 4.4 g/L. For normalization purposes, all subsequent steps used 1 mg of total protein for all the samples.

TOTAL PROTEIN ASSAY

Total protein of each diluted NAF sample was quantified by using a Coomassie (Bradford) protein assay reagent (Pierce).

ACETONE PRECIPITATION

We added 6 volumes of cold acetone and inverted the tubes 3 times. The samples were incubated at −20 °C for 3 h to precipitate the proteins. The acetone was removed by centrifugation and the pellet was reconstituted in 8 mol/L urea.

GEL FILTRATION

We performed gel filtration using a TSK-Gel G3000SW column (0.75 × 60 cm) (Tosoh Bioscience) attached to an Agilent 1100 HPLC system. The column was equilibrated with phosphate/sulfate buffer (100 mmol/L NaH_2PO_4, 100 mmol/L Na_2SO_4, pH 6.8). A 1-h fractionation protocol was performed at a flow rate of 0.5 mL/min, collecting eluted fractions at 1-min intervals. The eluate was monitored at a wavelength of 280 nm. The fractions were pooled according to the chromatogram in 4 pools of approximately similar protein content and lyophilized to dryness. Before each run, a protein gel filtration standard (Bio-Rad) was injected to evaluate column performance.

BUFFER EXCHANGE USING NAP5 COLUMNS

For buffer exchange using NAP5 columns (GE Healthcare), we followed the manufacture's instructions.

STRONG ANION EXCHANGE–LIQUID CHROMATOGRAPHY

Strong anion exchange–liquid chromatography (LC) was performed using a Mono Q 4.6/100 PE high-performance column (GE Healthcare) connected to an Agilent 1100 HPLC system. The column was equilibrated sequentially with 20 mmol/L Tris-HCl (pH 8.3). 20 mmol/L Tris-HCl + 1.0 mol/L NaCl (pH 8.0), and 20 mmol/L Tris-HCl (pH 8.3), with 5 column volumes each. A 1-h fractionation protocol was performed at a flow rate of 2 mL/min, with a linear 1 mol/L NaCl gradient (0%–60%), collecting eluted fractions at 1-min intervals. The eluate was monitored at a wavelength of 280 nm. Before each run, to evaluate column perfor-

mance, we injected a protein anion exchange standard (Bio-Rad).

TRYPSIN DIGESTION

The lyophilized proteins were denatured with 8 mol/L urea and reduced with dithiothreitol (final concentration, 13 mmol/L; Sigma). After reduction, the samples were alkylated with iodoacetamide (final concentration, 125 mmol/l; Sigma) and desalted using a NAP5 column (GE Healthcare). The samples were lyophilized and resuspended in trypsin buffer (trypsin/protein ratio 1:50; 120 μL of 50 mmol/L ammonium bicarbonate, 100 μL of methanol, 150 μL of water) overnight at 37 °C.

STRONG CATION-EXCHANGE–LIQUID CHROMATOGRAPHY

The samples were directly loaded onto a PolySULFO-ETHYL A™ column (The Nest Group), containing a hydrophilic, anionic polymer (poly-2-sulfoethyl aspartamide) with a pore size of 200-Å and a diameter of 5 μm. A 1-h fractionation method was performed using an Agilent 1100 HPLC system at a flow rate of 200 μL/min. A linear gradient of mobile phase B (0.26 mol/L formic acid in 10% acetonitrile and 1 mol/L ammonium formate) was applied and the eluate was monitored at 280 nm. Twelve fractions were collected every 5 min. Before each run, a protein cation-exchange standard (Bio-Rad) was applied to evaluate column performance.

