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Research Article

Proteomics analysis of human breast milk to assess breast cancer risk

Detection of breast cancer (BC) in young women is challenging because mammography, the most common tool for detecting BC, is not effective on the dense breast tissue characteristic of young women. In addition to the limited means for detecting their BC, young women face a transient increased risk of pregnancy-associated BC. As a consequence, reproductively active women could benefit significantly from a tool that provides them with accurate risk assessment and early detection of BC. One potential method for detection of BC is biochemical monitoring of proteins and other molecules in bodily fluids such as serum, nipple aspirate, ductal lavage, tear, urine, saliva and breast milk. Of all these fluids, only breast milk provides access to a large volume of breast tissue, in the form of exfoliated epithelial cells, and to the local breast environment, in the form of molecules in the milk. Thus, analysis of breast milk is a non-invasive method with significant potential for assessing BC risk. Here we analyzed human breast milk by mass spectrometry (MS)-based proteomics to build a biomarker signature for early detection of BC. Ten milk samples from eight women provided five paired-groups (cancer versus control) for analysis of dysregulatedproteins: two *within* woman comparisons (milk from a diseased breast versus a healthy breast of the same woman) and three *across* women comparisons (milk from a woman with cancer versus a woman without cancer). Despite a wide range in the time between milk donation and cancer diagnosis (cancer diagnosis occurred from 1 month *before* to 24 months *after* milk donation), the levels of some proteins differed significantly between cancer and control in several of the five comparison groups. These pilot data are supportive of the idea that molecular analysis of breast milk will identify proteins informative for early detection and accurate assessment of BC risk, and warrant further research. Data are available via ProteomeXchange with identifier PXD007066.

Keywords:
Biomarkers / Breast cancer / Breast milk / Mass spectrometry / Proteomics
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1 Introduction

BC is the second most common type of cancer, exceeded only by skin cancers, and the second leading cause of cancer death, exceeded only by lung cancer, among American women [1]. These statistics make BC a major focus of research. BC in young, reproductively active women is a particularly challenging disease for several reasons. First, breast tissue is denser in young women making mammography, the most frequent method of early detection, ineffective in young women [2]. Second, the relationship between full-term pregnancy and BC risk is complicated. While a woman's ***lifetime risk*** of developing BC ***decreases with each full-term pregnancy***, she experiences a ***transient increased*** risk of developing BC, known as pregnancy-associated BC, which is thought to last ***up to five***

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Abbreviations: **BC**, breast cancer; **DTT**, Dithiothreitol; **ELISA**, enzyme-linked immunosorbent assay; **GO**, gene ontology; **IAA**, iodoacetamide; **MS**, mass spectrometry; **MS/MS**, tandem mass spectrometry; **nanoLC-MS/MS**, nanoliquid chromatography tandem mass spectrometry; **NCBI**, National Center for Biotechnology Information; **pkl**, peak list; **PLGS**, ProteinLynx Global Server; **RT-PCR**, Reverse transcription polymerase chain reaction; **SDS-PAGE**, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Color Online: See the article online to view Fig. 1–4 in color.

years after giving birth [3]. Finally, because BC risk increases with age, a woman's risk of developing pregnancy-associated BC increases with age at childbirth. At present there are no tools for accurately assessing BC risk in young women. Given that early detection is key to reducing BC related deaths [1], it is important to discover biomarkers for the disease.

A biomarker is a molecule, such as a protein, detected in bodily fluids, that is representative of a specific status, process, abnormality, or disease. For instance, alterations in protein levels (upregulation or downregulation of proteins) can be considered as a marker for BC diagnosis. Different types of bodily fluids have been analyzed for biomarkers of BC, such as blood/plasma/serum [4–9], nipple aspirate fluid and ductal lavage fluid [10–14], tears [15, 16], urine [17–20], saliva [21–24], and breast milk [25–28].

Human breast milk has been proposed as a suitable bodily fluid for discovering biomarkers useful in early detection of BC, as well as for assessing future risk [2, 3, 26, 28–32]. Breast milk contains secreted proteins, immune cells and exfoliated epithelial cells originating throughout the organ. Milk can be collected non-invasively, from each breast, and is available at a critical time-period in the breast development of premenopausal women [32]. Aberrant protein levels in milk may represent secreted proteins from precancerous/cancer epithelial cells or a response of the immune cells and local environment (e.g., stroma cells) to the presence of disease.

Among the different analytical methods currently used for protein identification and characterization, as well as for determination of the alterations in the levels of proteins, MS based proteomics is one of the most accurate methods and therefore ideal for the analysis of various breast milk samples [33–36]. MS has the potential not only for identification and quantitation of proteins (canonical proteins), but also of their isoforms and variants and modified proteins (protein species) that resulted from the differential expression of genes (alternative splicing), RNA editing, or post-translational modifications (PTMs) of proteins such as truncation, phosphorylation, acetylation or glycosylation [37–39].

Here, we fractionated the proteins in breast milk based on their molecular weight using gel electrophoresis (SDS-PAGE), then digested the protein bands with trypsin, analyzed them by nanoLC-MS/MS, and conducted bioinformatics analysis. In this pilot study, we analyzed a total of ten milk samples from eight women. The ten milk samples provided five pairs of "BC versus control" comparisons as follows: two women who had cancer in one breast donated milk from each breast for *within woman comparisons*, and three women with BC and three women without BC provided a sample of milk mixed from their left and right breasts for three *across women comparisons*. The time between milk donation and diagnosis of BC ranged greatly with diagnosis occurring from 1 month before to 24 months after the milk donation. Despite this wide, several proteins were dysregulated between the cancer and control milk samples in multiple paired comparisons. For instance, proteins from the human chorionic gonadotropin (hCG) family, lipoproteins and tenascin C (hexabrachion) isoform CRA_a are among the upregulated proteins in cancer milk samples, and members from the casein family, xanthine oxidoreductase, human bile salt and mannose receptor are among the downregulated proteins in the cancer milk samples. These preliminary results are encouraging and support the suggestion that proteins in breast milk may serve as biomarkers of BC risk.

2 Materials and methods

2.1 Human subjects and milk samples

Ten breast milk samples from an archived bank at the University of Massachusetts Amherst (UMass-Amherst) were selected for proteomics analysis. The parent study, approved by the UMass-Amherst Institutional Review Board, included secondary analyses at collaborating laboratories. Subject enrollment and collection of breast milk samples have been described previously [26, 28]. Briefly, fresh and/or frozen milk samples were collected along with demographic data from women residing across the United States. Targeted recruitment of women who had a previous biopsy, together with annual follow-up gathering information on breast health, resulted in a subset of women who either had, or developed BC. Breast biopsy reports were obtained and diagnoses were extracted. Eight women, five diagnosed with BC and three with no cancer diagnoses, provided a total of ten breast milk samples. Two women diagnosed with cancer provided a milk sample from both the breast with cancer and the cancer-free breast. The remaining three women diagnosed with BC provided a sample containing a mixture of the milk from the diseased and disease-free breasts. Finally, samples representing a mixture of milk from the left and right breasts were selected from three cancer-free women. Subject demographics are shown in Table 1.

