

Serum Proteomic Signatures of Male Breast Cancer

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Abstract. *Background:* To date, the elucidation of serum protein alterations in male breast cancer (MBC) has not been extensively studied, due to the rarity of the disease. *Materials and Methods:* In the present work, two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) were employed to detect differences in serum protein expression between patients with MBC and healthy controls. *Results:* A panel of differentially expressed serum proteins was identified, including proteins involved in the regulation of the cell cycle [e.g. cell division cycle 7-related protein kinase (CDC7)], in mitochondrial function [e.g. mitochondrial aldehyde dehydrogenase (ALDH2) and dimethyladenosine transferase 1 (TFB1M)], in lipid metabolism and transport [e.g. apolipoprotein A-I (APOA1) and E (APOE)], in apoptosis and immune response [e.g. CD5 antigen-like (CD5L), clusterin (CLUS) and C-C motif chemokine 14 (CCL14)], in transcription (e.g. protein SSX3 and signal transducer and activator of transcription 3 (STAT3)), in invasion and metastasis (e.g. alpha-2-HS-glycoprotein (FETUA)), in estrogen synthesis [aromatase (CYP19A1)] and other diverse biological roles [e.g. actin-related protein 2/3 complex subunit 4 (ARPC4), dual specificity mitogen-activated protein kinase kinase 4 (MP2K4), ectoderm-neural cortex protein 1 (ENC1), and matrix metalloproteinase-27

(MMP27)]. Conclusion: These findings provide valuable insight into the distinct clinicopathological features of MBC and indicate that select serum proteomic markers may help improve MBC management.

Male breast cancer (MBC) is an uncommon malignancy accounting for fewer than 1% of all male cancers (1), with an incidence of less than one per 100,000 men-years, which is considerably low compared to female breast cancer (FBC) (2). The heterogeneity of breast cancer is well established. Recent advances in genomic technologies have helped researchers expand their insight into the genetic basis of cancer and have provided them with an important tool for the discovery of novel biomarkers (3). Still, we presently know that the genomic approach has its limitations; gene- or transcript-level analysis may not directly reflect cellular functions, due to key biological mechanisms such as alternative splicing and post-translational modification of proteins (4).

It is currently considered that in contrast to the genome, the proteome itself reveals a more dynamic state of the cell, since changes in the mutational landscape of the genome are translated into an aberrantly regulated proteome (5). Therefore, proteomic technologies, including mass spectrometry-generated data, aim to identify variations in proteins circulating in the blood which can serve as a less invasive alternative to histopathological assessment. The proteomic approach may have the ability to transform the clinical management of disease and discover serum biomarkers that can be integrated into personalized patient care (6).

Data on protein alterations in male breast carcinogenesis is scarce, due to the rarity of the disease. It is interesting that although some studies have identified similarities between breast cancer in men and in postmenopausal women (7), there is increasing evidence suggesting that the disease in men differs both causally, as well as in the clinicopathological characteristics and in outcomes (8). Consequently, data on proteomics in women should not be extrapolated to men due

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to the fundamental differences between the two entities. There is an immense need for personalized management of MBC; in consequence, a panel of indicators obtained by a simple blood withdrawal, a so-called liquid biopsy, would represent a significant advance.

To the best of our knowledge, there is very limited information regarding proteomic MBC biomarkers circulating in human serum, which represents an attractive, easily-accessible source for biomarker discovery. In the current study, two-dimensional gel electrophoresis (2-DE) separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometric (MALDI-TOF-MS) analysis were employed to monitor differences in serum protein expression between patients with MBC and healthy individuals. Our aim was to identify a panel of candidate proteins that could be used to better understand MBC pathology, in order to improve MBC management and to advance oncological medical care.

Materials and Methods

Patients. Eleven male patients with sporadic breast cancer (MBC) and eight healthy individuals, who were attending the First Propaedeutic Surgical Department at Hippokration General Hospital and the Department of Clinical Therapeutics at the Alexandra Hospital in Athens, Greece, were enrolled in this study. The control group consisted of age-matched healthy individuals, admitted for a routine annual physical examination, who had no family history of malignancy. Participation was voluntary and informed consent was provided by each participant. The study was approved by the Ethical Committees of the participating hospitals (approval number ES66/28-5-2015). MBC was diagnosed based on a combination of standard clinical, radiological and histological criteria (9). The main demographic and clinical characteristics of the MBC patients are presented in Table I.

