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Identification of vitronectin as a potential non-invasive biomarker of metastatic breast cancer using a label-free LC–MS/MS approach

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Abstract

Background Breast cancer (BC) is a complex heterogenous disease that is a leading cause of death in women. For patients with early stage disease following primary BC therapy, approximately 30% will develop metastatic BC (MBC). The median survival of MBC patients is ~ 2–3 yr. While the early detection and monitoring of BC progression have improved prognosis and reduced BC-related mortality, there is a lack of long-term surveillance strategies for monitoring patients for recurrence of MBC. The aim of our study was to identify non-invasive urinary biomarkers for detection and monitoring of MBC.

Methods We have conducted a comparative label-free LC–MS/MS analysis of the urinary proteome of patients with MBC and healthy age-matched, sex-matched controls (HC). A hybrid quadrupole time of flight (Q-Tof™) mass spectrometer was used for urine analysis via liquid chromatography (LC) with tandem mass spectrometry (MS/MS). Retrospective analysis of urine samples from MBC and locally invasive breast cancer (IBC) patients as well as HC was conducted. Diagnostic accuracies of candidate markers were validated using independent training and validation sets according to the REMARK criteria.

Results Using this approach, we have identified 212 urinary proteins of which 83 and 25 were unique to the MBC and HC groups, respectively. Upregulated proteins in the MBC cohort were associated with angiogenesis, Ca^{2+} homeostasis, apoptosis, proteolysis, extracellular matrix regulation, cell adhesion and protein synthesis pathways. A specific non-invasive metastasis signature comprised of candidate biomarkers (urinary CALB1, S100A8, ZAG, VTN and TN) were validated and analyzed via monospecific ELISA assays. Urinary vitronectin (uVTN) levels correlated with disease status and were significantly higher in samples from MBC compared to those from IBC patients and HC. uVTN alone (cutoff > 500 ng/ml) could discriminate between HC and MBC groups ($AUC = 0.782$, $P < 0.001$). Longitudinal analysis of samples from MBC patients indicated a strong correlation between uVTN levels and disease status.

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Conclusions Our findings suggest that uVTN is a promising and non-invasive biomarker for the diagnosis and monitoring of MBC. While future validation in larger cohorts should be done, these results identify a novel urinary protein that represents the first non-invasive diagnostic test for monitoring BC progression and recurrence.

Keywords Liquid chromatography with tandem mass spectrometry (LC–MS/MS), REMARK, Urinary biomarkers, Metastatic breast cancer, Surveillance, Vitronectin

Background

Breast cancer (BC) is a major public health problem being the most common cancer for women in the United States (http//seer.cancer.gov) and the leading cause of cancer death among women worldwide [1–3]. With the widespread use of mammographic screening, primary BC can now be detected at early time points such as ductal carcinoma in-situ (DCIS) or locally invasive BC (IBC; Stages I-III). Once BC is biopsied and diagnosed pathologically, treatment includes surgical excision and/or neoadjuvant therapies [4]. While ~10% of all metastatic BC is found at the time of initial diagnosis (de novo MBC) [5], it is estimated that up to 30% women with initially locally confined, non-metastatic disease will develop recurrent disease at distant sites (rMBC) [6–10] with bone, brain or viscera being the common sites for BC metastasis [11, 12]. While the 5-yr overall survival (OS) for BC patients has improved for localized and regionally metastasized BC (~ 99% and ~86% OS respectively), in the case of recurrent metastatic BC (rMBC), the OS remains significantly lower (≤ 30%) [13] and median survival after rMBC diagnosis is quite dismal at ~2–3 yr [14–16]. In rMBC, skeletal locations are often the first and most common sites for metastasis as compared to brain or visceral metastases [12, 17]. In addition, underlying obesity and higher BMI (body mass index) in BC patients markedly increases not only the risk of developing BC, but also the incidence of distant metastasis [18, 19]. Distinct BC subtypes differ in their metastatic behavior, for example, luminal A/B BC is predisposed to bone metastasis whereas HER2 + and TNBC BC subtypes are significantly associated with liver and brain metastasis, respectively [20–22].

Despite the risk of relapsing with distant metastases, current strategies for monitoring BC recurrence or metastasis are inadequate. For patients with hormone-receptor negative BC the risk of recurrence is typically high during the first two years after initial diagnosis [11], whereas for patients diagnosed with hormone-receptor positive disease the annual risk of relapse remains constant and may occur up to a decade later [23]. The National Comprehensive Cancer Network (NCCN) post-therapy follow-up guidelines for women with BC are confined to a physical examination and symptom assessment followed by an annual mammography or MRI imaging for patients at a higher risk of recurrence [24] conducted semiannually for the first 5 years. Current surveillance strategies for detecting distant rMBC lack any form of systemic surveillance [14, 16, 25] in the form of circulating tumor markers or whole body imaging such as PET/CT or ultrasound. While early randomized clinical trials did not demonstrate any benefit from screening for BC recurrence [26, 27] on the OS of BC patients, these studies were conducted when few treatments were available for rMBC or oligometastatic disease. Recent advances in treatment of local, regional and contralateral recurrences as well as oligometastatic disease [28, 29] now offer the possibility of improving OS of such patients and are therefore transforming BC follow up strategies. While genotyping of the primary breast tumor can establish a patient's personal risk score to determine the potential of a future metastatic relapse (Oncotype Dx, MammaPrint) [30–32] and the presence of certain tumor mutations may predict the risk of bone metastasis [33, 34], however, routine full-body imaging procedures or FDA-approved clinical biomarkers that would allow monitoring of patients for MBC recurrence are currently lacking. Identifying BC patients with asymptomatic recurrence or oligometastatic disease early would enable the use of effective systemic therapies or other treatments earlier and improve the long term outcome for these patients [35]. This underscores an urgent need for tools providing successful early detection and metastasis prediction via systemic monitoring for potential relapses in a secure, easy and cost-efficient way for BC patients, their families and physicians [16, 35].