Of the five women with BC, three were diagnosed after they donated milk (cancer diagnosis was between 6 and 24 months after milk collection), and two women were diagnosed before they donated milk (cancer diagnosis was between one and two months before milk collection). Importantly, only samples 1 through four are each from a single breast. Samples 5 through ten are mixtures of milk from the left and right breasts.

2.2 Reagents and proteomics analysis

Unless otherwise stated, reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO). An overview of the MS-based proteomic analysis of milk samples and data processing is shown in Supporting Information Fig. 1. Briefly, protein concentration was determined using the Bradford assay and for each milk sample 400 μg of protein were separated using 11% SDS-PAGE. Coomassie Blue stained gel bands were then excised and subjected to in-gel trypsin digestion. An overview of the in-gel trypsin digestion protocol is shown in Supporting Information Fig. 2.

Table 1. Subject demographics and sample characteristics

Age (years)	Age at first birth	Number of live births	Baby's age (days)	BC diagnosis	Family history of BC	Milk sample ID[a)]	Timing of cancer diagnosis
35	33	2	180	DCIS[b)]	yes	**1**[a)]	24 months **after** milk donation
						2	**NA**
35	31	3	21	Invasive carcinoma	yes	**3**[a)]	2 months **before** milk donation
						4	**NA**
33	31	2	90	Invasive & DCIS	no	**5**[a)]	22 months **after** milk donation
38	37	1	210	no	yes	**6**	**NA**
28	28	1	480	Invasive ductal carcinoma	yes	**7**[a)]	1 month **before** milk donation
24	23	1	270	no	no	**8**	**NA**
38	19	2	180	Invasive & DCIS	no	**9**[a)]	6 months **after** milk donation
29	28	1	210	no	no	**10**	**NA**

a) Indicates that the milk came from a breast (samples 1 and 3) or a woman (samples 5, 7, and 9) with BC
b) DCIS = ductal carcinoma in situ

Briefly, the gel bands were cut into small pieces, followed by washing, reduction of disulfide bonds using dithiothreitol, and subsequent alkylation of the reduced cysteine residues using iodoacetamide. The gel pieces were then subjected to overnight in-gel trypsin digestion and the resulting peptides were extracted and cleaned using Zip-Tip reversed phase chromatography (C18 Ziptip™; Millipore, Billerica, MA) to remove possible contaminations. The peptide mixture was then solubilized and analyzed by nanoliquid chromatography tandem mass spectrometry (nanoLC-MS/MS) using a nanoACQUITY® UPLC coupled with a QTOF Ultima API mass spectrometer (Waters, Milford, MA), as previously described [40]. The resulting raw data files were processed and converted to pkl (peak list) files using ProteinLynx Global Server (PLGS version 2.4, Waters) software as previously described [41] using the following parameters: polynomial order 5 with a threshold of 35% for background subtraction, smoothing of two, with a window of three channels and Savitzky-Golay as smoothing type and top 80% of peaks considered for centroid calculation along with four channels as a minimum peak width at half height. A database search was performed on the pkl files to identify proteins using in-house Mascot server (www.matrixscience.com, Matrix Science, London, UK, version 2.5.1). The MASCOT parameters used for database search, were as follows: National Center for Biotechnology Information (NCBI), NCBI_20150706 (69 146 588 sequences; 2 4782 014 966 residues) database, selected for homo sapiens (human) (312 165 sequences), trypsin enzyme, fixed modification = cysteine carbamidomethyl, variable modifications = lysine acetylation, methionine oxidation, serine, threonine and tyrosine phosphorylation, peptide mass tolerance of ±1.3 Da (one ^{13}C isotope), fragment mass tolerance of ±0.8 Da, maximum missed cleavages = 1. The outcome files from Mascot were submitted to the Scaffold software (Scaffold version 4.2.1, Proteome Software Inc., Portland, OR) for quantitation and statistical analysis, as well as validation of MS/MS based peptide and protein identification. Peptide threshold of 20% probability was considered as the acceptable identification by the Scaffold Local FDR algorithm. Protein threshold of 90% probability with minimum two peptides identified was considered as acceptable criteria for protein identification using Protein Prophet algorithm [42]. Protein annotation was done with the gene ontology (GO) terms from NCBI (downloaded March 21 and 22, 2016) [43]. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [44–47] partner repository with the dataset identifier PXD007066.

2.3 Statistical analysis

Fisher's exact test was applied to compare the relative abundance between two samples (BC and control). The *p*-value calculation by Fisher's exact test is described elsewhere [48]. Comparisons with p-value <0.05 were considered statistically significant.

2.4 Data sharing

MS and MS/MS raw data files, the Scaffold file, and MASCOT database search HTML files are available upon request, according to Clarkson University' Material Transfer Agreement.

3 Results and discussion

The image of the SDS-PAGE separation of proteins for each of the ten milk samples demonstrates that some differences in protein pattern can be detected directly by visual inspection (Fig. 1). The most divergent sample, # 3, is from the milk of a woman diagnosed with BC just 2 months prior to collection of the milk. It is highly likely that this pattern reflects primarily proteins involved in wound healing related to the biopsy. Interestingly, however, sample # 7, is similarly from a woman diagnosed with cancer just one month before donating her milk sample, yet by visual inspection sample 7 diverges little from the others. One explanation for the discrepancy between samples 3 and 7 may be that sample 3 is milk collected solely

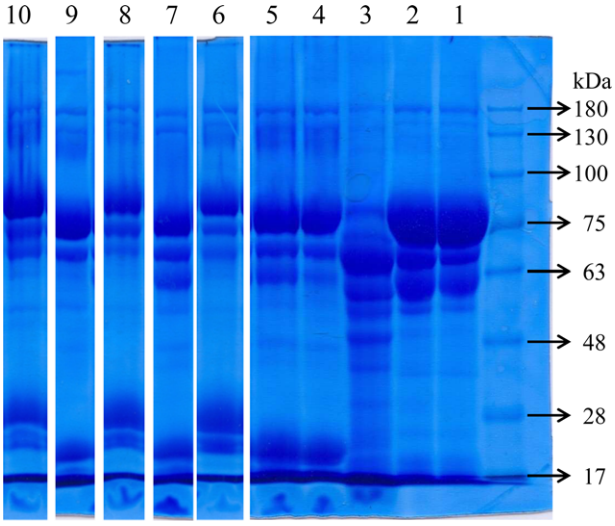


Figure 1. SDS-PAGE of milk samples. 400 μg of protein was separated in each lane. The lanes were cropped in order to have each paired comparison next to each other.