Proteomic analysis. A blood sample was collected from each patient at the time of diagnosis. Following centrifugation, serum was collected and stored at -80°C until use. The protein concentration was determined with a Bioanalyzer system (Agilent Technologies Inc., Waldbornn, Germany), using Protein 200 plus kit (Agilent Technologies Inc.). 2-DE was performed as previously described (10). Gel images were scanned in a GS-800 Calibrated Densitometer (Bio-Rad, Hercules, CA, USA) and images were stored in digital format for further analysis.

Image analysis. Protein spots from all gels analyzed were detected, aligned, matched and quantified using PD-Quest v8.0 image processing software (Bio-Rad) according to the manufacturer's instructions. Manual inspection of the spots was carried out to verify the accuracy of matching. Spot volume was used to quantify protein expression. Normalization of each individual spot was performed according to the total quantity of the valid spots in each gel, after subtraction of the background values. Optical density (O.D.) level (%) of each protein from the control and MBC groups was determined separately and calculated as the sum of the percentage volume of all spots from all gels containing the same protein.

Table I. *Clinical characteristics of patients with male breast cancer.*

Variable	N (%)
Age at diagnosis	
≤ 50 Years	2 (18.2)
> 50 Years	9 (81.8)
Race	
Caucasian	11 (100.0)
Other	0 (0)
Tumor size	
T1	5 (45.5)
T2	4 (36.4)
T3	1 (9.1)
Tx	1 (9.1)
Tumor type and grade	
Ductal, I	2 (18.9)
Ductal, II	6 (54.6)
Ductal, III	3 (27.3)
Lobular	0 (0)
Lymph nodes	
N0	8 (72.7)
N1	2 (18.2)
N2	0 (0)
N3	0 (0)
Nx	1 (9.1)
ER	
Positive	10 (90.9)
Negative	1 (9.1)
PR	
Positive	9 (81.9)
Negative	2 (18.2)
HER2/neu	
Positive	1 (9.1)
Negative	10 (90.9)
Ki-67	
Low ($< 15\%$)	7 (63.6)
Moderate (16-30%)	4 (36.4)
High ($> 30\%$)	0 (0)

ER: Estrogen receptor; PR: progesterone receptor; HER2/neu: human epidermal growth factor receptor 2; Ki-67: proliferation marker protein Ki-67.

Selection of protein spots for MS identification was based upon O.D. alterations observed between the two groups. A minimum of 1.5-fold change in the expression level was used as a selection criterion.

Protein identification by MALDI-TOF-MS. For MALDI-TOF-MS analysis, gel spots of interest were manually annotated using Melanie 4.02 software and excised from 2-DE gels using Proteiner SPII (Bruker Daltonics, Bremen, Germany). Gel pieces were then placed into 96-well microtiter plates, destained with 180 μl of 30% acetonitrile (ACN) in 50 mM ammonium bicarbonate and dried in a speed vacuum concentrator (MaxiDry Plus; Heto, Allerød, Denmark). In-gel digestion was performed with 0.01 $\mu\text{g}/\mu\text{l}$ trypsin (Roche Diagnostics, Basel, Switzerland) for 16 h at room temperature. Next, 10 μl of 50% ACN containing 0.1% trifluoroacetic acid, were added to each dried gel piece and tryptic digested peptides were extracted. Tryptic peptide mixtures (1 μl) were applied on an anchor chip MALDI plate mixed with 1 μl of matrix solution, consisting of 0.08%

α -cyano-4-hydroxycinnamic acid (Sigma–Aldrich, St. Louis, MO, USA) and internal standard peptides *des*-Arg-bradykinin (904.4681 Da; Sigma–Aldrich) and adrenocorticotrophic hormone fragment 18–39 (2465.1989 Da; Sigma–Aldrich) in 50% distilled water, 50% ACN and 0.1% trifluoroacetic acid. Peptide mixtures were analyzed in a MALDI-TOF MS as described by Vaiopoulou *et al.* (10). Peptide masses were compared with the theoretical peptide masses of all available proteins from *Homo sapiens* in the SWISS-PROT and TrEMBL databases. Stringent criteria were used for protein identification with a maximum allowed mass error of 25 ppm and a minimum of four matching peptides. probability score with $p < 0.05$ was used as the criterion for affirmative protein identification. Monoisotopic masses were used, and one missed trypsin-cleavage site was calculated for proteolytic products. Search parameters included potential residue mass modification for carbamidomethylation and oxidation. Any redundancy of proteins that appeared in the database under different names and accession numbers was eliminated. If more than one protein was identified under one spot, the single protein member with the highest protein score was singled out from the multi-protein family.