As a liquid biopsy approach, urine can be used for the non-invasive diagnosis of a variety of cancer types. Urine is obtained non-invasively, can be collected repeatedly, without the need of a healthcare practitioner and allows for serial sampling and multimodal analysis. Urine contains a variety of proteins, polypeptides, cell-free DNA (cfDNA) and extracellular vesicles that can serve as disease sentinels. Urinary proteomic studies have been successfully used to identify novel markers of cancer diagnosis and progression [36–38]. We have previously reported the discovery and validation of non-invasive urinary biomarkers for BC as well as other types of cancer [16, 39–44]. While some proteins in urine have been reported in cases of BC-related bone turnover or skeletal

metastasis [45–49], none of these proteins have been translated to the clinic and, to date, no biomarkers exist for BC metastasis.

The goal of this study was to identify and characterize the urinary proteome of patients with MBC, compare it to that of HC via a label free LC–MS/MS proteomics technique and identify novel proteins associated with MBC, followed by a two-step validation according to the REMARK ("REporting recommendations for tumor MARKer prognostic studies) guidelines [50, 51] in order to identify novel, non-invasive biomarker(s) to monitor BC recurrence. Using this approach, our findings suggest that urinary vitronectin (uVTN) may serve as a biomarker for the detection of MBC. While future validation in larger cohorts should be done, these results identify a novel urinary protein that represents the first non-invasive diagnostic test for monitoring BC progression and recurrence.

Methods

Study population

One hundred and forty-three samples were analyzed ($n=143$) including samples from patients diagnosed with IBC ($n=50$), MBC ($n=43$) and age- and sex-matched healthy controls ($n=50$) (Supplemental Table S1). All diagnoses were confirmed by biopsy. Specimens were obtained prior to surgical or other therapeutic intervention according to the bioethical guidelines of the Institutional Review Board at Mount Auburn Hospital and Boston Children's Hospital.

Urine collection and processing

All samples were collected and handled according to the institutional bioethical guidelines pertaining to discarded materials as previously reported by us [43, 44, 52]. Urine was tested for the presence of blood and leukocytes using Multistix Strips (Bayer, Elkhart, IN) as previously reported [43, 44, 52] and positive samples were excluded from the study.

Multidimensional liquid chromatography with tandem-mass spectrometry (LC–MS/MS)

For proteomics analysis, urine samples from HC and MBC samples ($n=1$/group, ~10–20 ml urine/sample, ~1 mg total protein/sample) were analyzed in the following manner (i) unfractionated or (ii) sequentially fractionated (Blue Sepharose FF, Protein A Sepharose FF, Pharmacia) to remove abundant proteins such as albumin and immunoglobulin prior to analyses by mass spectrometry. Urine samples were desalted and concentrated using ultrafiltration with 5 kDa MWCO Vivaspin columns. Precipitates formed during the process were removed by a centrifugation at 10,000 rpm for 10 min. Protein concentration of urine was determined via the Bradford method using bovine serum albumin as the standard as previously reported [43, 44, 52]. The concentrated urine protein samples (25 µL) were spiked with 25 µL of 0.2% aqueous solution of (w/v) RapiGest™ (Waters Corporation, Milford, MA). Sample was heated to denature the proteins (80 °C for 15 min). After cooling 2.5 µL of 100 mM dithiothreitol (Sigma-Aldrich, St. Louis, MO) was added to reduce the protein disulfide bonds (60 °C for 30 min). The proteins were subsequently alkylated by adding 2.5 µL of 200 mM iodoacetamide (room temperature, 30 min). Digestion was performed with Promega sequencing grade trypsin (20 µg trypsin vial was resuspended in 45 µL of 0.1% acetic acid; 1 µL of trypsin was added to each sample) at 37 °C for 8 h. The digestion mixture was acidified with 1 µL of 99% formic acid to hydrolyze the RapiGest detergent prior to LC/MS analysis [53]. The digested peptide sample (62 µL) was diluted with 150 µL of 0.1% formic acid in 30% acetonitrile and purified using Oasis MCX 96-well µElution Plate (removal of RapiGest residues and buffers was crucial to maintain the quality of nanoLC MS analysis). The Oasis MCX 96-well plate wells were conditioned with 200 µL of methanol and equilibrated with 200 µL of 0.1% FA in water prior to sample loading. Loaded wells were washed with 200 µL of 0.1% FA in water followed by 200 µL of methanol. The peptides were eluted with 2× 50 µL of 5% ammonium hydroxide solution (20-fold dilution of concentrated 28% ammonium hydroxide) in methanol. Samples were evaporated to dryness and reconstituted in 20 µL of 0.1% FA in 1% acetonitrile. 2 µL of sample was injected for nanoLC MS/MS analysis (for example, see Supplemental Figure S1). nanoLC MS/MS experiments were performed using a NanoAcquity UPLC system equipped with a NanoAcquity Binary Solvent Manager pump, Sample Manager and Column Heater. An ACQUITY UPLC M-Class Peptide BEH C18 Column, 130 Å, 1.7 µm, 75 µm X 100 mm (Waters) was operated at 40° C at a flow rate 0.3 µL/min. Solvent A was 0.1% FA in water, solvent B was 0.1% FA in acetonitrile. The gradient was 0–40% B in 1–97 min followed by a 3 min wash with 80% B and column equilibration at initial gradient conditions for 15 min.