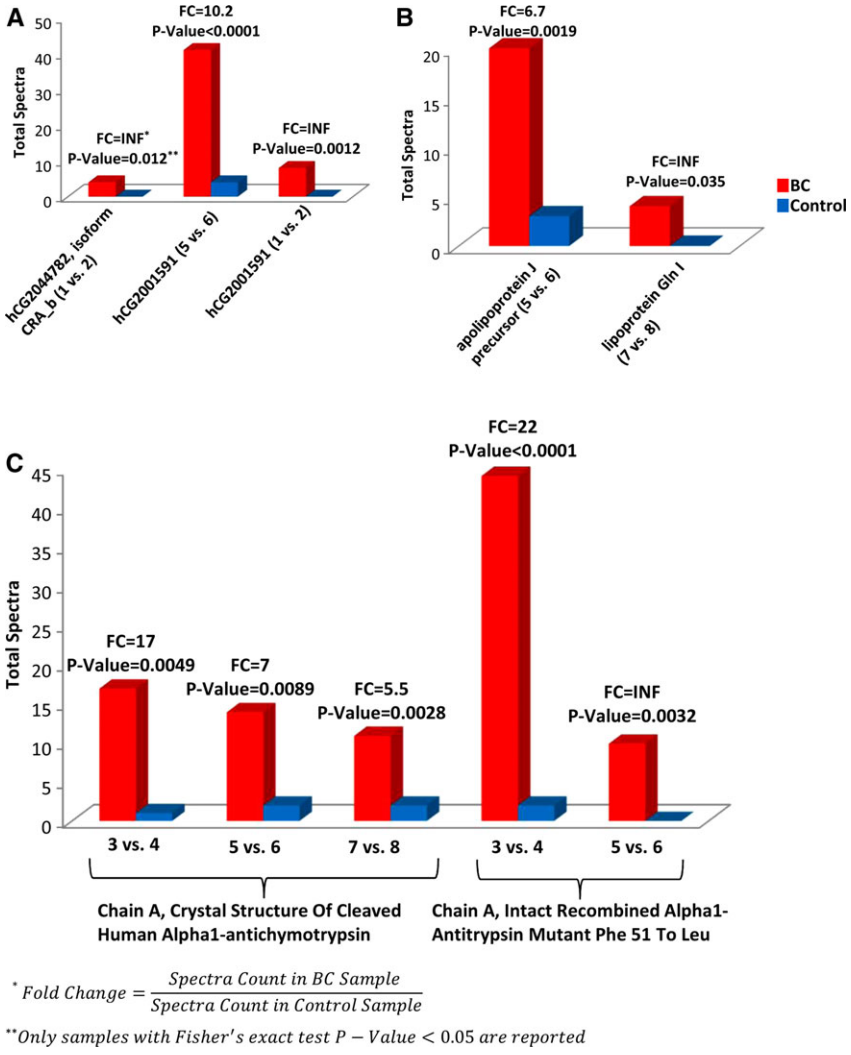
from the breast with cancer, whereas sample 7 represents a mixture of the diseased and disease-free breast, possibly resulting in a dilution of proteins related to the biopsy and wound healing.

To assess the extent to which proteins known to be associated with BC can be detected in breast milk, samples from each lane were cut into small bands, digested with trypsin and analyzed by nanoLC-MS/MS. Protein classification based on cellular component using Scaffold™ software demonstrated that both intra- and extracellular proteins were detected in cancer and control milk samples (data not shown). Given the small sample size, we conducted five ***paired*** comparisons between cancer and non-cancer samples. NanoLC-MS/MS analysis for each pair of samples was done at the same timeframe to avoid any instrumentational inconsistency and eliminate operational inaccuracies. For the *within* woman comparisons, milk from the diseased and healthy breasts (control) from the same woman provided two pairs as shown in Table 1: sample 1 (BC) vs. sample 2 (control from the same woman), and sample 3 (BC) versus sample 4 (control from the same woman). The remaining randomly assigned pairs were *across* women comparisons: a milk sample (mixed from the left and right breasts) from a woman diagnosed with cancer and a milk sample (mixed from the left and right breasts) from a women without a diagnosis of BC. As shown in Table 1, there were three randomly assigned across women pairs: sample 5 (BC) versus sample 6 (control from a different woman), sample 7 (BC) versus sample 8 (control from a different woman), and sample 9 (BC) versus sample 10 (control from a different woman). Dysregulated proteins were categorized into two groups: (i) proteins that were upregulated or downregulated in more than one paired comparison, and (ii) proteins that were upregulated or downregulated in only one of the 5 paired comparisons. We first discuss the proteins that differed between cancer and control in more than one of the paired comparisons.

3.1 Proteins dysregulated in more than one paired comparison between cancer and control milk samples

3.1.1 Proteins upregulated in more than one paired comparison

Numerous proteins were upregulated in the cancer milk sample as compared to its matched control in more than one of the five paired comparisons. Figure 2 shows the statistically significant (p-value < 0.05) upregulated proteins that have been previously associated with cancer and tumor development. Among the proteins upregulated in multiple paired comparisons of BC and control milk samples are proteins from the human chorionic gonadotropin (hCG) family, proteins from lipoproteins family, and proteins from Alpha1-antichymotrypsin and Alpha1-antitrypsin family (Fig. 2). hCG, upregulated in two of the paired comparisons (Fig. 2A), is a hormone produced during early stages of pregnancy. hCG free β is produced in cancer cell lines, such as cervical, bladder, ovarian, brain, colorectal, uterine, lung and BC [49]. In a different study, βhCG has been considered as a tumor biomarker by showing higher levels in serum of patients with specific types of brain, uterus and embryonal tumors [50]. Apolipoproteins, upregulated in two comparisons (Fig. 2B), are lipid transporters, and high levels of apolipoprotein D (from the same family) has been reported in BC [51, 52]. Apolipoprotein D has been observed in BC tumors using immunohistochemistry on 36 BC samples acquired after removing the tumor from randomly chosen patients. Based on the immunohistochemical staining, only less than 1% of BC samples showed negative results for apolipoprotein D [51]. In a different study of 163 BC tumor samples, the level of apolipoprotein D was measured using immunoperoxidase staining and only 36.8% showed negative results for apolipoprotein D, whereas the rest of them showed strong, moderate, or weak positive staining results [52]. Antichymotrypsin, a protease inhibitor was upregulated in three comparisons (Fig. 2C). α1-antichymotrypsin gene has shown to be associated with BC tumor prognosis in a study which was done on 110 BC tumor samples. The patients with higher mRNA levels of this gene, resulted from reverse transcription polymerase chain reaction (RT-PCR) method, showed higher 5-year disease free survival rate [53]. Presence of α1-antichymotrypsin has been also shown in lung cancer, both in different lung cancer cell lines using RT-PCR, Western blotting and immunohistochemistry, as well as in lung cancer tumor tissues using immunohistochemistry. One hundred and seventy lung cancer samples were analyzed, in which 52% showed positive immunohistochemical results of α1-antichymotrypsin [54]. In a different study, 164 Prostate cancer tissue samples from 42 patients, as well as 127 samples of 20 patients with benign prostate