MASS SPECTROMETRY

The fractions were desalted by using a ZipTip C_{18} pipette tip (Millipore) and eluted in 5 μL of buffer (64.5% acetonitrile, 35.4% water, 0.1% formic acid, and 0.02% trifluoroacetic acid). We added 80 μL of buffer (95% water, 0.1% formic acid, 5% acetonitrile, and 0.02% trifluoroacetic acid) to each sample and loaded 40 μL from a 96-well microplate autosampler onto a 2-cm C_{18} trap column, packed with Varian Pursuit (5 μm C_{18}), using the EASY-nLC system (Proxeon Biosystems). Peptides were eluted from the trap column onto a resolving 5-cm analytical C_{18} column packed with Varian Pursuit (3 μm C_{18}) with an 8-μm tip (New Objective). This LC setup was coupled online to an LTQ-Orbitrap XL (Thermo Fisher Scientific) mass spectrometer using a nanoelectrospray ionization source (Proxeon Biosystems). Each fraction underwent a 54-min gradient, and eluted peptides were subjected to 1 full MS scan (450–1450 m/z) in the Orbitrap at 60 000 resolution, followed by top 6 data-dependent MS/MS scans in the linear ion trap (LTQ Orbitrap). With the use of charge-state screening and preview mode, unassigned charge states as well as charges 1+ and 4+ were ignored.

DATA ANALYSIS

Data files were created by use of Mascot Daemon (version 2.2.0) and extract_msn. The resulting mass spectra from each fraction were analyzed using Mascot (Matrix Science; version 2.2) and X!Tandem (Global Proteome Machine Manager, version 2006.06.01) search engines on the nonredundant International Protein Index human database (version 3.46, 144 158 protein sequences), which included the forward and reversed sequences for calculating false-positive rates. False-positive rates were calculated as number of proteins identified by searching the reverse sequences ($\times$2) divided by the total number of identified proteins. The resulting Mascot and X!Tandem search result files were loaded into Scaffold (version 2.0, Proteome Software) to cross-validate Mascot and X!Tandem data files. We used a normalized spectral counting method for spectral counting analysis performed using Scaffold. Spectrum reports were exported from Scaffold and uploaded into an in-house program for additional data analysis.

KALLIKREIN 10 ELISA IMMUNOASSAY

The concentration of the kallikrein 10 (KLK10) in NAF samples was measured by a sandwich-type immunoassay as previously described (13).

Results

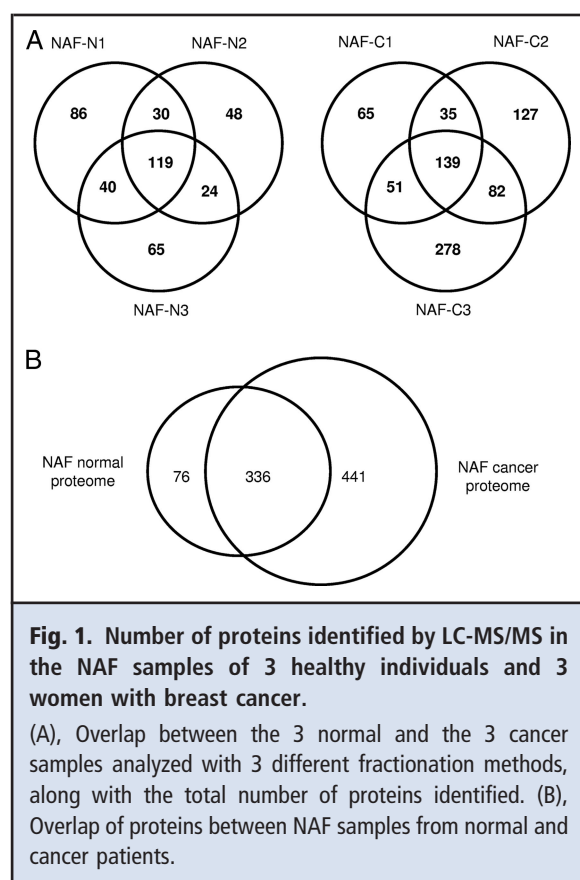### IDENTIFICATION OF PROTEINS BY MS–GEL FILTRATION

After the sample preparation method outlined in Methods, tryptic peptides from samples NAF-N1 and NAF-C1 were analyzed by MS. We identified 1520 and 1902 peptides in NAF-C1 and NAF-N1, respectively, which correspond to 303 and 290 nonredundant proteins, with a false-positive rates of 0.4% and 0.7% (see online Supplementary Tables 2 and 3).