Biomarker analysis

For analysis, urine samples were thawed on ice and repeat freeze–thaw cycles were avoided as previously reported [39, 42, 43, 52]. Monospecific ELISAs were used to quantify levels of vitronectin (Human Vitronectin, Catalog #: ELH-VTN-1, RayBiotech, Peachtree Corners, GA), tetranectin (Human CLEC3B/Tetranectin, Catalog #: LS-F2916-1, LifeSpan BioScience Inc., Lynwood, WA), zinc-alpha-2-glycoprotein (Human ZAG, Catalog #: EIA-ZAG-2, RayBiotech), Calgranulin A/S100 A8) (human

Calgranulin A, Catalog #: DS8900, R&D Systems, Inc., Minneapolis, MN) and Calbindin D (Quantikine Human Calbindin D ELISA, Catalog #: DCALD0, R&D Systems, Inc.) as previously reported [41, 44, 52]. Specimens were analyzed in duplicate and CV values < 20% were used to reduce variation. Standards, urine specimens and reagents were prepared according to the manufacturer's instructions.

Data analysis and bioinformatics

LC MS data were collected in the data independent MS/MS mode proposed by Silva et al [54]. and Geromanos et al [55].. This data collection method uses an alternate scanning between low energy (intact peptide precursor MS data) and elevated energy (fragmented peptides MS/MS data). The assignment of fragments to precursor data is achieved via time alignment of precursor and fragment data in the LC chromatogram domain. In order to eliminate run to run peptide/protein identification variability usually observed for tandem mass spectrometry (MS/MS), alternative low and elevated energy scanning modes were used for parallel monitoring of precursors and their fragment ions. The precursors and their corresponding high-energy ions were linked via a prototype in-house programmed software (Waters Corporation) using accurate mass and retention time parameters. The LC MS experiments were conducted in triplicate analyses. The false positive identification rate was 0% when considering only the proteins identified in 2 out of 3 LC MS experiments [53]. Proteomics data including peptide precursor and fragmentation data are provided in Supplemental Table S4. Peptides were searched against the Swiss Prot Human Database with a subset of 735 human proteins (Supplemental Table S5). The false positive identification rate was assessed by inserting an equal number of random protein sequences (735) into the database of 735 urine proteins.

The Ion Accounting (IA) algorithm was used for database search [56]. The IA approach matches the theoretical tryptic peptide b/y fragment ions with its precursor ion signal starting from the highest abundance signals. Due to the probability of multiple precursors eluting at any given time, the matched b/y ions are assigned to a dominant precursor first followed by the precursor and b/y ions then being removed from the list and the peptide identification search cycle is repeated with the subsequent dominant ion precursor present in the MS spectrum (and b/y ions in MS/MS spectrum). B/y ions are produced by collision induced fragmentation of the peptides and are two fragments generated by the breakage of the peptide bond(s) and can be used to elucidate the primary peptide sequence. The rules for confident identification include the robustness of the matches in the replicate experiments. We accepted the peptide identifications based on mass precursor (MS data) and at least three MS/MS fragments derived from the precursor all detected within the 10 ppm mass accuracy. The peptide identifications were validated by replicate experiments. Only peptides detected in at least two out of three LC–MS/MS replicate experiments were considered as confident hits and counted towards protein identification. The implementation of the IA algorithm was described by Li et al. [56]. The data independent MS/MS data acquisition can provide quantitative information about the proteins (Silva et al [54]. and Geromanos et al. [55]), however, we did not perform MS quantitation in this experiment. Instead, we utilized the selective depletion of the dominant proteins from the sample prior to tryptic digestion. This well accepted strategy decreases the potential for peptide MS signal suppression of the low abundance proteins due to the overwhelming signals of the dominant proteins. Targeted removal of the dominant proteins from the sample increases the chances for identification of the low abundance proteins of interest.

We applied the STRING: functional protein association network analysis to determine global protein networks including both physical and functional interactions of MBC biomarker candidates included in the general metastasis signature identified in this study. The STRING database v11 (https://string-db.org/) compiles, scores and integrates protein–protein association networks based on experimental data, literature survey and computational predictions [57]. The following parameters were noted: number of nodes identified:17, number of edges:33, average node degree:3.88, average local clustering coefficient:0.544, expected number of edges:4 and PPI enrichment P-value: $< 1.0e^{-16}$.

Statistical analysis

Samples were analyzed in a double-blind manner. To determine statistically significant differences for experiments with two groups, the nonparametric Mann-Whitney U-test was applied to compare median and interquartile range for urinary biomarkers between MBC and HC groups. To determine statistically significant differences for experiments across the 3 groups (HC, IBC, MBC) the Mann–Whitney using the Kruskal–Wallis test was performed [58]. Receiver operating characteristic (ROC) curve analysis was applied to determine area under the curve (AUC) to assess predictive accuracy and Youden's J-index was used to identify the optimal threshold biomarker cutoff value for maximizing optimal classification accuracy of cancer patients and healthy controls. The likelihood ratio for a positive test was calculated as sensitivity/(1-specificity) [59]. Samples were randomly assigned as part of a training set (~ 70%) or a downstream