$^{*}\ Fold\ Change = \frac{Spectra\ Count\ in\ BC\ Sample}{Spectra\ Count\ in\ Control\ Sample}$

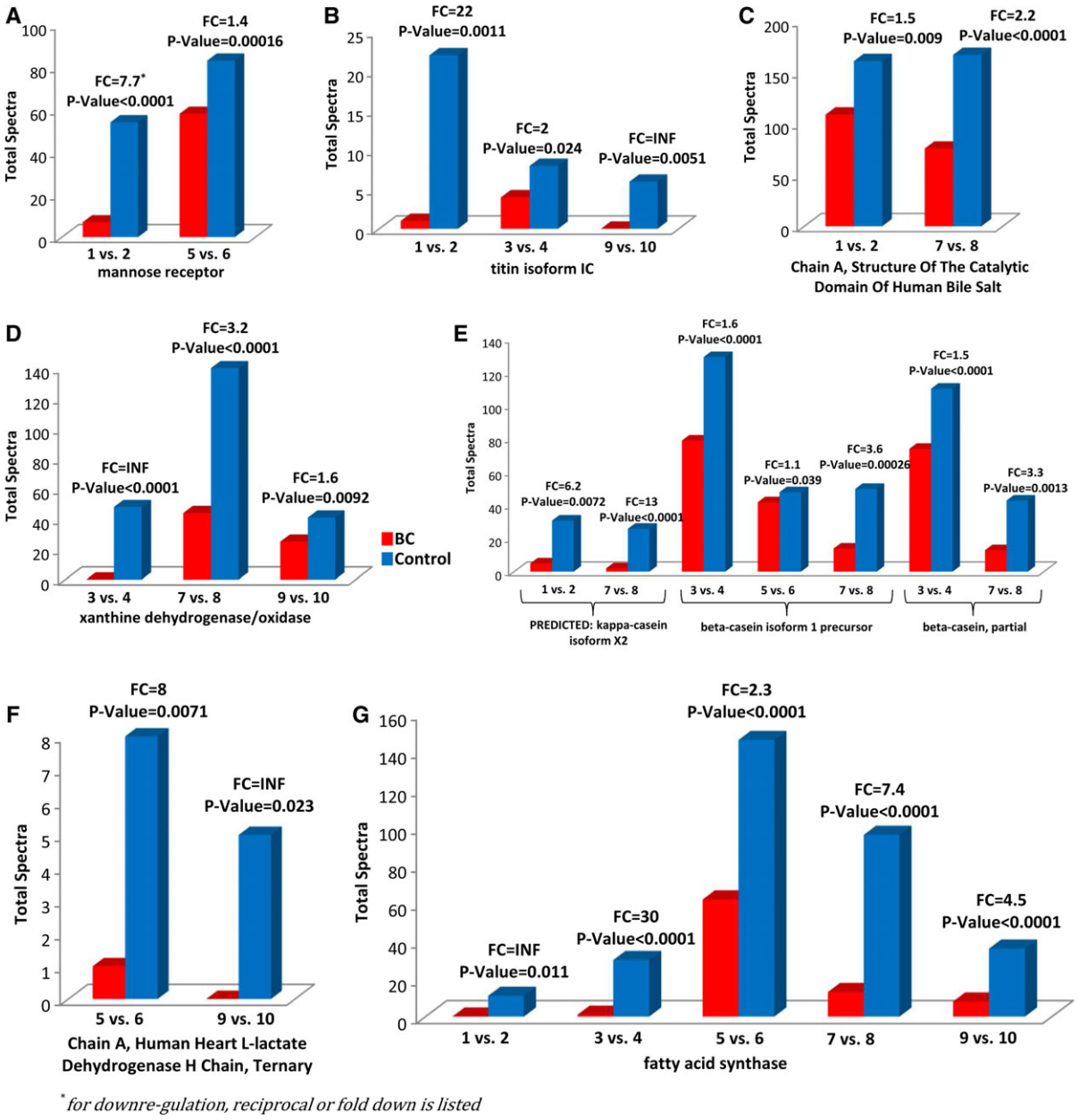
$^{**}Only\ samples\ with\ Fisher's\ exact\ test\ P - Value < 0.05\ are\ reported$

Figure 2. Upregulated proteins in multiple BC samples versus controls. The y-axis shows the total spectra counts for each protein in BC samples versus controls resulted from Scaffold ™ software. Spectra counts for BC samples are shown in red and for control sample are shown in blue. The Fisher's exact test p-value and the fold change (which is the spectra count in BC sample divided by spectra count in respected control sample) both calculated by Scaffold ™ software are shown in figure for each comparison. (A) Upregulation of hCG proteins in multiple BC samples. (B) Upregulation of lipoproteins in multiple BC samples. (C) Upregulation of antitrypsin and antichymotrypsin in multiple BC samples.

hyperplasia were analyzed performing immunohistochemistry and only eight samples among 42, did not show the positive results, while the normal prostate tissue and benign prostate hyperplasia tissues showed negative results for α1-antichymotrypsin [55].

