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DISSERTATION

Optimizing methodologies for clinical proteomics

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List of Abbreviations

4PL - 4 Parameter Logistic nonlinear regression model; **BCG** - Bacillus Calmette Guérin; **CKD** – Chronic kidney Disease; **ELISA** - Enzyme-Linked Immunosorbent Assay; **FASP** - Filter Aided Sample Preparation; **FDA** - US Food and Drug Administration; **FISH** - Fluorescence In Situ Hybridization; **H2B** - Histone H2B; **iTRAQ** - Isobaric Tags for Relative and Absolute Quantitation; **LC-MS/MS** - Liquid Chromatography coupled to tandem Mass Spectrometry; **LFQ** - Label-Free Quantification; **NIF-1** - NRC-Interacting Factor 1; **NMIBC** – Non-Muscle Invasive Bladder Cancer; **MIBC** – Muscle Invasive Bladder Cancer; **MS** – Mass Spectrometry; **PFN-1** - Profilin-1; **ppm** – part per million; **PR3** - Proteinase-3; **SDS-PAGE** - Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis; **SPARC** - Secreted Protein Acidic and Rich in Cysteine; **SLIT-2** - Slit homolog 2 protein; **UBC** - Urothelial Bladder Cancer

Abstract (English)

Introduction: Frequent monitoring of patients with **U**rothelial **B**ladder **C**ancer (UBC) is required due to high disease relapse rates. This leads to increased associated healthcare costs and moderated patient compliance. A prevalent need for urine biomarkers which will enable the timely diagnosis of UBC in a non-invasive manner remains. Moreover, tumour invasion results in poor prognosis, because of limited treatment options. Thus a thorough understanding of the underlying molecular processes is needed to guide the development of therapeutic approaches. To assess both clinical demands, the application of proteomics technologies appears to be advantageous. Due to the high complexity of the biological specimens, a thorough investigation on the optimization of the analytical and post-analytical steps is required. In this thesis, we aim at optimising the methodologies for urine and tissue quantitative analysis.

Methods: Twelve commercially available **E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssays (ELISAs) were utilized to measure UBC biomarkers in urine. Analysis of the urine and tissue proteomes was performed using **L**iquid **C**hromatography coupled to tandem **M**ass **s**pectrometry (LC-MS/MS). Both analytical workflows were optimized, considering type of the material used.

Results: In the first part, special emphasis was placed on the assessment of the analytical performance of ELISA assays. Based on the standard curve evaluation, reproducibility, recovery and linearity analysis, only three out of twelve evaluated assays comply with the U.S. **F**ood and **D**rug **A**dministration (FDA) guidelines.

The second part of the thesis was focused on optimization of the sample preparation strategies for urine proteome analysis. Comparison of four depletion kits, targeting the removal of highly abundant proteins in urine, revealed high reproducibility of all the methods used, usually accompanied with good depletion efficiency. However, application of depletion had no impact on the number of proteins identified by LC-MS/MS.

In the third part of the thesis, label-free and label-based (**I**sobaric **T**ags for **R**elative and **A**bsolute **Q**uantitation, iTRAQ) quantification methods were evaluated, aiming at selecting the most suitable method for quantitative tissue proteomic analysis. Both label-free and iTRAQ (when preceded by fractionation) provided similar protein identification rate. However, the use of the label-free approach showed an improved sequence coverage and detection rate of differentially abundant proteins.

Conclusions: Difficulties in developing ELISA assays in compliance with the regulatory agency guidelines for analytical validation, imply the need for application of alternative analytical platforms. Although, the mass spectrometry-based platforms seems to be advantageous, the success of each approach depends on the optimization of analytical and pre-analytical steps.

Abstract (German)

Einleitung: Aufgrund der hohen Rezidivraten ist eine häufige Überwachung der Patienten mit **U**rothelialen **B**lasenkrebs (UBC) erforderlich, dies führt zu einer Erhöhung der Kosten im Gesundheitswesen und vermindert die Patienten-Compliance. Ein aktueller Bedarf an nicht-invasiven Harn-Biomarkern, welche die rechtzeitige Diagnose von primären und rezidivierenden UBC erleichtern, bleibt unerfüllt. Eine Tumorinvasion führt zu einer schlechten Prognose, so dass ein gründliches Verständnis der zugrunde liegenden molekularen Prozesse offensichtlich notwendig ist, um die Entwicklung geeigneter therapeutischer Ansätze durchzuführen. Um beide klinische Anforderungen zu bewerten, scheint die Anwendung von Proteomics-Technologien vorteilhaft. Auf Grund der heterogenen Natur der Krankheit und der hohen Komplexität der biologischen Materialien ist eine gründliche Untersuchung der Optimierung der analytischen und postanalytischen Schritte erforderlich. Daher ist das Ziel dieser Arbeit, verschiedene Methoden für die Urin- und Gewebe quantitative Analyse zu optimieren.

Methode: Zwölf kommerziell erhältliche Immunoassays (**E**nzyme **L**inked **I**mmuno**s**orbent **A**ssay - ELISA) wurden verwendet, um UBC Biomarker im Urin zu messen. Es wurde parallel eine Proteomanalyse von Urin und Gewebeproben mittels Flüssigkeitschromatographie-Massenspektrometrie (LC-MS/MS) durchgeführt. Beide analytische Workflows wurden unter Berücksichtigung des verwendeten Materials optimiert.

Ergebnisse: Im ersten Teil wurde besonderer Wert auf die Beurteilung der analytischen Leistungsfähigkeit von ELISA-Tests gelegt. Basierend auf der Standardkurvenauswertung, Reproduzierbarkeit, Recovery- und Linearitäts Analyse entsprachen nur 3 von 12 ausgewerteten Assays den amerikanische **F**ood and **D**rug-**A**dministation (FDA)-Richtlinien, dies deutet Beschränkungen von ELISA-basierten Assays an.

Der zweite Teil der Arbeit widmete sich der Optimierung der Strategien für die Probenvorbereitung. Der Vergleich von vier Depletion-Kits, welche die Entfernung von hochkonzentrierten Proteinen im Urin ermöglichen, ergab eine hohe Reproduzierbarkeit aller Methoden, welche in der Regel mit einer guten Verarmungseffizienz begleitet waren. Jedoch hatte die Anwendung der Depletion-Kits keine Auswirkung auf die Anzahl der Proteine, die durch LC-MS / MS identifiziert wurden.

Im dritten Teil der Arbeit wurden markierungsfreie und Label-basierte (**I**sobaric **T**ags for **R**elative and **A**bsolute **Q**uantitation, iTRAQ) Quantifizierungsmethoden ausgewertet mit dem Ziel, die am besten geeigneten Methoden für die quantitative Gewebe Proteomanalyse auszuwählen.

Sowohl markierungsfreie als auch iTRAQ (angewendet nach der Fraktionierung) Methoden lieferten eine ähnliche Protein Erkennungsrate. Jedoch wurde mit der Verwendung des markierungsfreien Ansatzes eine verbesserte Sequenzabdeckung und Erkennungsrate von differentiell angereicherten Proteinen erreicht.

Schlussfolgerungen: Die Unzulänglichkeit der ELISA-Assays bei der erfolgreichen Erfüllung der Richtlinien der Aufsichtsbehörde über die analytische Validierung impliziert die Anwendung einer, alternativen Analyseplattform, um Proteine zu analysieren und zu messen, Massenspektrometrie-basierte Plattformen scheinen vorteilhaft zu sein. Allerdings hängt der Erfolg der MS-basierten Ansätzen stark von der Optimierung der analytischen und pre-analytischen Schritte ab.

1. Introduction

Urothelial **B**ladder **C**ancer (UBC) is one of the most common cause of deaths among malignancies of the genitourinary system. More precisely, approximately 429,000 individuals worldwide were diagnosed with UBC [1] and 165,000 patients worldwide succumbed to the disease in 2012 [1]. Around 80% of patients that are initially diagnosed with UBC harbour **N**on-**M**uscle **I**nvasive **B**ladder **C**ancer (NMIBC i.e. stages pTa/pT1 and CIS), while the remaining patients exhibit **M**uscle-**I**nvasive **B**ladder **C**ancer (MIBC, stages ≥ pT2) [2]. Currently, the typical procedure to diagnose UBC relies on invasive cystoscopy and urinary cytology. Even though, these modalities are considered as "gold standard" for UBC diagnosis, sub-optimal diagnostic accuracy has been reported, particularly for urinary cytology. In a meta-analysis by Mowatt et al [3], the use of white-light-cystoscopy resulted in sensitivity of 71% (49–93%) and specificity of 72% (47–96%), whereas the application of urinary cytology resulted in sensitivity of 44% (38–51%) and specificity of 96% (94–98%).

In an effort to address the clinical demand for more accurate diagnostic tools and reduce the patients' burden related to the invasive character of cystoscopy, several new non-invasive diagnostic assays have been developed and already approved by the US **F**ood and **D**rug **A**dministration (FDA). This includes immunoassays (e.g. BTA stat®, NMP22®, ImmunoCyt™/uCyt+™) and a **F**luorescence **I**n **S**itu **H**ybridization (FISH)-based assay (i.e. UroVysion™). Although, the diagnostic performance of these tests appeared to be promising, further investigations in independent and appropriately selected patient populations failed to reproduce the initial results [4-6]. Therefore, the clinical applicability of these assays has become questionable. At the same time, the impact of accurate and timely diagnosis is particularly reflected by disease outcome. The 5-year survival rate for patients with localized disease is 69.9%, while in case of distant metastases, the survival drastically decreases to 5.4% [7]. Standard treatment for NMIBC is the transurethral resection of the tumour followed by intravesical instillations of chemotherapy or **B**acillus **C**almette **G**uérin (BCG), while for MIBC a radical cystectomy is performed [8, 9]. However, limitations of the available therapeutic options underscore the unmet need for the identification of new targets for therapeutic intervention.

The failure of the commercially available assays is related to both technical and conceptual limitations. Technical limitations of antibody-based assays generally include questionable specificity of the antibodies, lengthy development process of the novel

assays, high cross reactivity and limited multiplex capability [10]. In addition, when diagnosis of heterogeneous diseases such as UBC is attempted, a single biomarker may not be efficient to detect the disease. In such a case, the possibility to measure multiple biomarkers simultaneously is of high relevance. Furthermore, emerging evidence from recent sequencing analysis of bladder tumor specimens indicates a high phenotypic variability. Therefore, to improve the characterization of the disease at the molecular level, omics platforms seem more appropriate. Particularly, the application of high resolution proteomics techniques allows for the dynamic characterization of a biological system, including possible environmental factors that likely affect gene expression.

Currently, **L**iquid **C**hromatography coupled to tandem **M**ass **S**pectrometry (LC-MS/MS) is one of the most commonly applied platforms for the identification of molecular determinants of disease. Recent advances in separation techniques as well as sample preparation procedures enabled a significant increase in the depth of the proteome analysis, improving thus the identification of low abundance proteins. Importantly and in order to obtain high quality and biologically meaningful results through the application of these highly sophisticated platforms, a thorough investigation on possible adjustments and/ or optimization methodologies is required. This includes acquisition, post-acquisition analytical steps and statistical analysis. Particularly, in the context of clinical proteomics, the reproducibility, repeatability and stability of the analytical method is of paramount importance to assess the differences between control and disease condition.

For this purpose, in the present study, we aimed at optimising the methodologies for urine and tissue quantitative analysis, focusing on the assessment and adjustment of analytical procedures as well as data acquisition strategies. In the first part, the analytical performance of conventional antibody-based **E**nzyme-**L**inked **I**mmuno**s**orbent **A**ssays (ELISA's) was critically evaluated based on the FDA guidelines for Bioanalytical Method Validation [11] (**Publication 1** [12]). For that purpose, a total of 12 ELISA assays were used to assess the selected biomarker candidates for UBC in urine. However, the analytical performance in 75% of the employed kits did not comply with the FDA guidelines. The suboptimal performance of the presented immuno-based assays can be attributed to high complexity of the analyzed material and the variability in the compounds of urine matrix. Both the specificity of the antibodies as well as the efficiency of the binding to the antigens, have a significant impact on the performance of the ELISA assays. In addition, presence of protein isoforms that may have a different affinity to the antibody, have an effect on the linearity of the test. Challenges associated with development of

ELISA assays to measure urine biomarkers underscores the need for further improvements. Nowadays, **M**ass **S**pectrometry (MS) -based approaches have become a promising alternative and are now more commonly applied for many indications in the clinic [13].

Following this, the subsequent part of this PhD thesis was focused on the application of “state-of-the art” MS-based platforms to analyze urine and tissue samples (**Publication 2** [14] and **Publication 3** [15]**,** respectively). On one hand, proteomic profiling of urine samples is considered as a mine of information about non-invasive biomarker candidates; on the other hand, global analysis of tissue samples is a suitable way to investigate disease associated mechanisms. In the second part of the thesis, we investigated the potential impact of the complexity of urine proteome on the results from high-resolution LC-MS/MS analysis. In general, the presence of highly abundant urine proteins affects the accuracy of the quantification and hamper the identification of low abundance proteins, which could be of used as putative biomarkers. To address this issue, four commercially available depletion kits were tested. Unfractionated material served as a reference for this study (**Publication 2** [14]). Even though, efficient depletion of most of the targeted proteins was achieved and the obtained results were highly reproducible, overall the application of the depletion methods did not bring any added value in the number of peptides/ proteins that were identified during the LC-MS/MS analysis. Therefore, the analysis of unfractionated urine samples appears to be beneficial in this studied context.

The third part of the thesis was devoted to the comparison of quantification methods routinely applied in quantitative proteomics i.e. **L**abel-**F**ree **Q**uantification (LFQ) and label-based (i.e. **I**sobaric **T**ags for **R**elative and **A**bsolute **Q**uantitation, iTRAQ) approach (**Publication 3** [15]). Since there is no consensus on which of these two strategies is best suited for the detection of differentially abundant proteins in tissue samples, comprehensive comparison of both methods was attempted. Both methods (LFQ and iTRAQ, when combined with fractionation) resulted in the identification of comparable numbers of proteins. In addition, the protein sequence coverage and the capability to detect differentially abundant proteins were advanced when the LFQ approach was applied. Therefore, the LFQ approach as optimized in this study appears to be preferable, when a more comprehensive characterization of the disease-associated mechanisms is attempted.

2. Methods

2.1. Clinical samples

Urine samples from UBC patients were collected at the Urology clinic of the Laikon University Hospital, Athens, Greece according to the local ethics regulations. As control samples, urine samples from patients suffering from benign urological conditions (hernia, cystitis, benign prostate hyperplasia etc) were considered [12]. Urine samples from **C**hronic **K**idney **D**isease (CKD) patients and normal individuals were collected in accordance to the local ethics committee of Macedonia Academy of Science and Arts [14]. UBC tissue specimens were collected from patients undergoing transurethral resection of bladder cancer at the Urology clinic of the Laikon University Hospital, Athens, Greece and at the Department of Urology and Urological Oncology in Hannover Medicine School in Germany [15]. A written informed consent was obtained from all individuals.

2.1.1. Urine samples

Measurement of the urine levels of the selected biomarkers was performed using commercially available ELISA assays in a total of 167 urine samples [12]. This includes 53 urine samples from patients harboring benign urological diseases (control group) as well as 114 urine samples from patients with primary tumor in the bladder (case group i.e. stage pTa n=46, stage pT1 n=36 and sage pT2+: n=32). Protein concentration, pH and hematuria were determined using Bradford assay and standard urine analysis strips from EMAPOL, respectively. The evaluation of the abundant protein depletion strategies prior to proteome analysis by MS was performed using pooled urine samples with both high protein content (from patients with CKD stage IV) and low protein content (from normal controls) [14]. Around 30 mL of urine samples in each pool was obtained. Cell debris were removed using the centrifugation at 1,000 x g for 10 min at 4°C. Supernatant was collected, aliquoted and stored at -20^{o}C until used.

2.1.2. Tissue samples

Tumor specimens from 8 patients undergoing transurethral resection of bladder cancer were employed for the analysis including NMIBC (stage pTa, n=4) and MIBC cases (stage pT2+, n=4) [15]. Staging relied on TNM classification system [16].

2.2. ELISA assays

The analytical validation was performed for 12 commercially accessible ELISA kits. The following assays were tested: (1) **S**ecreted **P**rotein **A**cidic and **R**ich in **C**ysteine

(SPARC) - R&D Systems Inc., Catalogue no. DSP00, (2) **SLIT** homolog **2** protein (SLIT-2) - Cloud Clone Corp., Catalogue no. SEA672Hu, (3, 4) Histone **H2B** (H2B) - US Biological Life Sciences, Catalogue no. 025705 and Cloud Clone Corp., Catalogue no. SEA356Hu, (5, 6) Survivin - Enzo Life Sciences AG, Catalogue no. ADI-900-111 and R&D Systems Inc., Catalogue no. DSV00, (7-9) **P**ro**f**ili**n-1** (PFN-1) - USCN LIFE, Catalogue no. E2122h, US Biological Life Sciences, Catalogue no. 027613 and Cloud Clone Corp., Catalogue no. SEC233Hu, (10, 11) **N**RC-**I**nteracting **F**actor **1** (NIF-1) - Cusabio Biotech CO. LTD, Catalogue no. CSB-EL026683HU and USCN LIFE, Catalogue no. E1019h, (12) **Pr**oteinase-**3** (PR3) - Cusabio Biotech CO. LTD, Catalogue no. CSB- E13058h. All measurements were performed using ELx800 plate reader (BioTek Instruments). Assessment of the ELISA kits' analytical performance in urine relied on the evaluation of the standard curve, recovery, reproducibility and linearity. Performance of the assays was considered as satisfactory when the following criteria were met: a) a good fit of standard curve to the **4 P**arameter **L**ogistic nonlinear regression model was achieved (4PL) (R^2> 0.95), b) the percentage of the recovery was in the range between 80 to 120%, c) intra-assay coefficient of variation was in the range of 0 to 20% and d) linearity analysis was resulting in a linear fit with R^2>0.9 and a slope of 0.9-1.0. More details on the ELISA assays is reported by Chatziharalambous et al [12].

2.3. Proteomic analysis

2.3.1. Sample preparation

2.3.1.1. Tissue proteomics

Bladder cancer tissue samples (~20 mg) were homogenized in 150 μL of lysis buffer (4% SDS, 0.1M DTE, 0.1M Tris-HCl pH 7.6) using a blade homogenizer in combination with sonication. Cell debris and undissolved materials were removed by centrifugation at 13,000 rpm for 10 minutes. Protein concentration was assessed using Bradford assay (BioRad) [15]. Subsequently, **F**ilter **A**ided **S**ample **P**reparation (FASP) method was used to digest extracted proteins [17], separately for label-free and label-based analysis.

2.3.1.2. Urine proteomics

Amicon Ultra Centrifugal Filter Units (3 kDa cut-off, Millipore) were applied for both buffer exchange and subsequent concentration of 500 μL of urine aliquots. For the purpose of buffer exchange, the solutions as recommended by the manufacturer were used. Subsequently, depletion of highly abundant proteins was performed using four

commercially available assays including immuno-based (Seppro IgY14, Sigma-Aldrich; ProteoPrep, Sigma-Aldrich; SpinTrap, GE Healthcare) and ion-exchange kit (ProteoSpin, Norgen Biotek). The samples were subsequently processed using the manufacturer's recommended protocols, with some minor modifications. Each experiment was performed in five technical replicates per condition (for high or low protein content). A more detailed description can be found in Filip et al [14]. The protein concentration was determined by the Bradford assay (BioRad) and the protein extracts were in parallel analyzed by **S**odium **D**odecyl **S**ulfate **P**oly**a**crylamide **G**el **E**lectrophoresis (SDS-PAGE) and processed by FASP prior to LC-MS/MS analysis (as described by Filip et al [14]).

2.3.2. Protein digestion

The bladder tumor and the urine protein extracts were processed using the FASP protocol as described previously [17] with some minor modifications. Briefly, the protein extracts were subjected to buffer exchange in Amicon Ultra Centrifugal filter devices (0.5 mL, 30 kDa cut off, Millipore) at 13,000 rpm for 15 minutes at room temperature conditions. Buffer exchange with 8M urea buffer and 50 mM NH_4HCO_3 buffer as well as alkylation of the proteins with iodoacetamide, prior to protein digestion were performed. An overnight digestion with trypsin was conducted (trypsin to protein ratio - 1:100), followed by peptide elution by centrifugation. For performing iTRAQ labelling, the tissue protein extracts were processed by FASP, as described above, with some additional modifications including replacement of 50 mM NH_4HCO_3 buffer with 50 mM $C_7H_{17}NO_3$, reduction of the volume of the solution containing trypsin and decreased volume of the eluted peptides. More information about the protein digestion protocol can be found in manuscript by Latosinska et al [15].

2.3.3. iTRAQ labeling

100 μg of tryptic digested peptides (per sample) were labeled using the 8-plex iTRAQ Reagent kit (AB Sciex) followed the manufacturer‘s recommendations. 80 μg of peptide mixture was purified using Pierce C18 Tips (Thermo Scientific), following the manufacturer‘s instructions; while the remaining peptide mixture (~700 μg) was purified and pre-fractionated using a high pH reverse phase chromatography on a Dionex P680 HPLC system. Detailed description is provided in Latosinska et al. [15].

2.3.4. LC-MS/MS analysis

All protein digests were analyzed on a Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK), after loading onto a Dionex 0.1×20 mm 5 μm C18 nano

trap column at a flow rate of 5 µl/min in 98% 0.1% formic acid and 2% acetonitrile, sample was eluted onto an Acclaim PepMap C18 nano column 75 µm×50 cm (Dionex, Sunnyvale, CA, USA), 2 µm 100 Å at a flow rate of 0.3 µl/min. The eluent was ionized using a Proxeon nano spray ESI source operating in positive ion mode into an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). Additional information about LC-MS/MS analysis of urine and tissue proteome is described by Filip et al [14] and Latosinska et al [15], respectively.

2.3.5. Data processing and analysis

Processing of the raw MS data files was conducted using Proteome Discoverer v. 1.4 (Thermo Scientific). The protein identification relied on the database search against Human Swiss-Prot Database [18, 19] using the Sequest search engine [20]. The evaluation of the false discovery rate was performed by using the Percolator node [21]. The identified peptides were filtered based on mass deviation being below 5 ppm between the experimental and the theoretical mass, false discovery rate being below 1% and peptide rank being up to 5. In order to increase the consistency of the data, only peptides that were reported in more than 60% of the samples (in at least one analyzed group) were included for analysis. Subsequently, the Occam Razor rule [22] was applied to assign the identified peptides to proteins. More information about the processing of urine and tissue proteomics data is presented by Filip et al [14] and Latosinska et al [15], respectively.

2.3.6. Relative quantification

Quantitative proteomics analysis of urine and tissue was performed using label-free approach. Specifically, the quantification process was based on the peak area of the precursor ions (i.e. area under the curve), which was calculated based on the extracted ion chromatogram during the data processing through the Proteome Discoverer Software. Additionally, for the tissue protein digests labeled with iTRAQ reagents, quantification using a label-based approach was followed according to the reporter ion intensities in Proteome Discoverer Software. The data were normalized based on the **p**art **p**er **m**illion (ppm)-normalization method. A detailed description of the quantification strategies is provided by Filip et al [14] (for proteomic analysis of urine samples) and Latosinska et al [15] (for proteomic analysis of tissue specimens).

3. Results

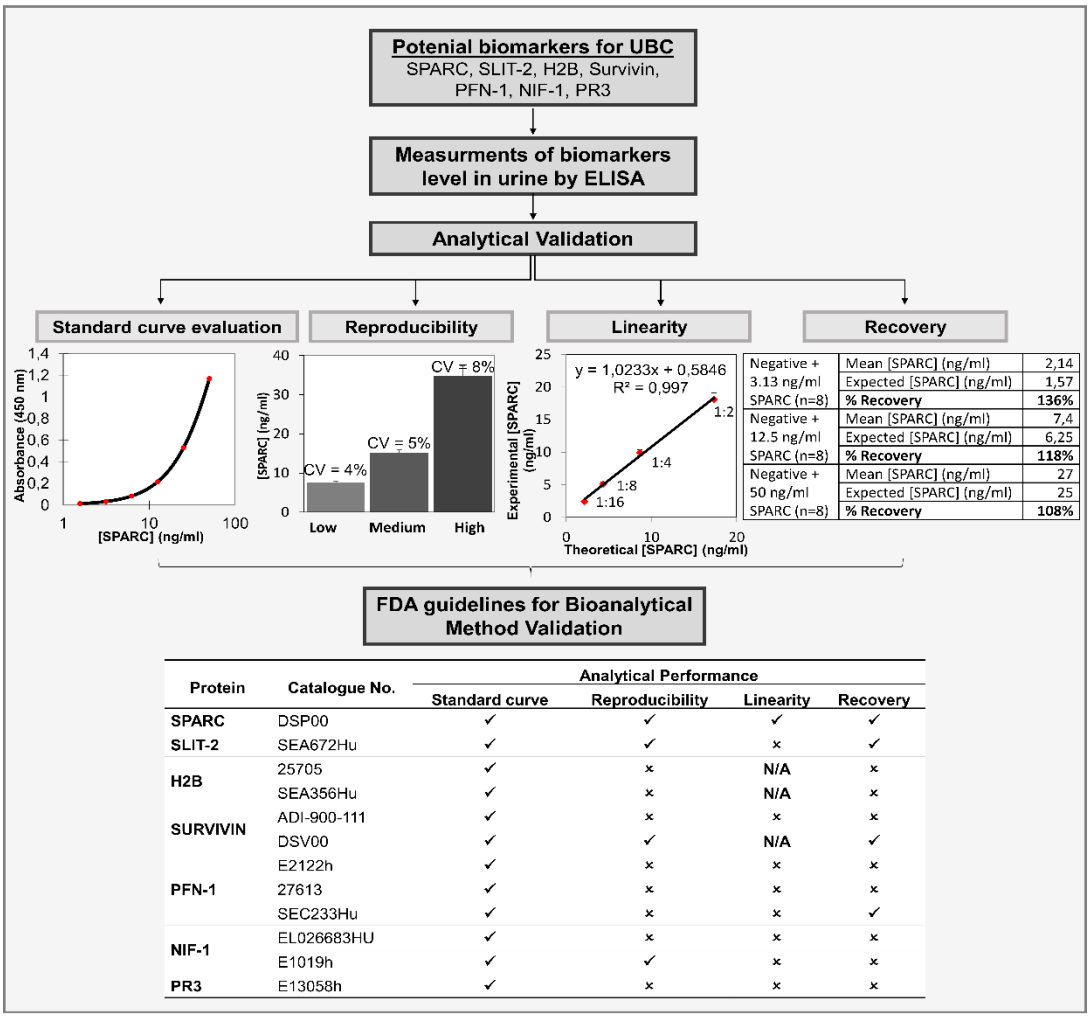
3.1. Publication 1: Analytical performance of ELISA assays in urine: one more bottleneck towards biomarker validation and clinical implementation.

The discriminatory capacity of some selected putative UBC biomarkers was assessed in urine using typical biochemical assays, like ELISA. The analysis was conducted for seven candidate biomarkers (i.e. SPARC, SLIT-2, H2B, Survivin, PFN-1, NIF-1, PR3) using a total of 12 commercially available ELISA kits, aiming at the evaluation of their analytical performance in urine and the validation of its discriminatory potential. As presented in **Figure 1**, the analytical performance of the selected ELISA assays in urine was extensively investigated, focusing on the standard curve estimation, recovery, reproducibility and linearity analyses. The performance of the assays was assessed according to the FDA guidelines for Analytical Validation [11]. Only assays complying with at least three evaluation criteria were considered as successful. Satisfactory analytical performance was reported for 3 out of 12 ELISA tests (25%) targeting SPARC, Survivin and SLIT-2; while the remaining assays for NIF-1, PFN-1, PR3 and H2B showed insufficient analytical performance. For the latter, even though the standard curve validation was evaluated as successful, the assays failed to show good reproducibility, or recovery and linearity in the performed studies.

On the basis of these results, two successfully validated assays were further used to measure the level of SLIT-2 and SPARC in a larger sample set. Significant increase in the level of both SLIT-2 and SPARC was demonstrated in UBC (pT2+) vs. other conditions ($p<0.05$). Interestingly, a successive increase of the SLIT-2 level in urine was observed along with the advancement of tumor Grade, but this difference showed no statistical significance. When analyzing SPARC, Grade 2 UBC tumors showed higher mean values of the urine levels of this particular protein, compared to Grade 1 and Grade 3 UBC tumors, again though with the observation not being statistically significant. In both assays, for SPARC and SLIT-2 ELISA measurements, very high standard deviation was reported. Due to high inter-patients variability and limited statistical power of the study (for grade analysis: n=79 for SLIT-2, n=40 for SPARC), a more thorough investigation of the association of SPARC and SLIT-2 with tumor grade is now ongoing.

Conclusively, a thorough analytical validation of the analytical assays creates a solid, evidence-based background, which is required for reliable measurements of the biomarker candidates. This study pointed out several challenges associated with the

development of antibody-based tests for clinical use, being related to the limited assay reproducibility, linearity and recovery. This indicates a clear need for adoption and development of alternative strategies to measure urine biomarkers in clinical settings.



Protein	Catalogue No.	Analytical Performance			
		Standard curve	Reproducibility	Linearity	Recovery
SPARC	DSP00	✓	✓	✓	✓
SLIT-2	SEA672Hu	✓	✓	×	✓
H2B	25705	✓	×	N/A	×
	SEA356Hu	✓	×	N/A	×
SURVIVIN	ADI-900-111	✓	×	×	×
	DSV00	✓	✓	N/A	✓
PFN-1	E2122h	✓	×	×	×
	27613	✓	×	×	×
	SEC233Hu	✓	×	×	✓
NIF-1	EL026683HU	✓	×	×	×
	E1019h	✓	✓	×	×
PR3	E13058h	✓	×	×	×

Figure 1. Study design and summary of the assessment of the analytical performance for 12 assays, modified from Chatziharalambous et al [12]. As an example, analytical performance is presented for SPARC assay. (✓) Satisfactory and poor (×) analytical performance are indicated. As shown in the graph, the assays targeting SPARC, SLIT-2 and Survivin showed satisfactory analytical performance, being in compliance with FDA guidelines.

3.2. Publication 2: Comparison of Depletion Strategies for the Enrichment of Low-Abundance Proteins in Urine

In order to overcome the presented limitations associated with the application of ELISA assays, mass spectrometry analysis was applied. Considering the complexity of the urine proteome and in an effort to improve the identification of the potential biomarkers

in urine, a comparative analysis of four depletion strategies including immuno-based and ion-exchange was attempted. For this analysis an initial volume of 500 μL was used. As reference condition, unfractionated material was utilized. An overview on the workflow followed is depicted on **Figure 2**.