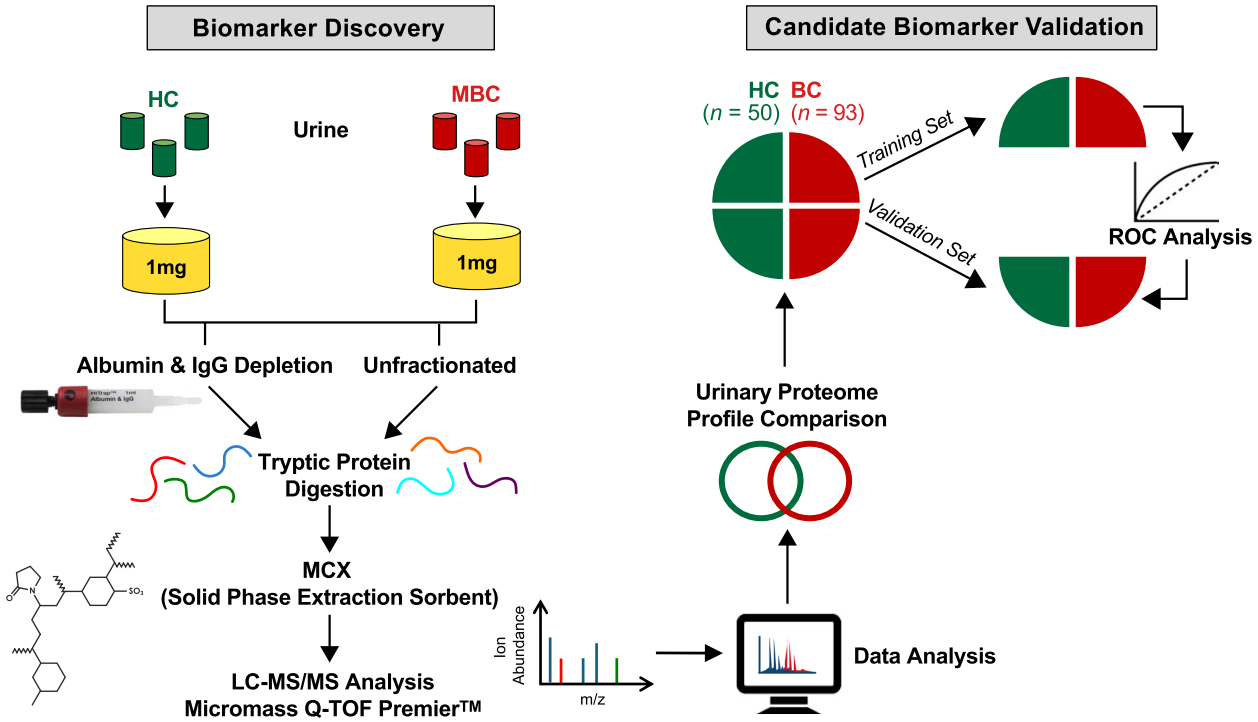

Fig. 1 Workflow of identification and validation of urinary biomarkers for MBC

independent validation set (~ 30%). Using data from training and validation sets, multivariable logistic regression modeling was implemented with age and VTN concentration (ng/ml) as covariates to obtain probabilities of MBC for combinations of age and biomarker values with 95% confidence intervals. Statistical analysis was conducted using Stata software (version 17, Stata Corp LLC, College Station, Texas).

Results

Discovery and selection of urinary biomarkers of MBC via LC–MS/MS

To discover novel urinary biomarkers for metastatic breast cancer (MBC), we conducted a comparative analysis of the global urinary proteome of patients with MBC and HC (Workflow; Fig. 1). Using this stringent approach, we have identified 212 proteins in urine from MBC patients and HC combined. Fifty two proteins were common between the groups and 25 were uniquely present in HC whereas 83 were only identified in the MBC group (Fig. 2A, Supplemental Table S2). Since albumin and IgG comprise the most abundant proteins in urine [60] and their presence could potentially mask the identification of low-abundance proteins, we also conducted stepwise depletion of these two proteins prior to MS analysis of urine samples. Interestingly, the number of unique peptides identified did not differ significantly between albumin- and immunoglobulin-depleted versus undepleted samples (Supplemental Fig. S2). We focused on the proteins uniquely identified in the MBC group which contribute to metastasis-associated functions such as cell proliferation, metabolism and extracellular matrix (ECM) turnover (Fig. 2B). Bioinformatic analysis of the data using the PANTHER classification system was used to place the identified proteins into putative functional categories (Fig. 2C). Proteins associated with angiogenesis, transcription, stress response, proteolysis and cellular adhesion were upregulated whereas proteins

(See figure on next page.)

Fig. 2 Selection of MBC biomarker candidates. Venn diagram indicating proteins identified in MBC and HC groups (**A**). Functional distribution of proteins identified uniquely in the MBC group (**B**). Bioinformatic analysis of the data using the PANTHER classification system to identify putative functional categories of identified proteins (**C**). General metastasis signature from the proteins uniquely identified in the MBC samples (**D**). Workflow for candidate biomarker selection and validation using the REMARK criteria (**E**). Preliminary analysis of uZAG, uVTN and uTN indicated significant differences between HC ($n = 13–20$) and MBC ($n = 14–19$) groups (**F–H**). Mean ± SD, groups compared via Nonparametric Mann Whitney test (ns, not significant, *$P = 0.018$, **$P = 0.0005$)