Western blot analysis. Total proteins (10 μ g) of control and MBC serum samples were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (Mini-PROTEAN® TGX™ Precast Protein Gels; Bio-Rad) under reducing conditions and electroblotted to Porablot polyvinylidene difluoride membranes (Macherey-Nagel, Düren, Germany). After blocking with 5% non-fat dried milk in a solution of 20 mM Tris/pH 7.6, 137 mM NaCl and 0.1% Tween20 (TBST) for 1 h at room temperature, membranes were washed with TBST and incubated overnight at 4°C with primary antibody against apolipoprotein E (APOE; dilution 1:600; Santa Cruz Biotechnology, Dallas, TX, USA). Next, membranes were washed with TBST and incubated with a goat anti-mouse horseradish peroxidase-conjugated secondary antibody (A00160, dilution 1:1000; GenScript Biotech Corp., Piscataway, NJ, USA). After a final wash with TBST solution, proteins were detected by ECL western blotting detection system (Thermo Scientific, Rockford, IL, USA). Western blots were scanned with a GS-800 calibrated densitometer (Bio-Rad). Band quantification was performed with Quantity One image processing software (Bio-Rad). Human IgG protein was used as an internal control to ensure equal sample loading (sc-69786, dilution 1:400; Santa Cruz Biotechnology).

Enzyme-linked immunosorbent assay (ELISA). Aromatase ELISA kit was purchased from Fine Biotech (EH2665; Fine Test, Wuhan, Hubei, P. R. China). The assay was performed following the kit instructions. Plates were read on a Multiskan® GO Microplate Reader (Thermo Fisher, Basingstoke, UK) with appropriate baseline correction. ELISA values were normalized for each 96-well plate, using standard solutions provided in the kit. The samples were not diluted, since the target protein concentration was very low (≤ 0.156 ng/ml).

Pathway analysis. Functional relationships analysis of the differentially expressed proteins was performed using the STRING v.10 database (Search Tool for the Retrieval of Interacting Genes/Proteins, <http://string-db.org>). The simplified version of the produced network, which involved both up- and down-regulated proteins, was adopted. The PANTHER database was used to reveal the molecular function and signaling pathway associated with each identified protein (<http://panther.appliedbiosystems.com/>).

Statistical analysis. To ensure confidence in our experimental approach, we employed an experimental design which involved duplicate 2-DE gels per sample and separate preparations for each replicate sample per experiment. Comparisons were performed between samples (MBC patients versus healthy controls). Mean densitometric values of all spots corresponding to a specific protein from each group were first checked for normal distribution using unequal variances. Data with normally distributed densitometric values were exported to Microsoft Excel 2016 software (Microsoft Corp., Redmond, WA, USA) and compared with the two pair *t*-test assuming unequal variance. Means of spot intensities for proteins with non-normally distributed values were compared for statistical significance with the Mann–Whitney nonparametric test (GraphPad Instat 3 software; GraphPad Software Inc., La Jolla, CA, USA). Statistical significance (alpha-level) was defined as $p < 0.05$. To control the false discovery rate (FDR), individual alpha-levels for each spot were adjusted following the FDR correction procedure.

In western blot and ELISA experiments, protein quantification was performed by three independent experiments for each protein analyzed. Mean optical density for each protein of the control and MBC groups were compared with independent samples *t*-test assuming unequal variances in Microsoft Excel 2016 (Microsoft Corp.). A $p < 0.05$ was considered statistically significant.

Results

Proteomic analysis. In order to detect serum proteins differentially expressed in the serum of patients with MBC and healthy controls, each protein sample was separated by 2-DE (Figure 1). Statistical analysis of the resulting 2-DE gels revealed 504 protein spots differentially expressed between the two groups. Two hundred and fifty-two proteins corresponding to the 504 spots were identified since more than one spot was related to the same protein. Table II focuses on the 42 proteins differentially expressed in all samples ($n=11$) of the patient group, which fulfilled the following requirements: Mascot Score >40 and an expression level of >1.5 or <0.5 . This table provides their identities, theoretical pI, molecular weight, MASCOT score, protein coverage, and expression levels as calculated with PDQuest 8.0 software. An expression level >1.5 indicates overexpression and that of <0.5 shows underexpression. Mascot scores >40 indicate identity or extensive homology at the $p < 0.05$ level.

Western blot analysis. The differential expression of APOE was further confirmed by western blot analysis using appropriate antibodies for both MBC and control samples. Optical density measurements of the bands revealed that there was a 1.89-fold increase ($p=0.0017$) in the amount of APOE in serum collected from patients with MBC compared to healthy controls (Figure 2).