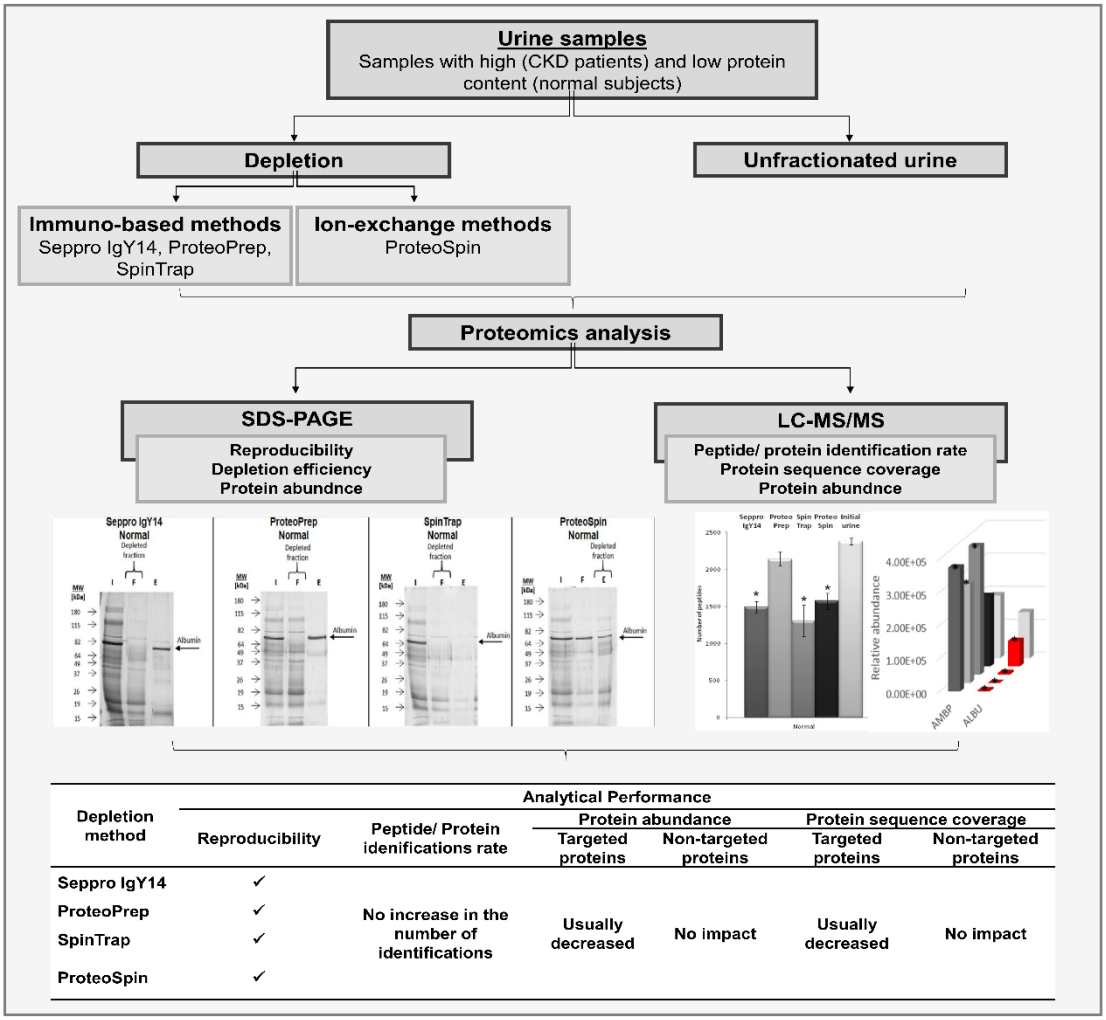
The reproducibility and the efficiency of the depletion methods was assessed using SDS-PAGE. In all cases, reproducible results were generated, with the highest depletion efficiency observed for immuno-based methods, in comparison to the ion-exchange strategy. Apparently, application of the depletion strategies does not increase the number of peptides and proteins that were identified during LC-MS/MS analysis. Specifically, in the case of samples with low protein concentration (normal group), the highest number of peptides was reported for unfractionated urine sample (~ 2,400 peptides); while for most of the depletion strategies tested (i.e. Seppro IgY14, ProteoSpin and SpinTrap), the number of peptides identified in depleted samples was significantly lower (~1,500 peptides) in comparison to reference condition. Along these lines, analysis of the urine samples with high protein content (i.e. disease group) revealed similar number of peptides identified in fractionated and unfractionated samples (~1,250 peptides). In addition, a similar number of proteins that were consistently identified in at least 60% of analyzed samples per methods was observed for both fractionated and unfractionated samples. Importantly, the identification rate of the proteins in normal urine was around 2- fold higher than in diseased samples. Highly abundant urine proteins such as albumin, uromodulin and beta-2-microglobulin were found to be overlapping between both types of the analyzed samples (fractionated/ unfractionated).

We further evaluated the impact of the depletion methods on the sequence coverage of targeted and non-targeted proteins. For the aforementioned targeted proteins, application of the depletion methods for urine samples with low protein content was associated with decrease in their sequence coverage, although the decrease in the coverage varied widely in a range between 5% to 90%. In the case of the samples with high protein content, decrease in the coverage of all targeted proteins was observed after the application of SpinTrap and ProteoPrep. Of note, application of Seppro IgY14 and ProteoSpin kit did not have any impact on the sequence coverage of albumin, immunoglobulin alpha and alpha-1-acid glycoprotein 1 as well as alpha-1-antitrypsin, respectively. On the contrary, sequence coverage of the proteins, which were not targeted by depletion kits, remains at the same level for fractionated and unfractionated samples.

Furthermore, the impact of the depletion strategies on the relative protein abundance was also studied. It has been shown that for the depletion targets, the application of the immuno-based methods resulted in a reduction of their relative abundance for both type of the analysed samples (urine from normal and CKD patients). However, for those samples that were fractionated with the ion-exchange method, the removal of serotransferrin (for normal urine) as well as alpha-1-antitrypsin and albumin (for CKD) was not efficient. In addition, there was no obvious trend in the relative protein abundance, when the non-target proteins were evaluated. Since the application of the depletion methods did not generally result in a significant improvement either in the protein sequence coverage or relative abundance for non-targeted proteins, the use of these methods to study urine proteome could not be justified, at least in the presented setting.

To further elaborate on that concept, extensive evaluation of the relative abundance of albumin (i.e. protein targeted by all depletion methods) was conducted, prior and after the application of the four kits. Significant reduction in the albumin levels was reported for normal samples (independently of the applied approach with ~98% and 45% decrease for immuno-based and ion-exchange methods, respectively), while in the case of urine samples from diseased patients, the application of SpinTrap, ProteoPrep and Seppro IgY14 allowed for the most efficient depletion of this protein (showing 95%, 91% and 63% decrease, respectively). However, a low depletion efficiency was reported for ProteoSpin kit. Based on the presented results, the depletion of albumin using immuno-based kits appears to be more efficient in comparison to the ion-exchange-based method. This results are in line with the analysis of the depletion efficiency using SDS-PAGE.

Conclusively, with the use of several depletion methods an effective removal of highly abundant proteins in urine was achieved, as confirmed by the results that were obtained during SDS-PAGE and LC-MS/MS analysis. However, these results did not reflect any increase in identification rate at both the peptide and the protein level. Therefore, for the purpose of the identification of potential biomarker candidates in urine, the use of unfractionated material appears to be advantageous.



Depletion method	Analytical Performance					
	Reproducibility	Peptide/ Protein idenifications rate	Protein abundance		Protein sequence coverage	
			Targeted proteins	Non-targeted proteins	Targeted proteins	Non-targeted proteins
Seppro IgY14	✓	No increase in the number of identifications	Usually decreased	No impact	Usually decreased	No impact
ProteoPrep	✓					
SpinTrap	✓					
ProteoSpin	✓					

Figure 2. An overview on the workflow of urine proteomic study, modified from Filip et al [14]. The impact of the depletion on proteomic analysis of urine samples is presented.

3.3. Publication 3: Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis.

Proteomic analysis in tissue can serve as an excellent tool to identify potential drug-targets, since tissue proteins directly reflect the pathological alterations that occur under a pathological condition. To better display the differences between diseased and healthy conditions, quantitative proteomics seem one of the most appropriate approaches. However, it is not clear which quantification method is optimal for detection of biologically relevant differentially abundant proteins, particularly in complex clinical specimens like tissue. Therefore, in the study presented here, a comparison of commonly applied quantification strategies i.e. label-free and label-based (iTRAQ) was anticipated. Towards that end, bladder cancer tissue samples representing NMIBC and MIBC were analysed. For the label-based approach, two data-acquisition strategies were tested. The

first one relied on the direct analysis of the labelled peptides in a single LC-MS/MS run, thus limiting the costs of analysis. On the other hand, the second approach relied on the analysis of multiple samples, as a result of the application of additional peptide fractionation prior to LC-MS/MS. Collectively, in the context of this study a total of three experimental approaches were evaluated (label-free, unfractionated and fractionated iTRAQ), as also schematically represented in **Figure 3.**

The same protein extracts from different biological replicates were used for the analysis with all three strategies. Moreover, in order to obtain a representative output from each of the methods the application of routinely applied protocols for sample preparation and subsequent MS analysis was preferred. The results collected from all three methods were assessed according to the number of the identified proteins, the protein sequence coverage, and the ability to identify differentially expressed proteins as well as the reliability of the reported expression trends. In order to further increase the credibility of the findings, only proteins that were identified based on at least two unique peptides in at least of three out of four samples at least in one group (pTa or pT2+) were considered for further evaluation. Based on the above criteria, the application of LC-MS/MS analysis of tumor tissue samples allowed for identification of a total of 910, 1092 and 332 proteins using label-free, fractionated and unfractionated iTRAQ, respectively. The protein sequence coverage as obtained using the label-free method, outperformed both those derived based on the iTRAQ experiments (as depicted in **Figure 3**).

In the next step, the capability of the selected methods to detect differentially abundant proteins ($p<0.05$, t-test) was assessed. Although, a comparable number of proteins was identified using label-free and iTRAQ approach (when assisted by an additional pre-fractionation step), this was not accompanied by a comparable number of differentially expressed proteins, with higher number of differentially abundant proteins to be reported by using label-free approach. Specifically, a total of 77 (49/28 up/down-regulated proteins in pT2+/pTa), 45 (21/24 up/down-regulated proteins in pT2+/pTa) and 6 proteins (1/5 up/down-regulated proteins in pT2+/pTa) were detected using label-free, pre-fractioned iTRAQ and iTRAQ approach alone, respectively.

To further assess the validity of the proteomics output at the level of both protein identification as well as the differential expression, the collected data were cross-correlated with other publicly available data (Proteomics databases [23], Human Protein Atlas [24] and published data on the disease [25-27]). The vast majority of the proteins that were identified as differentially abundant (83% and 87% for label-free and iTRAQ,

when accompanied by fractionation) was found also in normal and/or tumorous urothelium in all data repositories. When comparing the trend of expression between the proteomics datasets of this study and the three previously published transcriptomics data [25-27], 34 out of 77 (44%) and 15 out of 45 (33%) proteins exhibited similar expression trend at the mRNA level, in label-free and iTRAQ (supported by fractionation), respectively.

Conclusively, the collected results indicated that two of the tested strategies (label-free and iTRAQ, when combined to peptide fractionation) allowed for the identification of a comparable number of proteins. In addition, the analysis of tissue proteomics data by using the label-free approach provides also an added value with respect to the sequence coverage (thus increasing also the reliability of protein identifications) and the capability to detect significantly altered features. Thus, the LFQ approach as optimized in this study appears to be preferable, when a comprehensive characterization of the disease-related features is attempted.

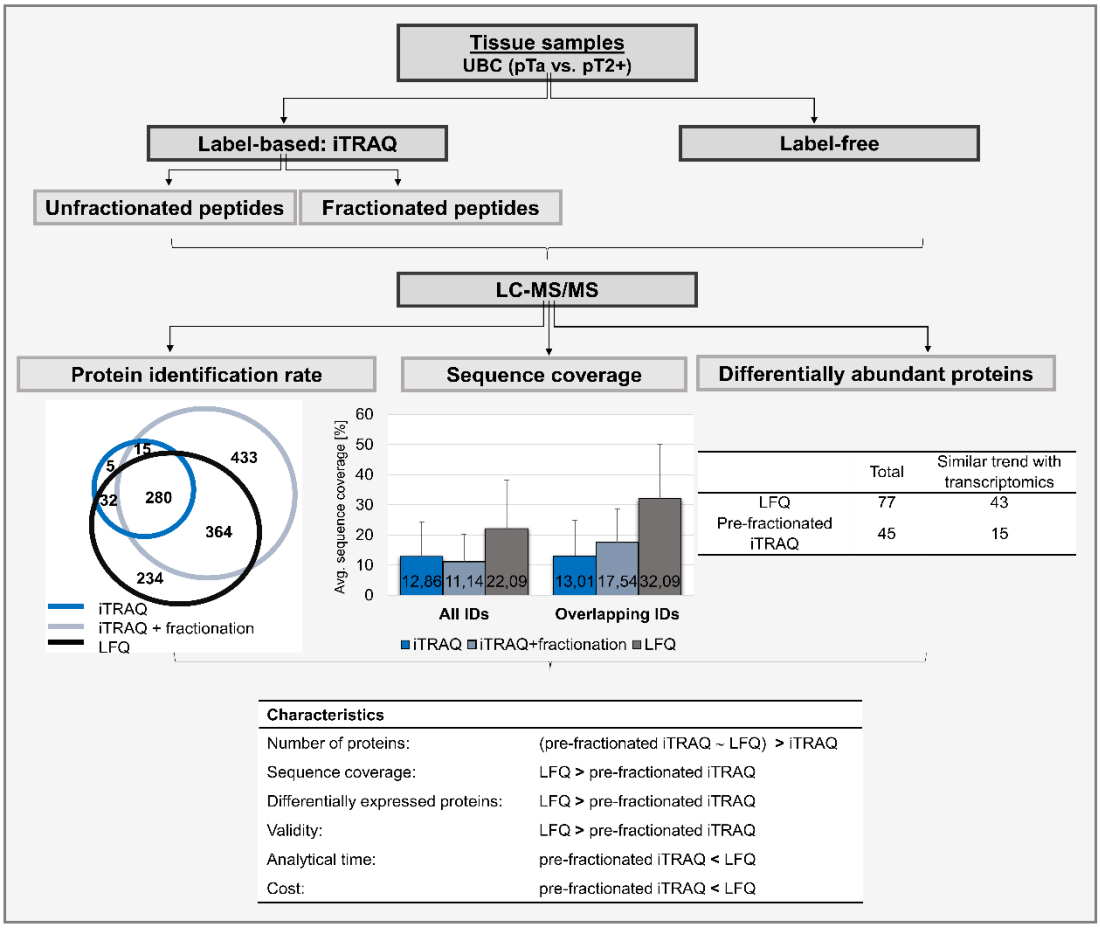


Figure 3. Study design modified from Latosinska et al. [15]. Graphical representation of the applied workflow for label-free (LFQ) and iTRAQ quantification as well as an overview on obtained results is presented.

4. Discussion

The introduction of highly sensitive proteomic platforms can facilitate the investigation of non-invasive biomarkers as diagnostic tools, as well as the discovery of novel potential drug targets for UBC treatment. Within this context and considering the highly sensitive technological platforms that are employed for urine and tissue proteomic analysis, in this thesis we aimed at optimizing methodologies that will yield reliable results with possible application in translational research. The analytical steps of: sample preparation, acquisition of the proteomics data, post-acquisition analytical steps and statistical analysis, were optimized.

For this purpose, we have analyzed previously identified biomarkers for UBC using conventional antibody based ELISA assays, routinely applicable in clinical practice. Considering the translational character of this research, and the potential application of the findings into clinics, FDA guidelines for Bioanalytical Method Validation were considered as criteria for assessment of the analytical performance of tested assays [11]. Although, twelve different kits were tested, most of them did not comply with the criteria outlined by FDA regarding the Analytical Validation. The challenges of the establishment of reproducible and precise urinary tests are related to the high complexity of urine sample [11]. Presence of proteins (> 1,500), inorganic ions and organic substances (i.e. urea, creatinine etc) along with cell/ cellular debris may have a substantial impact on the binding of the antibodies to targeted proteins during ELISA analysis [28]. In more details, low recovery as presented in many cases may be associated with the presence of different substances in urines (including organic and inorganic substances like salts etc), which may interfere with the recognition of specific antigen. On the other hand, non-specific protein binding may be reflected by obtaining higher recovery, than expected. Moreover, previous experiments measuring known amount of spiked protein in urine samples showed also the high variability in protein recovery, when the numerous proteins were spiked in urine samples. Variations between the reported recovery were also noted between assays, suggesting differences in the components of the urine matrix between samples, having an impact on the accuracy of protein quantification [29].

The limited analytical validity of ELISA based assays in urine have been also reported by other investigators. Kift et al observed a limited performance of ELISA kit detecting Neutrophil gelatinase-associated lipocalin, as reflected by the evaluation of recovery and linearity of the assay [30]. The presented observations underscored the

variability associated with the performance of immuno-based assays in urine. Therefore, the analytical validation of the assays is a prerequisite, particularly when the analysis of valuable clinical material is targeted. All of these shortcomings manifest the difficulties related to the establishment of analytically valid urine tests using antibodies to measure putative biomarkers, being also applicable for clinical purposes.

Nowadays, mass-spectrometry based platforms have become a promising alternative to assess biomarkers in urine or to investigate disease-associated mechanisms in tissue. Following this principle, at first place we focused on the analysis of the urine proteome. Considering the challenges associated with the high complexity of urine [31], and the broad dynamic range of the protein concentration, four depletion strategies were tested aiming at the removal of highly abundant proteins in urine samples. The conducted analysis demonstrated better depletion efficiency for immuno-based kits in comparison to the ion-exchange method, as a result of higher specificity in comparison to the former methods [32]. Moreover, it has been noted that higher number of proteins was identified when low protein content samples are analyzed, indicating that regardless from the different depletion methods tested, the masking effect that is associated with the presence of highly abundant proteins is sustained. Overall, in this study, the utilization of selected depletion kits did not result in any improvement either in the sequence or proteome coverage. The results presented in our study could be to some extent explained by the following factors: a) due to the high complexity of the urine proteome, the impact of the depletion on individual proteins cannot be easily predicted, b) the tested depletion kits might not be so efficient to observe a significant effect on the coverage and c) the low peptide concentration, could be below of the limit of the detection for the MS technology. The obtained results are partially in line with other previous investigations. Based on two-dimensional gel electrophoresis analysis, the application of immuno-based depletion methods did not have a significant impact on the number of unique proteins that was reported [33, 34]. On the contrary, in the study by Kushnir et al, an increase in the number of the identified proteins was observed upon depletion of the urine samples from CKD patients using MARS column [35]. However, the total number of the identified proteins was lower than in our analysis. The validity of the proteins identified in this study is supported by the high overlap between the 100 proteins that were identified in normal urine and other investigations of urine proteome from healthy individuals [28, 36, 37]. Collectively, considering the limited impact of the depletion on the results of the

proteomics analysis, at least when a limited volume of urine was used as initial material, the analysis of unfractionated urine appears preferable.

The second study utilizing MS-based approaches was devoted to the tissue proteomics analysis. Considering that tissue samples can directly present changes in the disease pathophysiology, quantitative tissue proteomic analysis presents several advantages for the discovery of putative biomarkers or drug targets. The present thesis was focused on an unbiased comparison of two quantification approaches i.e. label-free and iTRAQ (unfractionated and fractionated analysis) in order to determine which technique is better suited for the detection of differential protein expression in clinical samples. To the best of our knowledge, this concept was not explored in the past, since the previously published reports were mostly focused on the “technical” description of these two quantification methods, using also not that complex biological material like tissue [38-41]. In terms of the protein coverage, application of pre-fractionation of iTRAQ labeled peptides enables superior results over the conventional iTRAQ run; whereas the extent of the proteome coverage remains comparable with the label-free analysis. Similarly, in the study by Patel et al, a comparable number of proteins was identified between label-free and iTRAQ approach, when the latter was supported by an additional fractionation step [39]. Additionally, the added value of LFQ over the iTRAQ is reflected by a more confident protein identification (higher protein sequence coverage), that is in agreement with the already published results [38, 39]. Based on the obtained results, the label free approach appears to be the preferred option, when the detection of differential expression is the main objective of the study. Conclusively, label free quantitation may facilitate the characterization of the molecular mechanisms underlying pathological conditions. However, due to the possibility of detecting false positive changes, an increase in the studied sample size, the application of stringent statistical criteria (e.g. adjustment for multiple testing) and a further validation of findings are required.

In conclusion, thorough optimization of the methodologies that are used to analyze urine and tissue proteome is a first step and prerequisite for obtaining reliable data. Properly established analytical workflows can be subsequently applied for the discovery of novel biomarkers and potential therapeutic targets for the benefits of patients.

5. References

[1] Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D, Bray F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 2015; 136:E359-86.

[2] Nielsen ME, Smith AB, Meyer AM, Kuo TM, Tyree S, Kim WY, Milowsky MI, Pruthi RS, Millikan RC. Trends in stage-specific incidence rates for urothelial carcinoma of the bladder in the United States: 1988 to 2006. Cancer 2014; 120:86-95.

[3] Mowatt G, Zhu S, Kilonzo M, Boachie C, Fraser C, Griffiths TR, N'Dow J, Nabi G, Cook J, Vale L. Systematic review of the clinical effectiveness and cost-effectiveness of photodynamic diagnosis and urine biomarkers (FISH, ImmunoCyt, NMP22) and cytology for the detection and follow-up of bladder cancer. Health Technol Assess 2010; 14:1-331, iii-iv.

[4] Banek S, Schwentner C, Tager D, Pesch B, Nasterlack M, Leng G, Gawrych K, Bonberg N, Johnen G, Kluckert M, Gakis G, Todenhofer T, Hennenlotter J, Bruning T, Stenzl A, UroScreen Study G. Prospective evaluation of fluorescence-in situ-hybridization to detect bladder cancer: results from the UroScreen-Study. Urol Oncol 2013; 31:1656-62.

[5] Huber S, Schwentner C, Taeger D, Pesch B, Nasterlack M, Leng G, Mayer T, Gawrych K, Bonberg N, Pelster M, Johnen G, Bontrup H, Wellhausser H, Bierfreund HG, Wiens C, Bayer C, Eberle F, Scheuermann B, Kluckert M, Feil G, Bruning T, Stenzl A, UroScreen Study G. Nuclear matrix protein-22: a prospective evaluation in a population at risk for bladder cancer. Results from the UroScreen study. BJU Int 2012; 110:699-708.

[6] Odisho AY, Berry AB, Ahmad AE, Cooperberg MR, Carroll PR, Konety BR. Reflex ImmunoCyt testing for the diagnosis of bladder cancer in patients with atypical urine cytology. Eur Urol 2013; 63:936-40.

[7] Surveillance, Epidemiology, and End Results (SEER) Program: SEER Stat Fact Sheets, Bladder Cancer 2005-2011 and SEER Summary Stage 2000. (Accessed at http://seer.cancer.gov/statfacts/html/urinb.html).

[8] Babjuk M, Burger M, Zigeuner R, Shariat SF, van Rhijn BW, Comperat E, Sylvester RJ, Kaasinen E, Bohle A, Palou Redorta J, Roupret M, European Association of U. EAU guidelines on non-muscle-invasive urothelial carcinoma of the bladder: update 2013. Eur Urol 2013; 64:639-53.

[9] Witjes JA, Comperat E, Cowan NC, De Santis M, Gakis G, Lebret T, Ribal MJ, Van der Heijden AG, Sherif A, European Association of U. EAU guidelines on muscle-invasive and metastatic bladder cancer: summary of the 2013 guidelines. Eur Urol 2014; 65:778-92.

[10] Picotti P, Aebersold R. Selected reaction monitoring-based proteomics: workflows, potential, pitfalls and future directions. Nat Methods 2012; 9:555-66.

[11] U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM): Guidance for Industry, Bioanalytical Method Validation (Accessed May 2001, at http://www.fda.gov/).

[12] Chatziharalambous D, Lygirou V, Latosinska A, Stravodimos K, Vlahou A, Jankowski V, Zoidakis J. Analytical Performance of ELISA Assays in Urine: One More Bottleneck towards Biomarker Validation and Clinical Implementation. PLoS One 2016; 11:e0149471.

[13] Grebe SK, Singh RJ. LC-MS/MS in the Clinical Laboratory - Where to From Here? Clin Biochem Rev 2011; 32:5-31.

[14] Filip S, Vougas K, Zoidakis J, Latosinska A, Mullen W, Spasovski G, Mischak H, Vlahou A, Jankowski J. Comparison of Depletion Strategies for the Enrichment of Low-Abundance Proteins in Urine. PLoS One 2015; 10:e0133773.

[15] Latosinska A, Vougas K, Makridakis M, Klein J, Mullen W, Abbas M, Stravodimos K, Katafigiotis I, Merseburger AS, Zoidakis J, Mischak H, Vlahou A, Jankowski V. Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis. PLoS One 2015; 10:e0137048.

[16] Sobin L, Gospodarowicz K, Wittekind C. TNM Classification of Malignant Tumours, 7th edition. UICC International Union Against Cancer. Wiley-Blackwell 2009.

[17] Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods 2009; 6:359-62.

[18] Bairoch A, Apweiler R. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res 2000; 28:45-8.

[19] UniProt C. UniProt: a hub for protein information. Nucleic Acids Res 2015; 43:D204-12.

[20] Eng JK, McCormack AL, Yates JR. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom 1994; 5:976-89.

[21] Kall L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat Methods 2007; 4:923-5.

[22] Serang O, Noble W. A review of statistical methods for protein identification using tandem mass spectrometry. Stat Interface 2012; 5:3-20.

[23] Wilhelm M, Schlegl J, Hahne H, Moghaddas Gholami A, Lieberenz M, Savitski MM, Ziegler E, Butzmann L, Gessulat S, Marx H, Mathieson T, Lemeer S, Schnatbaum K, Reimer U, Wenschuh H, Mollenhauer M, Slotta-Huspenina J, Boese JH, Bantscheff M, Gerstmair A, Faerber F, Kuster B. Mass-spectrometry-based draft of the human proteome. Nature 2014; 509:582-7.

[24] Uhlen M, Bjorling E, Agaton C, Szigyarto CA, Amini B, Andersen E, Andersson AC, Angelidou P, Asplund A, Asplund C, Berglund L, Bergstrom K, Brumer H, Cerjan D, Ekstrom M, Elobeid A, Eriksson C, Fagerberg L, Falk R, Fall J, Forsberg M, Bjorklund MG, Gumbel K, Halimi A, Hallin I, Hamsten C, Hansson M, Hedhammar M, Hercules G, Kampf C, Larsson K, Lindskog M, Lodewyckx W, Lund J, Lundeberg J, Magnusson K, Malm E, Nilsson P, Odling J, Oksvold P, Olsson I, Oster E, Ottosson J, Paavilainen L, Persson A, Rimini R, Rockberg J, Runeson M, Sivertsson A, Skollermo A, Steen J, Stenvall M, Sterky F, Stromberg S, Sundberg M, Tegel H, Tourle S, Wahlund E, Walden A, Wan J, Wernerus H, Westberg J, Wester K, Wrethagen U, Xu LL, Hober S, Ponten F. A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics 2005; 4:1920-32.

[25] Dyrskjot L, Kruhoffer M, Thykjaer T, Marcussen N, Jensen JL, Moller K, Orntoft TF. Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. Cancer Res 2004; 64:4040-8.

[26] Kawakami K, Enokida H, Tachiwada T, Gotanda T, Tsuneyoshi K, Kubo H, Nishiyama K, Takiguchi M, Nakagawa M, Seki N. Identification of differentially expressed genes in human bladder cancer through genome-wide gene expression profiling. Oncol Rep 2006; 16:521-31.

[27] Liu Y, Noon AP, Aguiar Cabeza E, Shen J, Kuk C, Ilczynski C, Ni R, Sukhu B, Chan K, Barbosa-Morais NL, Hermanns T, Blencowe BJ, Azad A, van der Kwast TH, Catto JW, Zlotta AR, Wrana JL. Next-generation RNA Sequencing of Archival

Formalin-fixed Paraffin-embedded Urothelial Bladder Cancer. Eur Urol 2014; 66:982-6.

[28] Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 2006; 7:R80.

[29] Taylor TP, Janech MG, Slate EH, Lewis EC, Arthur JM, Oates JC. Overcoming the effects of matrix interference in the measurement of urine protein analytes. Biomark Insights 2012; 7:1-8.

[30] Kift RL, Messenger MP, Wind TC, Hepburn S, Wilson M, Thompson D, Smith MW, Sturgeon C, Lewington AJ, Selby PJ, Banks RE. A comparison of the analytical performance of five commercially available assays for neutrophil gelatinase-associated lipocalin using urine. Ann Clin Biochem 2013; 50:236-44.

[31] Wu J, Chen YD, Gu W. Urinary proteomics as a novel tool for biomarker discovery in kidney diseases. J Zhejiang Univ Sci B 2010; 11:227-37.

[32] Moser AC, Hage DS. Immunoaffinity chromatography: an introduction to applications and recent developments. Bioanalysis 2010; 2:769-90.

[33] Fountoulakis M, Juranville JF, Jiang L, Avila D, Roder D, Jakob P, Berndt P, Evers S, Langen H. Depletion of the high-abundance plasma proteins. Amino Acids 2004; 27:249-59.

[34] Tu C, Rudnick PA, Martinez MY, Cheek KL, Stein SE, Slebos RJ, Liebler DC. Depletion of abundant plasma proteins and limitations of plasma proteomics. J Proteome Res 2010; 9:4982-91.

[35] Kushnir MM, Mrozinski P, Rockwood AL, Crockett DK. A depletion strategy for improved detection of human proteins from urine. J Biomol Tech 2009; 20:101-8.

[36] Mischak H, Kolch W, Aivaliotis M, Bouyssie D, Court M, Dihazi H, Dihazi GH, Franke J, Garin J, Gonzalez de Peredo A, Iphofer A, Jansch L, Lacroix C, Makridakis M, Masselon C, Metzger J, Monsarrat B, Mrug M, Norling M, Novak J, Pich A, Pitt A, Bongcam-Rudloff E, Siwy J, Suzuki H, Thongboonkerd V, Wang LS, Zoidakis J, Zurbig P, Schanstra JP, Vlahou A. Comprehensive human urine standards for comparability and standardization in clinical proteome analysis. Proteomics Clin Appl 2010; 4:464-78.

[37] Zerefos PG, Aivaliotis M, Baumann M, Vlahou A. Analysis of the urine proteome via a combination of multi-dimensional approaches. Proteomics 2012; 12:391-400.

[38] Li Z, Adams RM, Chourey K, Hurst GB, Hettich RL, Pan C. Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. J Proteome Res 2012; 11:1582-90.

[39] Patel VJ, Thalassinos K, Slade SE, Connolly JB, Crombie A, Murrell JC, Scrivens JH. A comparison of labeling and label-free mass spectrometry-based proteomics approaches. J Proteome Res 2009; 8:3752-9.

[40] Sjodin MO, Wetterhall M, Kultima K, Artemenko K. Comparative study of label and label-free techniques using shotgun proteomics for relative protein quantification. J Chromatogr B Analyt Technol Biomed Life Sci 2013; 928:83-92.