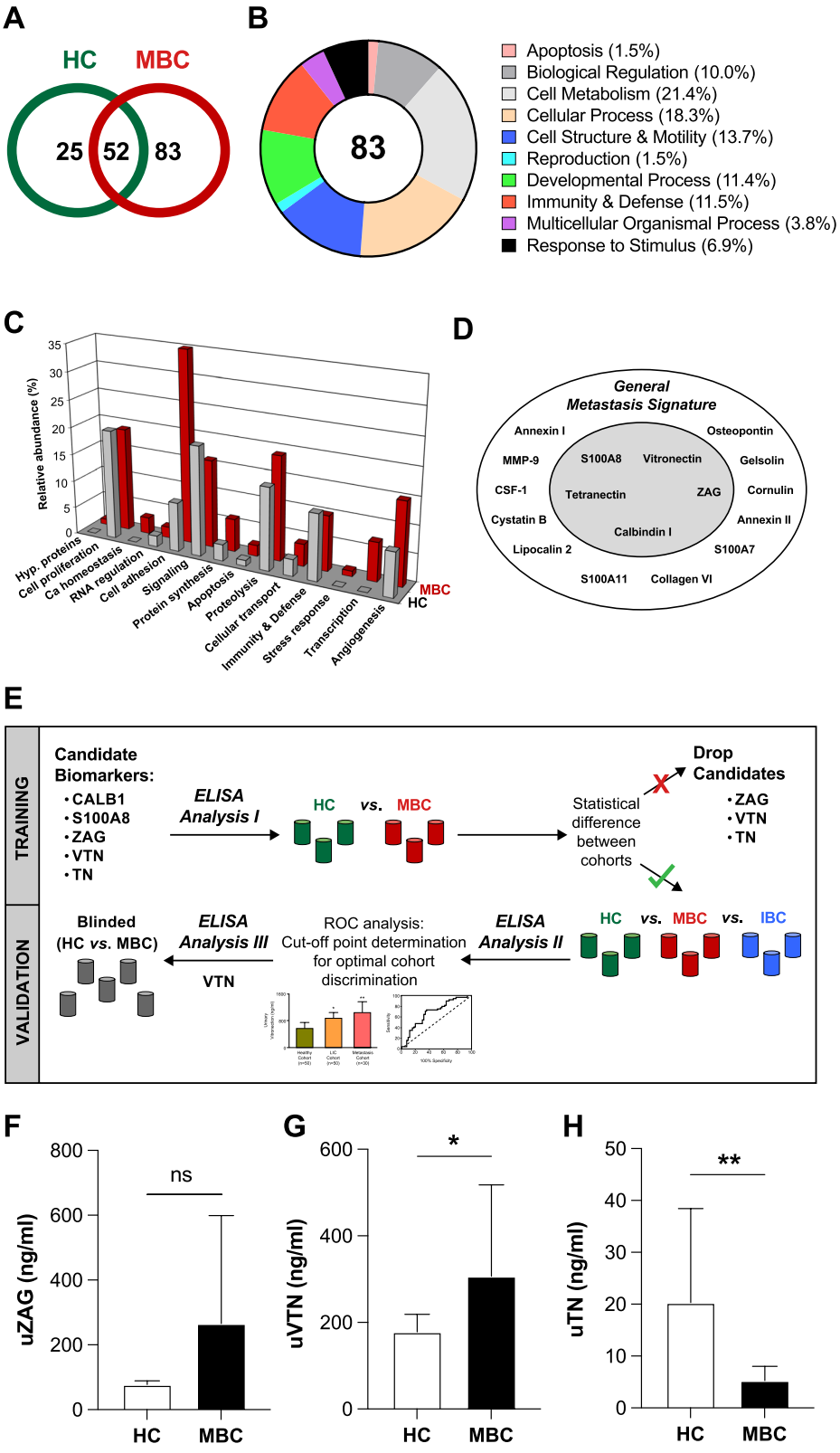

Fig. 2 (See legend on previous page.)

contributing to immunity and defense were downregulated in urine from MBC patients compared to HC (Fig. 2C). Approximately 15% of the unique proteins in MBC urines were involved in metabolic and cellular processes and in particular, calcium homeostasis and skeletal remodeling (Fig. 2C). Since we were interested in biomarkers of MBC, we next conducted in silico and literature reviews and narrowed down a general metastatic signature from the proteins that had been uniquely identified in the MBC samples (Fig. 2D).

STRING analysis of the general metastasis signature list indicated association between most of the identified candidates (Supplemental Fig. S3). Biological process enrichment pathways included cell adhesion, regulation of proteolysis, regulation of cell migration and wound healing. Among the 17 proteins were lipocalin 2 (neutrophil gelatinase-associated lipocalin, NGAL) and MMP-9, two proteins already identified as cancer biomarkers by us and others [39, 61–64] supporting the accuracy of the approach of this study. Annexin I, annexin II, CSF-1, cornulin, gelsolin, osteopontin, S100 A7, S100 A11, collagen VI and cystatin B were excluded from further validation due to either their known high-abundance and low specificity [65, 66] or the limited antibody/reagent or immunoadsorbent assay availability at the time of analysis. S100 A8, calbindin I (CALB1), tetranectin (TN), vitronectin (VTN) and zinc alpha 2-glycoprotein (ZAG) had not been previously investigated in urine of MBC patients and were included in our subsequent training and validation phases (Fig. 2D, Workflow; Fig. 2E).

For the initial candidate biomarker selection, a small number of HC ($n=$ 13–20) and MBC ($n=$ 14–19) urine samples were analyzed using quantitative ELISAs (Supplemental Table S3, Fig. 2F-H). Of the five candidates tested (Fig. 2E), CALBI and S100 A8 did not show statistically significant differences between the groups (Supplemental Table S3), whereas ZAG and VTN were 1.8—fourfold higher in the MBC cohort, whereas TN levels were 7.6-fold lower in the MBC groups compared to HC (Fig. 2F-H, Supplemental Table S3). Of the five biomarkers initially tested, since analysis indicated statistically significant differences in VTN and TN and an increasing trend in ZAG, these were then tested in the next phase of training and validation in a larger cohort of samples including HC, MBC as well as samples from patients with IBC.

Vitronectin is a biomarker of MBC

Next for the training set, we expanded the number of samples analyzed as indicated in Supplemental Table S1. We observed that uVTN levels were threefold higher in urine from MBC patients whereas uTN levels were sixfold higher in HC (Table 1 and Fig. 3A-B, D).

