Discovery and validation of novel protein biomarkers in ovarian cancer patient urine

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This is the author manuscript accepted for publication and has undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the [Version of Record](#). Please cite this article as [doi: 10.1002/prca.201700135](https://doi.org/10.1002/prca.201700135).

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Keywords: biomarkers, urinary system, Ovarian cancer

Abbreviations: LFQ, label-free quantitation; PRM, Parallel Reaction Monitoring; FDR, false discovery rate; ROC, receiver operating characteristic

ABSTRACT

Purpose

For the vast majority of ovarian cancer patients, optimal surgical debulking remains a key prognostic factor associated with improved survival. A standardized, biomarker-based test, to pre-operatively discriminate benign from malignant disease and inform appropriate patient triage, is highly desirable. However, no fit-for-purpose biomarkers have yet been identified.

Experimental Design

We conducted a pilot study consisting of 40 patient urine samples (20 from each group), using label-free quantitative (LFQ) mass spectrometry, to identify potential biomarker candidates in urine from individual ovarian cancer patients. To validate these changes, we used Parallel Reaction Monitoring (PRM) to investigate their abundance in an independent validation cohort (n=20) of patient urine samples.

Results

LFQ analyses identified 4394 proteins (17,027 peptides) in a discovery set of 20 urine samples. 23 proteins were significantly elevated in the malignant patient group compared to patients with benign disease. Several proteins, including LYPD1, LYVE1, PTMA and SCGB1A1 were confirmed to be enriched in the urine of ovarian cancer patients using PRM. We also identified the established ovarian cancer biomarkers WFDC2 (HE4) and Mesothelin (MSLN), validating our approach.

Conclusions and Clinical Relevance

This is the first application of a LFQ-PRM workflow to identify and validate ovarian cancer-specific biomarkers in patient urine samples.

INTRODUCTION

High grade serous ovarian cancers are typically diagnosed at an advanced stage, and account for around 90% of all ovarian cancer related deaths ^[1]. For the vast majority of these patients, optimal surgical debulking is strong prognosticator positively associated with survival ^[2]; thus, the identification of malignancy, and rapid referral for specialist treatment, is crucial to maximize the impact of primary therapies. The most widely used triage system is the Risk of Malignancy Index (RMI), incorporating CA125 measurement, menopausal status and ultrasound imaging to predict malignancy^[3]. However, a lack of availability and poor imaging standardization can significantly impact RMI implementation and scoring ^[4]. The combined measurement of CA125 and HE4 in serum, the only FDA-approved biomarkers for ovarian cancer, can also be used (with menopausal status) to calculate the Risk of Malignancy Algorithm (ROMA) and stratify patients into high- and low-risk groups ^[5]. CA125 and HE4 together can also provide improved accuracy for monitoring tumor progression or recurrence. However, neither marker alone is appropriate for diagnosis; and HE4 remains an expensive test, with limited availability ^[6]. Accordingly, there remains an unmet clinical

need for new biomarkers that achieve rapid, inexpensive and minimally invasive testing to monitor high-risk populations, detect tumor recurrence, and pre-operatively discriminate between benign and malignant ovarian disease [7].

Numerous studies have sought to identify disease biomarkers in urine, with a view to the development of clinically relevant diagnostic tests [8, 9]. As a sample type, urine offers simplified, non-invasive sample collection and storage relative to blood samples [10]; moreover, urine proteins are stable over time, increasing the consistency of data in both discovery experiments and the routine measurement of analytes [11]. The urine proteome lacks very high abundant proteins to that of plasma, which typically hamper proteomic analysis [12], thus avoiding the need for extensive protein depletion [13]. Several FDA-approved urine protein biomarkers are now in clinical use for monitoring bladder cancer and the early detection of acute kidney injury [14], supporting the use of urine-derived proteins as biomarkers of disease. In recent years, both human epididymis protein 4 (HE4/WFDC2) and mesothelin (MSLN) have been extensively investigated for their diagnostic potential as urine-based disease markers for ovarian cancer [15, 16] and can correctly discriminate malignancy at similar efficacy to serum-based testing of HE4 and MSLN [8].