[41] Trinh HV, Grossmann J, Gehrig P, Roschitzki B, Schlapbach R, Greber UF, Hemmi S. iTRAQ-Based and Label-Free Proteomics Approaches for Studies of Human Adenovirus Infections. Int J Proteomics 2013; 2013:581862.

6. Appendix

6.1. Affidative

I, **Agnieszka Latosinska** certify under penalty of perjury by my own signature that I have submitted the thesis on the topic **Optimizing methodologies for clinical proteomics** I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are, as such, in proper citations (see "uniform requirements for manuscripts (URM)" the ICMJE www.icmje.org) indicated. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

Date _____ Signature

6.2. Statement of Originality

Agnieszka Latosinska had the following share in the following publications:

Publication 1: Chatziharalambous D, Lygirou V, **Latosinska A**, Stravodimos K, Vlahou A, Jankowski V, Zoidakis J. Analytical performance of ELISA assays in urine: one more bottleneck towards biomarker validation and clinical implementation. PLoS One. 2016

Contribution in detail: Participated in data interpretation, contributed in the revision of the manuscript, critically evaluated the manuscript

Publication 2: Filip S, Vougas K, Zoidakis J, **Latosinska A**, Mullen W, Spasovski G, Mischak H, Vlahou A, Jankowski J. Comparison of Depletion Strategies for the Enrichment of Low-Abundance Proteins in Urine. PLoS One. 2015

Contribution in detail: Analysed the data (data processing and data analysis), wrote the manuscript, contributed in the revision of the manuscript, critically evaluated the manuscript

Publication 3: Latosinska A, Vougas K, Makridakis M, Klein J, Mullen W, Abbas M, Stravodimos K, Katafigiotis I, Merseburger AS, Zoidakis J, Mischak H, Vlahou A, Jankowski V. Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis. PLoS One. 2015

Contribution in detail: Conceived and designed the experiments, performed the experiments (sample preparation), analysed the data (data processing and data analysis), participated in data interpretation, wrote and revised the manuscript

Signature, date and stamp of the supervising University teacher

_____

Signature of the doctoral candidate

_____

6.3. Selected publications

PLOS ONE

RESEARCH ARTICLE

Analytical Performance of ELISA Assays in Urine: One More Bottleneck towards Biomarker Validation and Clinical Implementation

Despina Chatziharalambous[1], Vasiliki Lygirou[1], Agnieszka Latosinska[1,3], Konstantinos Stravodimos[2], Antonia Vlahou[1], Vera Jankowski[4], Jerome Zoidakis[1]*

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Abstract

ELISA is the main approach for the sensitive quantification of protein biomarkers in body fluids and is currently employed in clinical laboratories for the measurement of clinical markers. As such, it also constitutes the main methodological approach for biomarker validation and further qualification. For the latter, specific assay performance requirements have to be met, as described in respective guidelines of regulatory agencies. Even though many clinical ELISA assays in serum are regularly used, ELISA clinical applications in urine are significantly less. The scope of our study was to evaluate ELISA assay analytical performance in urine for a series of potential biomarkers for bladder cancer, as a first step towards their large scale clinical validation. Seven biomarkers (Secreted protein acidic and rich in cysteine, Survivin, Slit homolog 2 protein, NRC-Interacting Factor 1, Histone 2B, Proteinase-3 and Profilin-1) previously described in the literature as having differential expression in bladder cancer were included in the study. A total of 11 commercially available ELISA tests for these markers were tested by standard curve analysis, assay reproducibility, linearity and spiking experiments. The results show disappointing performance with coefficients of variation>20% for the vast majority of the tests performed. Only 3 assays (for Secreted protein acidic and rich in cysteine, Survivin and Slit homolog 2 protein) passed the accuracy thresholds and were found suitable for further application in marker quantification. These results collectively reflect the difficulties in developing urine-based ELISA assays of sufficient analytical performance for clinical application, presumably attributed to the urine matrix itself and/or presence of markers in various isoforms.

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Introduction

To establish a protein as a disease biomarker, its accurate, sensitive and reproducible detection and quantification in large numbers of samples representing the biomarker context of use is necessary. The most common methods for protein biomarker validation are affinity-based assays, such as enzyme-linked immunosorbent assays (ELISAs). ELISAs have high sensitivity and reasonable specificity for the detection of protein amounts with concentration ranges of ng/ml to pg/ml in serum. [1] Major limitations of this approach are the restricted number of validated ELISAs for human proteins, the costly and lengthy development of novel assays, and the limited multiplexing due to antibody (Ab) cross-reactivity. [2] These issues hinder the rapid validation of putative biomarkers derived from high-throughput proteomic and genomic studies. [3]

Research based on urine proteomics is crucial for the discovery of disease biomarkers especially of the renal and urogenital systems. In these latter cases, urine is apparently the most appropriate body fluid that can actually be examined for detecting changes related to pathophysiology as it is the filtrate of blood by the kidneys in direct contact with the bladder containing many soluble biomarker proteins. In addition, urine is easily available and can be collected frequently and in a non-invasive way; consisting collectively an appropriate specimen for proteomic biomarker research. [4,5]

Along these lines major efforts have been invested in recent years in biomarker investigations in urine for multiple diseases. [6,7] Bladder cancer (BC) is a major research area where introduction of effective biomarkers is expected to be of major impact on patient management: BC has the highest recurrence rate (approximately 30–70%) among all malignancies and requires extensive patient monitoring for several years. The gold standard for BC initial diagnosis and follow up is cystoscopy (endoscopic examination of the bladder), which is invasive and expensive. Urine cytology which is also used in the clinical setting lacks sensitivity for low grade tumors and is characterized by inter-observer variability. [8] Thus, non-invasive approaches with high sensitivity and specificity for early detection of primary tumors and recurrences are needed. [9,10] An effective BC biomarker could allow reducing the number of unnecessary cystoscopies especially among patients with low risk disease and as a result improve the patients' quality of life.

As a result of extensive research, several biomarker candidates have been identified following analysis of the urine proteome of bladder cancer patients. [11–15] Nevertheless, despite these efforts, no clinical implementation has been achieved yet, in most part due to lack of appropriate validation studies establishing the biomarker context of use. [16,17] As a first step towards the validation of previously discovered BC biomarker candidates, the objective of this study was to evaluate the analytical performance of ELISA assays in urine. Biomarker candidates include the: NRC-Interacting Factor 1 (NIF-1), Histone 2B (H2B), Profilin-1 (PFN-1), Slit homolog 2 protein (SLIT-2), Proteinase-3 (PR3), and Secreted protein acidic and rich in cysteine (SPARC) and Survivin. [12,18–20] In several cases (NIF-1, H2B, PFN-1) the association of these proteins with BC at the tissue level has been proven [11,12] and initial verification studies in urine have shown discriminatory potential of these marker for bladder cancer detection. [12,18,19] Survivin, has been described in multiple studies as a bladder cancer biomarker, in most cases, based on RT-PCR measurements, [20] but also based on ELISA. [21] Nevertheless, no clear added value for the use of this marker has been demonstrated, in part due to suboptimal assays for its measurement. [20,22]

In this study, extensive analytical validation of commercially available ELISA assays for these markers in urine was performed according to FDA guidelines, as a first step towards the

validation of their clinical use. [23] This is particularly interesting since few studies on the analytical performance of ELISA assays in urine are available. [2]

Materials and Methods

Urine samples

Urine samples from benign cases and BC patients were collected at the Urology clinic of the Laikon University Hospital, Athens, Greece in accordance to the local ethics regulations. The Ethics committee of Laikon Hospital (protocol number EΣ618) specifically approved the research for this study. In all cases, written consent forms were obtained.

The patients were selected according to the following criteria. Cases had bladder cancer primary tumors; controls suffered from benign urological conditions (hernia, etc).

Clinical data on the urine samples are presented in Table A in S7 File.

The samples were thawed, centrifuged at 2000 rpm for 10 min, and the supernatant was aliquoted to volumes ranging from 0.1 to 1.0 ml. Samples were stored at -20°C and aliquots were thawed for ELISA assays and pH/protein/hematuria determination. Thawed aliquots were not reused. The pH and hematuria of the urine samples was measured by using standard urine analysis strips from EMAPOL and are presented in Table A in S7 File. The protein concentration of the urine samples was measured by the Bradford assay.

ELISA assays

The following commercially available ELISA kits were tested:

- SPARC: R&D Systems Inc., Minneapolis, MN 55413, USA (Catalogue no. DSP00)
- SLIT-2: Cloud Clone Corp., Houston, TX 77082, USA (Catalogue no. SEA672Hu)
- H2B: US Biological Life Sciences, Swampscott, Massachusetts 01907, USA (Catalogue no. 025705) and Cloud Clone Corp., Houston, TX 77082, USA (Catalogue no. SEA356Hu)
- Survivin: Enzo Life Sciences AG, Postfach CH-4415 Lausen/Switzerland (Catalogue no. ADI-900-111), R&D Systems Inc., Minneapolis, MN 55413, USA (Catalogue no. DSV00)
- PFN-1: USCN LIFE, WUHAN EIAAB SCIENCE CO. LTD, Optics Valley, Wuhan, China (Catalogue no. E2122h); US Biological Life Sciences, Swampscott, Massachussetts 01907, USA (Catalogue no. 027613) and Cloud Clone Corp., Houston, TX 77082, USA (USCN Life Science Inc., Catalogue no. SEC233Hu)
- NIF-1: Cusabio Biotech CO. LTD, Wuhan, Hubei Province 430206, P.R.China (Catalogue no. CSB-EL026683HU) and USCN LIFE, WUHAN EIAAB SCIENCE CO. LTD, Optics Valley, Wuhan, China (Catalogue no. E1019h)
- PR3: Cusabio Biotech CO. LTD, Wuhan, Hubei Province 430206, P.R.China (Catalogue no. CSB- E13058h)

The type of plate reader used was ELx800 (BioTek Instruments).

Standard curve validation. Blanks and standards were assayed according to the manufacturer's instructions in each case. All assays were performed in duplicate and in at least 2 different days. The mean values of Absorbance vs. Concentration were plotted and a 4 Parameter Logistic (4PL) nonlinear regression model) fit was applied ($R^2 > 0.95$ was acceptable).

Recovery. A negative urine sample was spiked with 3 different standards containing high, medium and low concentration of the marker, in 4 replicates each time. The standard protein

provided by each ELISA manufacturer was used for the spiking experiments. The % recovery was calculated and the acceptable range was 80 to 120%.

Reproducibility. Three urine samples containing high, medium and low concentration of the marker were selected and at least five technical replicates were assayed to calculate the coefficient of variation (CV %) for intra-assay reproducibility. The acceptable range of CV was 0–20%.

The inter-assay reproducibility was evaluated only for the SLIT-2, Survivin, and SPARC since these assays had satisfactory intra-assay reproducibility. Aliquots were used in order to avoid freeze/thaw cycles.

Linearity. A urine sample with high marker concentration based on the present study and a published report [11] was selected and serial dilutions (1:2 to 1:32) were performed. Each linearity tests was performed in at least 4 replicates and the experimental versus theoretical concentrations were plotted. The acceptable range was a linear fit with $R^2>0.9$ and a slope of 0.9–1.0.

Limit of Detection (LOD) and Limit of Quantitation (LOQ). The LOD was provided by each ELISA kit manufacturer. The LOQ was determined by interpolating the absorbance of the lowest or highest standard on the standard curve.

Biomarker evaluation. The t-test was used to evaluate statistical differences between groups (benign controls and tumor stages; tumor grades 1, 2, 3). The effect of hematuria on ELISA results for SPARC, SLIT-2, and Survivin was assessed by the chi-square test.

Results

Most of the selected proteins had shown discriminatory power as BC biomarkers based on previous studies [11,12] However, no data in urine were available for SLIT-2 and SPARC; thereby these two proteins were initially tested in a small number of BC urine samples and controls (n = 167). In both cases, significantly higher levels of these proteins in BC samples compared to controls were obtained underscoring the need for their further validation. (Figures A, B in S1 File)

As summarized in Table 1, a total of three ELISA kits targeting respectively SPARC, Survivin and SLIT-2 successfully passed the analytical evaluation tests, whereas a total of 8 assays for NIF-1, PFN-1, PR3 and H2B showed poor analytical performance (Table 1). SPARC (R&D Systems, DSP00) and PR3 (Cusabio Biotech Co. LTD, E13058h) results are presented as examples of successful or poor analytical validation performance respectively (Figs 1–3, Tables 2 and 3), and detailed experimental data for each kit can be found in the supplementary information section. For SPARC, the standards yielded reproducible results and a good fit to the 4 Parameter Logistic (4PL) nonlinear regression model (Fig 1A) Similarly, for PR3, the standards yielded reproducible results and a good fit to the 4PL nonlinear regression model (Fig 1B). In contrast to SPARC, the PR3 assay failed the rest of the analytical performance tests. For SPARC, the % recovery for the medium and high standard was 118% and 108% respectively passing the acceptance threshold (Table 2). Nevertheless, recovery was 136% for the low SPARC levels, reflecting potential inaccuracies in the marker measurements at low concentrations. (Table 2)

In contrast, for PR3, the % recovery for the low, medium, and high standards was 269%, 135%, and 126% respectively (Table 3) clearly exceeding the allowed acceptable recovery range.

When tested for reproducibility in measurement using high, medium and low biomarker concentrations, as described in Materials and Methods section satisfactory CVs were obtained for SPARC. For the low [SPARC] sample a CV of 4%, for the medium [SPARC] sample a CV of 5% and for the high [SPARC] sample, a CV of 8% was obtained. (Fig 2A)

Table 1. Summary of analytical performance.

Protein	Company	Catalogue number	Analytical performance
SPARC	R&D Systems	DSP00	Successful in all assays
SLIT-2	Cloud Clone Corp.	SEA672Hu	Failed in linearity assay
H2B	US Biological Life Sciences	25705	Failed in recovery and reproducibility assays (linearity not possible)
	Cloud Clone Corp.	SEA356Hu	Failed in recovery and reproducibility assays (linearity not possible)
SURVIVIN	Enzo Life Sciences	ADI-900-111	Failed in recovery, reproducibility and linearity assays
	R&D Systems	DSV00	Successful in all assays (linearity not possible)
PFN-1	USCN LIFE	E2122h	Failed in recovery, reproducibility and linearity assays
	US Biological Life Sciences	27613	Failed in recovery, reproducibility and linearity assays
	Cloud Clone Corp.	SEC233Hu	Failed in reproducibility and linearity assays
NIF-1	CUSABIO	EL026683HU	Failed in recovery, reproducibility and linearity assays
	USCN LIFE	E1019h	Failed in recovery and linearity assays
PROTEINASE 3	CUSABIO	E13058h	Failed in recovery, reproducibility and linearity assays

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For PR3, the CV% was above the acceptable 20% limit for the medium and high [PR3] samples (24% and 21% respectively). The low [PR3] sample had a satisfactory CV (7%). (Fig 2B)

When further tested for linearity the performance of the ELISA assay for SPARC was excellent from dilution 1:2 up to 1:16 (R^2 = 0.997 and a slope of 1.023). (Fig 3A) The respective values for PR3 were R^2 = 0.965 which is acceptable, and a slope of 1.46 which is not acceptable. (Fig 3B)

The majority of the remaining ELISA kits, even though successful for the standard curve validation, failed either in reproducibility, or in recovery and linearity studies. For example, in the spiking experiments of pure recombinant standards to negative urine samples, extremely low (e.g. PFN-1 Elisa kit by US Biological, Figure G in S2 File) or high (e.g. NIF-1 Elisa kit by CUSABIO, Figure I in S2 File) % recoveries were obtained. It is important to note that particularly poor results were obtained in the linearity test for most of the assays (Figures A-G in S4 File).

The inter-assay reproducibility was evaluated for SLIT-2, Survivin, and SPARC since only these assays had satisfactory intra-assay reproducibility. The CVs of the inter-assay reproducibility for these 3 ELISA kits are reported in Table A in S9 File. For SPARC the CVs of the urine samples with low, medium and high concentration were 29%, 9% and 34% respectively. For SLIT-2 the CVs of the urine samples with low, medium and high concentration were 43%, 34% and 11% respectively. For Survivin only the CV of a low concentration urine sample could be assessed and was determined to be 41% (the available clinical urine samples were either

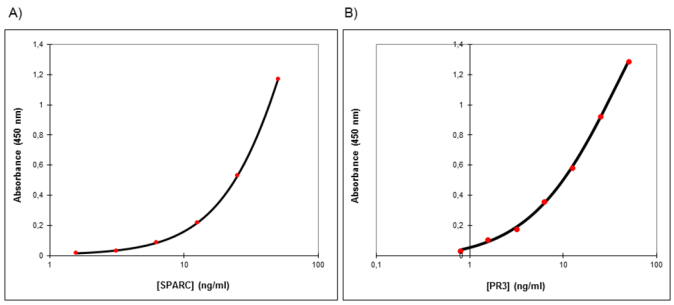

Fig 1. Standard curve validation of A) SPARC (R^2 = 0.999) and B) PR3 (R^2 = 0.996).

doi:10.1371/journal.pone.0149471.g001

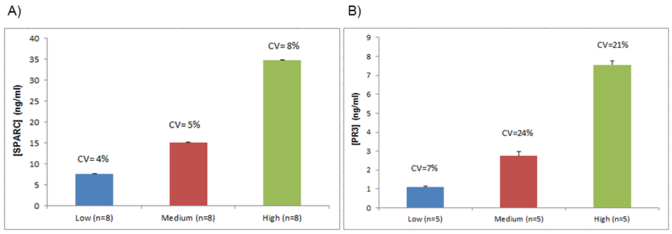

Fig 2. Reproducibility study results of A) SPARC and B) PR3. Three urine samples with low, medium and high concentration were used.

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negative or had low Survivin concentration). Aliquots were used in order to avoid freeze/thaw cycles.

The LOD and LOQ for each ELISA kit are listed in Table A in S10 File.

Hemoglobin released from erythrocyte lysis was measured by standard urine analysis strips (EMAPOL) and its effect on the ELISA assay is reported. (Figure A in S5 File, Table A in S11 File, Table A in S12 File, Table A in S13 File). Hematuria affected significantly only the Survivin ELISA assay as it is was determined by the chi-square statistical test.

The values of the SPARC and SLIT-2 ELISA kits and their dependence on tumor grade are presented. (Figures A, B in S6 File) There is a gradual increase in the SLIT-2 values when tumor grade increases but there is no statistically significant difference. In the case of SPARC Grade 2 tumors have higher mean value compared to G1 and G3 without any statistically significant difference. In both SPARC and SLIT-2 data the standard deviation is very high.

However given the limited number of samples analyzed, a more comprehensive multi-center study is under way for evaluating the effect of tumor grade on SPARC and SLIT-2 ELISA results.

Discussion

Recently, several urine-based bladder tumor markers have been evaluated and are implicated in non-invasive clinical tests for BC detection. [24,25] The commercially available ELISA assays include BTA, nuclear matrix protein 22, AccuDx, and UBC. Unfortunately these ELISA urine biomarkers do not have better performance than cystoscopy and are significantly affected by the presence of hematuria. There is no clearly demonstrated added value for using them in initial diagnosis or patient monitoring. [17]

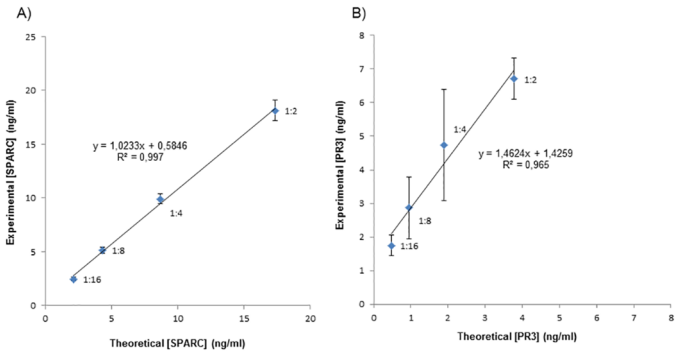

Fig 3. Linearity results of A) SPARC and B) PR3. For each biomarker a high concentration sample was serially diluted and theoretical values were compared to the experimental.

doi:10.1371/journal.pone.0149471.g003

Table 2. Recovery study results of SPARC. Negative urine samples were spiked with low, medium and high concentration of standard.

Negative + 3.13 ng/ml SPARC (n = 8)	Mean [SPARC] (ng/ml)	2.14
	Expected [SPARC] (ng/ml)	1.57
	% Recovery	***136%***
Negative + 12.5 ng/ml SPARC (n = 8)	Mean [SPARC] (ng/ml)	7.4
	Expected [SPARC] (ng/ml)	6.25
	% Recovery	***118%***
Negative + 50 ng/ml SPARC (n = 8)	Mean [SPARC] (ng/ml)	27
	Expected [SPARC] (ng/ml)	25
	% Recovery	***108%***

doi:10.1371/journal.pone.0149471.t002

Urine complexity hinders the development of methods for precise and reproducible protein quantitation. [5] Urine contains more than 1,500 proteins, the majority of which are extracellular and membrane bound along with cells and cellular debris, inorganic ions (K^+, Na^+, Cl^- and Ca^{2+}) and organic molecules such as creatinine, urea, and uric acid. All these substances can hinder the efficient binding of a protein to its corresponding antibody used in an ELISA assay. [12,26] Variability of urine matrix components such as electrolytes or pH can also have an effect on antibody binding and therefore on the performance of the immunoassay. [27] In the case of multiplex bead array assays, to compensate for the impact of matrix effects on biological fluids, manufacturers have developed standard sample diluents for serum, plasma, cultured cells. For urine, a diluent of phosphate buffered saline is recommended for use; however this does not resolve the issue of variability of urine matrix components as the measurements appear less stable compared to those in serum and plasma. [28] To our knowledge the only urinary protein measured by ELISA in clinical laboratories is albumin. [4]

Nevertheless even in the case of albumin measurements multiple limitations have been identified, mostly related to the presence of the protein in multiple isoforms. Many of these forms are considered different to those in plasma. Currently, a reference standard material for urine albumin is not available therefore serum albumin is used for calibration in urine assays. In healthy individuals serum albumin, when filtered and excreted in urine, is composed of a minor amount of intact protein (~4%) and a large amount of albumin fragments with MW in the 1–15 kDa range (~96%). [29] However, it was shown that diabetic nephropathy gradually increases the percentage of intact albumin in urine up to 35% in severe cases. [30] Conventional ELISA assays can detect only certain forms of albumin and the antibodies used fail to

Table 3. Recovery study results of PR3. Negative urine samples were spiked with low, medium and high concentration of standard.

Negative + 1.56 ng/ml PR3 (n = 4)	Mean [PR3] (ng/ml)	2.10
	Expected [PR3] (ng/ml)	0.78
	% Recovery	***269%***
Negative + 6.25 ng/ml PR3 (n = 4)	Mean [PR3] (ng/ml)	4.21
	Expected [PR3] (ng/ml)	3.13
	% Recovery	***135%***
Negative + 25 ng/ml PR3 (n = 4)	Mean [PR3] (ng/ml)	15.74
	Expected [PR3] (ng/ml)	12.50
	% Recovery	***126%***

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bind efficiently to many isoforms. [31] Despite these limitations Albumin ELISA is routinely used in clinical laboratories mainly for diagnosis of kidney diseases. [32]

In light of these findings for one of the most abundant urinary proteins it is imperative to evaluate the analytical performance of ELISA kits for the detection of candidate biomarkers in urine. The FDA guidelines for Bioanalytical Method Validation were followed. [23] Unfortunately, most ELISA assays used in this study did not pass these strict analytical criteria. Some explanations for these disappointing results are presented along with a comparison to previous urinary ELISA analytical performance studies.

Low recovery may be due to interference of antigen recognition caused by substances present in urine (salts, organic molecules, etc.). High recovery may be due to non-specific binding of proteins to the antibody immobilized on the ELISA plate. In a study by Taylor et al., in order to determine the degree of matrix interference in protein measurement in urine, known concentrations of 5 proteins (IL-6, IL-8, MCP1, MP1a and TNFα) were spiked in urine samples of 4 kidney disease patients and assayed 4 times each. High variability was observed in protein recovery in the urine samples even between assays indicating that matrix components differ among urine samples and also highlighting their ability to variably interfere in accurate protein measurement. [28]

Inter-assay reproducibility results were not acceptable (high CVs) for the three kits that yielded satisfactory intra-assay reproducibility (SPARC from R&D Systems, SLIT-2 from Cloud-Clone Corp. and Survivin from R&D Systems) (Table A in S9 File)

The failure of the linearity test is the major deficiency of most ELISA kits analyzed. A possible explanation of this deficiency is the fact that in urine proteins exist in multiple forms with different affinities for the ELISA antibodies. As it was determined for Albumin, urinary proteins are not present only as full length polypeptides but also as numerous low MW peptides and exhibit unique post-translational modifications (PTMs) different from those in plasma. [30,31,33] It is possible that some of these forms have higher Kd than the full length and do not bind to the Ab upon dilution resulting in lower signal. (Figures D, G in S3 File) Moreover, the linearity of the assay can be affected by the dilution of interfering salts and organic molecules. As a result protein-Ab binding is enhanced and a higher signal is obtained (Figures A, F in S3 File). In the case of Survivin and SLIT-2, urine sample desalting was performed before ELISA analysis. Unfortunately the desalting did not increase signal intensity and thus did not improve assay performance (data not shown). For the two H2B Elisa kits and the Survivin Elisa kit from R&D Systems the linearity tests could not be performed due to the unavailability of high concentration samples and the minimum detectable dose of each kit.

The poor performance of ELISA assays in urine presented in this study is not a unique occurrence. A comprehensive evaluation of the analytical performance of ELISA assays for Neutrophil gelatinase-associated lipocalin (NGAL) yielded poor results for recovery and linearity. These findings indicated the presence of variability in urinary immunoassay performance that needs to be taken into consideration in clinical sample analysis. [34]

The performance of SLIT-2 and SPARC in detecting BC recurrence and/or progression will be assessed in the context of a large clinical study involving prospectively collected samples. The effect of confounders, such as hematuria, on the ELISA assays and the diagnostic performance of SPARC and SLIT-2 individually or in combination will be evaluated.

The shortcomings of the assays presented in this article reflect the difficulties on developing robust ELISA in urine for clinical applications. An alternative to ELISA assays would be to develop MRM (Multiple Reaction Monitoring) methods for determining biomarker concentration in urine. Beasley-Green et al., employed isotope dilution–mass spectrometry (ID–MS) and multiple reaction monitoring (MRM) as a reference method to measure full-length albumin and its fragments in urine. The assay showed outstanding specificity, reproducibility and

sensitivity. Thus, MRM has the potential to be applied in the clinical setting for biomarker measurements. [35]

Supporting Information

S1 File. Preliminary clinical data of SLIT-2 (Figure A), SPARC (Figure B)($^{*}p \leq 0.05$).
(DOCX)

S2 File. Standard curve validation of SLIT-2 (Cloud-Clone Corp. USCN Life Science Inc., SEA672Hu) (Figure A) H2B (US Biological Life Sciences, 025705) (Figure B) H2B (Cloud-Clone Corp. USCN Life Science Inc., SEA356Hu) (Figure C) Survivin (Enzo Life Sciences, ADI-900-111) (Figure D) Survivin (R&D Systems Inc., DSV00) (Figure E) PFN-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E2122h) (Figure F) PFN-1 (US Biological Life Sciences, 027613) (Figure G) PFN-1 (Cloud-Clone Corp., USCN Life Science Inc., SEC233Hu) (Figure H) NIF-1 (Cusabio Biotech CO. LTD, CSB-EL026683HU) (Figure I) NIF-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E1019h) (Figure J).
(DOCX)

S3 File. Reproducibility study results of SLIT-2 (Cloud-Clone Corp. USCN Life Science Inc., SEA672Hu) (Figure A) H2B (US Biological Life Sciences, 025705) (Figure B) H2B (Cloud-Clone Corp. USCN Life Science Inc., SEA356Hu) (Figure C) Survivin (Enzo Life Sciences, ADI-900-111) (Figure D) Survivin (R&D Systems Inc., DSV00) (Figure E) PFN-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E2122h) (Figure F) PFN-1 (US Biological Life Sciences, 027613) (Figure G) PFN-1 (Cloud-Clone Corp., USCN Life Science Inc., SEC233Hu) (Figure H) NIF-1 (Cusabio Biotech CO. LTD, CSB-EL026683HU) (Figure I) NIF-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E1019h) (Figure J).
(DOCX)

S4 File. Linearity results of SLIT-2 (Cloud-Clone Corp. USCN Life Science Inc., SEA672Hu) (Figure A) Survivin (Enzo Life Sciences, ADI-900-111) (Figure B) PFN-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E2122h) (Figure C) PFN-1 (US Biological Life Sciences, 027613) (Figure D) PFN-1 (Cloud-Clone Corp., USCN Life Science Inc., SEC233Hu) (Figure E) NIF-1 (Cusabio Biotech CO. LTD, CSB-EL026683HU) (Figure F) NIF-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E1019h) (Figure G).
(DOCX)

S5 File. Bar plot results of SPARC (R&D Systems), SLIT-2 (Cloud Clone Corp.) and SURVIVIN (R&D Systems) ELISA (positive/negative) relative to the presence/absence of hematuria (* $p<0.05$). (Figure A).
(DOCX)

S6 File. ELISA results of SPARC (Figure A) and SLIT-2 (Figure B) relative to cancer grade.
(DOCX)

S7 File. Urine strip analysis, ELISA results, and clinical data for urine samples. (Table A).
(DOCX)

S8 File. Recovery study results of SLIT-2 (Cloud-Clone Corp. USCN Life Science Inc., SEA672Hu) (Table A) H2B (US Biological Life Sciences, 025705) (Table B) H2B (Cloud-Clone Corp. USCN Life Science Inc., SEA356Hu) (Table C) Survivin (Enzo Life Sciences, ADI-900-111) (Table D) Survivin (R&D Systems Inc., DSV00) (Table E) PFN-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E2122h) (Table F) PFN-1 (US Biological Life Sciences, 027613) (Table G) PFN-1 (Cloud-Clone Corp., USCN Life Science Inc.,

SEC233Hu) (Table H) NIF-1 (Cusabio Biotech CO. LTD, CSB-EL026683HU) (Table I) NIF-1 (USCN Life, WUHAN EIAAB SCIENCE CO. LTD, E1019h) (Table J).
(DOCX)

S9 File. Inter-assay reproducibility results for SPARC, SLIT-2 and SURVIVIN. (Table A).
(DOCX)

S10 File. LOD and LOQ information for all the ELISA kits. (Table A).
(DOCX)

S11 File. Chi-square test results of SPARC and hematuria. (Table A).
(DOCX)

S12 File. Chi-square test results of SLIT-2 and hematuria. (Table A).
(DOCX)

S13 File. Chi-square test results of SURVIVIN and hematuria. (Table A).
(DOCX)

Author Contributions

Conceived and designed the experiments: DC VL KS AV JZ. Performed the experiments: DC VL KS AV JZ. Analyzed the data: DC VL KS AV JZ. Contributed reagents/materials/analysis tools: KS AV JZ. Wrote the paper: DC AV JZ. Critically read the manuscript: AL VJ.