IDENTIFICATION OF PROTEINS BY MS–ACETONE PRECIPITATION

NAF-N2 and NAF-C2 were treated with acetone to precipitate proteins. The pellets were reconstituted in urea, and samples were subjected to trypsin digestion and strong cation-exchange chromatography. Proteomic analysis revealed 1234 and 1807 peptides in NAF-N2 and NAF-C2, respectively, which correspond to 231 and 389 proteins. The false-positive rate was 0.5% for both samples (see online Supplementary Tables 4 and 5).

IDENTIFICATION OF PROTEINS BY MS–ANION-EXCHANGE CHROMATOGRAPHY

Samples NAF-N3 and NAF-C3 were desalted with NAP5 columns and subjected to anion-exchange chromatography. After trypsin digestion of pooled fractions, strong cation-exchange chromatography, and MS analysis, 1427 and 2707 peptides were identified in

Fig. 1. Number of proteins identified by LC-MS/MS in the NAF samples of 3 healthy individuals and 3 women with breast cancer.

(A), Overlap between the 3 normal and the 3 cancer samples analyzed with 3 different fractionation methods, along with the total number of proteins identified. (B), Overlap of proteins between NAF samples from normal and cancer patients.

NAF-N3 and NAF-C3, respectively, corresponding to 264 and 559 proteins, with false-positive rates of 0.7% and 0.4% (see online Supplementary Tables 6 and 7).

IDENTIFICATION OF HUMAN KLK10 BY ELISA
KLK10 was identified by MS in NAF-C3. Online Supplementary Fig. 1 illustrates the sequence of the protein along with the peptides identified. Enzyme immunoassay confirmed that KLK10 was present at a concentration of 10 μg/L.

NAF PROTEOMES OF NORMAL AND CANCER PATIENTS
We compiled the data with NAFs from normal and cancer patients. Fig. 1A shows the overlap of proteins identified by the 3 different fractionation methods. In total, 412 proteins in NAF from healthy individuals and 777 proteins in NAF from cancer patients were identified. The lists of proteins along with the number of peptides identified are shown in online Supplementary Tables 8 and 9. The overlap of proteins between NAF samples from normal and cancer patients is shown in Fig. 1B and online Supplementary Table 10. Online Supplementary Table 11 represents a NAF proteome database of 896 proteins, 854 of which were

identified in this study and 42 in other studies. Approximately half of the proteins in our study were identified with greater than or equal to 2 peptide hits, and these proteins are depicted in online Supplementary Table 12. Online Supplementary Table 13 displays the number of normalized spectra of the 2 groups (normal and cancer) along with the fold change of the relative abundance of proteins identified in the cancer group compared with the normal group, as calculated by using Scaffold.

CELLULAR LOCALIZATION OF IDENTIFIED PROTEINS
The cellular localization of identified proteins based on gene ontology is shown in online Supplementary Fig. 2. These categories for cellular localization are nonexclusive (i.e., a number of candidates were found to be localized in more than one cellular compartment). More than 50% of all proteins were classified as extracellular and/or membrane bound.