Label-free quantitative mass spectrometry (LFQ), whilst well-established as a proteomic discovery tool, has seen only limited application in the analysis of ovarian cancer-related proteomic changes to date [17]. Only a single study has applied LFQ to examine the urine peptidome of ovarian cancer patients *versus* healthy controls [18]. In previous work, we used isotope-coded protein labeling (ICPL) and MALDI MS/MS to identify urinary biomarkers of ovarian cancer [19]. Whilst several potential disease biomarkers – both established and novel –

were identified, both the dynamic range of the ICPL-MALDI approach and our ability to accurately quantitate changes was limited [19].

Here we report a pilot study applying LFQ, followed by targeted parallel reaction monitoring (PRM) to discover and validate ovarian cancer-related protein changes in urine samples from multiple individual patients. Discovery experiments using LFQ were conducted in a cohort of patients (n=20; cohort 1), and were followed by PRM validation using an independent patient cohort (n=20; cohort 2). The workflow identified a number of novel proteins that were significantly upregulated in the validation disease cohort. This is the first use of LFQ-PRM for ovarian cancer biomarker discovery, and establishes the methodology required for subsequent studies to evaluate specific disease biomarkers of ovarian cancer.

Materials & Methods

Clinical samples

Urine was accessed from bio-banked samples, collected prospectively from women undergoing surgery for suspected gynaecological malignancies during the period 2008 – 2015. Information regarding collection and purification of urine proteins is located in supplementary information. Ethical approval for this study was obtained from the Southern Health Human Research Ethics Committee (HREC certificate #06032C), and all participants providing prior informed written consent. Sample details are summarized in Table 1.

Label Free Quantitation Mass Spectrometry and data analysis

Urine protein samples were processed in 20µg aliquots resuspended in 6M Urea, 100 mM DTT and 100 mM Tris-HCl (pH 7.0) and digested with trypsin using the Filter Aided Sample Preparation (FASP) column protocol as described previously [20] (Expedeon Inc., CA, USA). Peptide

concentrations were measured using a NanoDrop 2000 (Thermo-Fisher) at 205 nm, and acidified with trifluoroacetic acid (TFA; final 0.1%). Solvent was removed in a CentriVap concentrator (Labconco) and peptides were resuspended in MilliQ water containing 3% acetonitrile (ACN) and 0.1% formic acid (FA) at a final concentration of 100 ng/μl. A 5μl aliquot of each sample was analyzed by nanoflow liquid chromatography tandem-mass spectrometry (LC-MS/MS) on a nanoAcquity system (Waters, Milford, MA, USA) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) through a nanoelectrospray ion source (Thermo Fisher Scientific). Peptides were loaded on a 20mm X 180μm trap column (2G-V/MTrap 5μm Symmetry C18) at 1% buffer B, and separated by reverse-phase chromatography using a 250 mm X 75μm column (1.7μm BEH130 C18) on a 120 minute linear gradient from 1% to 35% buffer B (A: Milli-Q water, 3% ACN, 0.1% FA; B: 99.9% ACN, 0.1% FA) at 500nl/min constant flow rate. The Q-Exactive was operated in a data-dependent acquisition (DDA) mode, switching automatically between one full-scan and subsequent MS/MS scans of the ten most abundant peaks. The instrument was controlled using Exactive series version 2.1 and Xcalibur 3.0. Full-scans (m/z 350–1,850) were acquired with a resolution of 70,000 at 200 m/z. The 10 most intense ions were sequentially isolated with a target value of 10000 ions and an isolation width of 3 m/z and fragmented using HCD with normalized collision energy of 27 and stepped collision energy of 15%. Maximum ion accumulation times were set to 50ms for full MS scan and 150ms for MS/MS. Underfill ratio was set to 2% and dynamic exclusion was set to 60sec.

Raw files were analyzed using MaxQuant^[21] (version 1.5.1.2), The database search was performed using the Ludwig NR non-redundant protein database (version Q114, http://www.wehi.edu.au/faculty/advanced_research_technologies/proteomics/wehi_systems_biology_mascot_server/) with strict trypsin specificity allowing up to 2 missed cleavages. The minimum required peptide length was set to 7 amino acids.