Table 1 Comparison of Urinary Biomarkers Between MBC and HC Cohorts (Training)

Variable	HC ($n=30$)	IBC ($n=30$)	MBC (n = 29)	*P*
Age, mean ± SD	52 ± 7	52 ± 7	62 ± 9	0.03*
Vitronectin, ng/ml	300 (140–840)	600 (320–1000)	1000 (400–1600)	0.05*
ZAG, ng/ml	20.1 (0–95)	0 (0–156.1)	85 (0–1596)	0.24
Tetranectin, ng/ml	6.4 (0–21.5)	0 (0–12.7)	0.0 (0–2.7)	0.04*

Biomaker data are median (interquartile range). *P* values using Kruskal–Wallis test. *Statistically significant

While uZAG levels trended fourfold higher in MBC samples, the differences were not significant (Table 1 and Fig. 3A). In order to evaluate the statistical parameters for clinical relevance, the ROC curve was plotted for the levels of uVTN and uTN in MBC patients and HC. Using logistic regression, we determined that uVTN > 500 ng/ml or uTN < 2.6 ng/ml were associated with sensitivity of ~ 60% and ~ 72% respectively for the presence of MBC in the training set (Table 2). Urinary VTN alone (cutoff > 500 ng/ml) displayed an AUC of 0.677 and 0.782 in the training and validation sets respectively and were associated with a sensitivity of 79% and a specificity of 65% for the detection of MBC (Table 3, Fig. 3E). However, uTN levels did not provide discrimination between HC and MBC groups in the validation set (Table 3, Fig. 3C).

Multivariable logistic regression of the combination of uVTN levels and age provided an accuracy of AUC = 0.728 and AUC = 0.781 in the training and validation cohorts, respectively. Using multivariable logistic regression, model-based probabilities of MBC (*vs.* HC) were calculated for combinations of age and uVTN (Supplemental Fig. S4 A, B). Older age and higher uVTN were associated with increased odds of MBC. It was estimated that odds for women of 40 years of age and uVTN of ~ 250 ng/ml are associated with a 19.8% model-based probability of MBC, whereas age 70 years and uVTN of ~ 1500 ng/ml are associated with an ~ 73.9% probability of MBC. Moreover, for women of a similar age, the probability of MBC increases significantly for higher levels of uVTN. For example, in the age category close to 50 years, a woman with a uVTN level of 250 ng/ml has a corresponding 28% probability of MBC whereas a woman with a level of 1500 ng/ml has an estimated 53.3% probability of MBC. Therefore, independent of age (i.e. for any given age), a higher level of uVTN (ng/ml) increases the probability that the sample was obtained from a woman with MBC than a HC (Supplemental Fig. S4 A, B). uTN (ng/ml) levels

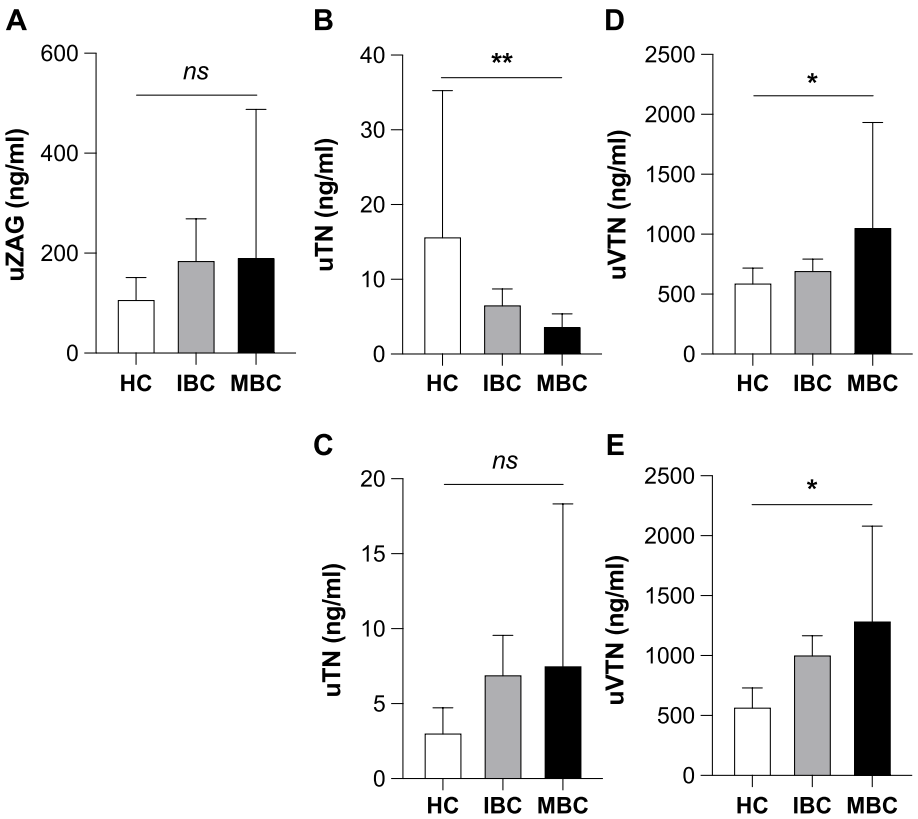

Fig. 3 Validation of MBC biomarkers. Urinary ZAG levels did not differ significantly between the groups (**A**). Urinary TN levels were significantly lower in IBC and MBC cohorts for the training set (**B**), however did not differ significantly in the validation set (**C**). Urinary vitronectin levels were significantly higher in IBC and MBC groups in both training (**D**) and validation sets (**E**). Training set included HC = 30, IBC = 30 and MBC = 29 samples and validation set included HC = 20, IBC = 20 and MBC = 14, respectively. Mean ± SD, groups compared via Kruskall-Wallis test (ns, not significant, $*P \leq 0.05$, $**P < 0.005$)

Table 2 Probability of MBC Based on Urinary Biomarkers (Training)