References

1. Huttenhain R, Malmstrom J, Picotti P, Aebersold R (2009) Perspectives of targeted mass spectrometry for protein biomarker verification. Curr Opin Chem Biol 13: 518–525. doi: 10.1016/j.cbpa.2009.09.014 PMID: 19818677
2. Whiteaker JR, Zhao L, Anderson L, Paulovich AG (2010) An automated and multiplexed method for high throughput peptide immunoaffinity enrichment and multiple reaction monitoring mass spectrometry-based quantification of protein biomarkers. Mol Cell Proteomics 9: 184–196. doi: 10.1074/mcp.M900254-MCP200 PMID: 19843560
3. Stoop MP, Coulier L, Rosenling T, Shi S, Smolinska AM, Buydens L, et al. (2010) Quantitative proteomics and metabolomics analysis of normal human cerebrospinal fluid samples. Mol Cell Proteomics 9: 2063–2075. doi: 10.1074/mcp.M900877-MCP200 PMID: 20811074
4. Gao Y (2015) Urine is a better biomarker source than blood especially for kidney diseases. Adv Exp Med Biol 845: 3–12. doi: 10.1007/978-94-017-9523-4_1 PMID: 25355564
5. Wu J, Chen YD, Gu W (2010) Urinary proteomics as a novel tool for biomarker discovery in kidney diseases. J Zhejiang Univ Sci B 11: 227–237. doi: 10.1631/jzus.B0900327 PMID: 20349519
6. Filip S, Zoidakis J, Vlahou A, Mischak H (2014) Advances in urinary proteome analysis and applications in systems biology. Bioanalysis 6: 2549–2569. doi: 10.4155/bio.14.210 PMID: 25411698
7. Mischak H, Ioannidis JP, Argiles A, Attwood TK, Bongcam-Rudloff E, Broenstrup M, et al. (2012) Implementation of proteomic biomarkers: making it work. Eur J Clin Invest 42: 1027–1036. doi: 10.1111/j.1365-2362.2012.02674.x PMID: 22519700
8. Yafi FA, Brimo F, Steinberg J, Aprikian AG, Tanguay S, Kassouf W (2015) Prospective analysis of sensitivity and specificity of urinary cytology and other urinary biomarkers for bladder cancer. Urol Oncol 33: 66 e25–31. doi: 10.1016/j.urolonc.2014.06.008 PMID: 25037483
9. Frantzi M, Latosinska A, Fluhe L, Hupe MC, Critselis E, Kramer MW, et al. (2015) Developing proteomic biomarkers for bladder cancer: towards clinical application. Nat Rev Urol 12: 317–330. doi: 10.1038/nrurol.2015.100 PMID: 26032553
10. Kaufman DS, Shipley WU, Feldman AS (2009) Bladder cancer. Lancet 374: 239–249. doi: 10.1016/S0140-6736(09)60491-8 PMID: 19520422
11. Frantzi M, Zoidakis J, Papadopoulos T, Zurbig P, Katafigiotis I, Stravodimos K, et al. (2013) IMAC fractionation in combination with LC-MS reveals H2B and NIF-1 peptides as potential bladder cancer biomarkers. J Proteome Res 12: 3969–3979. doi: 10.1021/pr400255h PMID: 23924207

12. Zoidakis J, Makridakis M, Zerefos PG, Bitsika V, Esteban S, Frantzi M, et al. (2012) Profilin 1 is a potential biomarker for bladder cancer aggressiveness. Mol Cell Proteomics 11: M111 009449.
13. Miremami J, Kyprianou N (2014) The promise of novel molecular markers in bladder cancer. Int J Mol Sci 15: 23897–23908. doi: 10.3390/ijms151223897 PMID: 25535079
14. Talukdar S, Emdad L, Das SK, Sarkar D, Fisher PB (2015) Noninvasive approaches for detecting and monitoring bladder cancer. Expert Rev Anticancer Ther 15: 283–294. doi: 10.1586/14737140.2015.989838 PMID: 25494295
15. Ye F, Wang L, Castillo-Martin M, McBride R, Galsky MD, Zhu J, et al. (2014) Biomarkers for bladder cancer management: present and future. Am J Clin Exp Urol 2: 1–14. PMID: 25374904
16. Cheung G, Sahai A, Billia M, Dasgupta P, Khan MS (2013) Recent advances in the diagnosis and treatment of bladder cancer. BMC Med 11: 13. doi: 10.1186/1741-7015-11-13 PMID: 23327481
17. Kluth LA, Black PC, Bochner BH, Catto J, Lerner SP, Stenzl A, et al. (2015) Prognostic and Prediction Tools in Bladder Cancer: A Comprehensive Review of the Literature. Eur Urol 68: 238–253. doi: 10.1016/j.eururo.2015.01.032 PMID: 25709027
18. Martyn-Hemphill C, Mak D, Khan MS, Challacombe BJ, Bishop CV (2013) Recent advances in diagnosis and treatment of transitional cell carcinoma of the bladder. Int J Surg 11: 749–752. doi: 10.1016/j.ijsu.2013.08.018 PMID: 24013072
19. Makridakis M, Roubelakis MG, Bitsika V, Dimuccio V, Samiotaki M, Kossida S, et al. (2010) Analysis of secreted proteins for the study of bladder cancer cell aggressiveness. J Proteome Res 9: 3243–3259. doi: 10.1021/pr100189d PMID: 20423150
20. Jeon C, Kim M, Kwak C, Kim HH, Ku JH (2013) Prognostic role of survivin in bladder cancer: a systematic review and meta-analysis. PLoS One 8: e76719. doi: 10.1371/journal.pone.0076719 PMID: 24204662
21. Li X, Wang Y, Xu J, Zhang Q (2013) Sandwich ELISA for detecting urinary Survivin in bladder cancer. Chin J Cancer Res 25: 375–381. doi: 10.3978/j.issn.1000-9604.2013.08.11 PMID: 23997523
22. Shariat SF, Casella R, Khoddami SM, Hernandez G, Sulser T, Gasser TC, et al. (2004) Urine detection of survivin is a sensitive marker for the noninvasive diagnosis of bladder cancer. J Urol 171: 626–630. PMID: 14713774
23. (2001) Guidance for Industry; Bioanalytical Method Validation. 1–20.
24. Tetu B (2009) Diagnosis of urothelial carcinoma from urine. Mod Pathol 22 Suppl 2: S53–59. doi: 10.1038/modpathol.2008.193 PMID: 19494853
25. Mbeutcha A, Lucca I, Mathieu R, Lotan Y, Shariat SF (2016) Current Status of Urinary Biomarkers for Detection and Surveillance of Bladder Cancer. Urol Clin North Am 43: 47–62. doi: 10.1016/j.ucl.2015.08.005 PMID: 26614028
26. Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M (2006) The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 7: R80. PMID: 16948836
27. Fichorova RN, Richardson-Harman N, Alfano M, Belec L, Carbonneil C, Chen S, et al. (2008) Biological and technical variables affecting immunoassay recovery of cytokines from human serum and simulated vaginal fluid: a multicenter study. Anal Chem 80: 4741–4751. doi: 10.1021/ac702628q PMID: 18484740
28. Taylor TP, Janech MG, Slate EH, Lewis EC, Arthur JM, Oates JC (2012) Overcoming the effects of matrix interference in the measurement of urine protein analytes. Biomark Insights 7: 1–8. doi: 10.4137/BMI.S8703 PMID: 22403482
29. Osicka TM, Panagiotopoulos S, Jerums G, Comper WD (1997) Fractional clearance of albumin is influenced by its degradation during renal passage. Clin Sci (Lond) 93: 557–564.
30. Osicka TM, Houlihan CA, Chan JG, Jerums G, Comper WD (2000) Albuminuria in patients with type 1 diabetes is directly linked to changes in the lysosome-mediated degradation of albumin during renal passage. Diabetes 49: 1579–1584. PMID: 10969843
31. Speeckaert MM, Speeckaert R, Van De Voorde L, Delanghe JR (2011) Immunochemically unreactive albumin in urine: fiction or reality? Crit Rev Clin Lab Sci 48: 87–96. doi: 10.3109/10408363.2011.591366 PMID: 21871001
32. Lopez-Giacoman S, Madero M (2015) Biomarkers in chronic kidney disease, from kidney function to kidney damage. World J Nephrol 4: 57–73. doi: 10.5527/wjn.v4.i1.57 PMID: 25664247
33. Miller WG, Bruns DE, Hortin GL, Sandberg S, Aakre KM, McQueen MJ, et al. (2009) Current issues in measurement and reporting of urinary albumin excretion. Clin Chem 55: 24–38. doi: 10.1373/clinchem.2008.106567 PMID: 19028824

34. Kift RL, Messenger MP, Wind TC, Hepburn S, Wilson M, Thompson D, et al. (2013) A comparison of the analytical performance of five commercially available assays for neutrophil gelatinase-associated lipocalin using urine. Ann Clin Biochem 50: 236–244. doi: 10.1258/acb.2012.012117 PMID: 23605129

35. Beasley-Green A, Burris NM, Bunk DM, Phinney KW (2014) Multiplexed LC-MS/MS assay for urine albumin. J Proteome Res 13: 3930–3939. doi: 10.1021/pr500204c PMID: 25057786

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RESEARCH ARTICLE

Comparison of Depletion Strategies for the Enrichment of Low-Abundance Proteins in Urine

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Abstract

Proteome analysis of complex biological samples for biomarker identification remains challenging, among others due to the extended range of protein concentrations. High-abundance proteins like albumin or IgG of plasma and urine, may interfere with the detection of potential disease biomarkers. Currently, several options are available for the depletion of abundant proteins in plasma. However, the applicability of these methods in urine has not been thoroughly investigated. In this study, we compared different, commercially available immunodepletion and ion-exchange based approaches on urine samples from both healthy subjects and CKD patients, for their reproducibility and efficiency in protein depletion. A starting urine volume of 500 μL was used to simulate conditions of a multi-institutional biomarker discovery study. All depletion approaches showed satisfactory reproducibility (n=5) in protein identification as well as protein abundance. Comparison of the depletion efficiency between the unfractionated and fractionated samples and the different depletion strategies, showed efficient depletion in all cases, with the exception of the ion-exchange kit. The depletion efficiency was found slightly higher in normal than in CKD samples and normal samples yielded more protein identifications than CKD samples when using both initial as well as corresponding depleted fractions. Along these lines, decrease in the amount of albumin and other targets as applicable, following depletion, was observed. Nevertheless, these depletion strategies did not yield a higher number of identifications in neither the urine from normal nor CKD patients. Collectively, when analyzing urine in the context of CKD biomarker identification, no added value of depletion strategies can be observed and analysis of unfractionated starting urine appears to be preferable.

decision to publish, or preparation of the manuscript. The specific roles of these authors are articulated in the 'author contributions' section.

Introduction

Advances in mass spectrometry (MS) have recently facilitated the development of high-throughput and sensitive analysis methods for proteomics investigations [1–3]. However, proteome analysis of complex biological samples remains challenging, among others due to the huge abundance differences among individual protein components; for example, in plasma, the presence of albumin or immunoglobulins (IgG) and other predominant proteins hinder the detection of less abundant proteins and reduces the efficiency of LC-MS/MS analysis [4]. This masking effect is also expected to be pronounced in the analysis of the urinary proteome of patients with chronic kidney disease (CKD) who present high levels of urinary albumin [5]. Furthermore, albumin abundance is highly variable between patients with CKD, even with the same disease etiology, which further complicates the analysis and comparison of the urinary protein content of these samples [6, 7]. Similarly to plasma [8], the range of protein concentration in urine spans several orders of magnitude [9, 10]. Due to the fact that the concentration of potential disease biomarkers might be relatively low, predominant proteins may mask them and make their identification challenging. Therefore, fractionation and depletion strategies are generally employed prior to MS analysis [11].

Currently, several fractionation methods for protein depletion are available. Some of them are based on the separation of proteins by physicochemical properties such as charge (ion-exchange [12]) or size (size-exclusion chromatography [13]), while others target specific protein groups or ligands, such as glycosyl groups in the case of glycoproteins [14] or biochemical properties (i.e. immunoaffinity [15]). These affinity chromatography methods are applicable for a rapid and selective depletion or enrichment of biomolecules from complex samples [16, 17]. The selection of a fractionation strategy depends on the specific study requirements. For example, combinatorial peptide ligand libraries, allow for the simultaneous depletion of highly-abundant proteins and enrichment of low-abundance targets, facilitating their detection by MS [18]. However, this approach requires relatively high amounts of starting material (hundreds of milliliters of urine) to ensure efficient enrichment of low-abundance proteins; otherwise, high- and medium-abundance proteins would not fully saturate their ligands and ultimately the elution would have the same profile as initial sample [19–21]. Since in most cases low volumes of urine (<1 mL) are available when investigating prospectively collected samples from clinical cohorts, combinatorial ligand peptide libraries do not appear to be applicable for analysis of such individual urine samples. [21]. Strategies based on the depletion of abundant proteins require lower initial material compared to combinatorial peptide ligand libraries [21, 22]. These strategies include immuno-based depletion methods involving selective binding of target proteins to the stationary phase based on affinity. They are considered to have high specificity and efficiency and achieve rapid purification or concentration of the analytes [15]. Another depletion strategy is based on ion-exchange chromatography relying on attraction of oppositely charged molecules as the basis for separation [12].

Depletion of abundant proteins appears especially relevant when investigating the urinary proteome of CKD patients, where the levels and variability of highly-abundant proteins noticeably increase with each stage of CKD [5]. On the other hand, depletion of abundant proteins causes co-depletion of several low-abundance proteins, hindering their detection [23–25]. Several protein depletion kits are commercially available. These kits are generally designated to be used for plasma samples and their application has been evaluated in several manuscripts (e.g. [22, 24, 26–28]). Kulloli et al. [28] applied a kit for depletion of 14 abundant proteins in plasma prior to analysis by LC-MS/MS. The depletion allowed to enrich the sample for low-abundance proteins and increased the number of identifications compared to the non-depleted sample (from approx. 71 to 130 proteins). Similarly, Tu et al. [26] observed a 25% increase in the

number of identifications when kits depleting 7 or 14 high-abundance proteins were applied prior to the LC-MS/MS analysis. However, the authors questioned the applicability of the depletion strategy for the identification of disease biomarkers in plasma, since the low-abundance proteins accounted only for 6% of total identifications and 50 of the proteins with the highest abundance accounted for 90% of total spectral counts. Along the same lines, two-dimensional gel electrophoresis (2DE) analyses of plasma samples, where depletion of abundant proteins strategy was applied, demonstrated an increase in the number of spots on the gel. Yet, most of the newly identified spots, represented different isoforms of high-abundance proteins (e.g. albumin, IgGs) [24, 27].

Various protein depletion kits have been also tested on urine samples [29–32]. Afkarian et al. [31] depleted albumin and IgG from urine of diabetic patients with or without nephropathy. Subsequently, iTRAQ labeling was performed and the samples were analyzed by 2D-LC-MS (MALDI-TOF/TOF). No increase in the number of identified proteins was observed in the depleted samples, regardless if the patient was normo- or macro-albuminuric. On the other hand, Kushnir et al. [30] reported a 2.5-fold increase in the number of protein identifications by LC-MS/MS after depleting 6 highly abundant proteins (albumin, IgG, alpha-1 antitrypsin, IgA, transferring and haptoglobin) using multiple affinity removal (MARS) column (Agilent Technologies, Santa Clara, CA). Abundant protein depletion strategies (14 MARS) in conjunction with iTRAQ labeling were also applied for the identification of potential bladder cancer biomarkers from urine [33]. The depletion strategy allowed increasing the number of identifications from approximately 300 proteins in the non-fractionated sample to 500, and the discovery of a potential biomarker panel for bladder cancer [33].

Collectively, based on the existing conflicting data it is presently unclear whether depletion strategies are of benefit when analyzing urine samples. In this study, we therefore aimed to assess the effectiveness of different commercially available depletion strategies for the proteome analysis of urine samples from CKD patients and healthy controls: four different strategies (three immunodepletion- and one ion-exchange-based) were applied prior to LC-MS/MS analysis. The efficiency of depletion, reproducibility, and the overall impact of each strategy on the number of protein identifications and relative protein quantification were assessed.

Materials and Methods

Sample characteristics

Second morning mid-stream urine samples were employed. To remove cell debris, urine was centrifuged at 1,000xg for 10 min at 4°C. Two pooled urine samples (with a final volume of approx. 30 mL each) corresponding, to normal and CKD (stage IV) were generated. Protein content was estimated by Bradford protein assay. To reduce freeze-thaw cycles to minimum, samples were aliquoted in 500 μL (40 aliquots per CKD and normal pool) and kept at -20°C until used. Sample collection was performed in accordance to local ethics requirements and the study was approved by the local ethics committee ("Macedonia Academy of Sciences and Arts"; ethics subcommittee for medicine, pharmacy, veterinary and stomatology: 07–65711, 1-04-2013). All individuals gave written informed consent.

Chromatography approaches

500 μL urine aliquots (corresponding to a protein content of 29 μg for normal and 437 μg for CKD sample) were subjected to buffer exchange applying buffers compatible with each depletion method according to the respective manufacturer, and concentrated to a final volume of 20 μL, using Amicon Ultra Centrifugal Filter Units (3kDa cut-off, Millipore).

Table 1. Characteristics of the applied depletion strategies.

Depletion kit	Company	Mechanism	Depleted proteins
Seppro IgY14	Sigma Aldrich	Immunodepletion	Albumin, IgG, α1-Antitrypsin, IgA, IgM, Transferrin, Haptoglobin, α2-Macroglobulin, Fibrinogen, Complement C3, α1-Acid Glycoprotein (Orosomucoid), HDL (Apolipoproteins A-I and A-II), LDL (mainly Apolipoprotein B)
ProteoPrep	Sigma Aldrich	Immunodepletion	Albumin, IgG
SpinTrap	GE Healthcare	Immunodepletion	Albumin, IgG
ProteoSpin	Norgen Biotek	Ion-exchange	Albumin, alpha-1-antitrypsin, transferrin and haptoglobin

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Such prepared samples were processed with four commercially available kits targeting the depletion of abundant proteins (Table 1) according to the manufacturers' protocols. To assess the reproducibility of each method, five technical replicates of each of the urine samples from healthy controls and from CKD patients per technique were prepared. Depleted samples were obtained either from the flow-through fraction for three immuno-based kits: Seppro IgY14 (Sigma-Aldrich, Saint Louis, MO, USA), ProteoPrep (Sigma-Aldrich, Saint Louis, MO, USA) and SpinTrap (GE Healthcare, Little Chalfont, UK) or in the elution fraction for the ion-exchange kit: ProteoSpin (Norgen Biotek, Thorold, Canada). Protein content after depletion was quantified by Bradford protein assay. The protocol for each depletion kit is briefly described below:

Seppro IgY14: (loading capacity: up to 1000 μg of total protein content) After buffer exchange to "Dilution Buffer" (100 mM Tris-Buffered Saline, Tris-HCl with 1.5 M NaCl, pH 7.4) and concentration to 20 μL, urine sample was further diluted with the "Dilution Buffer" to a final volume of 500 μL. Depletion column was centrifuged to remove the storage buffer and the sample was applied to the column. In brief, the sample was thoroughly mixed with the column resin and incubated on an end-to-end rotator for 15 minutes. This step ensures binding of target proteins to the resin. Afterwards, the sample was centrifuged and the first depleted fraction was collected. Subsequently, to increase the recovery rate of proteins not binding to the resin, 500 μL of "Dilution Buffer" was added onto the column and centrifuged once more. Two fractions (0.5 mL each), corresponding to depleted sample, were combined prior to filter-aided sample preparation (FASP) for LC-MS/MS analysis. The depleted sample was analyzed by SDS-PAGE and LC-MS/MS. To prepare the column for another use, bound proteins were stripped off the column resin by applying "Elution Buffer" (1 M glycine, pH 2.5) followed by 3 min incubation, according to the manufacturer's instructions. Afterwards, the column resin was rinsed and kept in the storage buffer until further use.

ProteoPrep: (loading capacity: up to 3000 μg of total protein content) After buffer-exchange to "Equilibration Buffer" (low ionic strength Tris buffer, pH 7.4) and concentration to 20 μL, the sample was further diluted with "Equilibration Buffer" to a final volume of 100 μL. Diluted sample was then loaded onto the equilibrated column (prepared according to the manufacturer's instructions) and incubated for 10 minutes to allow binding of the target proteins to the column resin. This step was repeated once. The sample was centrifuged and in order to collect remaining unbound proteins, 125 μL of "Equilibration Buffer" was added onto the column. The depleted sample comprised of the flow-through from previous step and the wash (in total 225 μL). The depleted sample was analyzed by both SDS-PAGE and LC-MS/MS. To collect bound proteins for analysis by SDS-PAGE, the column was eluted twice with 150 μL of "Protein Extraction Reagent" (40 mM Trizma Base, 7.0 M urea, 2.0 M thiourea and 1% C_7BzO detergent, pH 10.4). Elution fraction was also kept for further analysis by SDS-PAGE.

SpinTrap: (loading capacity: up to 3000 μg of total protein content) After buffer-exchange to "Binding Buffer" (20 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4) and concentration to 20 μL, the sample was diluted with the Binding Buffer to a final volume of 100 μL. The column was equilibrated, the sample was applied onto the column and incubated for 5 min. Unbound sample components were collected by centrifugation, and the column was washed twice with 100 μL of "Binding Buffer". The depleted sample comprised of these three collected fractions (flow-through of the loaded sample and two washes—300 μL) and was further analyzed by SDS-PAGE and LC-MS/MS. Bound proteins were eluted by adding 150 μL of "Elution Buffer" (0.1 M glycin-HCl, pH 2.7) twice. These obtained fractions (300 μL) were also combined and further analyzed by SDS-PAGE.

ProteoSpin: (loading capacity: up to 500 μg of total protein content) After buffer-exchange to "Binding Buffer" (20 mM sodium phosphate, 0.15 M sodium chloride, pH 7.4) and concentration to 20 μL, the sample was diluted with the "Column Activation and Wash Buffer" (composition not specified by the manufacturer) to a final volume of 500 μL. The column was activated followed by application of the diluted sample. During this step, the non-targeted proteins bind to the resin. Afterwards, the samples were centrifuged. The flow-through containing the highly-abundant target proteins was kept for SDS-PAGE analysis. The column was then washed twice with 500 μL of "Column Activation and Wash Buffer". 100 μL of the "Elution Buffer" (composition not specified by the manufacturer) was added and the column was centrifuged. This step was repeated twice. Collected fractions (200 μL) were combined. This depleted sample was analyzed by SDS-PAGE and LC-MS/MS.

1-dimensional gel electrophoresis (SDS-PAGE)

15μL of each chromatography fraction were loaded on a 10% acrylamide gel and SDS-PAGE was performed. The gels were stained with silver [34].

Sample preparation for LC-MS/MS

Urine samples (5 replicates each) prior to or after subjecting to fractionation (Table 1) were processed following the FASP protocol, commonly applied in our laboratory as described previously [35], with minor modifications. Specifically, in brief, samples were concentrated to a final volume of 50 μL using Amicon Ultra Centrifugal Filter Units (30kDa cut-off, Millipore, Billerica, MA, USA) at 13,000 rpm and incubated with 0.1 M 1,4-Dithioerythritol for 20 min. Subsequently, two centrifugal wash steps were performed by adding 200 μL urea buffer (8M urea in 0.1M TRIS-HCl, pH 8.5). After these centrifugation steps, protein alkylation was conducted by adding 100 μL of iodoacetamide solution (0.05M iodoacetamide in urea buffer) and incubating the mixture for 20 min in the dark. Afterwards, two additional washes with urea buffer were performed followed by two washes with ammonium bicarbonate (ABC) buffer (50mM NH_4HCO_3, pH 8). Overnight digestion was conducted by adding trypsin solution in ABC buffer (trypsin to protein ratio—1:100). Peptides were eluted by centrifugation followed by filter washing with 40 μL ABC solution. The peptide mixture was lyophilized and resuspended in 20 μL (for urine from healthy controls) and 200μL (for urine from CKD patients) of mobile phase A (0.1% formic acid), due to the different protein load of the two samples.

LC-MS/MS analysis

6μL (corresponding to 30% for normal and 3% for CKD samples of the respective total peptide mixtures) of the prepared peptide mixture were analyzed on a Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK). After loading onto a Dionex 0.1×20 mm 5 μm C18 nano trap column at a flow rate of 5 μl/min in 98% 0.1% formic acid and 2% acetonitrile,

sample was eluted onto an Acclaim PepMap C18 nano column 75 μm×50 cm (Dionex, Sunnyvale, CA, USA), 2 μm 100 Å at a flow rate of 0.3 μl/min. The trap and nano flow column were maintained at 35°C. The samples were eluted with a gradient of solvent A: 0.1% formic acid; solvent B: 100% acetonitrile, 0.1% formic acid, starting at 2%B for 10 min, rising to 5%B at 11 min, 15%B at 73 min and 55%B at 95 min. The column was then washed and re-equilibrated prior to injection of the next sample.

The eluant was ionized using a Proxeon nano spray ESI source operating in positive ion mode into an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). Ionization voltage was 2.2 kV and the capillary temperature was 250°C. The mass-spectrometer was operated in MS/MS mode scanning from 350 to 2,000 amu. The resolution of ions in MS1 was 60,000 and 15,000 for HCD MS2. The top 20 multiply charged ions were selected from each scan for MS/MS analysis using HCD at 35% collision energy.

Protein identification and data processing

Protein identification was performed using the SEQUEST search engine (Proteome Discoverer 1.4, Thermo Scientific). Protein search was performed against the SwissProt human protein database (30.10.2013) containing 20277 entries without protein isoforms. The following search parameters were applied: i) fragment mass tolerance: 0.05Da; ii) full tryptic digestion; iii) max missed cleavage sites: 2; iv) static modifications: carbamidomethylation of cysteine; v) dynamic modifications: oxidation of methionine; vi) event detector mass precision: 2 ppm; vii) min. precursor mass: 600 Da; viii) max. precursor mass: 5000 Da; ix) min. collision energy: 0 eV; x) max. collision energy 100 eV; xi) target FDR (strict): 0.01; xii) target FDR (relaxed): 0.05; xiii) FDR validation based on: q-Value. Obtained results were further processed by applying the following filters: i) high confidence (FDR <1%); ii) mass peak deviation: 5 ppm; iii) at least one unique peptide per protein; iv) peptide and protein grouping were enabled. Additionally, since the same peptide can be associated with two (or more) different sequences in different experiments and hence be "lost" for comparison, we initially collected information on the top5 ranked sequences. In the next steps using an in-house developed software (described in the next paragraph), these sequences were harmonized so that the most probable sequence per peptide is assigned, improving the data consistency.

Specifically, the list of peptides was exported from "Proteome Discoverer" and processed further as follows; For each spectrum, the corresponding sequence was defined based on the relative number of sequence identifications in each sample. The relative quantitative analysis was performed based on the peptide area values. Obtained sequences for all technical replicates were merged. Peptides were assigned to the corresponding proteins after merging the list of peptides from 5 technical replicates. Peptides corresponding to multiple proteins were assigned to the protein identified based on the highest number of peptides ("Occam's Razor rule" [36]). Due to a bug in "Proteome Discoverer", for a limited number of peptide identifications the area was not retrieved. If such situation occurred, missing values were replaced by the mean area for the group. Only peptides reported in more than 60% of the samples (3 out of 5 technical replicates) were considered for the calculations of the number of peptide and protein identifications, protein peak areas, sequence coverage, evaluation of consistency and statistical analysis.

Protein peak area was calculated based on the average of top three most abundant peptides for a given protein. Subsequently, normalization of the protein peak areas was conducted. Depletion targets and putative targets were excluded from calculating total sample peak area, since levels of these proteins change between each method applied, introducing bias and falsely increasing the abundance of other proteins. Therefore, the data were normalized based on non-target proteins, which, in principle, should remain unchanged. The validity of this method

was confirmed following a comparison of normalized values to ELISA measurements of albumin (data not shown). As putative depletion targets, we consider proteins with high homology to targeted proteins (Table 1), therefore of potential affinity to the corresponding antibody (for example, different complement factors—see S2 Table for the list of excluded proteins). Proteins identified with at least one unique peptide were included in the analysis.

$$\frac{Average\ protein\ area\ based\ on\ top\ 3\ peptides}{Total\ peak\ area\ (of\ non-targets)\ in\ the\ sample\ based\ on\ average\ of\ top\ 3\ peptides\ per\ protein} * 10\hat{}6$$

Immunoglobulin chains were combined into the following proteins, representing the abundant proteins from the group: Ig gamma-1 chain C region (comprising of lambda, gamma and kappa and heavy chains), Ig alpha-1 chain C region (comprising of Ig alpha chains and J chain) and Ig mu chain C region.

Statistical analysis was based on the unequal variance 2-tailed Student's t-test. Proteins with p-value $\leq$0.05 and ratio $\geq$1.5 or $\leq$0.66 were considered as statistically significant. Additionally, in the case of relative protein abundance, obtained p-values were adjusted by applying Benjamini-Hochberg correction for multiple testing.

Results

SDS-PAGE analysis

Four commercially available depletion kits were employed to estimate their efficiency and reproducibility in combination with LC-MS/MS analysis of urinary proteins. Five technical replicates were performed in each case, using urine from normal or CKD patients. In addition, 5 technical replicates of each of the urine from CKD and normal patients (unfractionated – starting material) were analyzed to assess effectiveness of protein depletion. Since the study aims at the evaluation of depletion strategies in biomarker discovery using samples from large clinical cohorts, where typically low-urine volumes are available per researcher, the analysis was performed using a starting volume of 500 μL (without targeting specific starting protein amounts, regularly not feasible in such studies).

Fractionation was performed according to the manufacturer's instructions with minor adaptations, as described in the Materials and Methods section. Bradford assay was performed to estimate the total protein content in urine samples after depletion. Protein amounts at different steps of the analysis, when determined, are presented in Fig 1. The total protein content prior to depletion was estimated at 29 μg (normal) and 437 μg (CKD). In the case of normal sample, the protein content after depletion was below the limit of detection, regardless of the method applied. For the CKD sample, after applying ProteoPrep and SpinTrap kits, the protein content was estimated at 48 μg and 65 μg respectively. The highest protein amount remaining in the sample after depletion was observed for ion-exchange-based ProteoSpin kit, (estimated at 135 μg). For Seppro IgY14, the respective protein content was below the limit of detection. As shown, protein measurements in the depleted fraction vary among different methods, as expected in part based on their specificity.

Depleted urine fractions were then subjected to SDS-PAGE analysis to investigate efficiency and reproducibility of each depletion strategy. Representative gel fractions per method are presented in Fig 2 and all of the analyzed SDS-PAGE gels are shown in S1–S5 Figs. Gel patterns of the depleted fractions indicate reproducibility in all cases (evidenced in S1–S5 Figs), as estimated by their high similarity among technical replicates. As shown based on this gel image analysis, the immuno-based methods appear to have a higher depletion efficiency compared to the ion-exchange strategy, in overall agreement with the measured protein concentration.