Carbamidomethylation of cysteine was set as a fixed modification while N-acetylation of proteins N-termini, oxidation of methionine and pyroglutamate at peptides N-termini Glu and Gln were set as variable modifications. During the main search, precursor ion mass error tolerance was set to 4.5 ppm and fragment ions were allowed a mass deviation of 20 ppm. PSM and protein identifications were filtered using a target-decoy approach at a false discovery rate (FDR) of 1%. The match between runs option was enabled with a match time window of 1 min and an alignment time window of 20 min. Further analysis was performed using a custom pipeline developed in Pipeline Pilot (Biovia) and R (see supplementary information for a detailed description). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE^[22] partner repository with the dataset identifier PXD007962.

Parallel Reaction Monitoring Mass Spectrometry and data analysis

PRM analyses were preceded by DDA to define peptide retention times. PRM samples were analyzed by nanoflow LC-MS/MS on a nanoAcquity system (Waters) coupled to a Q-Exactive mass spectrometer (Thermo Fisher Scientific) through a nanoelectrospray ion source (Thermo Fisher Scientific). Peptide mixtures were directly injected on a 250 mm x 75µm analytical column packed into a silica emitter tip (in-house; 1.7µm BEH130 C18) at 1% buffer B, and separated by reverse-phase chromatography on a 90 minute linear gradient from 1% to 35% buffer B at a 400nl/min constant flow rate. The PRM method combined two scan events starting with a full scan followed by up to 30 targeted MS/MS scans for charge 2+ and 3+ precursor ions from a scheduled precursor inclusion list with a ±5 min retention time window. Full-scans (m/z 350–1,850) were acquired with a resolution of 70,000 at 200 m/z. The PRM

scans were run at a resolution of 17,500 at m/z 200. The targeted peptides were isolated using a 2.4 m/z window. Fragmentation was performed with a normalized collision energy (NCE) of 27.

Skyline software [23] was used for analysis of PRM data. The quantification was based on the sum of the area under the curves (AUC) of three to six fragments of the selected peptides and the ratio between peptide fragments of each patient sample. Full scan data was searched using MaxQuant and feature intensities for PRM scans were normalized by converting the features identified in the allPeptides table to base 2 logarithms and then multiplying each feature value by the ratio of maximum median intensity of all replicates over median replicate intensity. Receiver operating characteristics (ROC) analysis was performed using the R package pROC [24]. ROC analysis was performed for the ovarian cancer patients against benign patients using the Log2 summed intensity values for peptides analyzed using PRM for each selected protein.

RESULTS

Experimental strategy and rationale

Urine proteomics has shown promise as a source of clinically relevant biomarkers of ovarian cancer [6]. In this study, we evaluated the application of label-free mass spectrometry and PRM to identify and validate new urinary biomarkers for the differentiation of benign from malignant ovarian disease. The overall strategy and simplified workflow is shown in Figure 1. In total, 40 urine samples (n=20 malignant, n=20 benign) were collected from individual patients for this study (Table 1) and randomly assigned into either discovery or validation sets. To provide

consistency at point-of-collection, urine samples were obtained intraoperatively from anaesthetized patients immediately prior to surgery, and processed identically. This standardization overcomes the variability introduced using typical collection methods (midstream, second morning void) reported in other proteomic studies [14]. Discovery experiments using LFQ were performed using one cohort (n=10 per group; cohort 1) to identify and prioritize proteins that were altered in malignant *versus* benign ovarian disease. The intensity distributions between patient samples demonstrated low systematic variation (Fig.2). PRM validation experiments were then performed using the independent second cohort (n=10 per group; cohort 2), providing strong evidence for selected proteins that could potentially indicate the presence of ovarian malignancy.

Identification of differentially expressed proteins by LFQ

Using the LFQ workflow we identified a total of 4394 proteins (17,027 peptides) in the discovery cohort samples, with 3750 proteins (15,666 peptides; CV 30.9%) identified from malignant and 3950 proteins (14,326 peptides; CV 21.7%) from benign groups (Fig.3A and 3B; Supplementary Table 1). Comparison of the identified species revealed an overlap of 75.2% amongst proteins (and 76.1% of identified peptides) between the two groups (Fig.3C and D). A total of 444 unique proteins (1361 peptides) and 644 unique proteins (2701 peptides) were associated with the malignant or benign sample groups, respectively.