ELISA. Serum aromatase (CYP19A1) concentrations in patients with MBC and healthy controls were measured using ELISA since CYP19A1 is an endoplasmic reticulum enzyme also detected in the serum. CYP19A1 was similarly

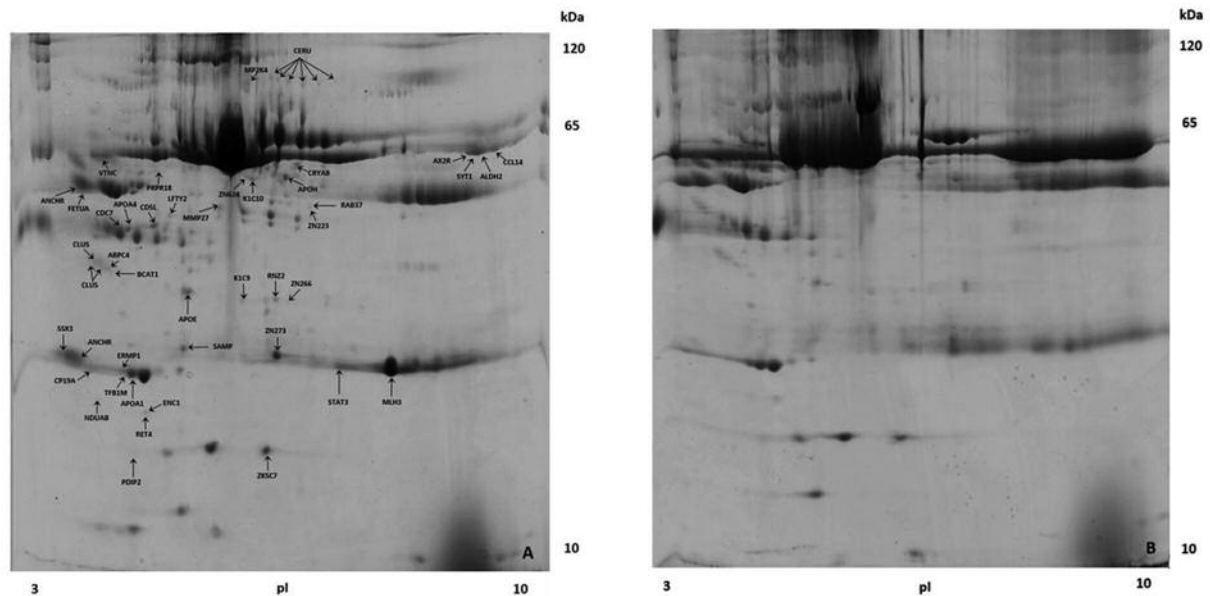


Figure 1. Serum samples from patients with male breast cancer (A) and healthy controls (B) were analyzed by two-dimensional gel electrophoresis (2-DE). The gels were stained with Coomassie blue and protein spots were excised and further analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry, as described in the Materials and Methods section. In the gel images, each spot represents a protein. Comparison of protein profiles revealed proteins differentially expressed between the two groups analyzed (arrows). Protein abbreviations used are presented in Table II.

expressed among patients with MBC and healthy individuals. The mean serum CYP19A1 level was 0.189 ± 0.115 ng/ml for patients with MBC and 0.193 ± 0.156 ng/ml for healthy controls ($p=0.95$).

Pathway analysis. The pathways which engage differentially expressed proteins identified in our samples were studied using the STRING database (Figure 3). For this interpretation, an analysis of 42 proteins was performed using the PANTHER classification system, which sorts the proteins into respective groups based on their biological process, and their involvement in different signaling pathways (Figure 4). As presented in Figure 4A, it is of great interest that the three major groups involved molecules related to cellular process (42.9%), metabolic process proteins (35.7%), and members of the response to stimulus process, factors that are known to be aberrant in cancer. Regarding the PANTHER pathway analysis, as illustrated in Figure 4B, six main pathways were identified. Specifically, 8.3% of proteins identified as being overexpressed in the MBC samples corresponded to proteins found in the angiogenesis pathway, 8.3% in the cholecystokinin receptor (CCKR) signaling map, 5.6% in the RAS pathway, 5.6% in the gonadotropin-releasing hormone receptor pathway, 5.6% in the epidermal growth factor receptor (EGFR) signaling pathway, and 5.6% in the inflammation mediated by chemokine and cytokine signaling pathway.