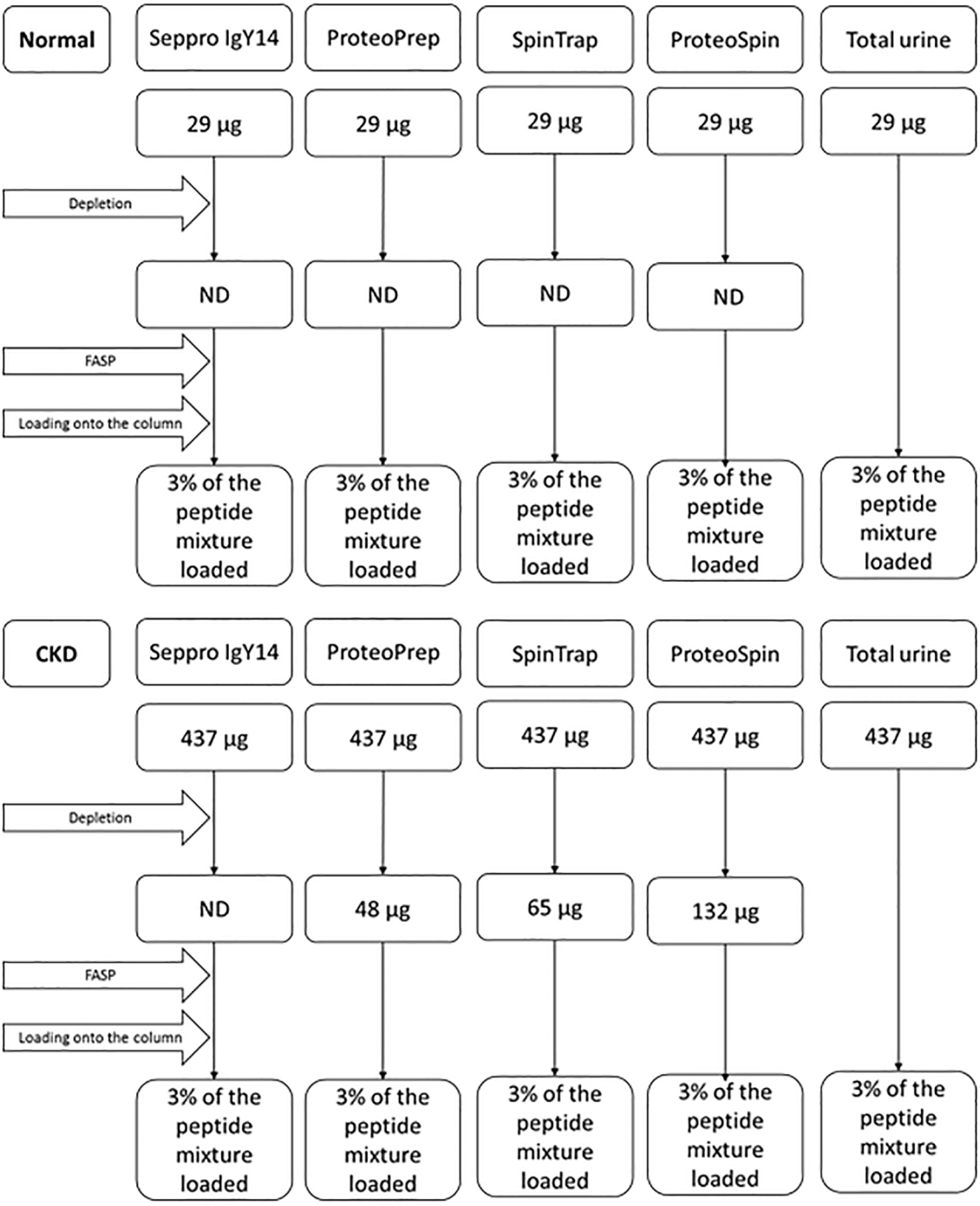

Fig 1. Protein amounts at different steps of the analysis as estimated by Bradford measurements. ND: not determined due to measurements being below the limit of detection (i.e. concentration < 0.2 µg/µL).

doi:10.1371/journal.pone.0133773.g001

Urine peptides and proteins identified by LC-MS/MS

Urine samples prior to or after depletion were processed according to the FASP protocol and analyzed by LC-MS/MS. The numbers of identified peptides per run for each of the five technical replicates per method were compared (Fig 3). For urine of healthy controls, the highest

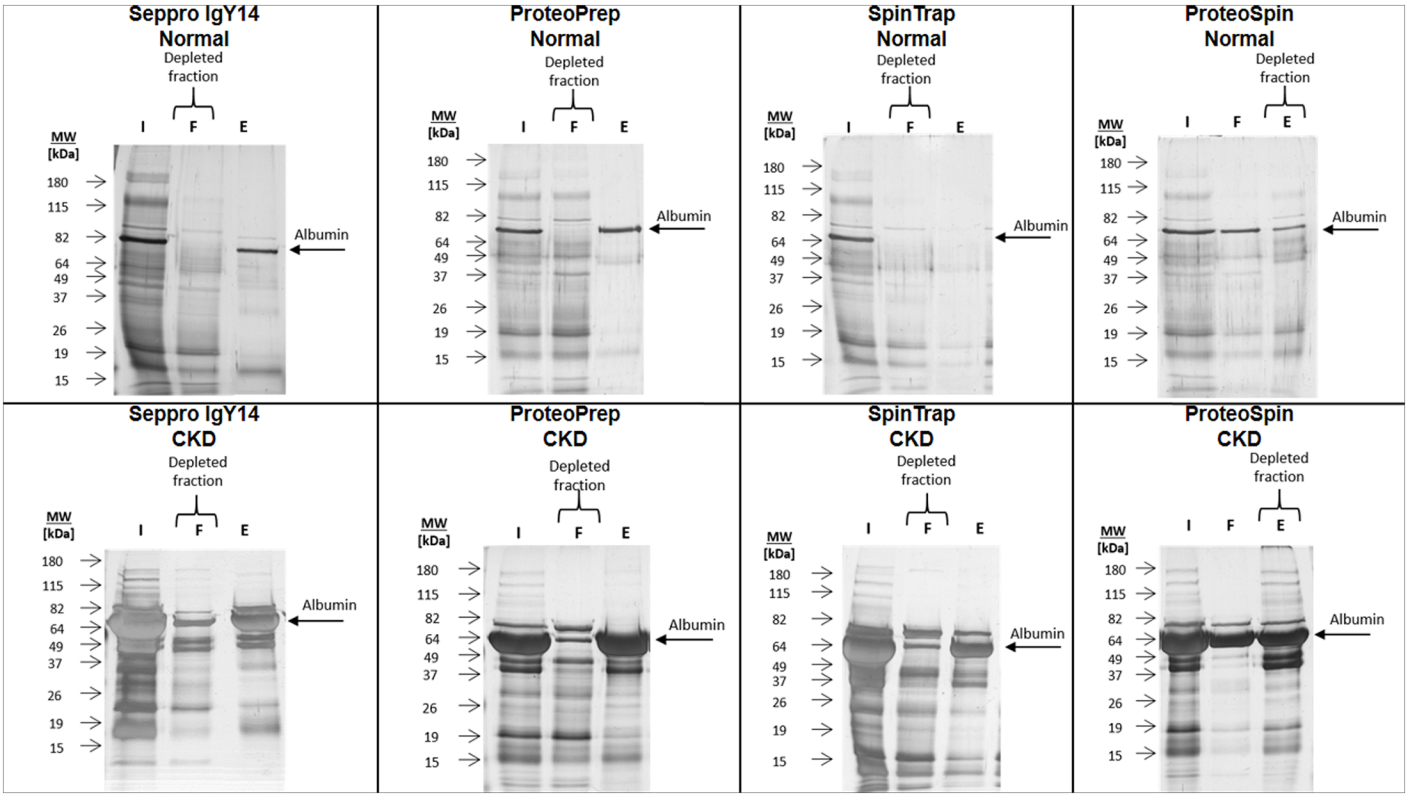

Fig 2. Representative SDS-PAGE results for fractionated and non-fractionated samples (normal and CKD). The figure represents initial urine, flow-through and elution for each of the depletion kits applied. The fractions representing depleted sample and albumin as a common protein depleted by all the kits are marked. I—Initial urine (non-fractionated sample); F—Flow-through fraction; E—Elution. The same protein amounts were loaded onto the gels for initial sample (lane 2 in all cases). Any observed differences in staining intensities are attributed to differences in the silver staining procedure.

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number of peptides was identified from the initial (unfractionated) sample (approx. 2,400 peptides) and in the depleted fraction processed by ProteoPrep kit (approx. 2,150 peptides), followed by ProteoSpin, Seppro IgY14 and SpinTrap kits (approx. 1,500 peptides). The most significant differences in the number of identifications, were found between initial urine and

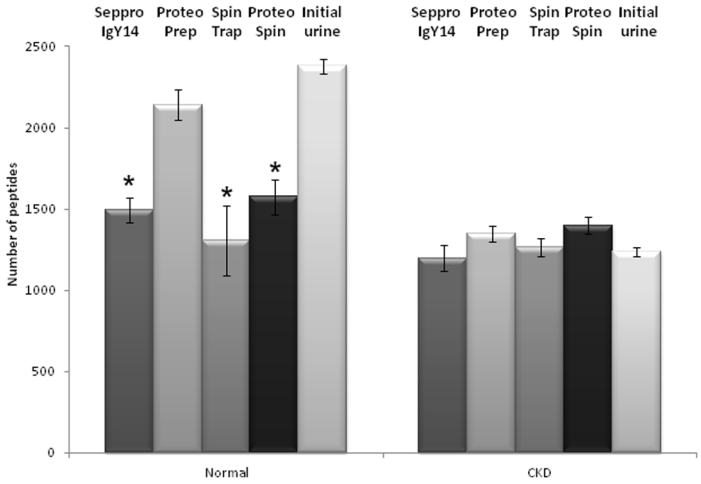

Fig 3. Average number of peptides identified per method.

doi:10.1371/journal.pone.0133773.g003

Table 2. Comparison of the number of peptide identifications, PSMs, search inputs and TICs for normal and CKD sample.

		Normal		
Analysis method	Average number of identified peptides	Average number of PSMs	Average number of Search inputs	Average total ion current [sum of the peak areas]
Seppro IgY14	1495	4978	15813	7.98E+10
ProteoPrep	2142	6263	18092	2.55E+11
SpinTrap	1306	4363	15685	9.07E+10
ProteoSpin	1575	5184	15725	6.73E+10
Total urine	2380	10650	21576	3.86E+11
		CKD		
Seppro IgY14	1197	5646	15905	5.06E+10
ProteoPrep	1350	6980	16628	9.02E+11
SpinTrap	1264	6667	16425	8.26E+10
ProteoSpin	1399	8772	19192	2.00E+11
Total urine	1234	9055	22455	4.34E+11

doi:10.1371/journal.pone.0133773.t002

Seppro IgY14, SpinTrap and ProteoSpin kits (p-value $\leq$ 0.0002). In the case of urine from CKD patients, no significant difference in the number of detected peptides could be observed when comparing the output of the different methods (approx. 1250 peptides in the unfractionated and all depleted fractions).

To rule out that differences in the number of identifications is related to undersampling and/or MS data quality, we investigated the number of obtained peptides, number of PSMs, search inputs (MS/MS scans), and total ion currents (TICs) obtained in each case. As demonstrated in Table 2, the average numbers of PSMs, search inputs and TICs were comparable among CKD and normal samples per depletion strategy. Nevertheless, in the case of CKD, the number of peptide identifications is lower compared to the respective number from normal. This suggests that for CKD, a larger fraction of the MS/MS scans is on the same, highly-abundant peptides.

Comparable numbers of proteins identified in at least three out of five replicates per technique were detected in all cases (approx. 390 in normal and 160 in CKD samples). Overall, more proteins were detectable in the normal urine than in CKD sample (p-value = 0.0002). This observation applies for both total urine and fractionated samples (Table 3). All techniques were found to be reproducible in terms of received protein identifications, as shown in Table 3. In all cases, at least 80% of identified proteins were detected in all 5 replicates.

Table 3. Total number (sum) of identified proteins per depletion strategy for normal and CKD sample (in at least 3, 4 and 5 technical replicates). For both depleted and non-depleted sample the number of identifications is higher in normal than in CKD urine.

		Normal			
Name of the kit	Seppro IgY14	ProteoPrep	SpinTrap	ProteoSpin	Total urine
Proteins identified in 5 replicates	287	387	265	276	362
Proteins identified in 4 replicates	321	420	299	315	397
Proteins identified in 3 replicates	354	466	352	361	431
		CKD			
Name of the kit	Seppro IgY14	ProtoPrep	SpinTrap	ProteoSpin	Total urine
Proteins identified in 5 replicates	113	151	159	116	132
Proteins identified in 4 replicates	124	164	172	126	146
Proteins identified in 3 replicates	137	172	185	139	159

doi:10.1371/journal.pone.0133773.t003

Among the detected proteins, 33% and 36%, which correspond to 205 proteins (normal) or 90 proteins (CKD), are identified by all methods (S6 Fig). These include many highly-abundant proteins such as albumin, vitamin D-binding protein, clusterin, zinc-alpha-2-glycoprotein, uromodulin and beta-2-microglobulin (S2 Table). This "core proteome" corresponds to 53% (+/-7%; normal) and 58% (+/-8%; CKD) of total identifications received per method. In fact, these common proteins correspond to approx. 95% of the total protein peak area in all analyzed samples. The percentage of identified proteins that are unique per analysis method is low, in the range of 10–15% (S2 Table).

To confirm efficiency of analysis, the applied LC-MS/MS protocol was compared to various alternative experimental conditions including: Top 20 versus Top 10 or Top 7 MS/MS analysis; injection of 1 versus 4 μg of protein. In all cases no substantial difference to the presented data could be observed. Importantly, the applied protocol provided average numbers of received MS/MS scans similar to numbers reported in published high resolution datasets [37, 38].

Changes in protein sequence coverage after protein depletion

Peptide sequences per protein identified from the five technical replicates were combined and used for coverage calculations (S2 Table). The coverage from depleted samples was compared with the coverage from initial samples ($\log_2$ ratio depleted/initial urine) (S7–S10 Figs). For all samples from healthy controls, the depletion reduced sequence coverage of protein targets compared to the undepleted urine (from 5% reduction for IgG, up to 90% for serotransferrin in Seppro IgY14 kit). Similarly, in the case of CKD samples, sequence coverage slightly decreased for all depletion targets after application of the albumin and IgG depletion kits (ProteoPrep and SpinTrap) (S8 and S9 Figs). Decrease in the sequence coverage of three target proteins was not observed after fractionation through Seppro IgY14 (S7 Fig): albumin, alpha-1-acid glycoprotein 1 and immunoglobulin alpha. Similarly, sequence coverage did not decrease for alpha-1-antitrypsin after applying ProteoSpin kit (S10 Figs). Among the non-target proteins, no clear trend or impact on sequence coverage could be observed following application of depletion strategies (S7–S10 Figs).

To further investigate this issue, the number of PSMs in relation to sequence coverage was studied. A positive correlation between protein sequence coverage and PSMs could be observed in all cases: if the sequence coverage for a given protein was higher in the depleted sample compared to the unfractionated urine, so was the number of respective PSMs. Similarly, decrease in protein sequence coverage was associated with lower number of PSMs (data not shown). This correlation was in the range of 60%-70% for normal and 70%-80% for CKD samples.

Changes in relative abundance after protein depletion

To estimate the variability in protein abundance between technical replicates, the coefficient of variation for the 50 most abundant proteins from each sample and for the whole protein dataset was calculated (Fig 4). The list of 50 most abundant proteins per method tested is summarized in S3 Table. In the normal urine sample, higher variability was observed for Seppro IgY14, SpinTrap and ProteoSpin (CVs in the range of 26% for 50 most abundant and 40% for whole dataset). ProteoPrep and initial urine demonstrated variabilities in the range of 14% for the 50 most abundant, and 30% for the whole dataset. In the case of CKD samples, all of the analysis strategies demonstrated similar CVs (approx. 10% for 50 most abundant and 28% for the whole dataset), with the exception of Seppro IgY14, which showed a higher CV (27% for the 50 most abundant and 40% for the whole dataset). In all cases the variability increases (by approxiamately 16% for the 50 most abundant proteins) when low-abundance proteins are included in the CV calculations.

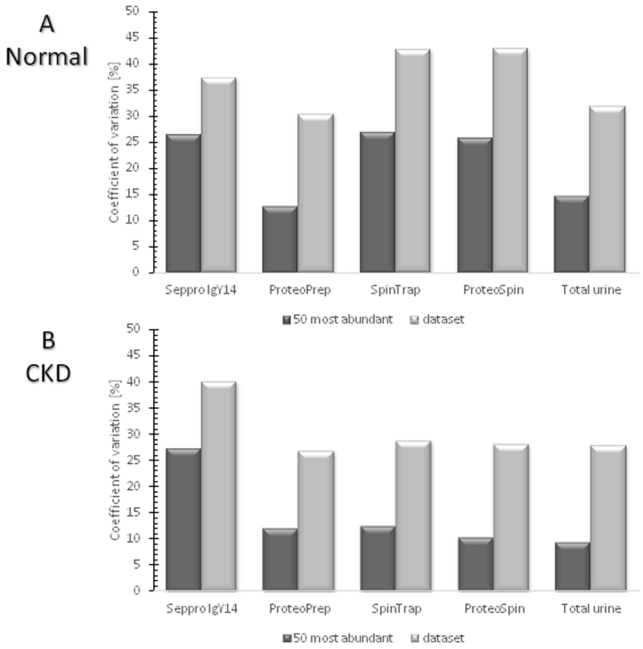

Fig 4. Coefficient of variation for 50 most abundant proteins and whole dataset for A) Normal, B) CKD urine. Normal samples appear having higher variability compared to the CKD samples, nevertheless this difference is not significant. Additionally and as expected, the variability increases when low-abundance proteins are included in the CV calculations.

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To evaluate the effect of depletion on relative abundance of proteins, a comparison of relative abundance of individual proteins between a depletion method and the undepleted urine was conducted. The enrichment or depletion of proteins was calculated based on the $\log_2$ ratio of signal intensity in the depleted against initial urine (S11–S14 Figs). Additionally, in Fig 5 the relative abundance of 20 most abundant proteins from undepleted urine (for normal and CKD) was compared to their abundance from corresponding depleted fractions. In the case of depletion targets, the application of immuno-based methods resulted in the reduction of their relative abundance. This observation is valid for urine from both normal and CKD patients. However, for the ion-exchange method (S14 Fig), the depletion was not efficient for serotransferrin in normal urine and for alpha-1-antitrypsin and albumin for CKD. When non-target proteins were compared, no clear trend in the abundance (increase or decrease) was observed. Collectively, similarly to protein sequence coverage, protein depletion had a variable impact on protein abundance, suggesting no added value of these strategies for the analysis of urine samples.

The depletion efficiency of the tested kits was also further estimated as follows: the relative abundance of albumin, as a target for all depletion kits, was compared before and after application of the fractionation strategies. As shown in Fig 5, significant depletion of Albumin was observed for normal samples: (approx. 98% decrease for all three immuno-based methods and 45% decrease for ion-exchange). For the urine from CKD patients, the most efficient depletion was observed for the albumin and IgG depletion kits: SpinTrap ProteoPrep and (95% and 91% decrease respectively), followed by the Seppro IgY14 (63% decrease). The depletion was inefficient in case of using ion-exchange ProteoSpin kit. Collectively, immuno-based methods outperformed the ion-exchange-based strategy in depleting albumin. Additionally, all three immuno-depletion kits depleted albumin with similar efficiency in the case of normal samples, whereas albumin and IgG depletion kits (ProteoPrep and SpinTrap) demonstrated higher

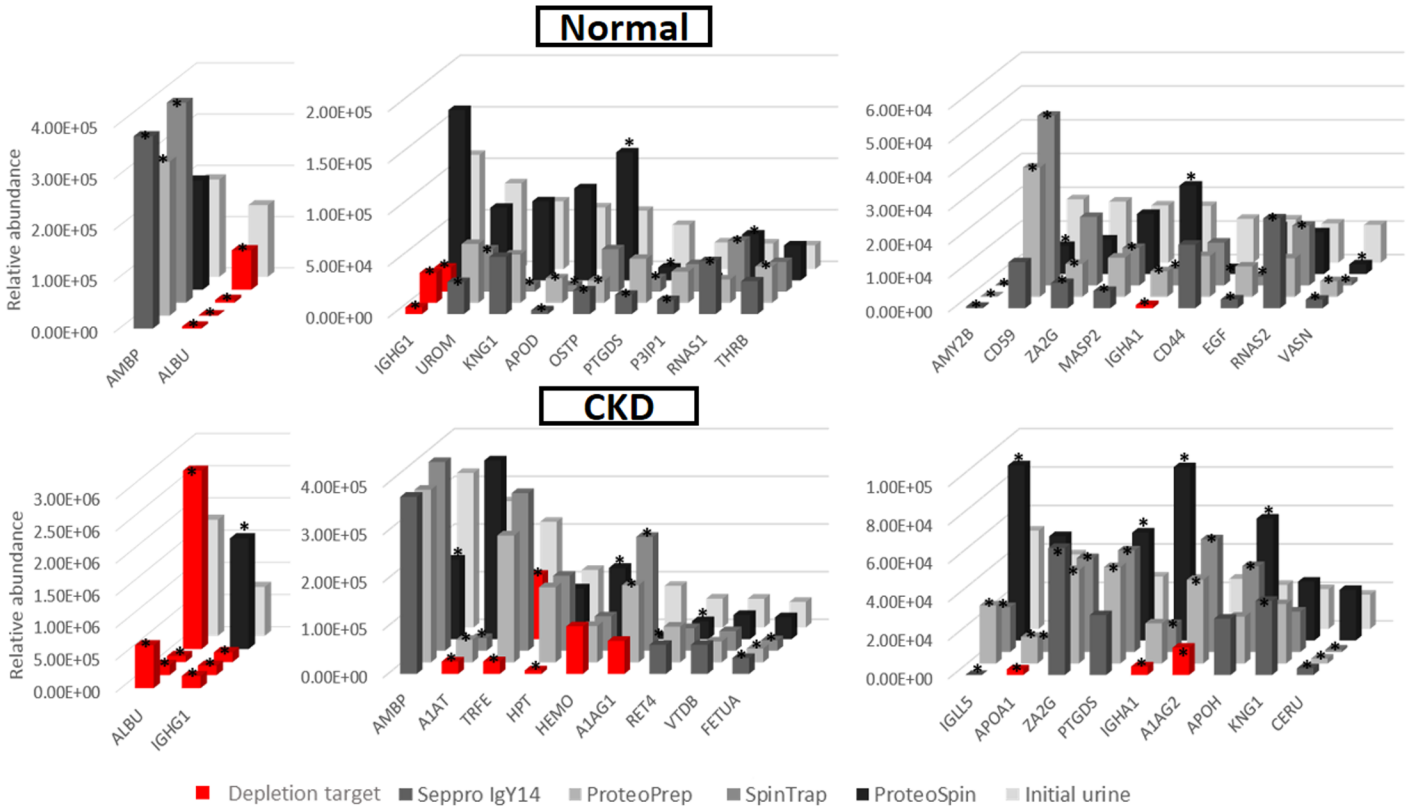

Fig 5. Relative abundance of 20 most abundant proteins derived from undepleted urine and comparison of their abundance with corresponding depleted fractions for urine from healthy controls and CKD patients. Efficient depletion of target proteins is observable for all methods, with the exception of albumin for ProteoSpin in CKD sample. * Denotes significant changes compared to initial urine. ABMP: protein AMBP, ALBU: albumin, IGHG1: Ig gamma-1 chain region, UROM: uromodulin, KNG1: kininogen 1, APOD: apolipoprotein D, OSTP: osteopontin, PTGDS: prostaglandin-H2 D-isomerase, P3IP1: phosphoinositide-3-kinase-interacting protein 1, RNAS1: ribonuclease pancreatic, THRB: prothrombin, AMY2B: alpha-amylase 2B, CD59: CD59 glycoprotein, ZA2G: zinc-alpha-2-glycoprotein, MASP2: mannan-binding lectin serine protease 2, IGHA1: Ig alpha-1 chain C region, CD44: CD44 antigen, EGF: pro-epidermal growth factor, RNAS2: non-secretory ribonuclease, VASN: vasorin, A1AT: alpha-1-antitrypsin, TRFE: serotransferrin, HPT: haptoglobin, HEMO: hemopexin, A1AG1: alpha-1-acid glycoprotein 1, RET4: retinol-binding protein 4, VTDB: vitamin D-binding protein, FETUA: alpha-2-HS-glycoprotein, IGLL5: immunoglobulin lambda-like polypeptide 5, APOA1: apolipoprotein A-I, A1AG2: alpha-1-acid glycoprotein 2, APOH: beta-2-glycoprotein 1, CERU: ceruloplasmin.

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depletion effectiveness compared to Seppro IgY14 for CKD. These results are in agreement with the SDS-PAGE analysis (Fig 2), where the highest albumin band intensity reduction was observed for ProteoPrep and SpinTrap, followed by Seppro IgY14 (see SDS-PAGE Analysis section). Of note, the relative abundance of Albumin based on MS data is noticeably higher in the initial CKD sample (approx. 65% of the total peak area) compared to normal (approx. 25% of the total peak area).

Discussion

The main goal of the study was to evaluate the applicability of depletion of abundant proteins in urine samples from CKD patients and controls, at starting volumes regularly available from large clinical cohorts, using commercially available kits, originally designed for plasma. Based on the gel profiles from SDS-PAGE and the number of identified peptides from LC-MS/MS, each depletion strategy is reproducible, and in the case of normal samples, albumin as a target protein is efficiently depleted. For CKD samples, immunodepletion kits efficiently depleted

albumin and the highest efficiency was observed for albumin and IgG depletion kits (ProteoPrep and SpinTrap) followed by Seppro IgY14 (Fig 2 and Fig 5).

The reduced efficiency of Seppro IgY14 may be attributed to potential column overloading-even though this was not expected to be the case based on the manufacturer's instructions: Seppro IgY14 is designed to work with plasma, where the concentration of highly-abundant proteins is substantial. Additionally, the loaded protein amount in this study (437 μg) was not even half of the column binding capacity (1 mg max. column binding capacity). The reason(s) of the lower efficiency of Seppro IgY14 in depleting albumin in CKD urine is still unknown. The ion-exchange-based ProteoSpin kit was found to be the least efficient in eliminating target proteins from both normal and CKD urine. This was expected due to the highly-specific nature of immuno-based mechanism employed in the other kits [15].

Regardless whether a depletion method was applied or not, the number of protein identifications from LC-MS/MS analysis were comparable. In all cases, in the urine from CKD patients fewer proteins were identified in comparison to urine from healthy controls, even though the number of PSMs, MS/MS scans and TICs were similar per method. This may indicate that, even upon depletion, the potential masking effect from highly abundant proteins still exists. After depletion of the target highly-abundant proteins, other non-targeted high and medium-abundance molecules (e.g. protein AMBP, vitamin D-binding proteins, zinc-alpha-2-glycoprotein, uromodulin) likely maintain the masking effect. Alternatively, a large number of proteins may be below the limit of detection (estimated at low femtomole range for the applied mass spectrometer) and therefore, any positive impact of depletion on proteome coverage cannot be observed. Collectively, comparable numbers of received identifications between different strategies, as well as the presence of unique proteins in both fractionated and initial urine indicate no benefit of depletion for biomarker identification purposes.

Our results are not in agreement with Kushnir et al. [30] findings, where the employment of a multiple affinity removal (MARS) column allowed increasing the number of identifications in urine from 60 to 142 in CKD patients. Still, in our presented study the number of protein identifications is higher in comparison, possibly a result of a less sensitive instrument used by the authors (Q-TOF equipped with a ChipCube). The immuno-based depletion strategies were also evaluated in 2D gel proteomics experiments [24, 27, 39, 40]. In these cases the number of unique identifications did not change significantly following depletion.

In order to evaluate the validity of the obtained protein identifications from urine from healthy controls, 100 most abundant (as the most reliable) proteins from each analysis method (i.e. undepleted and fractionated samples), were compared with the identifications from three manuscripts reporting on the analysis of urine proteome from healthy individuals [41–43]. In each case, approx. 90 out of the 100 most abundant proteins identified in the present study were also reported in these manuscripts. When expanding the comparison from the 100 most abundant to the whole dataset an overlap of approx. 60%, for the normal samples was observed, similar to the overlap of protein identifications between the three different studies. These similarities between different datasets representing normal urine support the validity of our data. To estimate the validity of obtained identifications from CKD samples, proteins from all CKD datasets were compared with molecules associated with renal diseases reported in the literature [44–46]. Due to the too small sample size tested to evaluate differential expression of these molecules, our focus was set only on their presence. Several of these disease-associated proteins were identified in all datasets (i.e. albumin, neutrophil gelatinase-associated lipocalin, cystatin C, osteopontin, clusterin, beta-2-microglobulin). A few were unique for applied strategies: metalloproteinase inhibitor 1 was present in three kits (Seppro IgY14, SpinTrap and ProteoSpin), fatty acid-binding protein was unique for unfractionated sample and connective tissue growth factor for SpinTrap kit.

Based on the available literature data and in line to our observations, a number of non-targeted proteins are also depleted to some degree [23–25], negatively affecting the analysis. This effect may be related to the fact that targeted proteins may form stable complexes with non-targeted proteins resulting in their co-depletion. This co-depletion mechanism was observed in a number of studies (i.e. [23–25]). For example, Granger et al. [23] demonstrated that depletion of albumin removed also low-abundance proteins including cytokines from plasma samples. Similarly, Stempfer et al. [25], spiked 6 recombinant cytokines in serum samples and showed that application of depletion methods reduced the cytokine levels.

The application of depletion strategies did not improve the proteome or sequence coverage. Given that the overall data quality and quantity (as reflected by the number of MS/MS scans—Table 2) were not significantly affected following fractionation, the fact that no clear increase in proteome and sequence coverage could be observed may be attributed to the following factors: proteome complexity rendering effects of depletion per protein are unpredictable, lack of sufficient depletion to generate an observable impact on coverage as well as peptides (even if enriched) still remaining below the limit of detection (i.e. undersampling at an individual protein level).

Comparison of changes in protein abundance for overlapping identifications prior to and after depletion was also performed by Tu et al. [26] for plasma samples using MARS columns. In contrast to the present study, where no clear advantage of protein depletion was observed, the authors found that most of the non-targeted proteins were enriched after depletion. This discrepancy may be related to: i) depletion was evaluated in plasma samples, not urine, and ii) significantly higher starting protein content was used.

Target proteins were less efficiently depleted in the urine from CKD patients compared to normal, regardless of the depletion strategy applied, even though the protein content loaded onto the depletion column was always (according to the manufacturers protocols) below their loading capacity. However, it may be that the actual loading capacity is lower than claimed.

In conclusion, the depletion of abundant proteins does not present an added value for the study of the urine proteome, at least when starting with small urine volumes (less than 1 mL), regularly available in large clinical studies. No significant improvement in the number of identifications, protein sequence coverage or relative abundance in comparison to the undepleted samples were detected using different methods in the current study. Moreover, the depletion introduced additional variability. Depletion of targeted proteins was substantially more efficient in normal than for CKD samples, suggesting that additional disease-related factors may impair the depletion efficiency. Therefore, for the urinary proteomics studies especially in the context of CKD, analysis of total rather than depleted urine appears preferable.