OVERLAP WITH PLASMA PROTEOME
The NAF proteome from this study was compared with the Plasma Proteome Database (http://plasmaproteome database.org). A total of 330 proteins have also been identified in plasma (see online Supplementary Table 11). For the proteins not identified in the plasma
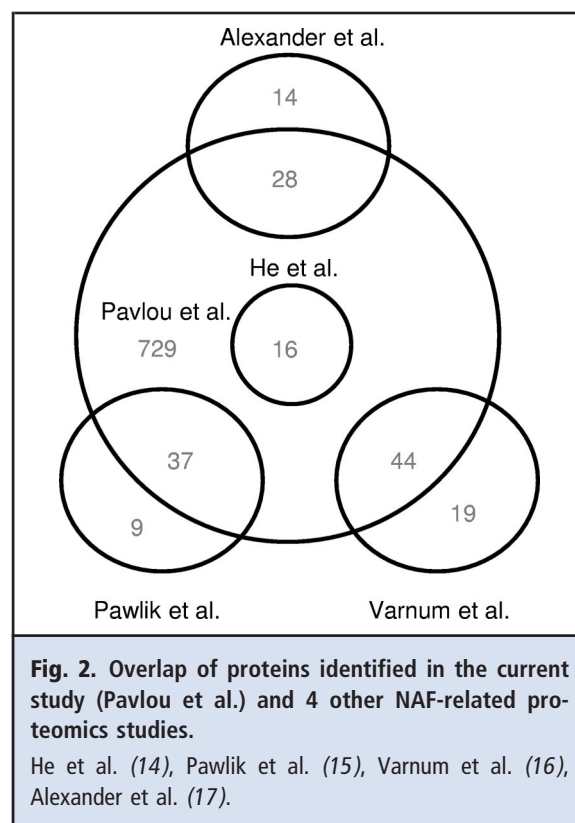

Fig. 2. Overlap of proteins identified in the current study (Pavlou et al.) and 4 other NAF-related proteomics studies.
He et al. (14), Pawlik et al. (15), Varnum et al. (16), Alexander et al. (17).

Table 1. Currently available biomarkers for breast cancer.		
Cancer marker	**Proposed uses**	**References**
Cancer antigen (CA) 15.3[a]	Monitoring therapy in advanced stages and postoperative surveillance in patients with no evidence of the disease.	Duffy (24)
Carcinoembryonic antigen (CEA)	Monitoring therapy in advanced stages and postoperative surveillance in patients with no evidence of the disease. (when CA 15.3 is not elevated).	Duffy (24)
Tissue plasminogen activator[a]	Monitoring therapy in advanced stages and postoperative surveillance in patients with no evidence of the disease (when CA 15.3 and CEA are not elevated).	Nicolini et al. (25), Soletormos et al. (26)
Urokinase-type plasminogen activator (uPA)[a]	Determining prognosis.	Duffy (27), Look et al. (28), Janicke et al. (29)
Plasminogen activator inhibitor-1	Determining prognosis (in combination with uPA).	Duffy (27)
Cathepsin-D[a]	Determining prognosis.	Foekens et al. (30)
HER-2/neu[b]	Determining prognosis and selecting patients for treatment with trastuzumab (herceptin).	Ross et al. (31), Slamon et al. (32)
Estrogen receptor	Predicting response in hormonal therapy.	Duffy (37)
Progesterone receptor	Predicting response in hormonal therapy (in combination with estrogen receptor).	Ravdin et al. (38)

[a] Proteins identified in this study.
[b] HER-2/neu, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog.

protein database, we performed Unigene analysis to identify genes that were breast specific (see online Supplementary Table 14). Immunoglobulins and hypothetical and putative uncharacterized proteins were excluded from this list.

COMPARISON OF THE IDENTIFIED PROTEINS WITH OTHER REPORTED PROTEINS

He et al. analyzed NAF samples from healthy individuals and women with breast cancer by using SELDI-TOF-MS (14). These investigators identified 16 proteins that differed between samples from cancer patients and individuals. All 16 of these proteins were found in our study. Using isotope-coded affinity-tag technology, Pawlik et al. also identified proteins differentially expressed in healthy women compared with women with breast cancer (15); 37 of 46 proteins Pawlik et al. identified were also found in our study. Varnum et al. performed proteomic characterization of NAF samples and identified 63 proteins (16), which had a 70% overlap with our data (44 of 63 proteins were in common). Finally, Alexander et al. coupled 2-dimensional polyacrylamide gel electrophoresis MALDI-TOF-MS to analyze a single NAF sample from a healthy individual, and they identified 42 proteins (17); 28 of them were also found in our study. Fig. 2 summarizes these comparisons.