3.1.2 Proteins downregulated in more than one paired comparison

Proteins downregulated in multiple comparisons are shown in Fig. 3. These proteins include mannose receptor, titin isoform IC, human bile salt, xanthine dehydrogenase/oxidase, members of the casein family, human heart L-lactate dehydrogenase H Chain ternary, and fatty acid synthase. Mannose receptor (downregulated in two comparisons; Fig. 3A) is a glycoprotein potentially involved in migration of macrophages [56], which plays a role in tumor development, and has been shown to be downregulated during activation of macrophages [56]. Titin (downregulated in three comparisons; Fig. 3B) is a striated muscle protein. Based on NCBI, A variation of the gene that encodes this protein has been implicated with breast size and BC [57]. Although titin is downregulated in three different BC versus control comparisons, it is upregulated in the cancer sample of one comparison (sample 7 versus sample 8; data not shown). Human bile salts (downregulated in two comparisons; Fig. 3C) play a role in fat digestion and low production of bile acids has been reported for a specific type of BC due to downregulation in the pathway of bile acids synthesis. In this study, 250 BC tumor samples were analyzed using microarray method to monitor gene expression in metabolic reactions and down-regulation in the biosynthesis of bile acids was observed [58]. Xanthine oxidoreductases (XORs; downregulated in three comparisons; Fig. 3D) are enzymes whose main function is in purine catabolism but also are involved in uric acid production, which has antioxidant activity and as a result, a cancer inhibitory effect [59]. Downregulation of XOR has been observed in aggressive BC in a study on 1262 BC patients. The levels of XOR were followed-up for 9.5 years and using immunohistochemistry, decrement of XOR was observed in %50 of samples and also in 7% of samples, no XOR was detectable [60]. In a different



*for downre-gulation, reciprocal or fold down is listed

Figure 3. Downregulated proteins in BC samples versus Controls. The y-axis shows the total spectra counts for each protein in BC samples versus controls resulted from Scaffold ™ software. Spectra counts for BC samples are shown in red and for control samples are shown in blue. The Fisher's exact test p-value and the reciprocal of fold change (fold down) are reported for all of the comparisons. (A) Downregulation of mannose receptor in multiple BC samples. (B) Downregulation of titin isoform IC in multiple BC samples. (C) Downregulation of bile salt in multiple BC samples. (D) Downregulation of xanthine dehydrogenase/oxidase in multiple BC samples. (E) Downregulation of casein proteins in multiple BC samples. (F) Downregulation of heart L-lactate dehydrogenase in multiple BC samples. (G) Downregulation of fatty acid synthase in multiple BC samples.

study, in vitro and in vivo experiments on human BC cells were done to indicate the relationship between XOR and cancer development. Moreover, XOR was inhibited in a mouse model which caused more development in BC tumor and it has been suggested that increased XOR may inhibit BC [61]. Interestingly, one of the most abundant proteins in milk was downregulated in the BC milk samples. As seen in Fig. 3E, several proteins from the casein family were downregulated in four of the five BC versus control comparisons. Caseins have multiple functions, for instance, beta-casein precursor provides amino acids for growth and was shown to be downregulated in human breast tumors [62]. In this study, 127 breast tumors (40 benign and the rest malignant) were analyzed by immunohistochemical techniques using four different anti-casein monoclonal antibodies. Only two of benign and one of malignant samples showed positive results for staining. Except from breast tumors, an extra of 42 tumor samples from different carcinomas were analyzed and there

was no positive staining results observed in those tumors as well [62]. It is also worth to mention that, alpha s1 casein has been reported to have antioxidant activities [63]. This protein is involved in transportation of calcium phosphate in milk and might be also involved in regulation of immune responses [64]. Lower levels of alpha s1 casein in normal and prostate cancer tissues, compared to benign prostate hyperplasia patients have been reported and as a result, it has been considered as a potential biomarker for diagnosis of benign prostate hyperplasia. In this study, using immunohistochemistry, the positive staining results of alpha s1 casein were as follow: zero of ten controls, 3 of 30 prostate cancer and 20 out of 22 benign prostate hyperplasia samples [65]. Figure 3F shows reduced levels of lactate dehydrogenase in two of the comparisons. Synthesis of lactate dehydrogenase, an enzyme that catalyzes lactate conversion to pyruvate, is altered by promoter methylation and reduced levels has been reported in BC [66]. In this study, low level of lactate dehydrogenase was observed both in BC cell lines and tumor tissues. Using RT-PCR method, lower level of lactate dehydrogenase was observed in three different BC cell lines (in two cases the enzyme and in one case the mRNA). Using immunohistochemistry, 26 BC tissue samples and eight samples of adjacent ductal carcinoma in situ lesions were analyzed. Negative results for lactate dehydrogenase B was observed in 23 out of 26 and four out of eight samples [66]. Finally, in our study, fatty acid synthase was downregulated in all five BC milk samples; Fig. 3G. However, this enzyme has been shown to be upregulated in BC [67–69], High level of fatty acid synthase was observed in several different types of cancers, malignant tumors and even several benign tumors [67] as well as in the serum samples of BC patients compared to controls [68]. In a different study, using enzyme-linked immunosorbent assay (ELISA) and Western blot analysis, upregulation of fatty acid synthase has been shown in two different BC cell lines and serum samples from 22 BC patients versus 25 control samples [69].

3.1.3 Proteins dysregulated in only one paired comparison between cancer and control milk samples

As discussed earlier, several proteins were dysregulated in only one of the five comparisons. While protein differences observed in just one of the comparisons are less likely to serve as biomarkers of risk or cancer, we report these differences as exploratory results. Of particular interest and potential value are the comparisons of the milk obtained from **both the diseased and healthy breasts of individual women**; samples 1 and 2, and samples 3 and 4. Comparisons ***within*** individuals are more robust as differences in genetic background are eliminated providing greater ability to detect cancer-related patterns. It is important to note that the timing between the milk donation and diagnosis of BC differed dramatically between these two sets of milk samples. For samples 1 and 2 the woman was diagnosed with cancer 24 months after she 1 donated her milk, whereas for samples 3 and 4 the woman was diagnosed with cancer 2 months before she donated her milk. Therefore, differences in protein levels can be considered to be signs of risk/early cancer or cancer, respectively.

3.1.4 A dysregulated protein specific to the diseased breast of a woman who was diagnosed with BC 24 months after donating breast milk from her left and right breast

Figure 4A shows that transcobalamin-1 is significantly downregulated in the milk from the breast with cancer (sample 1 versus 2). Transcobalamin-1 is precursor protein that binds to cobalamin (vitamin B12) and plays a role in the protection and transportation of vitamin B12. The relationship between changes in vitamin B12 related genes and gastric cancer has been reported previously [70]. Eight vitamin B-12 single-nucleotide polymorphisms (SNPs) from four different genes were analyzed using genotyping in 492 gastric cancer cases compared to 550 controls. The variations of these SNPs could be a marker for gastric cancer risk [70].