Supporting Information

S1 Table. Peptide lists for all analysis methods.
(XLSX)

S2 Table. Lists of common and unique identifications for depleted samples and initial urine for all strategies.
(XLSX)

S3 Table. 50 most abundant proteins for each depletion strategy and unfractionated sample for normal and CKD urine. X denotes that the protein was found as one of the 50 most abundant in the respective analysis method.
(XLSX)

S1 Fig. SDS-PAGE gel profiles for Seppro IgY14 depletion kit for normal samples: Depleted fractions and albumin as a target protein are marked. M—molecular size marker. I—initial urine. F—Flow-through fraction. W—Wash. E—Elution. 1–4 –consecutive numbers of flow-through/wash/elution within one replicate.
(TIF)

S2 Fig. SDS-PAGE gel profiles for Seppro IgY14 depletion kit for CKD samples: Depleted fractions and albumin as a target protein are marked. M—molecular size marker. I—initial urine. F—Flow-through fraction. W—Wash. E—Elution. 1–4 –consecutive numbers of flow-through/wash/elution within one replicate.
(TIF)

S3 Fig. SDS-PAGE gel profiles for ProtePrep depletion kit. Depleted fractions and albumin as a target protein are marked. M—molecular size marker. I—initial urine. F—Flow-through fraction. E—Elution. I-V—number of technical replicate.
(TIF)

S4 Fig. SDS-PAGE gel profiles for SpinTrap depletion kit. Depleted fractions and albumin as a target protein are marked. M—molecular size marker. I—initial urine. F—Flow-through fraction. E—Elution. I-V—number of technical replicate.
(TIF)

S5 Fig. SDS-PAGE gel profiles for ProteoSpin depletion kit. Depleted fractions and albumin as a target protein are marked. M—molecular size marker. I—initial urine. F—Flow-through fraction. E—Elution. I-V—number of technical replicate.
(TIF)

S6 Fig. Venn Diagram [47]: unique identifications for urine from A) normal B) CKD patients. In total 612 and 251 unique proteins, in at least three out of five replicates, were identified in normal and CKD samples respectively. Approximately 33% of the identifications are shared between non-depleted and depleted urine in normal or CKD sample.
(TIF)

S7 Fig. Changes in protein sequence coverage for overlapping identifications between Seppro IgY14 depleted sample and initial urine. X axis represents the protein sequence coverage of the initial urine. The changes after applying the depletion strategy are presented on Y-axis (with $\log_2$ scale) as a ratio of depleted versus non-depleted sample. Proteins, with increased sequence coverage are presented above the ratio of 0 on the Y-scale and with decreased below the ratio of 0. Proteins with a ratio of 0 show the same coverage in the initial and depleted sample. Sequence coverage for immunoglobulins is presented as an average coverage for all proteins combined in the group. Protein targets for depletion kit are marked as red dots (see Table 1). Protein targets for which the protein sequence coverage increased after depletion are marked by an arrow.
(TIF)

S8 Fig. Changes in protein sequence coverage for overlapping identifications between ProteoPrep depleted sample and initial urine. X axis represents the protein sequence coverage of the initial urine. The changes after applying the depletion strategy are presented on Y-axis (with $\log_2$ scale) as a ratio of depleted versus non-depleted sample. Proteins, with increased sequence coverage are presented above the ratio of 0 on the Y-scale and with decreased below the ratio of 0. Proteins with a ratio of 0 show the same coverage in the initial and depleted sample. Sequence coverage for immunoglobulins is presented as an average

coverage for all proteins combined in the group. Protein targets for depletion kit are marked as red dots (see Table 1).
(TIF)

S9 Fig. Changes in protein sequence coverage for overlapping identifications between SpinTrap depleted sample and initial urine. X axis represents the protein sequence coverage of the initial urine. The changes after applying the depletion strategy are presented on Y-axis (with $\log_2$ scale) as a ratio of depleted versus non-depleted sample. Proteins, with increased sequence coverage are presented above the ratio of 0 on the Y-scale and with decreased below the ratio of 0. Proteins with a ratio of 0 show the same coverage in the initial and depleted sample. Sequence coverage for immunoglobulins is presented as an average coverage for all proteins combined in the group. Protein targets for depletion kit are marked as red dots (see Table 1).
(TIF)

S10 Fig. Changes in protein sequence coverage for overlapping identifications between ProteoSpin depleted sample and initial urine. X axis represents the protein sequence coverage of the initial urine. The changes after applying the depletion strategy are presented on Y-axis (with $\log_2$ scale) as a ratio of depleted versus non-depleted sample. Proteins, with increased sequence coverage are presented above the ratio of 0 on the Y-scale and with decreased below the ratio of 0. Proteins with a ratio of 0 show the same coverage in the initial and depleted sample. Sequence coverage for immunoglobulins is presented as an average coverage for all proteins combined in the group. Protein targets for depletion kit are marked as red dots (see Table 1). Protein targets for which the protein sequence coverage increased after depletion are marked by an arrow.
(TIF)

S11 Fig. Changes in protein abundance for overlapping identifications between Seppro IgY14 depleted sample and initial urine. The scatterplots present the protein relative abundance changes after protein depletion in comparison to the initial sample. X axis represents the normalized protein abundance for initial urine in logarithmic scale ($\log_2$). Proteins on the Y axis ($\log_2$ scale) above a ratio of 0 are enriched in comparison to initial urine, while those below the ratio of 0 are depleted. Proteins with a ratio 0 show the same relative abundance in the initial and depleted sample. Protein abundance for immunoglobulins is presented as a sum of the abundance for all combined proteins in the group. Protein targets for depletion kit are marked as red dots (see Table 1).
(TIF)

S12 Fig. Changes in protein abundance for overlapping identifications between ProteoPrep depleted sample and initial urine. The scatterplots present the protein relative abundance changes after protein depletion in comparison to the initial sample. X axis represents the normalized protein abundance for initial urine in logarithmic scale ($\log_2$). Proteins on the Y axis ($\log_2$ scale) above a ratio of 0 are enriched in comparison to initial urine, while those below the ratio of 0 are depleted. Proteins with a ratio 0 show the same relative abundance in the initial and depleted sample. Protein abundance for immunoglobulins is presented as a sum of the abundance for all combined proteins in the group. Protein targets for depletion kit are marked as red dots (see Table 1).
(TIF)

S13 Fig. Changes in protein abundance for overlapping identifications between SpinTrap depleted sample and initial urine. The scatterplots present the protein relative abundance

changes after protein depletion in comparison to the initial sample. X axis represents the normalized protein abundance for initial urine in logarithmic scale (log_2). Proteins on the Y axis (log_2 scale) above a ratio of 0 are enriched in comparison to initial urine, while those below the ratio of 0 are depleted. Proteins with a ratio 0 show the same relative abundance in the initial and depleted sample. Protein abundance for immunoglobulins is presented as a sum of the abundance for all combined proteins in the group. Protein targets for depletion kit are marked as red dots (see Table 1).
(TIF)

S14 Fig. Changes in protein abundance for overlapping identifications between ProteoSpin depleted sample and initial urine. The scatterplots present the protein relative abundance changes after protein depletion in comparison to the initial sample. X axis represents the normalized protein abundance for initial urine in logarithmic scale (log_2). Proteins on the Y axis (log_2 scale) above a ratio of 0 are enriched in comparison to initial urine, while those below the ratio of 0 are depleted. Proteins with a ratio 0 show the same relative abundance in the initial and depleted sample. Protein abundance for immunoglobulins is presented as a sum of the abundance for all combined proteins in the group. Protein targets for depletion kit are marked as red dots (see Table 1). Protein targets for which the relative abundance increased after depletion are marked by an arrow.
(TIF)

Author Contributions

Conceived and designed the experiments: SF KV JZ WM HM AV. Performed the experiments: SF KV WM. Analyzed the data: SF AL. Contributed reagents/materials/analysis tools: KV WM AV. Wrote the paper: SF JZ AL GS HM AV JJ.

References

1. Yates JR, Ruse CI, Nakorchevsky A. Proteomics by mass spectrometry: approaches, advances, and applications. Annual review of biomedical engineering. 2009; 11:49–79. doi: 10.1146/annurev-bioeng-061008-124934 PMID: 19400705.
2. Law KP, Lim YP. Recent advances in mass spectrometry: data independent analysis and hyper reaction monitoring. Expert review of proteomics. 2013; 10(6):551–66. doi: 10.1586/14789450.2013.858022 PMID: 24206228.
3. Fliser D, Novak J, Thongboonkerd V, Argiles A, Jankowski V, Girolami MA, et al. Advances in urinary proteome analysis and biomarker discovery. Journal of the American Society of Nephrology: JASN. 2007; 18(4):1057–71. doi: 10.1681/ASN.2006090956 PMID: 17329573.
4. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Molecular & cellular proteomics: MCP. 2002; 1(11):845–67. PMID: 12488461.
5. Thomas R, Kanso A, Sedor JR. Chronic kidney disease and its complications. Primary care. 2008; 35 (2):329–44, vii. doi: 10.1016/j.pop.2008.01.008 PMID: 18486718; PubMed Central PMCID: PMC2474786.
6. Filip S, Pontillo C, Peter Schanstra J, Vlahou A, Mischak H, Klein J. Urinary proteomics and molecular determinants of chronic kidney disease: possible link to proteases. Expert review of proteomics. 2014:1–14. doi: 10.1586/14789450.2014.926224 PMID: 24957818.
7. Perkins BA, Ficociello LH, Roshan B, Warram JH, Krolewski AS. In patients with type 1 diabetes and new-onset microalbuminuria the development of advanced chronic kidney disease may not require progression to proteinuria. Kidney international. 2010; 77(1):57–64. doi: 10.1038/ki.2009.399 PMID: 19847154; PubMed Central PMCID: PMC3725722.
8. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: the long and uncertain path to clinical utility. Nature biotechnology. 2006; 24(8):971–83. doi: 10.1038/nbt1235 PMID: 16900146.
9. Decramer S, Gonzalez de Peredo A, Breuil B, Mischak H, Monsarrat B, Bascands JL, et al. Urine in clinical proteomics. Molecular & cellular proteomics: MCP. 2008; 7(10):1850–62. doi: 10.1074/mcp.R800001-MCP200 PMID: 18667409.

10. Hortin GL, Sviridov D. Diagnostic potential for urinary proteomics. Pharmacogenomics. 2007; 8 (3):237–55. doi: 10.2217/14622416.8.3.237 PMID: 17324112.

11. Doucette AA, Tran JC, Wall MJ, Fitzsimmons S. Intact proteome fractionation strategies compatible with mass spectrometry. Expert review of proteomics. 2011; 8(6):787–800. doi: 10.1586/epr.11.67 PMID: 22087661.

12. Jungbauer A, Hahn R. Ion-exchange chromatography. Methods in enzymology. 2009; 463:349–71. doi: 10.1016/S0076-6879(09)63022-6 PMID: 19892182.

13. Duong-Ly KC, Gabelli SB. Gel filtration chromatography (size exclusion chromatography) of proteins. Methods in enzymology. 2014; 541:105–14. doi: 10.1016/B978-0-12-420119-4.00009-4 PMID: 24674066.

14. Cheung RC, Wong JH, Ng TB. Immobilized metal ion affinity chromatography: a review on its applications. Applied microbiology and biotechnology. 2012; 96(6):1411–20. doi: 10.1007/s00253-012-4507-0 PMID: 23099912.

15. Moser AC, Hage DS. Immunoaffinity chromatography: an introduction to applications and recent developments. Bioanalysis. 2010; 2(4):769–90. doi: 10.4155/bio.10.31 PMID: 20640220; PubMed Central PMCID: PMC2903764.

16. Urh M, Simpson D, Zhao K. Affinity chromatography: general methods. Methods in enzymology. 2009; 463:417–38. doi: 10.1016/S0076-6879(09)63026-3 PMID: 19892186.

17. Filip S, Zoidakis J, Vlahou A, Mischak H. Advances in urinary proteome analysis and applications in systems biology. Bioanalysis. 2014; 6(19):2549–69. doi: 10.4155/bio.14.210 PMID: 25411698.

18. Righetti PG, Candiano G, Citterio A, Boschetti E. Combinatorial Peptide ligand libraries as a "trojan horse" in deep discovery proteomics. Analytical chemistry. 2015; 87(1):293–305. doi: 10.1021/ac502171b PMID: 25084147.

19. Righetti PG, Boschetti E. The ProteoMiner and the FortyNiners: searching for gold nuggets in the proteomic arena. Mass spectrometry reviews. 2008; 27(6):596–608. doi: 10.1002/mas.20178 PMID: 18481254.

20. Boschetti E, Giorgio Righetti P. Hexapeptide combinatorial ligand libraries: the march for the detection of the low-abundance proteome continues. BioTechniques. 2008; 44(5):663–5. doi: 10.2144/000112762 PMID: 18474042.

21. Righetti PG, Boschetti E. Sample treatment methods involving combinatorial Peptide ligand libraries for improved proteomes analyses. Methods in molecular biology. 2015; 1243:55–82. doi: 10.1007/978-1-4939-1872-0_4 PMID: 25384740.

22. Millioni R, Tolin S, Puricelli L, Sbrignadello S, Fadini GP, Tessari P, et al. High abundance proteins depletion vs low abundance proteins enrichment: comparison of methods to reduce the plasma proteome complexity. PloS one. 2011; 6(5):e19603. doi: 10.1371/journal.pone.0019603 PMID: 21573190; PubMed Central PMCID: PMC3087803.

23. Granger J, Siddiqui J, Copeland S, Remick D. Albumin depletion of human plasma also removes low abundance proteins including the cytokines. Proteomics. 2005; 5(18):4713–8. doi: 10.1002/pmic.200401331 PMID: 16281180.

24. Fountoulakis M, Juranville JF, Jiang L, Avila D, Roder D, Jakob P, et al. Depletion of the high-abundance plasma proteins. Amino acids. 2004; 27(3–4):249–59. doi: 10.1007/s00726-004-0141-1 PMID: 15592754.

25. Stempfer R, Kubicek M, Lang IM, Christa N, Gerner C. Quantitative assessment of human serum high-abundance protein depletion. Electrophoresis. 2008; 29(21):4316–23. doi: 10.1002/elps.200800211 PMID: 18956433.

26. Tu C, Rudnick PA, Martinez MY, Cheek KL, Stein SE, Slebos RJ, et al. Depletion of abundant plasma proteins and limitations of plasma proteomics. Journal of proteome research. 2010; 9(10):4982–91. doi: 10.1021/pr100646w PMID: 20677825; PubMed Central PMCID: PMC2948641.

27. Echan LA, Tang HY, Ali-Khan N, Lee K, Speicher DW. Depletion of multiple high-abundance proteins improves protein profiling capacities of human serum and plasma. Proteomics. 2005; 5(13):3292–303. doi: 10.1002/pmic.200401228 PMID: 16052620.

28. Kullolli M, Warren J, Arampatzidou M, Pitteri SJ. Performance evaluation of affinity ligands for depletion of abundant plasma proteins. Journal of chromatography B, Analytical technologies in the biomedical and life sciences. 2013; 939:10–6. doi: 10.1016/j.jchromb.2013.09.008 PMID: 24090752.

29. Magistroni R, Ligabue G, Lupo V, Furci L, Leonelli M, Manganelli L, et al. Proteomic analysis of urine from proteinuric patients shows a proteolitic activity directed against albumin. Nephrology, dialysis, transplantation: official publication of the European Dialysis and Transplant Association—European Renal Association. 2009; 24(5):1672–81. doi: 10.1093/ndt/gfp020 PMID: 19211645.

30. Kushnir MM, Mrozinski P, Rockwood AL, Crockett DK. A depletion strategy for improved detection of human proteins from urine. Journal of biomolecular techniques: JBT. 2009; 20(2):101–8. PMID: 19503621; PubMed Central PMCID: PMC2685607.

31. Afkarian M, Bhasin M, Dillon ST, Guerrero MC, Nelson RG, Knowler WC, et al. Optimizing a proteomics platform for urine biomarker discovery. Molecular & cellular proteomics: MCP. 2010; 9(10):2195–204. doi: 10.1074/mcp.M110.000992 PMID: 20511394; PubMed Central PMCID: PMC2957724.

32. Magagnotti C, Fermo I, Carletti RM, Ferrari M, Bachi A. Comparison of different depletion strategies for improving resolution of the human urine proteome. Clinical chemistry and laboratory medicine: CCLM / FESCC. 2010; 48(4):531–5. doi: 10.1515/CCLM.2010.109 PMID: 20148726.

33. Chen CL, Lin TS, Tsai CH, Wu CC, Chung T, Chien KY, et al. Identification of potential bladder cancer markers in urine by abundant-protein depletion coupled with quantitative proteomics. Journal of proteomics. 2013; 85:28–43. doi: 10.1016/j.jprot.2013.04.024 PMID: 23631828.

34. Chevallet M, Luche S, Rabilloud T. Silver staining of proteins in polyacrylamide gels. Nature protocols. 2006; 1(4):1852–8. doi: 10.1038/nprot.2006.288 PMID: 17487168; PubMed Central PMCID: PMC1971133.

35. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nature methods. 2009; 6(5):359–62. doi: 10.1038/nmeth.1322 PMID: 19377485.

36. Serang O, Noble W. A review of statistical methods for protein identification using tandem mass spectrometry. Statistics and its interface. 2012; 5(1):3–20. PMID: 22833779; PubMed Central PMCID: PMC3402235.

37. Goo YA, Cain K, Jarrett M, Smith L, Voss J, Tolentino E, et al. Urinary proteome analysis of irritable bowel syndrome (IBS) symptom subgroups. Journal of proteome research. 2012; 11(12):5650–62. doi: 10.1021/pr3004437 PMID: 22998556; PubMed Central PMCID: PMC3631108.

38. Goo YA, Tsai YS, Liu AY, Goodlett DR, Yang CC. Urinary proteomics evaluation in interstitial cystitis/painful bladder syndrome: a pilot study. International braz j urol: official journal of the Brazilian Society of Urology. 2010; 36(4):464–78; discussion 78–9, 79. PMID: 20815953.

39. Martin-Lorenzo M, Gonzalez-Calero L, Zubiri I, Diaz-Payno PJ, Sanz-Maroto A, Posada-Ayala M, et al. Urine 2DE proteome analysis in healthy condition and kidney disease. Electrophoresis. 2014; 35 (18):2634–41. doi: 10.1002/elps.201300601 PMID: 24913465.

40. Polaskova V, Kapur A, Khan A, Molloy MP, Baker MS. High-abundance protein depletion: comparison of methods for human plasma biomarker discovery. Electrophoresis. 2010; 31(3):471–82. doi: 10.1002/elps.200900286 PMID: 20119956.

41. Zerefos PG, Aivaliotis M, Baumann M, Vlahou A. Analysis of the urine proteome via a combination of multi-dimensional approaches. Proteomics. 2012; 12(3):391–400. doi: 10.1002/pmic.201100212 PMID: 22140069.

42. Mischak H, Kolch W, Aivaliotis M, Bouyssie D, Court M, Dihazi H, et al. Comprehensive human urine standards for comparability and standardization in clinical proteome analysis. Proteomics Clinical applications. 2010; 4(4):464–78. doi: 10.1002/prca.200900189 PMID: 21137064; PubMed Central PMCID: PMC3064949.

43. Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome biology. 2006; 7(9):R80. doi: 10.1186/gb-2006-7-9-R80 PMID: 16948836; PubMed Central PMCID: PMC1794545.

44. Brott DA, Adler SH, Arani R, Lovick SC, Pinches M, Furlong ST. Characterization of renal biomarkers for use in clinical trials: biomarker evaluation in healthy volunteers. Drug design, development and therapy. 2014; 8:227–37. doi: 10.2147/DDDT.S54956 PMID: 24611000; PubMed Central PMCID: PMC3928457.

45. Fassett RG, Venuthurupalli SK, Gobe GC, Coombes JS, Cooper MA, Hoy WE. Biomarkers in chronic kidney disease: a review. Kidney international. 2011; 80(8):806–21. doi: 10.1038/ki.2011.198 PMID: 21697815.

46. Mischak H, Delles C, Vlahou A, Vanholder R. Proteomic biomarkers in kidney disease: issues in development and implementation. Nature reviews Nephrology. 2015; 11(4):221–32. doi: 10.1038/nrneph.2014.247 PMID: 25643662.

47. Available: http://bioinformatics.psb.ugent.be/webtools/Venn/.

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RESEARCH ARTICLE

Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis

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Abstract

High resolution proteomics approaches have been successfully utilized for the comprehensive characterization of the cell proteome. However, in the case of quantitative proteomics an open question still remains, which quantification strategy is best suited for identification of biologically relevant changes, especially in clinical specimens. In this study, a thorough comparison of a label-free approach (intensity-based) and 8-plex iTRAQ was conducted as applied to the analysis of tumor tissue samples from non-muscle invasive and muscle-invasive bladder cancer. For the latter, two acquisition strategies were tested including analysis of unfractionated and fractioned iTRAQ-labeled peptides. To reduce variability, aliquots of the same protein extract were used as starting material, whereas to obtain representative results per method further sample processing and MS analysis were conducted according to routinely applied protocols. Considering only multiple-peptide identifications, LC-MS/MS analysis resulted in the identification of 910, 1092 and 332 proteins by label-free, fractionated and unfractionated iTRAQ, respectively. The label-free strategy provided higher protein sequence coverage compared to both iTRAQ experiments. Even though pre-fraction of the iTRAQ labeled peptides allowed for a higher number of identifications, this was not accompanied by a respective increase in the number of differentially expressed changes detected. Validity of the proteomics output related to protein identification and differential expression was determined by comparison to existing data in the field (Protein Atlas and published data on the disease). All methods predicted changes which to a large extent agreed with published data, with label-free providing a higher number of significant changes than iTRAQ. Conclusively, both label-free and iTRAQ (when combined to peptide

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fractionation) provide high proteome coverage and apparently valid predictions in terms of differential expression, nevertheless label-free provides higher sequence coverage and ultimately detects a higher number of differentially expressed proteins. The risk for receiving false associations still exists, particularly when analyzing highly heterogeneous biological samples, raising the need for the analysis of higher sample numbers and/or application of adjustment for multiple testing.

Introduction

Application of mass spectrometry-based quantitative approaches has largely contributed to the emerging role of proteomics [1]. Quantitative analysis has been widely applied in various proteomics fields such as a) clinical proteomics [2, 3], b) subcellular proteomics [4, 5] or c) interaction proteomics [6, 7]. Moreover, high-resolution, comparative proteomic studies have led to progress in system biology analysis, particularly in the context of elucidation of the mechanisms underlying pathophysiology of various diseases [8].

Currently, two main types of relative quantification strategies for MS-based proteomics analysis exist: a) label-based and b) label-free (LFQ) MS-based approaches [9]. In the label-based approach, the quantification relies on the introduction of stable isotopes. Depending on the methods for isotope incorporation into the peptides/proteins, several labeling protocols have been developed including a) metabolic labeling (stable isotope labeling of amino acids in cell culture), b) chemical labeling (isotope-coded affinity tag, isobaric tag for relative and absolute quantification (iTRAQ), tandem mass tag (TMT)), c) enzymatic labeling (oxygen isotope (^{18}O)) or d) external addition of the labeled synthetic peptides [9]. Label-based methods allow for the simultaneous analysis of multiple samples in a single MS run (multiplexing), resulting in reduced analytical variability. This is particularly relevant for the application of TMT and iTRAQ labeling, since up to eight (for iTRAQ) [10] or ten (for TMT) [11] samples can be analyzed simultaneously during a single experiment. In these cases, due to the isobaric nature of labels, labeled peptides appear as a single peak in the full MS scan. However, upon peptide fragmentation at the MS/MS level, the isotope-containing reporter ions are released and distinguished according to their masses based on the label composition.

On the other hand, the label-free approach does not utilize stable isotopes. In this case, the quantification is based on spectral counting and intensity-based measurements. In the former method, quantification occurs at the MS/MS level utilizing the number of fragmentation spectra assigned to peptides that belong to a particular protein. On the contrary, the intensity-based quantification method is applicable at the MS1 level and the quantification is based on the estimated area under the curve from the extracted ion chromatogram [9].

Both, iTRAQ and label-free quantification have been widely applied in proteomic research. Up to date, several studies have been published in order to evaluate their analytical performance including precision, accuracy of quantification, protein sequence coverage and quantification reproducibility [12–16]. In a few studies, an additional effort was made to evaluate the biological significance of the findings. These studies included evaluation of a) two *Chlamydomonas reinharditii* strains in the context of biofuel production [16], b) *Methylocella silverstris* bacterium cultured under various conditions [13] and c) adenovirus infection of human lung cells [15]. In the aforementioned studies, functional analysis of differentially expressed proteins identified in label-free and iTRAQ revealed the de-regulation of proteins associated with the studied process [13, 15, 16]. However, the contribution of the de-regulated proteins to

particular biological process varies between both approaches [16], likely as a result of the different analytical performance of both quantification strategies. Along the same lines, a comprehensive comparison of the two methods, as applied in the analysis of complex biological samples (such as tumors) has not been reported yet. Both strategies are advocated and might be used as complementary approaches [17, 18]. Importantly, performance achieved during the analysis of cell lines or bacterial strains (as has been reported so far) may not be representative when the biological variability and/or complexity of samples is high. Based on the above, knowledge on the performance of these quantification strategies would provide valuable guidance on which method to use when dealing with complex and heterogeneous material such as clinical samples.

In this manuscript, we describe a side-by-side comparison of the label-free and label-based (8-plex iTRAQ) methods, with the latter also preceded by an additional fractionation step. The central goal was to provide recommendations on which approach to use when investigating protein differential expression in samples typically used in clinical proteomics. In the presented study, bladder cancer (BCa) tissue specimens representing two different tumor stages (non-muscle invasive vs. muscle invasive) were evaluated. Specifically, the number of identified proteins, their sequence coverage, consistency of reported changes and reliability of findings as defined by agreement with existing transcriptomics data were assessed. To reduce quantification bias, we attempted to unify the sampling process by utilizing aliquots of the same tissue extracts to obtain as representative as possible results per method. Sample processing and analysis by mass spectrometry were performed according to regularly used/optimized protocols per method.

Materials and Methods

Clinical samples

Bladder cancer tissue specimens were collected from patients undergoing transurethral resection of bladder cancer in medical centers in Greece (Laikon Hospital, Athens) and Germany (Department of Urology and Urological Oncology, Hannover Medicine School). The studies were approved by the respective local ethics committees (for Athens E.S 618–2012 and for Hannover 614–2009) and all individuals gave written informed consent. Samples from tumor tissue from 8 patients were employed for the analysis including non-muscle invasive (stage pTa, n = 4) and muscle invasive bladder cancer cases (stage pT2+, n = 4). Tumor stage was determined according to TNM classification system [19].

Sample preparation

Approximately 20 mg of bladder cancer tissue was homogenized in 150 μL of lysis buffer (4% SDS, 0.1M DTE, 0.1M Tris-HCl pH 7.6) using blade homogenizer (three cycles of 30 – 40s) followed by sonication (15 s per sample). This protein extraction protocol was selected following preliminary experiments testing the performance of different homogenization means such as homogenization by using liquid nitrogen, Potter homogenizer or ultrasonication (data not shown). Undissolved materials were removed by centrifugation at 13000 rpm for 10 min. Protein concentration was determined by the Bradford assay (BioRad) and protein extracts were processed using the FASP [20], separately for LFQ and iTRAQ experiments.

Label free analysis. Equal amount of protein (200 μg) per sample prepared as described above was first subjected to buffer exchange in Amicon Ultra Centrifugal filter devices (0.5 mL, 30 kDa MWCO, Millipore) at 13 000 rpm for 15 min at room temperature. The protein extract was mixed with 200 μL of urea buffer (8M urea in 0.1M Tris-HCl pH 8.5) and centrifugal concentration was performed. The concentrate was then diluted with urea buffer and centrifugation was repeated. Subsequently, alkylation of proteins was performed by adding 100 μL of 0.1M iodoacetamide in urea buffer followed by 20 min incubation in the dark. Samples were

centrifuged at 13 000 rpm for 10 min. Additional series of washes were conducted with urea (twice) and ABC buffer (50 mM NH_4HCO_3 pH 8, twice). Overnight digestion was performed by adding 2 μg of trypsin (stock solution of 500 ng/μL) in 40 μL of ABC (trypsin to protein ratio 1:100). Peptides were eluted by centrifugation followed by washing with 40 μL of 50 mM NH_4HCO_3. Afterwards, samples were lyophilized.

8-plex iTRAQ labeling. 100 μg of protein extract was processed by FASP as described above with the following modifications a) 50 mM trietylamonium bicarbonate (TEAB) was used instead of ABC buffer, b) 1 μg of trypsin was added in 20 μL of 50 mM TEAB and c) peptides were eluted with 20 μL of 50 mM TEAB. Tryptic digest peptides were labeled using the 8-plex iTRAQ Reagent kit (AB Sciex) according to manufacturer instructions. Samples from non-invasive tumor tissue (pTa stage) were labeled using 113–116 tags, whereas for the invasive tumors (pT2+) 117–119 and 121 tags were used. Subsequently, 8 individual samples were mixed and lyophilized to dryness. To remove excess of the iTRAQ reagents, peptides were re-suspended in 0.1% formic acid and 80 μg were purified using Pierce C18 Tips, 100μL bed (Thermo Scientific) according to manufacturer instructions. As an alternative approach, a high pH reverse phase chromatography on a Dionex P680 HPLC system was applied to purify and pre-fractionate the remaining peptide mixture ($\sim$ 700 μg). Labeled peptides were lyophilized and redissolved in 250 μL of high pH buffer (0.05% NH_4OH, pH 9–9.5) by sonication in a water bath. The solution was filtered using syringe driven filter unit (0.22 μM PVDF). After loading of 200 μL onto an XBridge 4.6 x 150 mm C18 column (BEH Technology) at flow rate of 0.4 mL/min in 0.05% NH_4OH, the sample was eluted with a gradient of solvent A: 0.05% NH_4OH in water versus solvent B: 0.05% NH_4OH in 100% acetonitrile starting at 5% B for 15 min, then to 35%B at 25 min then to 80% B at 30 min followed by 5 min rinsing at 80% B. In total, 5 fractions of 1.2 mL were collected starting from 21 min up to 35 min of the gradient. Prior to the LC-MS/MS analysis, 3 of these fractions with the lowest peptide content (1, 4 and 5) were pooled.

LC-MS/MS analysis

10 μg of protein digest were loaded onto a Dionex Ultimate 3000 RSLS nano flow system (Dionex, Camberly UK). After loading onto a Dionex 0.1×20 mm 5 μm C18 nano trap column at a flow rate of 5 μl/min in 0.1% formic acid and 2% acetonitrile, samples were applied onto an Acclaim PepMap C18 nano column 75 μm×50 cm, 2 μm 100 Å at a flow rate of 0.3 μl/min. The trap and nano flow column were maintained at 35°C. The samples were eluted with a gradient of solvent A: 0.1% formic acid versus solvent B: 80% acetonitrile starting at 1% B for 5 min rising to 5% B at 10 min then to 25% B at 360 min and 65%B at 480 min.