We utilized a LFQ analysis pipeline (see materials and methods) to perform a pair-wise differential expression analysis of the two patient groups. Using this approach, 23 proteins were found to be significantly enriched in late stage relative to benign samples (Fig.4 and Supplementary Table 2 and

3). The majority of proteins highlighted have established links with ovarian cancer (references shown in Supplementary Table 3), demonstrating the effectiveness of the label-free quantitation approach to identify ovarian cancer-specific proteins enriched in the urine of patients.

Ten proteins showing significant differences between malignant and benign groups in the discovery cohort were selected for further validation (Supplementary Table 4), based on their observed enrichment in cancer patient urine and potential relevance to ovarian cancer. To evaluate their tumor specificity, we assessed their abundance and localization in ovarian tumor tissues *in silico* using data from the Human Protein Atlas (Supplementary Table 3)^[25]. Eight of the ten proteins were found to be expressed in ovarian tumor tissues, whilst two proteins (LYVE1 and SCGB1A1) were not stained; each of these, however, has previously been associated with ovarian cancers^[26, 27].

Validation of differentially expressed proteins using Parallel Reaction Monitoring

The validation of protein abundance changes in urine using antibody-based approaches is challenging^[19]. Indeed, we first attempted to validate several protein abundance changes using commercially available antibodies by western blotting, however, detection of target proteins at their expected sizes in urine samples was unsuccessful (Supplementary Figure 1). We therefore used targeted PRM to provide high sensitivity relative peptide quantification^[28]. Validation was performed using individual patient samples from cohort 2, comprising 10 benign and 10 malignant patients. The selection of quantotypic peptides^[29] for PRM required that (i) peptides were unique to the protein of interest; (ii) were previously identified in greater than 50% of samples in a patient group; (iii) were between 7-35 amino acids in length; and (iv) were not semi-tryptic.

Of the ten proteins identified as differentially expression in cohort 1 patients, significant differential expression of 7 of these candidate proteins was confirmed by PRM in the cohort 2 cancer patients for WFDC2, MSLN, LYPD1, SCGB1A1, IGFBP3, LYVE1 and PTMA (Fig.5, Table 2, Supplementary Fig.2). In the case of PTMA, only a single suitable peptide satisfied the criteria for quantotypic peptide inclusion (Table 2); nevertheless, this peptide was significantly elevated in cancer samples in agreement with the LFQ data. A comparison of the significantly differentially expressed proteins between the LFQ and PRM patient cohorts demonstrated good agreement between the two techniques (Table 3). Additionally, the degree of differential expression observed for these proteins was comparable to the established ovarian cancer urine biomarker, CA125 (Table 3, Supplementary Fig.3A). A ROC analysis was performed to assess the sensitivity and specificity of each protein in relation to ovarian cancer (Fig.6), with the results supporting the conclusions of the differential expression analysis. ROC analysis of the clinical CA-125 measurements demonstrated complete accuracy for classification of ovarian cancer patient within this cohort (Supplementary Fig.3B). These results demonstrate that we were able to quantify the reported ovarian cancer urine biomarkers, WFDC2 and MSLN ^[16], in addition to identifying five novel biomarkers that are enriched in the urine of ovarian cancer patients. Our success indicates that measuring expression of these proteins in a larger patient cohort may produce clinically applicable biomarkers of advanced ovarian disease.

DISCUSSION

The urine proteome holds significant interest as a potential source of biomarkers, and is an easily accessible fluid for diagnostic and prognostic evaluation. However, low sensitivity of detection and difficulties associated with validation can prevent the full exploitation of this bodily fluid as a novel

source of biomarkers ^[30]. Targeted mass-spectrometry methods, such as SRM and PRM, may help to increase detection sensitivity and also place less reliance on antibody validation ^[28, 30, 31].