Biomaker	AUC (95% CI)	Sensitivity (%)	Specificity (%)
Age, (> 56 y)	0.690 (0.553, 0.828)	62	71
Vitronectin, (> 500 ng/ml)	0.677 (0.536, 0.818)	60	60
Tetranectin, (< 2.6 ng/ml)	0.307 (0.179, 0.435)	72	60

Table 3 Probability of MBC Based on Urinary Biomakers (Validation)

Biomaker	AUC(95% CI)	Sensitivity (%)	Specificity (%)
Age, (> 56 y)	0.647 (0.460, 0.853)	54	65
Vitronectin, (> 500 ng/ml)	0.782 (0.624, 0.941)	79	65
Tetranectin, (< 2.6 ng/ml)	0.646(0.469, 0.824)	43	25

Area under the curve is the predictive accuracy as determined by receiver operating characteristic (ROC) curve analysis

were lower in women with IBC and MBC than HC but this was not a useful predictor.

Vitronectin levels may predict BC relapse and correlate with disease status in MBC

Analyzing publicly available gene expression datasets

via the R2 platform (Tumor Breast Relapse dataset Smid et al.'R2: Genomics Analysis and Visualization Platform (http://r2.amc.nl), we found that high VTN expression in breast tumor tissues is associated with shorter relapse-free survival in BC patients (Supplemental Fig. S5 A). Median relapse-free survival was estimated to be ~19.5 months for patients with high tumor VTN expression levels (≥ 1.5 RU, Supplemental Fig. S5 A) compared to ~29 months for those with low VTN expression. Finally, in a small subset of MBC patients, we measured uVTN levels over a follow up period. Longitudinal analysis of uVTN indicated that in 50% (5/10) of patients, uVTN levels correlated with disease status (Supplemental Fig. S5B). Longitudinal uVTN levels were reduced in patients with stable disease or those with a clinical therapeutic response, whereas clinical disease progression was associated with increased uVTN levels (Supplemental Fig. S5B).

Discussion

While there have been many advances in BC diagnosis in recent years, circulating biomarkers that would enable the monitoring of patients for MBC recurrence are currently lacking. Urine serves as a non-invasive source for liquid biopsies and is therefore ideal for the frequent monitoring and clinical management of patients. In this study, we report that uVTN is a novel biomarker that can identify the presence of MBC and may be helpful in identifying BC recurrence and progression. In the context of this study, discovery of urinary biomarkers was conducted via label-free Q-Tof LC–MS/MS analysis. This approach is useful for the simple and robust identification of previously unknown protein biomarkers or confirmation of putative biomarkers when contrasting the HC samples to MBC samples. In addition, data-independent MS/MS acquisition enables direct quantitation of the proteins in the sample without a need for isotopic labeling [54]. From the 83 unique proteins identified in the MBC samples and using in silico/literature-based analyses, we created a general metastasis signature for MBC. A major challenge in proteomic biomarker discovery lies in the validation phase – where there may be a lack of immunological assays that can quantitatively measure candidate markers. Based on abundance, specificity and reagent availability, we tested a subset of candidate biomarker proteins (CALB1, S100 A8, ZAG, VTN and TN) in a stepwise manner in independent training and validation cohorts as per the REMARK criteria [51]. While CALB1 and S100 A8 did not show significant differences between MBC and HC groups, ZAG, VTN and TN were further analyzed in a larger training sample set. Quantification through mono-specific ELISA analysis confirmed significantly upregulated uVTN in IBC and MBC patients as compared to HC. While uZAG levels trended 1.8-fold and fourfold higher in the initial biomarker selection phase and training cohort respectively, the differences were not statistically significant. uZAG has been previously shown to have discriminative potential for early prostate cancer using digital rectal exam (DRE) samples [67]. ZAG expression is upregulated in TNBC cells lines and has been linked to poor prognosis in TNBC patients [68]. ZAG expression has been reported to promote cancer-associated fibrosis and is linked to poor prognosis in TNBC patients [68]. Similarly, in the current study uTN levels were found to be 7.6-fold and sixfold lower in the initial biomarker selection phase and training cohort respectively, however, these findings were not replicated in the validation cohort where uTN did not provide discrimination between HC and MBC groups. We speculate that the most probable cause for these results may be a change in uTN detection methods, as the original vendor discontinued reagents necessitating a change in ELISA providers midpoint of our study. Future studies should reexamine the efficacy of uZAG and uTN as independent markers for MBC or in combination with uVTN using more robust detection methods and larger patient cohorts.

Our results determined that of the markers tested, uVTN levels were significantly higher in MBC patients compared to HC. Median urine concentrations of uVTN in both the training and the validation sets were 2.7- and 4.2-fold higher compared to HC respectively. Based on our independent validation set, we found that uVTN could discriminate between MBC and HC with 0.782 accuracy (cutoff > 500 ng/ml) and 79% sensitivity and 65% specificity respectively. Currently there are very few to no diagnostics available or being used for the detection and/or longitudinal monitoring of BC recurrence. Recently, the utility of several circulating tumor DNA (ctDNA) assays has been explored for the detection of BC relapse or of molecular residual disease [69–71]. While the classical serum-based BC markers such as carbohydrate antigen 15–3 (CA15-3), carcinoembryonic antigen (CEA) and carbohydrate antigen 125 (CA125) are not routinely recommended for detecting BC or monitoring therapy, they have been evaluated independently and in combination for BC surveillance. Serial evaluation of combined CA15-3 and CEA levels was shown to be a significant predictor of BC recurrence [72]. Individual serum markers tissue polypeptide specific antigen (TPS), CA15-3, CEA and CA125 when evaluated for diagnostic value in distant MBC displayed AUC of 0.754, 0.821, 0.755 and 0.651 respectively [73]. Serum periostin (POSTN), AUC 0.78, has been described as a novel biomarker complementing CA15-3 and CEA for MBC prediction, indicating that elevated levels were associated with poor overall

survival and progression-free survival [74]. Therefore, in this context, uVTN displays informative predictive accuracy with the added advantage of providing truly non-invasive testing of patients and the potential for accurate monitoring of disease progression with serial urine testing in a setting where no such options exist.