Discussion

We attempted to characterize the serum protein profile of patients with MBC using the MALDI-TOF-MS technique. According to our results, several proteins were differentially expressed in patients with MBC compared to controls. Among the proteins identified in our study, 38 were found to be up-regulated in all samples of the MBC group. Additionally, we found that spots corresponding to four proteins were detected only in patients with MBC. These results indicate that select serum proteomic markers might help elucidate the differences between patients with MBC and healthy individuals.

Very limited studies on breast cancer have focused on the male population and there is a lack of information regarding validated proteomic biomarkers in patients with MBC. Most proteomic studies refer to women and have identified possible biomarkers (11, 12), but few have been validated so as to achieve clinical application (13). Regarding MBC, as far as we are aware of, only one single study has been performed using MS-based technologies. Chahed *et al.* performed a thorough proteomic study of protein-expression alterations in MBC tissues (14), where overexpression of multiple proteins in male breast tumors was identified. To our knowledge, however, there are no studies attempting to mine the entire serum proteome of patients with MBC, as a less invasive approach for the discovery of candidate biomarkers.

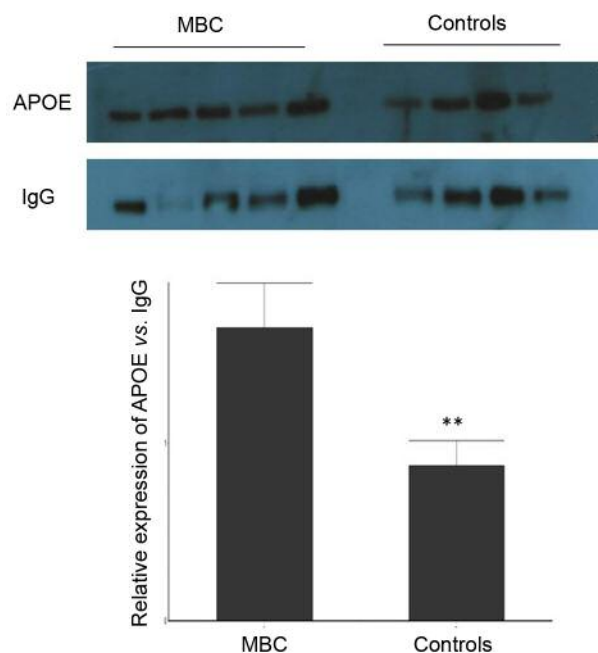


Figure 2. Confirmation of the differential expression of apolipoprotein E by western blot analysis. Quantification of protein content was performed using scanning densitometry. Each bar represents the mean optical density \pm SD of three independent experiments. **Differences were significant at $p < 0.0017$.

Differential expression of several proteins that carry out diverse biological roles was identified. Of these, we focused our discussion on the most clinically important ones. Concerning proteins involved in the regulation of the cell cycle, cell division cycle 7-related protein kinase (CDC7) is implicated in cell division, cell-cycle checkpoint mechanisms, and cancer progression. Our findings, suggest that this protein is overexpressed in MBC, and are in line with previous results on FBC (15). It has also been reported that high level expression of CDC7 is linked to unfavorable prognosis (16). Interestingly, a phase 1 study investigating a CDC7 inhibitor (TAK-931) in patients with advanced non-hematological malignancies is currently ongoing (17).

Aberrant expression of several mitochondrial proteins was also observed. Mitochondrial aldehyde dehydrogenase (ALDH2) belongs to the ALDH family of enzymes. Recent evidence reveals ALDH's potential role as a cancer stem cell marker (18). Consequently, high expression levels of ALDH directly correlate with poor clinical outcome in patients with breast cancer, since cancer stem cells have been implicated in breast tumor initiation, metastatic spread and recurrence (19). Additionally, our results showed overexpression of mitochondrial dimethyladenosine transferase 1 (TFB1M), which is consistent with previous findings revealing elevated

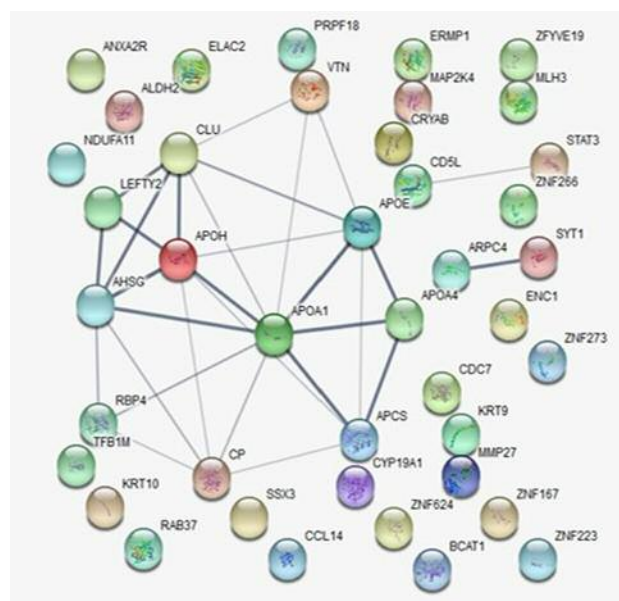


Figure 3. The figure depicts the interactions of the proteins identified as being differentially expressed in our study. Thicker network lines demonstrate stronger protein relation as well as neighboring positions. Protein abbreviations used are presented in Table II.