3.1.5 Dysregulated proteins specific to the diseased breast of a woman who was diagnosed with BC 2 months before donating breast milk from her left and right breast

Seven proteins were uniquely dysregulated in the comparison of samples 3 and 4 (Fig. 4B). Six upregulated proteins included myeloperoxidase isoform CRA_b, beta-actin-like protein 2, 92 kDa type IV collagenase, fibrinogen gamma, macroglobulin alpha2, and annexin A1. Myeloperoxidase, an enzyme involved in the production of reactive oxygen species resulting in DNA damage. Upregulation of myeloperoxidase has been observed in the early stages of ovarian carcinoma [71]. 305 blood samples from patients with ovarian tumors (230 cancer, 75 in early stages) as well as 299 control samples were analyzed. Performing genotyping, higher levels of polymorphism of myeloperoxidase's gene has been observed in early stages of ovarian cancer compared to control samples (83.3 versus 62%). Also the higher levels of myeloperoxidase have been observed in ovarian cancer tissue by performing Immunohistochemistry [71]. Actins are a wide group of proteins that play different roles in cellular processes. The relationship between actin and BC can be described as the role of actin in the development of tumors due to the fact that the progression of tumor cells relies on gene expression and nuclear actin's upregulation in cancer, alters the performance of RNA polymerase and chromatin structure and as a consequence, the gene expression in cancer cells [72]. Using immunohistochemistry and biochemical analysis, it has been shown that 72 and 92 kDa isoforms of type IV collagenase participate in tumor development and metastasis in human and rat models respectively [73]. Moreover, in a different study, higher activity (4–10 fold higher) of this enzyme has been reported in BC ZR75-31A cell line compared to

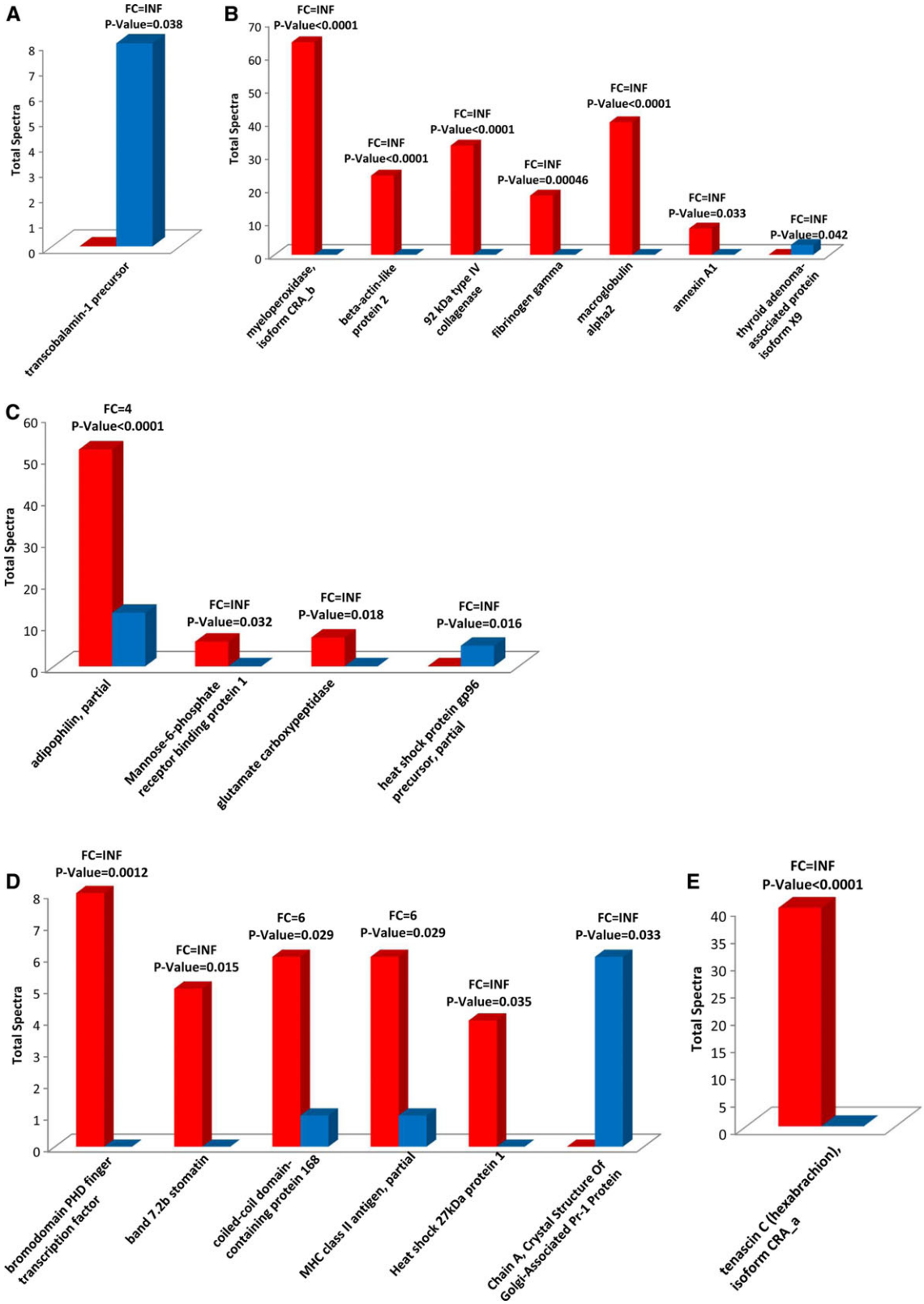


Figure 4. Dysregulated proteins in individual BC samples versus Controls. (A) Dysregulated proteins in sample 1 (BC) versus sample 2 (Control) (table 1). (B) Dysregulated proteins in sample 3 (BC) versus sample 4 (Control) (table 1). (C) Dysregulated proteins in sample 5 (BC) versus sample 6 (Control) (table 1). (D) Dysregulated proteins in sample 7 (BC) versus sample 8 (Control) (table 1). (E) Dysregulated proteins in sample 9 (BC) versus sample 10 (Control) (Table 1).

stromal cells [74]. Using Northern blot analysis, high levels of 92 kDa isoforms of type IV collagenase in human BC MDA-MB-435 cell line also has been reported [75]. Additionally, collagenase-1, from the same family, has been shown to be upregulated in MDA-MB-231 BC invasive cell line using scanning electron microscopy and confocal laser scanning microscopy [76]. Fibrinogens are involved in wound healing and cell migration. Also, it has been shown that fibrinogens play a role in BC tumor development and metastasis [77]. Aberrant production of extracellular matrix components such as fibrinogens, causes the development of malignancy through its effects on cell proliferation [77]. Macroglobulin alpha2 is in the family of proteins that includes protease inhibitors and it is produced by cancer cells in colon carcinoma [78] In a study in rat model. Using immunohistochemistry and in situ hybridization, it has been shown that the production of macroglobulin alpha2 in rat's liver is due to the presence of colon cancer cells and it is not from the hepatocytes [78]. Finally, it has been shown that annexin A1 is involved in tumor development. 135 BC samples, 20 samples of benign tumors and 20 control samples were analyzed using immunohistochemistry. This study showed various alterations in annexin A1 in different stages of progression for different tumors. Lower levels were observed in higher stages of tumor development, however, higher levels were observed in axillary lymph node metastasis [79]. A tatal of 175 samples, including 135 BC, 20 benign breast lesions and 20 controls were analyzed using immunohistochemistry. The negative result of staining was observed for 56.3% cases of BC. The level of annexin was negatively correlated with the stage of the disease. The relationship between tumor prognosis, patients' survival and levels of annexin A1 was investigated in a different study as well. The patients' data were from ten different studies including almost 6000 cases of BC. Using immunohistochemistry, higher levels of annexin A1 were observed in tumors with lower prognosis [80].