To our knowledge, VTN has not been validated in the urine of advanced BC patients despite its association with BC and metastatic progression. Upregulated VTN serum levels have been reported in IBC patients as compared to controls, suggesting VTN as a potential BC biomarker [75, 76]. Serum VTN and $\alpha_V\beta_3$ levels were reported to be higher in Stage I-II BC patients compared to those with benign lesions [77]. VTN serum levels have also been reported to predict metastasis in early-stage melanoma. Average VTN levels were found to be higher in early-stage melanoma patients that went on to develop metastatic disease within a two-year follow-up and correlated with poor outcome [78]. VTN is a ~ 75 kDa glycoprotein present in the ECM and plays a key role in the development and progression of solid tumors including BC by promoting cell migration, invasion and differentiation of cancer stem cells [79–81]. Interestingly, MMP-2 cleavage of VTN facilitated its activation and binding to a5β1 and aVβ3 integrin receptors and promoted its effect on cancer cell migration and a blockage of VTN and its receptors in ovarian cancer cells were reported to significantly inhibit cell migration [81]. Neutralization of VTN and its receptors in ovarian cancer cells was reported to significantly inhibit cell migration [80]. VTN also promoted VEGF-induced angiogenesis [82]. VTN is a component of mineralized bone and plays a role in bone metabolism [83]. Given the timing and frequency of osseous metastases associated with BC, validation of VTN in this patient population would be of interest in the future. Taken together, the urinary MBC signature proteins identified and validated in the current study, uVTN, uTN and uZAG, are known to play an active role in promoting tumor cell malignancy and their presence in urine may therefore serve as sentinels of BC disease status, tumor growth and metastasis.

Early diagnosis of asymptomatic recurrence may improve long-term outcome for MBC patients. For example, serial biomarker monitoring after primary surgery or adjuvant therapy could potentially identify recurrence at an early stage with minimal disease burden when treatment can lead to successful disease control for MBC patients. Therefore, identification of optimal biomarkers with high specificity and sensitivity for BC recurrence and that may be repeatedly and non-invasively analyzed along with imaging during surveillance/follow up of BC patients are urgently needed. In the current study, STRING network protein–protein interaction enrichment analysis (p-value: $< 1.0e^{-16}$) applied to the general metastasis signature indicated significantly more interactions than expected for a random group of proteins. Such an enrichment indicates that these proteins may be biologically connected as a group. Therefore, it would be useful to explore these interacting biomarkers such as MMP9, LCN2, osteopontin and Annexin I or II as independent markers or a panel of markers for MBC discrimination in future studies.

While the findings represented here are promising, our study includes several limitations that should be considered. First, the study has been conducted with retrospective samples derived from patients with known MBC diagnosed using conventional imaging and other methods. A prospective, unbiased patient cohort would be important for the assessment of MBC markers, ideally one including patients pre- and post-treatment of primary BC as well as the longitudinal assessment of patients who progress to MBC versus those who do not. Second, combined testing of uVTN with conventional methods such as imaging or serum markers such as CA15-3, CEA etc. may provide better sensitivity. Finally, our study may be limited by the sample size, in particular of the validation cohort.

Conclusions

Our findings suggest that uVTN has the potential to serve as a non-invasive biomarker for the detection of MBC. While further validation in larger sample cohorts should be done, our results demonstrate that using a global proteomics approach combined with stepwise analysis of candidate markers is useful in identifying promising biomarkers for the early detection of BC recurrence and metastasis.

Abbreviations

BC	Breast cancer
MBC	Metastatic breast cancer
HC	Healthy control
LC–MS/MS	Liquid Chromatography Tandem-Mass Spectrometry
IBC	Locally invasive breast cancer
IA	Ion Accounting
REMARK	REporting recommendations for tumor MARKer prognostic studies
CALB1	Calbindin 1
S100 A8	S100 calcium-binding protein A8/ Calgranulin A
ZAG	Zinc-alpha-2-glycoprotein
VTN	Vitronectin
TN	Tetranectin

Supplementary Information

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Supplementary Material 1.

Supplementary Material 2.

Supplementary Material 3.

Authors' contributions
Author's contributions: Conceptualization: MAM, RR, ES Methodology: MAM, RR, ES, PO, MG, SG, GL, AD, AE, RA, SP Investigation: MAM, RR, ES, PO, MG, SG, GL, AD, AE, RA, SS, DZ, ML, SP Visualization: MAM, RR, ES, PO, MG, SG, GL, JG, AD, AE, RA, SS, DZ Funding acquisition: MAM Project administration: MAM, MG, SP Supervision: MAM, RR, MG, SG, GL, JG, SP, DZ Writing – original draft, review and editing: MAM, RR, ES, PO, MG, SP, DZ, SS.

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Data availability
The datasets supporting the conclusions of this article are included within the article and its additional files and/or are available from the corresponding author upon reasonable request. Proteomics data including peptide precursor and fragmentation data are provided in Supplemental Table S4. Peptides were searched against the Swiss Prot Human Database with a subset of 735 human proteins (Supplemental Table S5).

Declarations

Ethics approval and consent to participate
Specimens were obtained prior to surgical or other therapeutic intervention according to the bioethical guidelines of the Institutional Review Board at Mount Auburn Hospital and Boston Children's Hospital.

Consent for publication
Not appliable.

Competing interests
The authors declare no competing interests.

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