expression of transcription factors associated with mitochondrial biogenesis in FBC, including TFB1M (20).

In our study, proteins involved in lipid metabolism and transport were found to be up-regulated in the MBC group, including proteins with potential clinical significance, namely apolipoprotein A-I (APOA1) and APOE. This comes as no surprise since research has shown that the dysmetabolism of lipids or lipoproteins circulating in the blood may influence carcinogenesis through inflammation and oxidative stress pathways (21). The role of APOA1 in cancer is under investigation. Results of previous studies on the relationship between APOA1 and breast cancer are mixed. High levels of APOA1 were associated with higher incidence rates of breast cancer (22), while another study indicated an inverse correlation between APOA1 levels and breast malignancies (23). Serum APOE may play diverse roles in several biological processes and reports show that it is up-regulated in various malignancies, including breast cancer, possibly acting as a defense mechanism (24, 25). Xu *et al.* not only showed that serum APOE levels were elevated in patients with breast cancer compared to healthy controls, which is in line with our own results, but also that patients with high serum APOE levels had a worse prognosis, indicating that serum APOE could be an independent prognostic variable for breast cancer (26). All these findings suggest that serum APOE may serve as a non-invasive serological biomarker for distinguishing patients with breast

Table II. *Proteins differentially present in the serum of patients with male breast cancer (MBC) and healthy individuals. Only proteins identified/present in all samples (n=11) with a MASCOT Score >40 and an expression level of >1.5 or <0.5 are included in the table, consisting initially of 252 entries.*

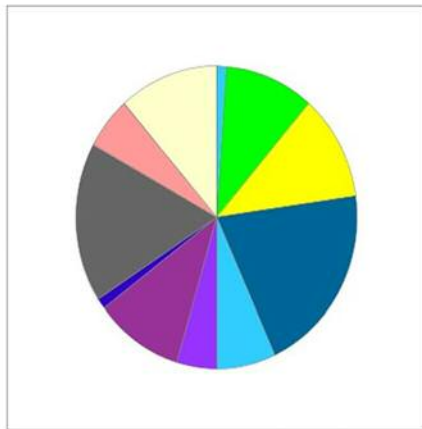
Full protein name	Abbreviation	Protein MW	pI	MASCOT Score	Coverage	Mean ratio*
Abscission/NoCut checkpoint regulator	ANCHR	52,427	5.47	40	14	5.42
Aldehyde dehydrogenase, mitochondrial	ALDH2	56,859	6.76	40	17	1.89
Alpha-2-HS-glycoprotein	FETUA	40,098	5.40	47	17	9.91
Alpha-crystallin B chain	CRYAB	20,146	6.92	41	27	2.77
Annexin-2 receptor	AX2R	22,181	4.73	42	26	2.42
Apolipoprotein A-I	APOA1	30,759	5.50	133	43	2.46
Apolipoprotein A-IV	APOA4	45,371	5.20	209	45	4.32
Apolipoprotein E	APOE	36,246	5.50	93	28	2.92
Aromatase	CYP19A1	58,358	7.84	43	17	1.55
Beta-2-glycoprotein 1	APOH	39,584	9.50	58	19	2.25
Branched-chain-amino-acid aminotransferase, cytosolic	BCAT1	43,509	5.00	40	9	2.91
C-C motif chemokine 14	CCL14	10,899	9.78	40	26	1.52
CD5 antigen-like	CD5L	39,603	5.20	79	15	3.66
Cell division cycle 7-related protein kinase	CDC7	64,646	9.77	41	13	4.32
Ceruloplasmin	CERU	12,2983	5.40	74	13	4.28
Clusterin	CLUS	53,031	5.90	50	11	2.36
Dimethyladenosine transferase 1, mitochondrial	TFB1M	39,860	10.05	52	28	2.39
DNA mismatch repair protein Mlh3	MLH3	16,6059	6.30	41	10	1.88
Endoplasmic reticulum metalloproteinase 1	ERMP1	10,1023	7.46	41	7	2.09
Keratin, type I cytoskeletal 10	K1C10	59,020	5.00	67	19	2.51
Keratin, type I cytoskeletal 9	K1C9	62,255	5.00	72	22	3.18
Left-right determination factor 2	LFTY2	41,407	10.20	40	15	1.80
NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 11	NDUAB	15,071	9.80	40	31	2.47
Polymerase delta-interacting protein 2	PDIP2	42,235	9.50	49	18	4.06
Pre-mRNA-splicing factor 18	PRP18	40,006	9.00	52	9	21.62
Protein SSX3	SSX3	21,740	9.40	40	23	2.46
Ras-related protein Rab-37	RAB37	25,028	5.96	48	36	1.90
Retinol-binding protein 4	RET4	23,337	5.70	67	34	1.64
Serum amyloid P-component	SAMP	25,485	6.10	82	26	1.61
Signal transducer and activator of transcription 3	STAT3	88,810	5.90	42	15	1.88
Synaptotagmin-1	SYT1	47,885	9.02	41	18	2.00
Vitronectin	VTNC	55,069	5.50	64	14	6.48
Zinc finger protein 223	ZN223	57,380	10.16	60	13	1.80
Zinc finger protein 266	ZN266	64,129	10.10	47	15	2.48
Zinc finger protein 273	ZN273	66,868	10.54	44	13	8.80
Zinc finger protein 624	ZN624	10,2544	10.11	44	10	1.58
Zinc finger protein with KRAB and SCAN domains 7	ZKSC7	86,918	7.60	51	6	1.95
Zinc phosphodiesterase ELAC protein 2	RNZZ	93,415	9.20	40	7	2.34
Actin-related protein 2/3 complex subunit 4	ARPC4	19,768	9.39	41	27	MBC only
Dual specificity mitogen-activated protein kinase kinase 4	MP2K4	44,716	9.10	44	22	MBC only
Ectoderm-neural cortex protein 1	ENC1	67,285	6.43	45	10	MBC only
Matrix metalloproteinase-27	MMP27	59,330	9.40	51	13	MBC only