In this pilot study, we have focused on patients diagnosed with late-stage (FIGO Stage III), high grade serous ovarian cancers (HGSOCs), compared to those with benign ovarian masses. HGSOC patients represent the largest proportion of ovarian cancers diagnosed ^[1], providing ample sample numbers for this pilot study and permitting discrete discovery and validation sets to be analyzed (4,394 non-redundant proteins / 17027 unique peptides in total; 1,388 non-redundant proteins / 9,190 unique peptides found in more than 50% of patient samples).

Amongst 23 proteins significantly increased in late stage ovarian cancer were the established ovarian cancer biomarkers WFDC2 (HE4) and mesothelin ^[16]. Each have well-documented roles in tumorigenesis, and their elevation is correlated with decreased survival rates and increased chemoresistance in epithelial ovarian cancer patients ^[8, 15, 16, 32]. Both have also been identified as potential urinary biomarkers for ovarian cancer. Urinary WFDC2 detection by ELISA, when combined with CA125, has been used to identify patients at high risk for primary platinum therapy resistance ^[32]. Urinary mesothelin detection by ELISA, in combination with CA125 detection, can aid in diagnosis and monitoring of ovarian cancer patients ^[16]. Their identification in urine using PRM, and their observed abundance increase in malignancy consistent with extensive literature, provides confidence in the use of our LFQ-PRM approach to identify urinary biomarkers.

Validation of protein abundance differences using antibody-based approaches is problematic [19]. Western blotting was unable to identify or validate protein changes, due to a lack of specificity and the detection of multiple reactive bands in urine samples. The most likely reason for this is the extensive proteolytic processing of proteins that occurs during prolonged incubation in the bladder, leading to the alteration and destruction of antigenic epitopes. Indeed, the majority of proteins in the urinary proteome are below 50 kDa due to degradation by endogenous proteases, presenting difficulties in generating the correct antibody specificity [11, 12, 33]. By contrast, a targeted MS workflow as used here can detect multiple peptides and proteoforms, without reliance on antibodies [28, 31]. An added advantage of this approach is that methods developed for successfully targeting peptides have the potential to be refined for use in a clinical setting, where selected reaction monitoring (SRM) is already in use [34].

Amongst the proteins enriched in ovarian cancer patient urine, we validated five proteins that may serve as novel urinary biomarkers. Uteroglobin (SCGB1A1) is multifunctional, hormonally regulated globular protein associated with a variety of cancers [27, 35, 36]. Displaying involvement in both hormonal response and immunity, its functions remain largely unknown [35]. In recent studies, expression analysis and immunohistochemical staining of ovarian tumor tissues identified no significant difference in uteroglobin levels between ovarian cancer tissue and normal ovarian tissue [27]. Our study and a previous analysis [18] identified the increased abundance of endogenous uteroglobin peptides in ovarian cancer patient urine samples, strongly supporting its potential as a urinary biomarker candidate for further investigation. Insulin-like growth factor-binding protein 3 (IGFBP3) is expressed in a wide variety of cancer types (including lung, liver and breast), and methylation of the *IGFBP3* gene has been identified as a prognostic marker of ovarian cancer progression [37]. LYPD1 (also known as putative HeLa

tumor suppressor (PHTS)) is a membrane bound member of the lymphocyte antigen-6 (Ly6)/urokinase-type plasminogen activator receptor (Ly-6/uPAR) protein family [38]. Ly6/uPAR proteins have diverse roles in cell migration, proliferation, immune cell maturation, macrophage activation and cytokine production [39]. *In silico* analysis of mRNA expression (from the Human Protein Atlas [25, 40]) suggests that LYPD1 is abundantly expressed in the fallopian tube, the potential origin of most high grade serous epithelial ovarian tumours [1]. Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) was previously investigated as part of a combined marker panel to evaluate lymphangiogenesis and prognosis in ovarian cancer [26]. Using immunohistochemical staining for LYVE1, Sundar *et al.* demonstrated positive reactivity in vascular endothelial cells in ovarian tumor tissues and identified lymphatic vessel density as a significant factor in progression-free survival of patients [26]. Prothymosin alpha (PTMA) is a nuclear oncoprotein commonly used as a prognostic marker in many tumour types [41]. In ovarian cancers, PTMA increases the CD8+ T-cell-mediated lysis of tumour cells, and inhibits tumour growth of orthotopic tumours in SCID mice [42].