Discussion

The composition of distal fluids is believed to mirror the entirety of tissues comprising an organism. It is known that changes in protein concentrations in blood, the ultimate distal fluid, reflect changes in physiological or pathological conditions (18). Plasma, however, contains many proteins, which can be present in concentrations that differ by more than 10 orders of magnitude. This high complexity makes plasma a less attractive source for the identification of biomarkers. Biological fluids such as NAF are still complex in terms of protein composition but they are organ specific; therefore their use in the discovery phase of biomarkers seems more appealing.

Proteomic analysis of NAF is challenging, owing to the presence of highly abundant proteins such as albumin and immunoglobulins (19). To reduce sample complexity and to increase the coverage of the NAF proteomes, we applied 3 different fractionation methods. The results were complementary, allowing the compilation of an extensive list including more than 800 NAF proteins. The relatively modest overlap of proteins identified by the 3 methods (approximately 20%–30%) could be explained in several ways. First, the different fractionation methods before MS analysis likely led to identification of different classes of proteins. Second, the samples were obtained from differ-

ent individuals, hence giving rise to interindividual variability. Two previous studies have highlighted the large variations of proteomic profiles of samples from different individuals *(20, 21)*. However, the more individuals included in such an analysis, the more complete the picture of the NAF proteome will be. Finally, although the LTQ Orbitrap is highly sensitive, many low-abundance proteins may remain undetected in large-scale proteomic analyzes, resulting in undersampling of the proteome.

In our study of the NAF proteome of breast cancer patients, we analyzed NAF obtained from the cancer-bearing breast in 2 patients with breast cancer and from the cancer-free breast in the remaining breast cancer patient. Although this sampling method (use of ipsilateral and contralateral breast NAF samples) may seem to be a limitation of our study, we noted that the levels of high-abundance proteins such as albumin were not significantly different among all 6 samples analyzed. Moreover, low-abundance cancer biomarkers were also found in the NAF of the contralateral breast suggesting that use of contralateral breast samples may be useful in identification of potential tumor biomarkers. Nevertheless, the limited number of samples we examined precludes meaningful comparisons of NAF proteomes between cancer-bearing and contralateral breast specimens.

The proteins found in NAF are either secreted by the epithelial cells that line the alveolar/ductal system or they circulate in blood and reach the ducts by diffusion *(7, 8)*. In accord with that observation, 40% of the NAF proteins have also been identified in the plasma of healthy individuals. Also, according to gene ontology, more than 50% of the proteins were classified as extracellular or bound to the plasma membrane, increasing the possibility that these proteins could enter the circulation. Moreover, we could assume that NAF proteins produced intraductally are breast-specific. Unigene analysis revealed that for the vast majority of these proteins (87%), mRNA transcripts have been detected in the mammary gland.

Because we assume that NAF is a promising source for breast-cancer–specific biomarkers, we were interested to see whether any of the established biomarkers was identified in our study. Table 1 depicts the currently available breast cancer biomarkers and indicates the proteins identified in our study. Table 2 shows the number and the sequence of the identified peptides that correspond to the proteins highlighted in Table 1. The percentage of sequence coverage for each of these proteins is also included.

Although interest in NAF has increased recently, only a few reports include lists of identified proteins *(14–17)*. Given that SELDI-TOF requires only 1 μL of sample and has high throughput *(12)*, it is not surpris-