One protein, thyroid adenoma-associated protein (THADA) isoform X9, was significantly downregulated in the milk from the breast with cancer (comparison of samples 3 and 4), as shown in Fig. 4B. Lower levels of THADA has been reported in thyroid tissue differentiation [81]. Eighty five thyroid hyper- and neoplasias samples versus 34 controls were analyzed using RT-PCR. The ratio of THADA level in tumor samples versus control was 1 to 45.94.

3.1.6 Dysregulated proteins specific to one of the three comparisons between the milk from a woman diagnosed with cancer and the milk from a woman without BC

As discussed in the beginning of the results section, because of the small sample size (ten milk samples), we conducted paired comparisons between cancer and control milk samples. The first two comparisons, between the diseased and healthy breasts of two individual women, provided a robust discovery platform. The next three paired comparisons, between milk from different women (one with cancer and one without), allowed us to increase the sample size of paired comparisons to 5, and provided the ability to determine the extent to which the altered levels of a particular protein was observed multiple times. Our final analysis is the most exploratory and results should be considered preliminary. Below we discuss proteins that were found to be significantly dysregulated in **only** one of the three randomly paired comparisons. As with the bilateral milk samples from women discussed above, the timing between milk donation and cancer diagnosis varied markedly among the three comparison groups, ranging from a cancer diagnosis one month before milk donation (sample 7) to a cancer diagnosis 6 months after milk donation (sample 9) and 22 months after milk donation (sample 5).

Figure 4C shows the proteins with altered levels in the comparison of sample 5 (BC) versus sample 6 (control). Three proteins, adipophilin, partial, Mannose-6-phosphate receptor binding protein 1, and glutamate carboxypeptidase were upregulated in the milk from the woman with cancer. Adipophilin is involved in the differentiation of adipose (fat storing tissues) and high levels of adipophilin have been shown to be associated with tumor development and human cancers [82, 83]. In a study on 117 samples of different types of cutaneous clear cell lesions, the level of adipophilin was identified using immunohistochemistry. In different carcinomas tested, positive results were observed in high percentage of samples (ranging from 62.5 to 100% in different groups, except for 36% in one group) and no positive results observed in controls [82]. In a different study, positive results for adipophilin were observed in hepatocellular carcinoma in a tumor tissue microarray [83]. It should be noted, however, that adipophilin was downregulated in sample 7 (BC) as compared to sample 8 (control) (data not shown), possibly related to the time between milk collection and cancer diagnosis (sample 5: cancer diagnosis was 22 months after milk donation; sample 7 cancer diagnosis was 1 month before milk donation). Mannose-6-phosphate receptor binding protein 1 (M6PRBP1), which is also known as Tail-Interacting Protein of 47 kDa (TIP47), is involved in transferring hydrolase enzymes by M6PR between golgi and endosomes. The relationship between glutamate carboxypeptidase II and BC and prostate cancer risk has been shown by dysregulation of the genes responsible for this protein [84]. Gene screening of glutamate carboxypeptidase II was done for 61 BC samples compared to 75 controls and 58 prostate cancer samples compared to 76 controls by performing PCR analysis. Glutamate carboxypeptidase 2, also known as PSMA (prostate specific membrane antigen) and folate hydrolase (FOLH1) showed high plasma levels in prostate and BC respectively [84] PSMA is considered as a prostate cancer biomarker and target for prostate cancer therapy [85]. There was also one downregulated protein specific to the comparison of sample 5 (BC) versus 6 (control). As shown in Fig. 4C, heat shock protein gp96 precursor, partial, is significantly lower in the milk from the woman with BC. The main function of this protein is in the folding of other proteins. However, other members of

heat shock protein family have been shown to be upregulated in cancer and play a role in immune system and metastasis [86]. The levels of glucose-regulated protein 94, which is an isoform of heat shock protein 90 were compared in two replicates of cancer tissues of prostate, BC metastatic to liver, and lung cancer versus normal tissues from the same person using Western blot analysis and no heat shock protein 90 was observed in controls, but there were positive results for cancer samples [86].