The accurate, pre-operative classification of ovarian adnexal masses can substantially improve the management of patients with suspected ovarian malignancy [43, 44], thus, biomarkers for rapid patient triage represent an important area of clinical need. Moreover, appropriate referral to a gynecologic oncology surgeon is clearly associated with improved outcomes for women with ovarian cancer [43, 45]. Using the LFQ-PRM workflow developed in this study, we identified and validated multiple discriminating biomarkers in urine samples. The identification of established ovarian candidate markers (WFDC2 and mesothelin) in our dataset validates this approach. These findings warrant further investigation, particularly in the context of monitoring therapeutic response and disease

recurrence. Ongoing studies will focus on expanding patient numbers, and also evaluate those rare patients diagnosed with early-stage (FIGO stage I) disease.

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ACKNOWLEDGEMENTS

The authors acknowledge Rhiannon Dudley for the collection of clinical samples. Funding support was provided by the Ovarian Cancer Research Foundation Inc., Australia (www.OCRF.com.au) and by the Victorian Government's Operational Infrastructure Support Program. Clinical samples were provided by the Ovarian Cancer Research Foundation Inc. sponsored Ovarian Cancer Tissue Biobank located at the Hudson Institute, Australia.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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FIGURE LEGENDS

Figure 1. Workflow describing LFQ and PRM analysis. Independent sample sets of 10 ovarian cancer and 10 benign urine samples were used for each workflow (total n=40). Proteins were digested using the FASP method, followed by nano-LC and high-resolution Orbitrap analysis. Label-free quantitation and Parallel Reaction Monitoring analysis were performed using MaxQuant and Skyline software.

Figure 2. Box plot of Log₂ intensity distributions of samples used in the LFQ discovery workflow. Results from patient samples demonstrated low systematic variation both within and between patient groups as determined by similar median values and quantiles.

Figure 3. Summary of protein and peptide counts after LC-MS/MS analysis. (A) Protein and (B) unique peptide identifications found per sample, and across each group. (C) Venn diagrams showing the distribution of unique proteins and (D) unique peptide identifications between benign and late stage cancer groups. A complete list of proteins corresponding to each group are shown in Supplementary Table 1.

Figure 4. Quantitative analysis of differentially expressed proteins in ovarian cancer. Highlighted proteins were significantly enriched in ovarian cancer patient urine. Protein intensity ratios with a fold change exceeding one standard deviation and a log₁₀ *p*-value <0.05 were considered significantly changed. Blue line = 1SD, red line = 2SD. A complete list of proteins corresponding to each group are shown in Supplementary Table 2.

Figure 5. Proteins found in benign and late stage disease validated by PRM. Box plot showing log₂ sum fragment area of identified peptides from seven proteins targeted by PRM. Averages (dotted line) and medians (solid line) were calculated on the sum fragment area for peptides

from each protein and the number of patients in each group (n=10). Seven proteins found in the initial LFQ were significantly increased (Wilcoxon rank sum test, $p < 0.05$) in late stage disease (n=10) compared to benign samples (n=10). The sum fragment area of each biological replicate for each peptide targeted by PRM is shown in Supplementary Fig.2. Significance of the PRM data was determined using a Wilcoxon rank sum test based on the summed protein area under the curve (AUC). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6. ROC analysis was performed for the ovarian cancer patients against benign patients using the Log2 summed intensity values for peptides analyzed using PRM for each selected protein.

Table 1. Summary of patient samples

<i>Patient groups</i>	Serous Cancer Late “late”	Benign - ovarian related “benign”
<i>Exp. Cohort 1 n=</i>	10	10
<i>Exp. Cohort 2 (PRM validation) n=</i>	10	10
<i>Stage</i>	3	n/a
<i>median age at diagnosis (Q3-Q1)</i>	73 (17)	69 (12)
<i>Median (Q3-Q1) CA125 (U/ml)</i>	575 (1315)	15 (24)
<i>Pathology</i>	Serous adenocarcinoma	Ovarian fibroma, simple cysts, serous cystadenoma

Table 2. Proteins increased in late stage ovarian cancer samples after LFQ and PRM analysis using an independent sample set. ¹ Human Protein Atlas available from www.proteinatlas.org ^[25, 40] , ²All links to Human Protein Atlas data are shown in Supplementary Table 2.