MASCOT search score is $-10 \log(p)$, where p is the probability that an observed match is a random event. Scores >40 indicate identity or extensive homology at the $p < 0.05$ level. *Expression levels were calculated relative to control densities. Expression level >1.5 shows overexpression, expression level <0.5 indicates underexpression.

cancer from healthy controls, highlighting its potential contribution in clinical practice.

Several of the proteins identified as being overexpressed in serum from patients with MBC seem to have an essential role in the mediation of cancer cell biology, including uncontrolled cell growth, invasion and metastasis. Alpha-2-

HS-glycoprotein (FETUA) is a multifunctional protein contributing to the modulation of many critical signaling pathways such as transforming growth factor beta (27). It has been demonstrated that purified FETUA mediates the adhesion and spreading of breast cancer cells *in vitro*, via a novel mechanism involving cellular exosomes (28).

A Biological processes

■ Biological adhesion (GO:0022610)
 ■ Biological regulation (GO:0065007)
 ■ Cellular component organization or biogenesis (GO:0071840)
 ■ Cellular process (GO:0009987)
 ■ Developmental process (GO:0032502)
 ■ Immune system process (GO:0002376)
 ■ Localization (GO:0051179)
 ■ Locomotion (GO:0040011)
 ■ Metabolic process (GO:0008152)
 ■ Multicellular organismal process (GO:0032501)
 ■ Response to stimulus (GO:0050896)