Figure 4D shows five upregulated and one downregulated proteins in sample 7 (BC) versus sample 8 (control). Five upregulated proteins are: bromodomain PHD finger transcription factor, band 7.2b stomatin, coiled-coil domain-containing protein 168, MHC class II antigen, partial, heat shock 27 kDa protein 1. Bromodomain PHD finger transcription factor has been identified to play a possible role in transcription. Upregulation of this protein has been reported in colorectal cancer [87]. Twenty samples of colorectal cancer were compared with controls and the levels of mRNA and protein were analyzed using RT-PCR, Western blot analysis and immunohistochemistry. Higher levels of mRNA of bromodomain PHD finger transcription factor was observed in 85% of tumor samples compared to controls. The results of Western blot confirmed the mRNA expression results and also 67.6% of samples showed high levels resulted from immunohistochemistry [87]. Stomatin is a cell membrane protein whose function is unclear, but it may be involved in the regulation of ion channels and transporters. It has been reported that stomatin-like protein 2 is upregulated in ovarian cancer [88]. mRNA and protein levels of stomatin-like protein 2 were analyzed in five ovarian cancer cell lines compared to control cells, using RT-PCR and Western blot analysis. Eight tissue samples from ovarian cancer patients compared to control tissue samples from the same person were analyzed as well. The upregulation of stomatin-like protein 2 was observed both for m-RNA and protein. Using immunohistochemistry, more tissue samples were also analyzed and the upregulation percentages were as follow: 140 ovarian cancer samples (73.6% positive), 20 early stages ovarian cancer (45% positive), 20 benign tumors (30% positive) and 20 control samples (0% positive). This shows that the levels depend on the stage of the disease [88]. Mutations in the gene that encodes coiled-coil domain containing 37 which is from the same family as Coiled-coil domain containing 168 protein, has been reported in lung carcinoma [89]. Semi-quantitative RT-PCR analysis of the genome from human lung cancer cells as well as 21 lung cancer tissues compared to controls was done in order to investigate the methylation and alterations in gene expression [89]. MHC (major histocompatibility complex) is found on the antigen presenting cells, and helps201700123 to introduce the antigens to the cells of the immune system. In a BC study, 34 BC patients in different stages of the disease provided 41 samples of tumor infiltrating lymphocytes and after analysis, MHC-class-II restricted $CD4^+$ T cells have been observed in BC samples [90]. It also has been shown that MHC class II antigen is upregulated in the cell surface of lung cancer tumor cells [91]. Forty four lung cancer tissue samples were analyzed using immunohistochemistry and 11 samples showed positive results. The positive results only happened in the region of lymphocytic infiltration. Except from tissue samples, four lung cancer cell lines were also investigated using immunoelectron microscopy and high levels of MHC-class-II antigen was observed on the cell surface [91]. The 27-kDa heat-shock protein is involved in cell development and cell trafficking and causes apoptosis blockage which can clearly explains the relationship of this protein with cancer cells. To investigate the effect of 27-kDa heat-shock protein which is upregulated in early stages of BC, ER-positive MCF-7 cell line has been subjected to a vector containing the cDNA of this protein. The results showed more proliferation and cell lysis in the presence of 27-kDa heat-shock protein [92]. The one downregulated protein, golgi-Associated Pr-1 Protein is from the CAP (cysteine-rich secretory proteins, antigen 5, and pathogenesis-related 1 proteins) family of proteins. Dysregulation of these proteins has been reported in cell lines of prostate, lung, colon, bladder cancers and lymphomas as well as in tumor tissues of prostate and renal cancers [93].

Figure 4E shows the upregulated protein, tenascin C, in sample 9 (BC) versus sample 10 (control). As can be seen in Table 1, cancer diagnosis associated with sample 9 was made 6 months after the woman provided her milk sample. Of possible interest, tenascin C (hexabrachion) isoform CRA_a was visually observed as a distinct band on the SDS-PAGE for sample 9. Tenascin is an extracellular protein found in cells during embryonic development, but also implicated in wound healing and tumor formation. This protein or proteins from the same family are reported to be upregulated in various types of cancers [94–98]. Tenascin-C is involved in tumor development, signaling in cancer cells and progression of the disease (reviewed in Ref. 94). In a different study on 143 patients with BC, the level of tenascin-C has been analyzed by immunohistochemistry and the association of tenascin-C with proliferation has been confirmed [95]. Another study has been done on tenascin-C with the purpose of investigation of tenascin-C co- speciation with vimentin. In this study, 128 BC tissue samples as well as 13 cell lines (11 BC and two controls) have been analyzed by immunohistochemistry and Western blotting. 97% of tissue samples and eight BC cell lines showed positive staining for tenascin-C [96]. To investigate the alterations in tenascin-C levels in different stages of BC, the levels of this protein has been measured using RT-PCR, Southern blotting and immunohistochemistry. The analysis was done on four different BC cell lines and two control cell lines, as well as tissue samples including 35 BC tumor tissues, 13 samples of ductal carcinoma in situ, five samples of fibroadenomas and 15 controls. Positive results of tenascin-C were observed for two of the BC cell lines and the controls. In tumor samples more invasive samples showed higher level of tenascin-C [97]. Finally, the presence of tenascin-W in transgenic mice BC tumor tissues was investigated and compared with control using immunohistochemistry and western blot analysis. The results showed the higher levels of tenascin-W in metastatic tumors compared to nonmetastatic ones [98].

3.2 Limitations

The work presented here has some major limitations. With respect to the breast milk, the sample size is small (only five pairs), there is a wide range in the stage of lactation (as measured by baby's age), and the time between milk donation and BC diagnosis differs significantly among the five pairs. This variability in sample characteristics decreases the likelihood that the dysregulated proteins are specific to BC risk. Despite these limitations we still found several dysregulated proteins in all five of the cancer versus the control milk comparisons; a result that warrants further study in another sample set. Another major limitation is related to the proteins that are analyzed. In a proteomics experiment, we usually identify and quantify the proteins that are the most abundant in a sample (canonical proteins). However, some of the protein isoforms and variants and many modified proteins (protein species) [37–39] that resulted from the differential gene expression (alternative splicing), RNA editing, or PTMs are not analyzed, either because of the experimental setup (i.e. 1D-PAGE and MS analysis versus 2D-PAGE and MS analysis) or the bioinformatics analysis (not looking for protein phosphorylation or not distinguishing between the canonical and truncated protein) or a combination of various sample-related or analysis-related factors. Therefore, the reader should be aware of all these limitations.

4 Concluding remarks

Our mass spectrometry analysis of five pairs of cancer versus control breast milk samples identified dysregulated proteins. In this report we focused on those proteins that both differed significantly and were previously shown to play a role in tumor and/or cancer development. We first discussed proteins that were dysregulated in more than one comparison: three proteins were upregulated and proteins downregulated in cancer versus control samples (Figs. 2 and 3), and two of these proteins, casein and fatty acid synthase, were downregulated in four and five of the comparisons, respectively. These results support the idea that proteins in breast milk may serve as biomarkers of BC risk or early detection.

We also presented proteins that were dysregulated in only one of the five comparisons: a total of 15 upregulated and four downregulated proteins. It is important to note that the five paired comparisons differed on two major characteristics: 1) whether they were *within* or *across* woman comparisons, and 2) the time between milk donation and cancer diagnosis. Within woman comparisons (samples 1 versus 2, and 3 versus 4) provide the most robust format for detecting cancer-related biomarkers, as the genetic background of both the cancer and control sample is identical. However, of the two *within* woman comparisons, the cancer diagnosis occurred either, 24 months after (samples 1 and 2), or 2 months before (samples 3 and 4) milk donation. This disparity in relation between milk collection and cancer diagnosis makes it likely that the proteins identified will differ as they are probably related to the stage of the disease. Interestingly, the two comparisons with the greatest number of dysregulated proteins (six upregulated and one downregulated for *within* comparison three versus four, and five upregulated and one downregulated for *across* comparison 7 versus 8) had the shortest time between milk donation and cancer diagnosis. Furthermore, these are the only two comparisons in which the cancer diagnosis was before the milk donation raising the question of whether the dysregulated proteins were related to the biopsy itself. The analytical approach we used in this pilot study (*within* and *across* woman comparisons) took advantage of rare breast milk samples from women with cancer, and provided a robust platform for biomarker discovery.

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