Table 2. Number and sequences of identified peptides corresponding to the known breast cancer biomarkers found in this study.
Cancer marker with sequences of identified peptides
Urokinase-type plasminogen activator (11[a])
ALDPAGNESAYPPNGVECYSCVGLSR
CAPGVDVCTEAVGAVETIHGQFSLAVR
CNEGPILELENLPQNGR
DGVTGPGFTLSGSCCQGSR
GCGSGLPGKNDR
GCVQDEFCTR
GLDLHGLLAFIQLQQCAQDR
QGVEHEASRDEEPR
SDALQLGLGK
SPEEQCLDVVTHWIQEGEEGRPK
TDSCQGDSGGPLVCSLQGR
Cathepsin-D (14[a])
AIGAVPLIQGEYMIPCEK
AYWQVHLDQVEVASGLTLCK
DPDAQPGGELMLGGTDSK
EGCEAIVDTGTSLMVGPVDEVR
FDGILGMAYPR
ISVNNVLPVFDNLMQQK
KAYWQVHLDQVEVASGLTLCK
LLDIACWIHHK
LVDQNIFSFYLSR
QPGITFIAAK
TMSEVGGSVEDLIAK
VGFAEAARL
YSQAVPAVTEGIPEVLK
YYTVFDRDNNR
Cancer antigen 15.3 (5[a])
DISEMFLQIYK
DTYHPMSEYPTYHTHGR
EGTINVHDVETQFNQYK
NYGQLDIFPAR
QGGFLGLSNIK
Tissue plasminogen activator (7[a])
ATCYEDQGISYR
GGLFADIASHPWQAAIFAK
GTHSLTESGASCLPWNSMILIGK
LGLGNHNYCR
LLEIDSVGSEDSLDTSLR
VTNYLDWIR
VYTAQNPSAQALGLGK
[a] The number of unique and nonredundant, identified peptides from the analysis of all 6 NAF samples.

ing that a high number of NAF studies use that method. However, SELDI provides proteomic profiles rather than actual identification of proteins. By comparing the proteomic profiles of NAF samples from cancer-bearing breasts, contralateral breasts of women with unilateral cancer and samples from healthy individuals, Noble et al. identified 9 statistically significant peaks between healthy individuals and patients with breast cancer and 7 discriminatory peaks between the contralateral breasts and healthy individuals *(22)*. When Pawlik et al. compared pooled SELDI spectra from 23 women with early stage breast cancer with spectra of NAF samples from healthy individuals, they observed 24 differential peaks *(23)*. Following a similar approach, Paweletz et al. generated the protein profiles of NAF samples from 12 women with breast cancer and 15 without, and detected a signature of 2 peptides found specifically in the tumor samples and 2 peptides unique to the normal ones *(21)*. In a different study, Li et al. were able to generate reproducible protein profiles from 10 individuals (5 healthy individuals and 5 patients with breast cancer) starting with just 1 μg of total protein and ending up with 3 discriminating peaks between the 2 conditions *(33)*. It is worth mentioning that the results of the above studies do not overlap, raising concerns about the reproducibility and the reliability of their method *(34–36)*.

Our goal was to perform an in-depth proteomic analysis of NAF to shed light on the protein content of the microenvironment of normal and cancerous breast epithelium. We compiled a list of 896 proteins, which could be a valuable source for new breast cancer biomarkers (see online Supplementary Table 11). The vast majority of these proteins (n = 729) were identified in this study for the first time. The focus of the study was the qualitative analysis of NAF proteome. It is worth mentioning that the inability to detect a protein in a specimen does not necessarily mean that the protein is absent; it may be possible to attribute lack of detection to the fact that the concentration of this component in the specimen is below the analytical measuring range of the instrument. Therefore, concluding that proteins uniquely identified in the cancer samples are unique in cancer and vice versa may be misleading.

Studying the differential expression of proteins between 2 conditions (e.g., normal and cancer) is an interesting approach for the discovery of components that are involved in disease development. Spectral counting constitutes a label-free method for quantifying relative changes in protein abundance in MS-based proteomic analyses, and we have performed such an analysis for our study. However, there are a number of different ways to analyze a data set and currently there is no consensus as to which approach to use.

Our future directions include collection and analysis of larger numbers of NAF samples from patients with different subtypes of breast cancer along with samples from individuals without breast cancer. Quantitative comparisons of such proteins may lead to a better understanding of the mechanisms underlying breast cancer.

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