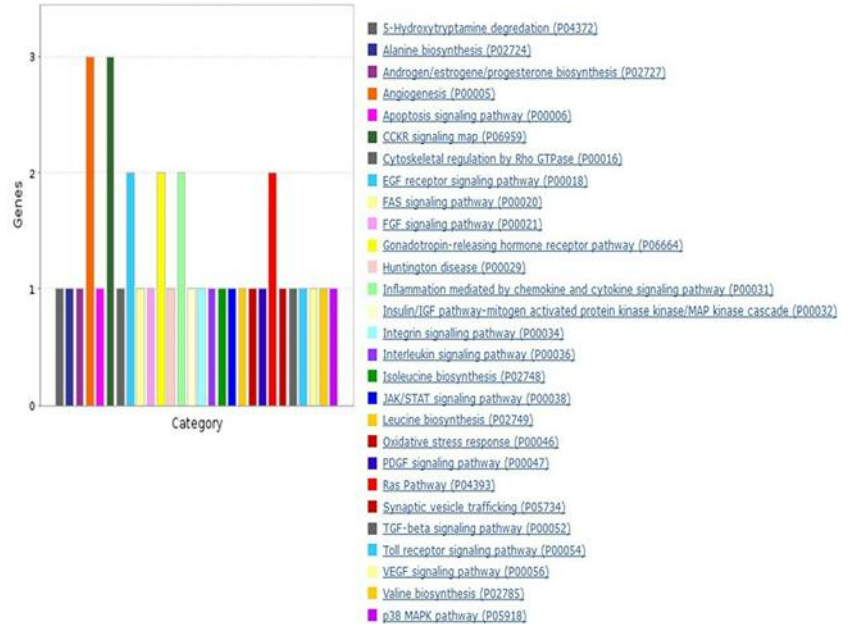
B Pathway analysis

Figure 4. *PANTHER* analysis. In total, 42 proteins were found to be differentially expressed between male breast cancer samples and healthy controls. A: Classification of these proteins into different groups based on their biological process. B: The different signaling pathway hits of these proteins.

Additionally, FETUA may act as a chemo-attractant for tumor cells along its concentration gradient and promote invasion (29). Our results concerning the aberrant expression of two different cytokeratins, K1C10 and K1C9, are consistent with an earlier study that reported the expression of key elements of the epithelial to mesenchymal transition process in breast tumor samples, including several cytokeratin forms and fragments (30).

Aromatase (CYP19A1) is responsible for the transformation of androgen precursors into estrogens. Estrogen receptor expression is highly positive in >90% of MBC cases and androgen receptor expression is present in as many as 87.6% of breast cancer cases in men (1, 31), and this may have important therapeutic implications in the follow-up of the disease. Aromatase inhibitors (AIs) are extensively used for treating hormone receptor-positive, postmenopausal women with breast cancer as well as patients with metastatic MBC. However, analyses of male patients show that men do not respond equally well when treated with AIs, while AIs can only be recommended in male patients in conjunction with a luteinizing hormone-releasing hormone agonist (32). In this setting, their use in MBC is not substantiated by data deriving from prospective clinical trials but is based on similarities to postmenopausal FBC. According to our results following MALDI-TOF-MS

analysis, serum CYP19A1 protein levels were similar between the two groups. This finding question the extrapolation of data and treatment effects of AIs from clinical studies in women, especially after considering the distinctive molecular and endocrine profile of the disease in men. ELISA assay confirmed our initial results, even though the target protein concentration was very low. The low levels detected may be due to tumor accumulation of these estrogens, since studies in postmenopausal women have shown that the levels of estrogens in breast cancer tissues can be several-fold higher than those in blood (33).

Finally, four proteins were detected only in the MBC group and not in the control samples: actin-related protein 2/3 complex subunit 4 (ARPC4), dual specificity mitogen-activated protein kinase kinase 4 (MP2K4), ectoderm-neural cortex protein 1 (ENC1), and matrix metalloproteinase-27 (MMP27). Specifically, ARPC4 contributes to several physiological processes, including cell migration; abnormally activated cell mobility may eventually result in a metastatic outcome (34). MP2K4 is a member of the MAPK signaling pathway. A report on the deletion and mutation of the *MAP2K4* gene in breast cancer (35) does not seem to concur with our results on MBC but suggests the implication of MP2K4 in tumor suppression. ENC1 has been previously detected in breast cancer cell lines, and its expression level

was higher compared to normal breast epithelial cells; our findings concurs well with this (36). The same authors suggested that ENCI enhances oxidative stress response in breast cancer cells *via* the nuclear factor (erythroid-derived 2)-like 2 (NRF2) pathway, identifying an important biological role of this protein. Regarding MMP27, although MMPs were initially identified as markers of poor prognosis for patients with breast cancer, subsequent clinical trials have been disappointing (37). Further research better defined the key role of MMPs as breast cancer promoters, highlighting the importance of these molecules (38).

Concerning the limitations of our study, we need to point out that the number of patients with MBC was small (n=11) due to the low incidence of breast cancer in men (fewer than one per 100,000 men in Europe) (2, 39). Despite the small number of patients tested and the potential influence of selection bias, we obtained reproducible results.

In conclusion, our results provide important evidence that there is a differential serum protein expression profile in men with breast cancer compared to healthy individuals. Our studies, therefore, address the need to manage MBC optimally, by showing the feasibility of identifying potential biomarkers associated with MBC using proteomics. This approach may ensure that the treatment of all patients with breast cancer, regardless of their sex, can be accomplished *via* a personalized approach.

Conflicts of Interest

The Authors declare that they have no potential conflicts of interest.

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