The eluent was ionized using a Proxeon nano spray ESI source operating in positive ion mode into an Orbitrap Velos FTMS (Thermo Finnigan, Bremen, Germany). Ionization voltage was 2.6 kV and the capillary temperature was 200°C. The mass spectrometer was operated in MS/MS mode scanning from 380 to 2000 m/z. The top 20 multiply charged ions were selected from each scan for MS/MS analysis using CID at 40% collision energy. The resolution in MS1 was 60,000 and 7,500 at m/z 400 for CID in MS2. For the iTRAQ samples, the top 20 multiply charged ions were selected from each scan for MS/MS analysis using HCD at 45% collision energy. AGC settings were 1,000,000 for full scan in the FTMS and 200,000 for MSn. Resolution in MS2 at m/z 115 was 16,300. Dynamic exclusion was enabled with a repeat count of 1, exclusion duration of 30 seconds.

Data processing

The processing of the individual raw MS data files was conducted using the commercially available software Proteome Discoverer v. 1.4.0.288 (Thermo Scientific). An event detection node

was used at a setting of 2 ppm along with the precursor ion peak detector node. Database search was carried out against Human Swiss-Prot Database (30/10/2013) [21, 22] containing only the canonical sequences with 20 277 entries using the Sequest search engine [23] implemented in Proteome Discoverer. The following search parameters were applied: a) precursor mass tolerance 10 ppm, b) fragment mass tolerance: 0.8 Da and 0.05 Da for label-free and iTRAQ experiments, respectively, c) fixed modification: carbamidomethylation of cysteine (C) and additionally for the labeling experiment an iTRAQ modification of N-terminus and lysine residues were added, d) variable modification: oxidation of methionine (M) and in the case of iTRAQ, the iTRAQ modification on tyrosine (Y) was added, e) allowing one missed cleavage site. The false discovery rate evaluation was performed by using the Percolator node [24] (Proteome Discoverer 1.4). To verify labeling efficiency, an additional search was performed by setting the iTRAQ 8-plex labels as variable modifications on N-terminus and Lysine (K). In parallel, the prevalence of the modifications (including oxidation, chemically induced cysteine modification, chemical and posttranslational modifications) was evaluated by using Preview™ node (v2.6.46, Protein Metrics Inc.) [25] incorporated in the Proteome Discoverer workflow. To this end, a search was performed for the selected data files from each experimental approach incorporating the modifications indicated above. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium [26] via the PRIDE partner repository with the dataset identifier PXD002170.

Protein Identification

The same selection criteria were applied for protein identification in both approaches. Identified peptides were initially filtered requiring mass deviation below 5 ppm between experimental and theoretical mass, false discovery rate below 1% (assigned in Proteome Discoverer as high confidence peptides) and peptide rank up to 5. Peptides were excluded if they contained an unknown amino acid (X) in the sequence or if the protein accession could not be mapped. In the case of the label-free approach, the list of the non-redundant peptides for the entire experiment was then generated, based on the individual datasets (due to its multiplexity nature, merging was not required in the case of iTRAQ). During the merging of the individual datasets from the label-free experiment, only peptides with an FDR$<$1% were included. FDR level was not assessed again after merging of the data. If sequences with identical number of modifications, although in different position, were reported, only one sequence was retained. For each spectrum (as defined by the same m/z and retention time), the best candidate sequence was defined based on the relative number of sequence identifications per sample (e.g. the sequence with the highest number of identifications was maintained). The confidence in the interpretation (based on the XCorr) was taken into consideration in cases where the same number of sequence identifications was reported. Additionally, only peptides consistently reported in more than 75% of the samples (at least in one group: pTa and/or pT2+) were considered as credible. Subsequently, peptides were assigned to the protein according to the Occam Razor principal [27]. All peptides derived from keratins were excluded as probable contaminations, and were not taken into consideration during the subsequent analysis. Only proteins identified based on $\geq$2 peptides were considered for further comparative analysis.

Relative Quantification

Label-free quantification. The peak area-based quantification uses precursor ions to assess the relative abundance of identified proteins in the label-free data. For each precursor ion, peak area (i.e. area under the curve) is calculated from the extracted ion chromatogram during data processing in Proteome Discoverer by using the Precursor Ions Area Detector

node. For the sequences for which no peptide area could be integrated by Proteome Discoverer (version 1.4; this is a well-known, but not yet corrected problem of this software), the absent values were replaced with the mean area values calculated in that group (pTa or pT2+). When the peptide was not identified in the particular sample, the missing values were replaced with zero. Part per million (ppm)-normalization was conducted for the selected peptides according to the following formula: Normalized peak area = (Peptide peak area/Total peak area)$\times 10^6$. Protein abundance in each sample was calculated as the sum of all normalized peptide areas for a given protein. Peptides matching to multiple protein IDs were included only for the quantification of the one protein indicated by the Occam Razor rule [27]. The mean protein abundance per groups was then calculated and the average values were log2 transformed. The log2 ratio was then calculated by the subtraction of the log2 transformed mean value obtained for case and controls [$\log_2 \frac{case}{control} = Log_2Avg.Cases - Log_2Avg.Controls$].

Label-based quantification. All quantification steps were performed using the Proteome Discoverer Software (version 1.4). The 8-plex iTRAQ quantification was performed based on the reporter ion intensities detected by the Reporter Ions Quantifier Node in Proteome Discoverer. The reporter ion intensities were corrected for the isotopic impurities using reporter ion isotopic distribution (S1 Table). When the individual reporter intensities were 0 (the reporter, or mass, tags are missing in the quantification spectrum), the minimal reported intensity was assigned to the respective peptide. To provide an accurate quantification of proteins, only peptide spectrum matches with co-isolation interference below 30% were included in the analysis [28]. Subsequently, for each distinct peptide the abundance was calculated as the median of reporter ions from all matching spectra, since median is more resistant to outliers. Spectra were grouped based on mass and sequence, without taking into consideration the peptide charge. In the case of modifications, the peptides were considered as distinct when modifications were different. The reporter ion intensities for each individual peptide were represented as a ratio of the particular reporter ion to the sum of all reporter (as in the case of Libra implemented in Trans Proteomic Pipeline Software [29]). To account for experimental biases (e.g. unequal loading), the quantification values for each channel were balanced to be equal to 12.5%, which corresponds to the contribution of 1 out of 8 labels for quantification. This is based on the assumption that the reporter ions are ionized with the same efficiency and in the case of equal loading comparable total intensity of reporter ions should be obtained for each label. For protein quantification, only unique peptides were taken into consideration. For each label, protein abundance was defined as the average of the peptide quantification values belonging to the given protein, which is expected to better reflect the overall change at the protein level (in comparison to using the median values), due to the expected ionization efficiency differences among different peptides. Subsequently, the average values were calculated for cases and controls, and these values were log2 transformed. The ratio was calculated by following subtraction of the mean value obtained for case and controls, as in the case of label-free approach. As an alternative quantification strategy (referred as analysis 2), balanced quantification values were employed to calculate the peptide ratio. The latter was expressed as a ratio of quantifications values corresponding to pT2+ vs. pTa samples. Similarly, only unique peptides were considered for protein quantification. Protein ratio was calculated by the averaging of all quantifiable peptide ratios belonging to each protein and the ratio values obtained were subsequently log2 transformed.

Statistical analysis

Statistical analysis was performed using SPSS Statistical Software (SPSS 17.0, IBM). For each quantification method, the p-value was calculated for the log2 transformed values by using

independent sample t-test. In the case of the alternative quantification approach tested for the iTRAQ (analysis 2), the p-value was calculated based on the normal distribution of the ratios by using R programming language. Proteins with a p-value below 0.05 were considered as statistically significant. Pearson correlation and regression analysis was calculated in MedCalc Version 12.1.0.0 (Mariakerke, Belgium).

Assessment of reliability of protein identification and differential expression

Validity of the received protein identification was assessed by comparison to expression data from urinary bladder and/ or bladder cancer tissue reported in the Human Protein Atlas (http://www.proteinatlas.org/ [30]), ProteomicsDB (http://www.proteomicsdb.org/ [31]) as well as transcriptomic resources (Bgee Database [32]). Credibility of the regulation trend (up-/ down-regulated in pT2+ vs. pTa), as obtained from the proteomic analysis, was evaluated based on comparison with the mRNA microarray data (GSE3167 [33]) deposited in the Gene Expression Omnibus [34] as well as literature [35, 36]. The former transcriptomic data obtained for the pT2+ and pTa bladder cancer stages were analyzed by the GEO2R [37], a web tool enabling statistical analysis of the data. The information about analyzed samples as well as the output from the GEO2R is presented in S2 Table. The expression trend reported in proteomics was considered valid when agreement between the proteomic and microarray data was observed.

Immunohistochemistry

Immunohistochemical evaluation of Annexin A6 expression (Annexin VI Antibody (N-19), polyclonal, anti-goat, Sc-1931, Santa Cruz, AB_630873 Antibody Registry ID, dilution 1:200) was performed on a tissue microarray containing 35 tissue samples (n = 11 non-cancerous bladder samples, n = 8 pTa tumors, n = 8 pT1 tumors, n = 8 pT2+ tumors). Further visualization was performed with diaminobenzidine according to the manufacturer instruction (ultraView Universal DAB Detection Kit) and subsequently sections were counterstained with hematoxylin. Quantification of the staining was performed with ImageJ software after application of color deconvolution [38]. Briefly, 5 images were acquired per section and 10 identical areas among the sections were selected for measurement. The optical density for the background was subtracted from all measurements.

Results

Three experimental approaches were evaluated (label-free, unfractionated and fractionated iTRAQ) aiming to select the optimal strategy for determination of protein differential expression in highly complex samples employed in clinical proteomics (i.e. non-muscle invasive (pTa) in comparison to muscle-invasive (pT2+) bladder cancer). The workflow for sample preparation and data analysis is depicted in Fig 1. Aliquots of the same protein extracts were used in all cases and all samples were processed by FASP. The lysis buffer was selected based on preliminary experiments which showed its efficiency (in terms of protein recovery and reproducibility) for bladder tissue (data not shown). In the case of iTRAQ some minor modifications of the classical FASP protocol were necessary to ensure compatibility with the subsequent labeling, as suggested by the manufacturer and described earlier [39–41]. These include a reduction of the initial amount of protein processed by FASP (from 200 μg used for LFQ to 100 μg for iTRAQ), and substitution of the ammonium bicarbonate buffer with triethylammonium bicarbonate to avoid interference of the former with labeling via interactions with the iTRAQ reagents.

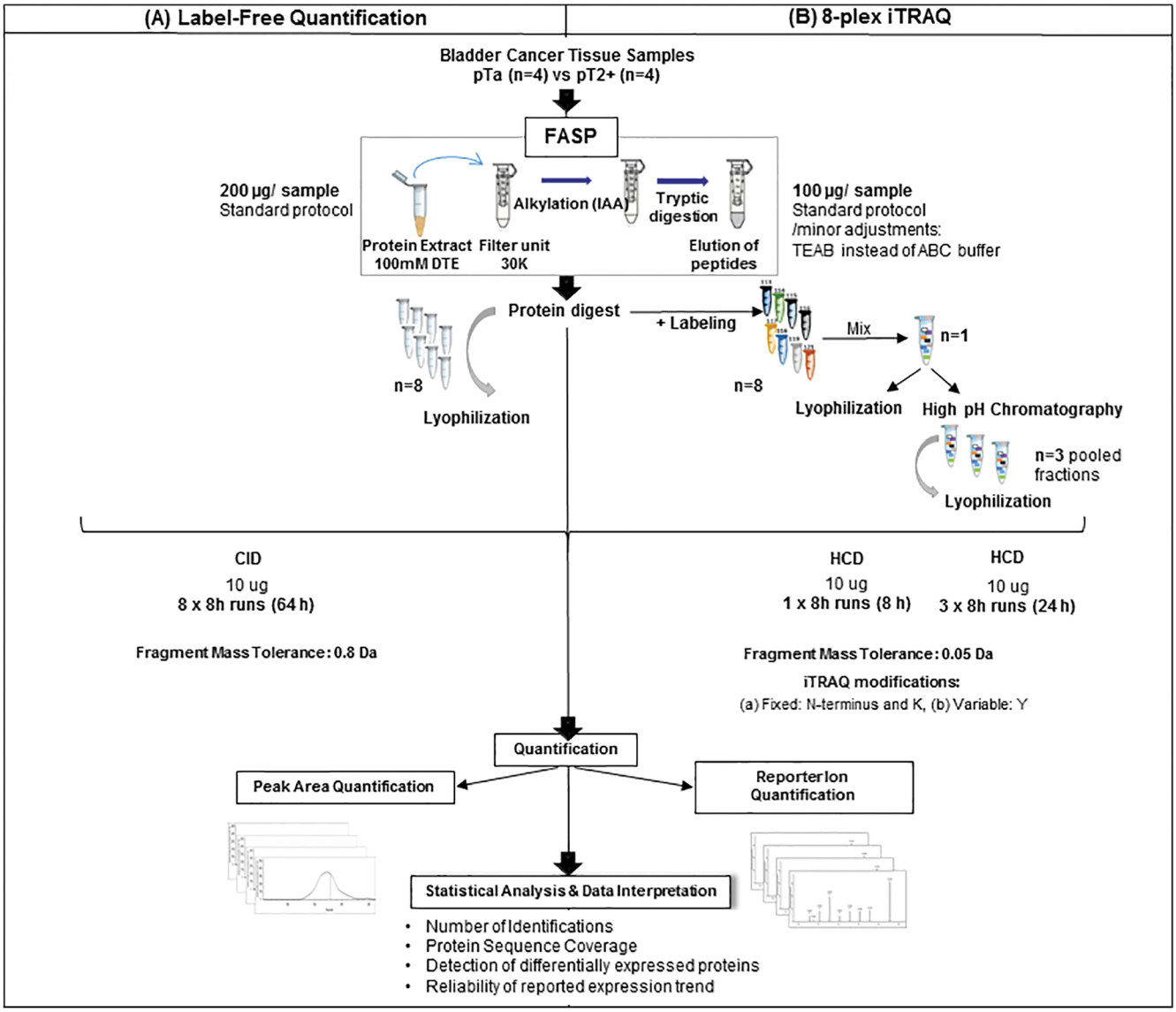

Fig 1. Experimental workflow. The applied workflow for sample preparation and data analysis for LFQ and iTRAQ quantification is graphically depicted.

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The impact of possible differences introduced by the sample preparation protocols was assessed based on the prevalence of defined modifications in both experiments. The analysis was conducted using Preview™ software [25] and the results were subsequently evaluated according to the percentage and the fraction of modified peptides (ratio of number of peptides containing modifications vs. peptides that could possibly contain modification). In general, a comparable percentage of modified peptides between both approaches (LFQ and iTRAQ) was obtained, as confirmed by regression analysis (S1 Fig). Most reported modifications were identical (S3 Table) and the frequency of appearance was generally low. For few modifications, there was a significant difference between the percentage of modified peptides in LFQ and iTRAQ including a) formation of Pyro-glu N-terminus, b) carbamylation of methionine, c) N-terminal acetylation of proteins, d) oxidation of methionine/ histidine/ tryptophan and e) carbamidomethylation artifacts.

Mass spectrometry and data analysis were performed according to optimized protocols for each approach, as described in Materials and Methods. In this way, we targeted to obtain unbiased and representative results for each approach.

Protein identification and Quantification

To ensure validity of the findings, only consistently detected peptides (in at least 75% of samples of one group (pTa or pT2+) were considered in the LFQ approach. As indicated in Materials and Methods, peptides representing possible contaminations such as keratins were completely removed from the datasets and thus, the corresponding proteins were not taken into consideration in further analysis. Based on this threshold, an average number of 5184±550 peptide and 1113±78 protein identifications, including single peptide hits, were reported per LC-MS/MS run (Table 1). In total the LFQ approach enabled the identification of 6871 peptides corresponding to 1346 proteins. For unfractionated iTRAQ, a total number of 1859 peptides and 664 proteins were reported; pre-fractionation of the labeled peptides increased the identification rate for both, peptides (6099) and proteins (2064). However, in the two iTRAQ experiments approximately 49% of the reported proteins were represented by a single peptide only. The respective percentage for LFQ was 32%. Upon exclusion of proteins represented by a single-peptide, a total of 332, 1092 and 910 protein identifications are received in the unfractionated, fractionated iTRAQ and LFQ experiments, respectively. The lists of identified peptides and proteins per technique (including also single-peptide identifications) are presented in S4 Table.

The obtained data were subsequently compared at both peptide (Fig 2A) and protein levels (Fig 2B). For this analysis and to increase reliability of findings only multiple peptides (≥ 2) identifications were taken into consideration. As represented in the Venn diagram (Fig 2A), 782 peptides were reported in all three approaches, which corresponds to 42% and 13% of the total number of the peptides detected by the non-fractionated and fractionated iTRAQ respectively and 11% of peptides identified by the label-free approach. When comparing the data at the protein level, 280 proteins were found to be detected by all acquisition methods (Fig 2B). This overlap corresponds to 84% of the proteins identified by iTRAQ unfractionated, 26% for iTRAQ-fractionated and 31% for LFQ. When comparing the proteins exclusively detected per method, an approximately two fold higher number of uniquely identified proteins (433 IDs) was obtained for the pre-fractionated iTRAQ sample as compared to the LFQ (234 IDs); a

Table 1. Overview of the number of peptides and the corresponding proteins as being identified in the individual MS-runs.

Method	Sample ID	# peptide groups	# protein groups
	3_pTa	5073	1096
	11_pTa	5269	1099
	16_pTa	5725	1185
	19_pTa	3931	937
LFQ	**Mean (SD)_pTa**	**5000 ± 763**	**1079 ± 103**
	9_pT2+	5360	1130
	12_pT2+	5112	1136
	15_pT2+	5516	1169
	17_pT2+	5485	1155
	Mean (SD)_pT2+	**5368 ± 184**	**1148 ± 18**
iTRAQ	10 µg	1859	664
	fractionation	6099	2064

doi:10.1371/journal.pone.0137048.t001

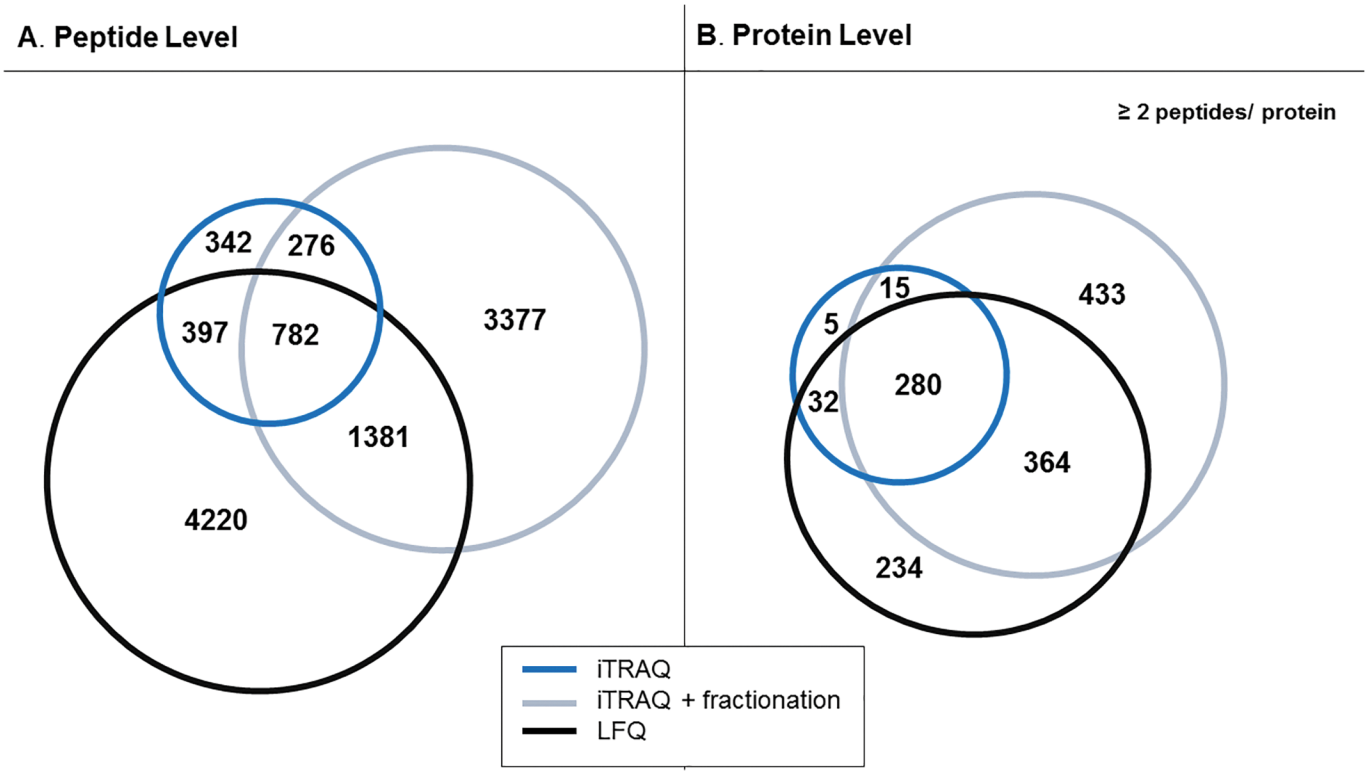

Fig 2. Comparison of peptide and protein identifications in iTRAQ and LFQ experiments. Venn diagrams representing the comparison of all identified peptides, without considering fixed/variable modifications (A), and proteins (B) from LFQ, fractionated/ unfractionated iTRAQ analysis.

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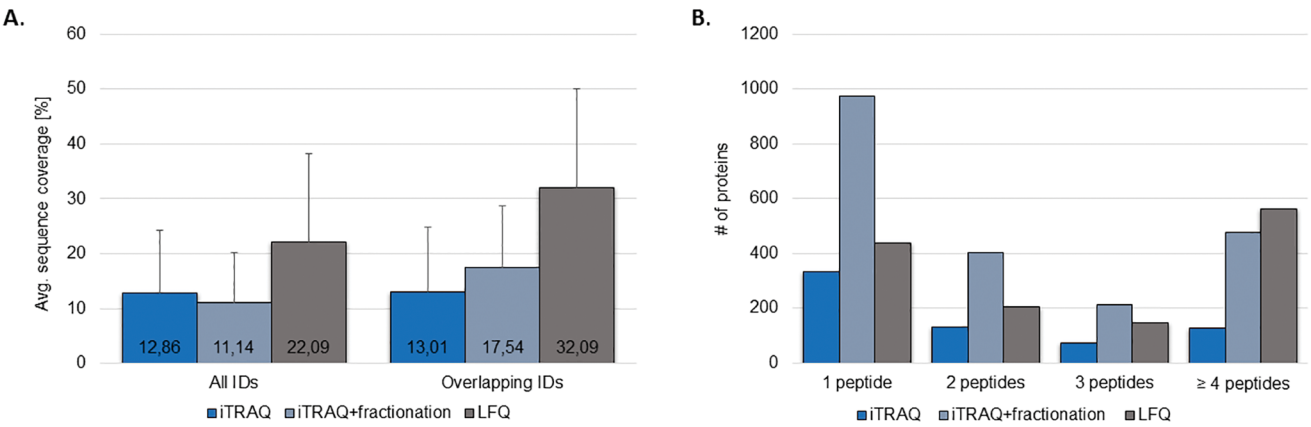
limited number of proteins exclusively identified in the unfractionated samples was also observed (5 IDs). Of note, in the LFQ approach all identified peptides and proteins have a quantification value, whereas 76% (1441 out of 1859 peptides) and 81% (4918 out of 6099 peptides) of the peptides detected by the iTRAQ experiments, without and with fractionation, respectively, could be quantified, as per the iTRAQ restrictions (unique labeled peptides detected in all clinical samples with a percentage of the co-isolation interference below 30% are considered as quantifiable in iTRAQ). At the protein level the vast majority of the proteins without a quantification value were single-peptide identifications. Of the proteins identified with at least two peptides, the quantification was not possible for 6 out of 332 and 14 out of 1092 proteins in the case of unfractionated and pre-fractionated iTRAQ sample, respectively.

In the case of the label-based approach, two methods were tested to calculate the abundance at protein level and subsequently assess the protein ratio. Either by calculating the average peptide quantification values (separately for each label) assigned to the protein (analysis 1) or by employing the averages of the individual peptide ratios (analysis 2) (as described in detailed in Materials and Methods). To exemplify differences related to the assessment of protein abundance, the two methods were tested for the data obtained from the fractionated iTRAQ sample. The obtained results are presented in S4 Table. The methods give highly comparable results: the ratios for de-regulated proteins (as indicated by the primary analysis, analysis 1) as well as for whole dataset (including proteins identified with $\geq$2 peptides) were significantly correlated between both methods, with the Pearson correlation coefficient of 0.99 ($p<0.0001$, S2 Fig). Moreover, comparable numbers of significant changes ($p<0.05$) were reported using both methods (45 and 48 for analysis 1 and 2, respectively).



C.

All protein identifications			Overlapping proteins		
iTRAQ	LFQ	iTRAQ-fractionated	iTRAQ	LFQ	iTRAQ-fractionated
5	7	5	5	13	8

Fig 3. Evaluation of protein sequence coverage for LFQ and iTRAQ. Average protein sequence coverage was compared for all identified proteins per technique as well as for the overlapping identifications (A). The total number of identified proteins based on the particular number of peptides (B) and the average number of peptides per protein are also presented (C).

doi:10.1371/journal.pone.0137048.g003

Coverage of protein sequence

The protein sequence coverage was calculated for all the identified proteins per method and for the overlapping identifications between the three approaches (Fig 3A). Only multiple-peptide identifications (≥2 peptides) were considered. For iTRAQ experiments, a similar protein sequence coverage was reported for the unfractionated (13%) and fractionated (11%) sample. In the case of the LFQ, a significantly higher sequence coverage (22%, $p<0.001$ independent sample t-test) in comparison to both iTRAQ approaches was observed. This difference was even more pronounced when considering the overlapping identifications among different methods (32% vs. 13%/ 18% for LFQ and non-/ fractionated iTRAQ, respectively) (Fig 3A). The above observations are further supported by the evaluation of the average number of peptides per identified protein (Fig 3C) as well as a comparison of the number of proteins identified based on particular number of peptides (i.e. 1, 2, 3 and ≥ 4 peptides/ protein) per technique (Fig 3B). As shown, comparable numbers of single-peptide identifications were reported in LFQ (436 IDs) and iTRAQ-unfractionated (332 IDs). Fractionation of the labeled peptides increased the number of single peptide identifications substantially to 972 IDs. At the same time, fractionation also resulted in a higher number of multiple-peptides identifications (≥ 2 peptides) in comparison to the unfractionated iTRAQ (Fig 3B), but still at an overall lower average number of peptides per protein in comparison to LFQ (Fig 3C).

Evaluation of differential expression

Assessment of the relative protein abundance is based on the comparison of the quantification results of pTa (control) versus pT2+ (case) groups. Statistical analysis was used as a criterion to define the altered protein abundance. Thus, proteins with p-value < 0.05 were considered as being significantly changed in the case vs. control group. Additionally, the expression trend

Table 2. Comparison of number of differentially expressed proteins identified by LFQ and iTRAQ approaches.

Regulation Trend (≥ 2 peptides)	LFQ	iTRAQ	iTRAQ + fractionation
# Up-regulated	49	1	21
# Down-regulated	28	5	24
Total	77	6	45

doi:10.1371/journal.pone.0137048.t002

(up- or down-regulation in the case group) is represented by the ratio indicating the changes in the abundance between pT2+ over pTa BCa samples. Based on the statistical analysis ($p<0.05$), LFQ enabled identification of a higher number of differentially expressed proteins (77 proteins, identification based on at least 2 peptides), even in comparison to pre-fractionation of iTRAQ (45 proteins). The distribution of up- and down-regulated proteins is presented in Table 2. Three of these proteins were statistically significant in all three methods (Fig 4). On the other hand, 65 and 32 proteins were found to be statistically significant only in LFQ and fractionated iTRAQ samples, respectively (Fig 4). Of the former (65 proteins), as presented in Table 3, 49 proteins were identified by the other techniques but a significant difference in the relative abundance could not be detected. In the case of fractionated iTRAQ, the majority of proteins reported as uniquely differentially expressed were not identified by other methods.

Proteins with p-value < 0.05 were considered as differentially expressed. Based on the reported ratios ($\log_2 \frac{case}{control} = Log_2Avg.Cases - Log_2Avg.Controls$) proteins were classified as up/down regulated in pT2+ group (case group).

As indicated above, a very low number of identifications along with a low number of differentially expressed proteins were reported for the unfractionated iTRAQ approach in comparison to the other two techniques. The dataset obtained for the fractionated iTRAQ sample was considered as more favorable for the label-based approach and was included in further analysis.

In total, significantly altered levels of abundance (at least according to one quantification strategy i.e. LFQ and fractionated iTRAQ) were observed for 71 proteins (out of the overlapping 644 proteins detected by both methods).

To further evaluate the consistency of the reported changes to these 71 proteins, S5 Table, the comparison of their regulation (up-/ down-regulated in pT2+) as reported by the different methods was performed. As shown (S5 Table), good consistency was observed: 66 proteins exhibit the same trend of expression, while 5 proteins appear to have inconsistent results (Table 4). Among the proteins exhibiting a consistent regulation, Annexin A6 was found to be increased using both proteomic approaches, but statistical significance was reached only for the case of LFQ. This expression trend was further confirmed by immunohistochemistry (Fig 5). For those 5 inconsistent quantification results observed between LFQ and iTRAQ, a comparison of the quantification values at the peptide level was conducted (Table 5). All of the proteins were quantified based on a comparable number of peptides. As presented in Table 5, the majority of the common peptides used for quantification were characterized by the opposite expression trend in the two methods.