Gene Name	Protein Name & accession	Human Protein Atlas: Cancer Tissue Expression ^{1,2} (See Supp Table 2 for links)	Human Protein Atlas: Normal expression ovary/fallopian tube ^{1,2}	Peptides used for PRM quantitation
IGFBP3	Insulin-like growth factor-binding protein 3 A6XND1	91% medium-high intensity	Medium – fallopian tube	ALAQCAPPPAVCAELVR CQPSPDEARPLQALLDGR EPGCGCCLTCALSEGQPCGIYTER
LYPD1	Ly6/PLAUR domain-containing protein 1 Q8N2G4	100% medium intensity	No data at protein level	EVMEQSAGIMYR SCASSAACLIASAGYQSFCSGPK
LYVE1	Lymphatic vessel endothelial hyaluronon acid receptor 1 Q9Y5Y7	Not detected at protein level – in contrast to ^[26]	No data at protein level	ANQQLNFTFAK DQVETALK IMGITLVSK LLGLSLAGK NGVGVLWIK
MSLN	Mesothelin; Megakaryocyte-potentiating factor; Mesothelin, cleaved form. Q13421-2	83% medium-high intensity	High – fallopian tube	EIDSLIFYK GHMSPQVATLIDR IQSFLGGAPTEDLK LLGPHVEGLK QLDVLYPK TDAVLPLTVAEVQK VNAIPFTYEQLDVLK
PTMA	Prothymosin alpha B8ZZQ6	100% medium intensity	High – fallopian tube	EVVEEAENGR
SCGB1A1	Uteroglobin P11684	Not detected at protein level – in contrast to	No data at protein level – in contrast to ^[27]	KLVDTLPOKPR LVDTLPOKPR VIETLLMDTPSSYEAMELFSPDQD MR

		[27]		
WFDC2	WAP four-disulfide core domain protein 2 (HE4) Q14508	92% medium- high intensity	Medium – fallopian tube	CCSAGCATFCSLPNDK CCSAGCATFCSLPNDKEGSCPQVNI NFPQLGLCR DQCQVDSQCPGQMK EGSCPQVNINFPQLGLCR VSCVTPNFK

Table 3. Comparison of significantly differentially enriched protein data between the LFQ patient cohort and PRM patient cohort. *CA125 was measured in the clinic as part of routine Ovarian Cancer diagnosis and treatment (not mass spectrometry analysis). CA125 measurements were taken from the identical patient cohort as those used for the PRM analysis.

<u>Protein</u>	<u>LFQ</u>		<u>PRM</u>	
	<u>Log2 Fold Change</u>	<u>P-Value</u>	<u>Log2 Fold Change</u>	<u>P-Value</u>
IGFBP3	1.69	0.0000429	0.96	0.043257
LYPD1	3.38	0.0002669	3.37	0.000725
LYVE1	1.6	0.0000015	1.81	0.028806
MSLN	2.15	0.0000014	3.59	0.00105
PTMA	2.57	0.0057344	2.26	0.000725
SCGB1A1	2.83	0.0022373	3.59	0.035463
WFDC2	2.92	<0.0000001	1.96	0.000205
*CA125			4.46	0.000021

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Title:

Discovery and Validation of Novel Protein Biomarkers in Ovarian Cancer Patient Urine

Date:

2018-05-01

Citation:

Sadow, J. J., Rainczuk, A., Infusini, G., Makanji, M., Bilandzic, M., Wilson, A. L., Fairweather, N., Stanton, P. G., Garama, D., Gough, D., Jobling, T. W., Webb, A. I. & Stephens, A. N. (2018). Discovery and Validation of Novel Protein Biomarkers in Ovarian Cancer Patient Urine. *PROTEOMICS CLINICAL APPLICATIONS*, 12 (3), <https://doi.org/10.1002/prca.201700135>.

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