Validity of protein differential expression

We next evaluated whether the proteins identified as differentially expressed were previously detected in normal and/or malignant urothelium using various proteomics resources i.e. Human Protein Atlas (HPA) [30], ProteomicsDB [31], and gene expression database [32] (S6 Table). The expression of almost all differentially expressed proteins (39 out of 45 in iTRAQ

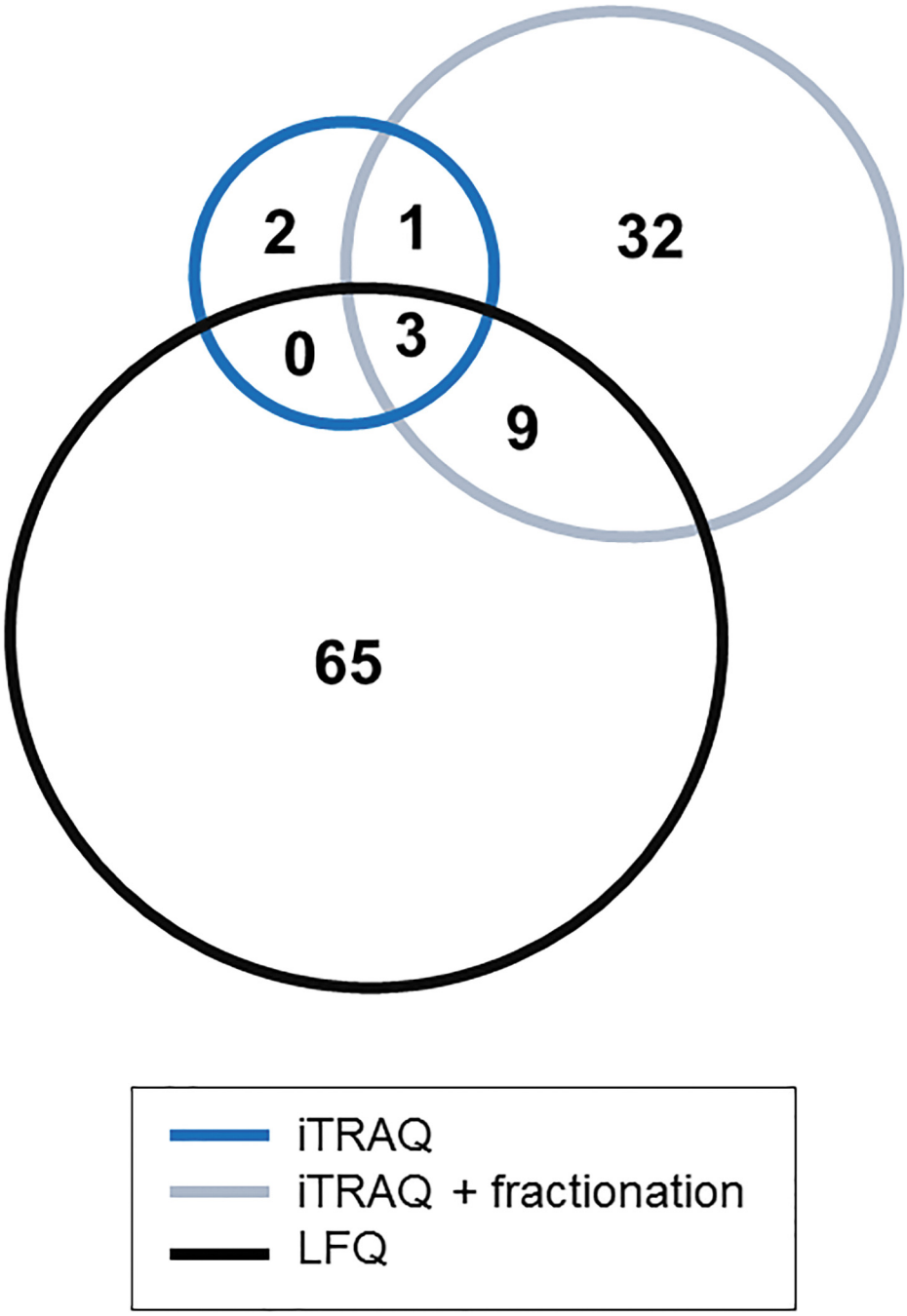


Fig 4. Comparison of differentially expressed proteins identified in both iTRAQ experiments and LFQ. Venn diagrams representing differentially expressed proteins found among the identified proteins after exclusion of single peptide hits.

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and 64 out of 77 in LFQ, respectively) have been confirmed in normal and/or tumorous urothelium in all data repositories.

In an effort to further investigate the validity of the proteomics results, we compared them to transcriptomics data [33, 35, 36] from comparison of BCa vs. normal tissue [35], high vs.

Table 3. Evaluation of the proteins with the altered abundance found as a unique based on the results obtained for three methods.

	LFQ	iTRAQ	iTRAQ + fractionation
Total number	65	2	32
Proteins identified in all three approaches	20	1	5
Proteins identified by two techniques	29	1	9
Exclusively identified	16	-	18

doi:10.1371/journal.pone.0137048.t003

low grade bladder cancer [36] and/or invasive (pT2+) vs. non-invasive (pTa) BCa (Gene Expression Omnibus ID: GSE3167 [33]) (S6 Table).

The summary of the obtained results for datasets derived from the fractionated iTRAQ and LFQ is presented in Table 6. Of the differentially expressed proteins detected by LFQ 44% (34/77) were also found to be differentially expressed at the mRNA level (S6 Table). In the case of significant changes according to fractionated iTRAQ, the expression trend of 15 out of 45 proteins (33%) was in agreement with the microarray data. As aforementioned, a comparison of the differentially expressed proteins revealed several proteins as being significant only according to one approach.

Of proteins that were detected in both approaches, but found as differentially expressed only based on one approach, 18 out of 45 for LFQ and 3 out of 14 for iTRAQ were also found to be differentially expressed at the mRNA level (Table 6, Overlapping IDs). In the case of the identifications solely detected by one technique (20 for LFQ and 19 for iTRAQ), the differential expression of 13 proteins (LFQ) and 9 proteins (iTRAQ) was supported by the mRNA data (Table 6, Unique IDs).

Discussion

Since the introduction of the iTRAQ labeling as a quantification strategy for shotgun proteomics [42], several studies have been published aiming at the comparative analysis of label-free and iTRAQ performance [12–16]. Considering the advantages of multiplexing, MS analytical time and the total cost of the experiments is relatively lower in the iTRAQ analysis as compared to the LFQ. Additionally, since all the samples are measured simultaneously in a single MS run, the inter-run variations of the protein identification and quantification caused by the data-dependent acquisition do not exist. Reports presented in the past focused on the detailed "technical" characterizations of the quantification strategies applied in proteomics (including

Table 4. List of proteins with conflicting expression trend.

	iTRAQ + fractionation				Label-free			
Protein Name	**#quantified peptides[a]**	**Log2Ratio**	**p-value**	**Regulation**	**# Peptides**	**Log2Ratio**	**p-value**	**Regulation**
Actin-related protein 2/3 complex subunit 3	3	-0,25	**0.04** [a]	down	2	0.37	0.47	up
Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 2	7	-0,06	0.58	down	6	1.23	**0.02** [a]	up
KH domain-containing, RNA-binding, signal transduction-associated protein 1	2	-0.25	0.10	down	4	0.63	**0.02** [a]	up
General vesicular transport factor p115	4	-0.10	0.57	down	3	0.52	**0.04** [a]	up
Heterochromatin protein 1-binding protein 3	3	-0,30	**0.04** [a]	down	6	0.72	0.17	up

Proteins that were found to be differentially expressed only according to one quantification method. Fold changes and p-values are reported.

[a] Differentially expressed proteins with p-value <0.05.

doi:10.1371/journal.pone.0137048.t004

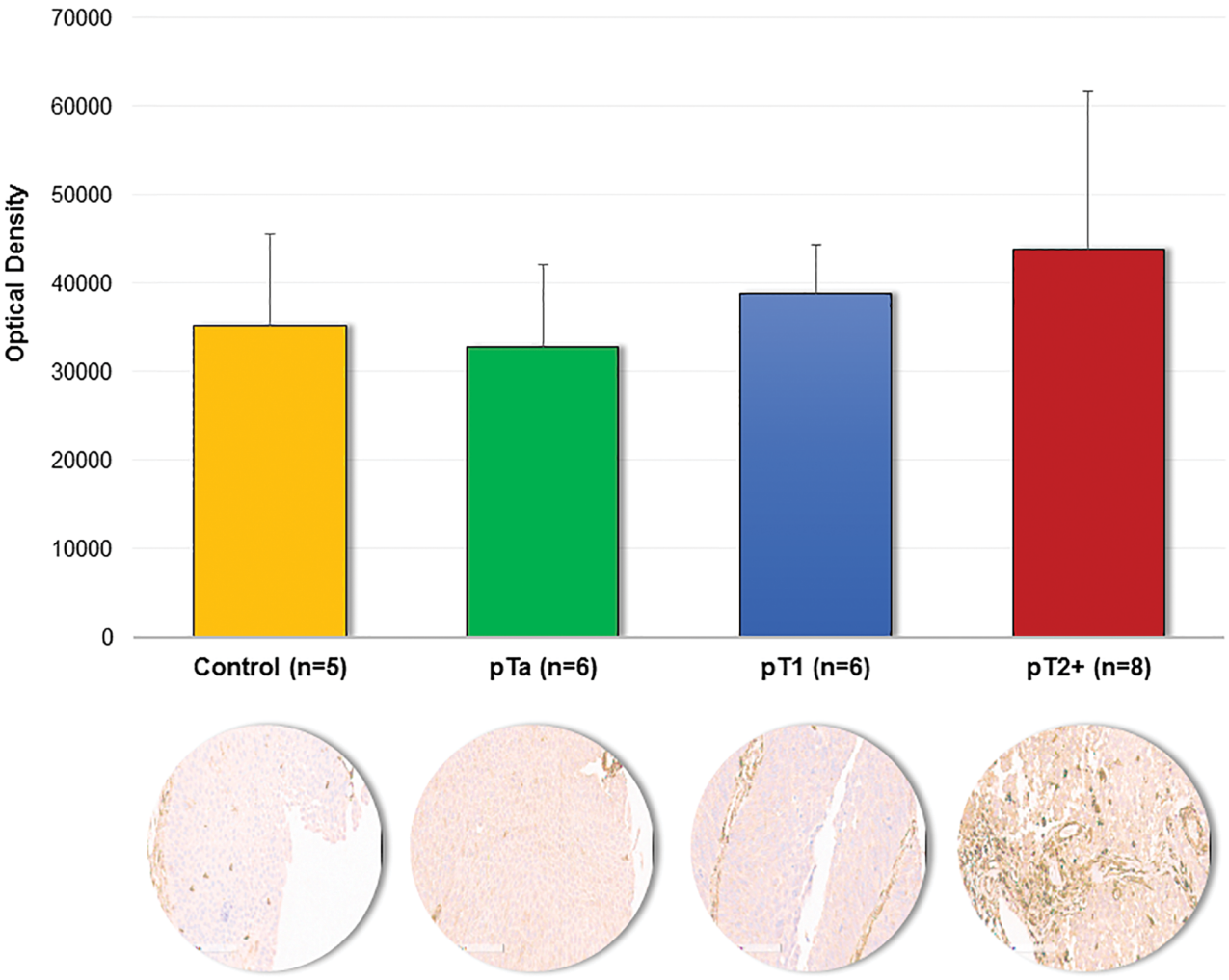

Fig 5. Immunohistochemical staining of Annexin A6. Quantification results obtained from non-cancerous tissue and bladder cancer tissues (pTa, pT1 and pT2+) along with the representative images of stained sections are presented. Quantification of the immunoreactivity was conducted by using Image J software followed by color deconvolution and background subtraction.

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reproducibility, accuracy and precision) [12–16]. To the best of our knowledge a comparison of LFQ and iTRAQ as applied in the investigation of complex clinical samples has not yet been presented, especially in the context of the detection of the differentially expressed proteins.

We selected bladder cancer tissue specimens from the non-invasive (pTa) and muscle-invasive disease (pT2+) as a prototypic model for comparing the ability of iTRAQ and LFQ to efficiently detect differentially expressed proteins. In addition to the single LC-MS/MS analysis of the iTRAQ sample, peptide fractionation prior to LC-MS/MS analysis by using a high pH chromatography was also tested. All experiments were conducted using optimized protocols per technique as reported in the literature, to enable unbiased comparison of the two approaches. However, in order to minimize potential influence of biological and analytical variability on the quantification results, we applied, as far as possible, comparable sample processing strategies. Specifically, the same tissue extracts were employed in all cases generated using a protocol (FASP) for bladder tissue optimized in our laboratory. The FASP approach was selected to enhance both homogenization and protein solubilization process in bladder tumor specimens. The analyzed tissue specimens are considered difficult to be homogenized, therefore

Table 5. Comparison of the quantification results at the peptide and protein level for identifications with conflicting expression trends between fractionated iTRAQ and LFQ.

Protein/ Peptide		Peptide Log2 Ratio (pT2+ vs. pTa)		Protein Log2 Ratio (pT2+ vs. pTa)	
Protein Name	**Peptide sequence used for quantification**	**iTRAQ + fractionation**	**LFQ**	**iTRAQ + fractionation**	**LFQ**
	eASYSLIR	-0.24	0.03	**-0,30**	**0.72**
	mDAILTEAIk	-0,37	0.32		
Heterochromatin protein 1-binding protein 3	tIPSWATLSASQLAR	-0.30	0.41		
	SSAVDPEPQVK	-	1.31		
	LEDVLPLAFTR	-	0.24		
	GASGSFVVVQK	-	5.12		
	aYLQQLR	0.01	0.61	**-0.25**	**0.37**
Actin-related protein 2/3 complex subunit 3	lIGNMALLPIR	-0.43	-0.06		
	lIGNmALLPIR	-0.34	-		
	sIVEEIEDLVAR	0,21	2.46	**-0.06**	**1.23**
	eDQVIQLMNAIFSk	-0.36	-		
	fELDTSER	0.15	-		
	nFESLSEAFSVASAAAVLSHNR	-0.12	-		
Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 2	qEIQHLFR	0.09	-		
	yHVPVVVVPEGSASDTHEQAILR	-0.38	0.62		
	LQVTNVLSQPLTQATVK	-	0.33		
	ISTEVGITNVDLSTVDKDQSIAPK	-	1.78		
	NPILWNVADVVIK	-	3.65		
	YIANTVELR	0.06	3.15		
	KDDEENYLDLFSHK	-	0.39	**-0.25**	**0.63**
	ILGPQGNTIK	-	0.55		
KH domain-containing, RNA-binding, signal transduction-associated protein 1	DSLDPSFTHAMQLLTAEIEK	-	2.42		
	SGSMDPSGAHPSVR	0.06	0.68		
	qPPLPHR	-0.56	-		
	SSQTSGTNEQSSAIVSAR	-	0.27	**-0.10**	**0.52**
	SQLNSQSVEITK	-	0.25		
General vesicular transport factor p115	NDGVLLLQALTR	-0,23	2.44		
	eQDLQLEELR	-0.47	-		
	qSEDLGSQFTEIFIk	0.31	-		
	vASSTLLDDRR	-0.00007	-		

Similarly to the calculation of the relative abundance at the protein level, the peptide ratio values were calculated based on the log-2 transformed average vales for cases (pT2+) and controls (pTa).

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application of buffers containing high concentration of strong detergents (as in the case of FASP lysis buffer) is advisable. Additionally, application of the same approach for sample preparation and processing is crucial to avoid introducing any analytical bias related to variability of starting material. Even though the FASP method may not be considered optimal for iTRAQ analysis, adaptations were made according to existing literature ([39–41] also described in Materials and Methods). The small difference in the amount of protein initially processed by FASP (200 μg/ sample in LFQ and 100 μg/ sample in iTRAQ) likely does not affect the obtained results, since the same amount of protein was finally loaded onto the LC-MS/MS. The lower

Table 6. Assessment of the validity of the differentially expressed proteins identified in proteomics experiments.

Overlapping IDs			
	Total	Similar expression trend with transcriptomics	Not conclusive
Significant only in LFQ	45	18	27
Significant only in iTRAQ	14	3	11
Significant in both metods	12	3	9
Unique IDs			
	Total	Similar expression trend with transcriptomics	Not conclusive
LFQ	20	13	7
iTRAQ	19	9	10

The validity of the findings was evaluated by comparing the observed expression trends in this proteomics experiment with several transcriptomic experiments [35, 36]. Comparison of the detected changes was performed for the differentially expressed proteins reported among overlapping identifications between iTRAQ and LFQ as well as for the proteins solely detected in one approach (unique IDs). Proteins exhibiting similar expression trend in transcriptomics are presented in the "similar expression trend" column. In the cases when the expression trend was not in accordance to mRNA expression level or the data were not available, the findings were classified as "not conclusive".

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starting material in the case of iTRAQ was selected as one vial of the reagent can label between 20 and 100 μg of protein digest. We avoided acetone precipitation, as this can introduce additional variability and peptide modifications [43]. To assess whether the utilization of different protocols could introduce unanticipated modifications, we assessed the presence of known modifications using the Preview™ software [25]. The identity and the prevalence of vast majority of known modifications was similar in the iTRAQ and the LFQ approach (S1 Fig, S3 Table). This indicates that differences in the sample processing likely did not affect the obtained results. Following tissue processing, all subsequent steps, including proteins digestion and mass spectrometry analysis were conducted according to optimized protocols per LFQ and iTRAQ as described in the literature, in order to obtain representative results per technique.

The pivotal goal of the study was an evaluation of both quantification approaches, label-based and label-free, particularly focusing on the number and credibility of the identified differentially abundant proteins. To address this objective, sequence and proteome coverage as well as the capability of both techniques to detect the significantly altered proteins and, more importantly, the credibility of the identified changes were evaluated. This latter assessment was based on the comparison with existing expression data at the mRNA [32] and protein levels. The later included data deposited in Human Protein Atlas ([30], http://www.proteinatlas.org/) and ProteomicsDB ([31], http://www.proteomicsdb.org/) as well as relevant scientific literature [35, 36]. Since not only protein identification, but also the quantification process might be uncertain for single-peptide hits, we decided to assess the performance for all three acquisition methods based on proteins represented by at least 2 peptides identified.

Comparison of proteome coverage

In our study, analysis of bladder tumor samples revealed a more than 2 times higher number of multiple-peptides based protein identifications in the label-free (910 IDs), than in the iTRAQ approach (332 IDs). However, this difference appears to be overcome when labeled peptides are pre-fractionated (1092 IDs). Along the same line, Patel et al. [13] reported a comparable number of proteins identified by both approaches during proteomics analysis of bacterium *Methylocella silverstris* (384 and 425 proteins were identified in iTRAQ and LFQ, respectively). In this report, the authors applied an additional peptide fractionation step prior to the MS analysis of iTRAQ samples [13]. In iTRAQ it was shown that, due to an increase in the average ion

charge state, there is a significant reduction in the number of identifications [44]. Further, the iTRAQ 8-*plex* was reported to result in reduced protein annotation rate in comparison to the iTRAQ 4-*plex* [10]. During fragmentation, the loss of fragments of the label tag from precursor ions may occur, which causes some difficulties in the interpretation of the fragmentation spectra by the current search engines leading to the reduced peptide scoring.

We used the same set of tissue extracts for both experiments and sample processing was comparable (including homogenization and protein digestion), which minimized the biological and sampling variability on the number of detected proteins by LFQ and iTRAQ. The differences in fragmentation type of precursor ions, number of MS runs and MS run time are a consequence of utilizing standardized protocols for each strategy. Therefore, the collected data should reflect the optimized performance of each individual approach per se.

As indicated above, different fragmentation methods for data acquisition were applied: HCD in iTRAQ and CID in label-free experiments. For iTRAQ, HCD is mandatory for quantification, since the low masses of the reporter ions prohibit their detection in the ion trap. This was accompanied with the application of different thresholds for the fragment mass tolerance. However, this difference has been shown to not have a marked effect on the data [45]. To assess the possible influence of fragmentation strategy on the observed differences in the number of identified proteins, one of the selected tissue extracts from the label-free analysis was analyzed in duplicate under both experimental conditions i.e. CID and HCD fragmentation. This approach resulted in comparable number of total protein identifications (2270 and 2564 for CID and HCD, respectively) (S7 Table). Another explanation of the lower number of proteins identified in iTRAQ is related to the number of MS runs conducted per experiment. Since data-dependent acquisition is, to some extent, a stochastic process, the number of conducted MS runs in an experiment has an impact on the total number of identified peptides/ proteins. In the case of the LC-MS/MS analysis, consistently detected peptides (minimum frequency of 75% in one group) from 8 runs contribute to the total number of identified proteins. On the other hand, due to the multiplex character of the iTRAQ approach, all samples are analyzed in a single MS run. Performing duplicate runs of the iTRAQ sample (but in lower quantity than used for the presented results, e.g. 6 μg) leads to a slight increase in the total number of the identified proteins from 707/ 663 (for 1st / 2nd run) to 861 (S8 Table). Nonetheless, the number of identifications is comparable with the results obtained when 10 μg of protein was analyzed in a single run (664 proteins).

Advantages of iTRAQ are reduced MS run time and the ability to analyze multiple samples in a single run. Even though multiplexing in the iTRAQ approach may reduce the cost of experiments (by an 8 fold reduction of the MS run time) as well as decreased the inter-run variability, the added value of these features appears to be limited. However, after application of the pre-fractionation step an improved identification rate is achieved, being similar to the results obtained for LFQ. At the same time an advantage of shorter MS analytical time is maintained. In addition, some complementarity of both approaches is demonstrated [17, 18].

Collectively, based on our results, application of the fractionation strategy prior MS run provides superior results over conventional iTRAQ, and matches the increased number of identification in LFQ in part brought about by the multiple MS runs.

Evaluation of quantification strategies

The quantification of the LFQ results was based on the sum of precursor ion areas for all peptides belonging to a protein. In iTRAQ the intensity of reporter ions is utilized. If the ion cannot be assessed, quantification is not possible [13]. This inability to perform quantification was experienced in about 6% of all peptides identified. The impaired quantification efficiency in

iTRAQ might be related to insufficient peptide labeling [13]. However, in our experiments high labeling efficiency was observed. As an example, in the case of the fractionated iTRAQ sample, among the group of 1181 unquantifiable peptides (out of 6099), in 75 of these peptides the quantification value was not reported. High labeling efficiency is also supported by the results obtained using the Preview software (Protein Metrics Inc) [25]. The efficiency of the labeling is estimated based on percentage of labeled peptides vs. all possible labeling targets, as assessed based on the 100 most representative proteins in the dataset [25]. In our case, ∼98% of peptides containing K and ∼100% of peptides N-termini were detected as labeled. Comparable results were also obtained during the additional search in Proteome Discoverer, where the iTRAQ labels (N-termini, K) were set as variable modification. Over 99% of reported peptides carried the modification on N-terminal residue and/or lysine. In our study, the vast majority of unquantifiable peptides resulted from the criteria applied for inclusion of peptides for quantification including percentage of co-isolation interference (939 out of 1181 unquantifiable peptides) and peptide sequence uniqueness (167 out of 1181 peptides). Specifically, co-isolation (precursor mixing) is a well-known problem in iTRAQ, caused by selection of the precursor ions in a user-defined m/z window [28, 46, 47]. In this case, the co-isolation of other precursor ions may lead to contribution of non-related reporter ions to quantification. This has an impact on the accuracy of the quantification process. Sandberg et al. evaluated the impact of precursor mixing on the accuracy of the quantification [28]. For this purpose, a lysate of the breast cancer cell line (MCF7) was spiked with 57 standards and the effect of precursor mixing was investigated by co-analyzing iTRAQ (8-plex) and TMT (6-plex) labeled peptides. The bigger impact of the quantification accuracy was observed for the lower abundant proteins, which are particularly interesting as biomarker candidates. To reduce the effect of the precursor mixing on quantification accuracy, only spectra with the percentage of the co-isolation interference below 30% were included, yielding a good quantification accuracy [28].

Two different data analysis strategies were employed for the iTRAQ experiment. To keep similar criteria for identification of differentially expressed proteins between both, iTRAQ and LFQ, we have calculated the protein abundance based on averaging the quantification values from associated peptides. However, this approach may affect one of the strengths of the iTRAQ i.e. quantification at the level of MS/MS spectrum for each individual peptide. Considering this fact, we compared the conducted data analysis with the classical approach, where the protein abundance is calculated as an average of the peptide ratios belonging to the protein. Both methods enabled detection of comparable numbers of differentially expressed proteins, with the obtained ratios being highly consistent between both approaches, confirming that the obtained results were not affected by the selection of the quantification workflow. To further evaluate the quantification results, addition of the internal standard could be of substantial help and has been proven to be successfully applied by Sjödin et al during the evaluation of quantification results from various label-based and label-free techniques [14].

Coverage of protein sequence

Our data demonstrate a general increase in protein sequence coverage in LFQ in comparison to both iTRAQ experiments, in agreement with previously published results [12, 13]. On the other hand, the limitation of MS-based approaches to identify low abundance proteins during global-proteomic analysis still exist [48]. Typically, difficulties in the detection of low abundance proteins are related to masking by proteins of higher abundance. During data-dependent acquisition, not all of the present ions are selected for fragmentation and usually, the ones excluded are the low-abundance peptides. As a result, the identification of low abundance proteins is often limited to single peptides. The additional pre-fractionation step resulted in an

increased number of identified proteins with a single peptide. Hence, the application of the fractionation method may have a clear added value especially for the identification of low abundance proteins. However, the credibility of these findings has to be carefully assessed by the other techniques.

Detection of differentially expressed proteins and their biological reliability

Differentially expressed proteins were defined based on the statistical analysis ($p<0.05$, independent sample t-test) to enable comparison based on the level of confidence. The fold change threshold was not taken into consideration, since the reported magnitude is not conclusive, particularly when comparing two quantification approaches which differ in performance. A meaningful comparison of the differential abundance based on the reported ratio appears questionable, since different thresholds are routinely applicable for each approach. On the other hand, even if a substantial change is observed, if it is not of statistical significance, then due to the low confidence should not be reported as a difference. However, when the interpretation of differentially expressed proteins in the specific biological context is of highest relevance, the assessment of the reliability of proteomic findings can be supported by volcano plots. The latter evaluation helps to eliminate apparent significant changes with low fold change, before the specific FDR is reached; thus the number of false positive changes will decrease in comparison to the analysis utilizing solely the level of significance as a criterion for defining differential expression.

A higher total number of changes was observed in the LFQ experiment (77 proteins) vs. both iTRAQ experiments (6 and 45 proteins were reported for the iTRAQ sample, without and with fractionation, respectively), which corresponds to 8% of total identifications in LFQ and 4% of quantified proteins in the fractionated iTRAQ sample. This trend was also observed previously by others (e.g. comparison of non-infected vs. infected with adenovirus human lung epithelial cells A549 [15] or *Chlamydomonas reinhardtii* sta6 and cw15 strains [16]).

In the data presented here, we observe that the highest fold change range of reported ratios was reported in label-free (-9.41 up to 9.33) and exceed the range observed in both iTRAQ experiments, being particularly prominent for the un-fractionated (-2.03 up to 1.88) vs. fractionated sample (-1.72 up to 2.60). This likely reflects underestimations of the ratios in iTRAQ due to isotopic impurities, sample complexity or efficiency of chromatography separation and is consistent with previous studies [16]. Overall, a good agreement in the measured relative abundance, as defined by the two strategies (e.g. up/ down regulated proteins according to the ratios), was observed. Preliminary results of immunohistochemical staining (IHC) for Annexin A6 confirm its increased expression in invasive tumors in line with the proteomics results (Fig 5).

Our analysis showed that among the reported significantly altered proteins (according to at least one method, LFQ or fractionated iTRAQ) only 5 proteins showed an inconsistency in the reported ratios. As reported in Tables 4 and 3 out of these 5 proteins were significantly differentially expressed only according to the LFQ analysis, and 2 proteins exhibited a significant change according to the fractionated iTRAQ. In general, in both approaches confidence in quantification results is supported by the comparable number of peptides contributing to protein quantification. On the other hand, since the confidence level of the differential expression for one of the methods is limited ($p>0.05$), the validity of the results from this particular method cannot be established. To evaluate the observed discrepancies, a literature search was conducted to examine concordance of our findings with published reports. However, the deregulation of these proteins has not been reported in the context of bladder cancer invasion (pT2+ vs. pTa). Thus, the reported changes in protein abundance has to be further verified in independent experiments such as Western Blotting or immunohistochemistry. Preliminary

results of IHC staining for Annexin A6 confirm its increased expression in invasive tumors in line with the proteomic quantification results (Fig 5).

To further assess the reliability of the protein identification, the expression of the identified proteins in urothelium and bladder tumor was checked based on available data from proteomics and transcriptomics resources (S6 Table). The presence of almost all proteins in urothelial epithelium and/or bladder tumor was confirmed, thus supporting the reliability of the identification process. As presented, most of these proteins have been previously identified by other MS-based or immune-based experiments in the bladder (according to ProteomicsDb [31] or Human Protein Atlas [30]).

To further assess the credibility of the observed significant changes, a comparison of protein expression trends with transcriptomic studies [33, 36] for the invasive (pT2+) vs. non-invasive (pTa) and high versus low grade bladder cancer was performed. With the knowledge that some of these comparisons may not entirely correspond to changes at the protein level related to invasive versus non-invasive cancers, a higher percentage of confirmed differentially expressed proteins was observed for LFQ (34 out of 77 proteins, 44%) versus fractionated iTRAQ (15 out of 45 proteins, 33%). These data also showed that among the overlapping proteins, but found to be significant only according to one method, the 40% and 21% of the changes, as respectively indicated by LFQ and iTRAQ approach, were confirmed by transcriptomic data. Along the same line, a higher percentage of the confirmed changes among the proteins solely detected by one approach, was reported for LFQ (65%) compared to fractionated iTRAQ (47%) (Table 6). Consequently, based on the higher number of confirmed changes, the LFQ appears to have a better capability to detect differentially altered proteins in comparison to iTRAQ. However, since many of the changes could not be supported by the transcriptomic data, to make up for the risk of false associations received by both techniques, analysis of a higher number of samples and/or more stringent statistical analysis (e.g. adjustment for multiple testing) may be helpful.

Conclusions

The presented work represents an unbiased comparison of two quantification approaches i.e. label-free and iTRAQ (unfractionated and fractionated analysis) in order to determine which technique is better suited for the detection of differential protein expression in clinical samples. In terms of the number of identified proteins, application of pre-fractionation of iTRAQ labeled peptides enables superior results over the conventional iTRAQ run; whereas the number of identified proteins was comparable to the LFQ. Based on the obtained results, the label free approach appears to be the preferred option, when the detection of differential expression is a main objective of the study. LFQ provides both a higher number and a higher percentage of differentially abundant proteins for which the change was also supported by the transcriptomics data. Additionally, the added value of LFQ over the iTRAQ is reflected by the more confident protein identification (higher protein sequence coverage). However, when time on the instrument or cost is a significant issue, iTRAQ may be the method of choice, when the pre-fractionation step is applied. Conclusively, label free quantitation may facilitate the characterization of the molecular mechanisms underlying pathological conditions. However, due to the possibility to detect false positive changes, an increase in the studied sample sizes, when possible application of stringent statistical criteria (e.g. adjustment for multiple testing) and further validation of findings are required.

Supporting Information

S1 Fig. Evaluation of the prevalence of modifications in label-based and label-free experiments using regression analysis (95% CI). Both analysis were performed based on average

percentage of recognized modifications [number of peptides containing modifications/ number of peptides that could possibly contain modification * 100%]. For iTRAQ, results obtained from the fractionation experiment are shown. The average percentage of modified peptides was assessed based on the values obtained separately for each fraction; whereas for the label free experiment 3 randomly selected samples were evaluated. For the purpose of this analysis, iTRAQ derived modifications were excluded (Lysine and N-terminus set to a fixed modification of 304). The modifications exhibiting significantly different prevalence in iTRAQ and LFQ were highlighted.
(TIF)

S2 Fig. Evaluation of protein quantification strategies for iTRAQ experiment. Pearson correlation analysis was performed for protein log2 ratios obtained for all quantified proteins (A) and significantly altered proteins, as indicated by analysis 1 (B). Only proteins identified with at least 2 peptides were included. In the case of analysis 1, protein abundance relied on the averages of peptide quantification values for each label and the protein ratio was calculated based on log2 transformed average values for cases and controls; whereas in the case of analysis 2, the protein ratio was calculated by averaging the ratios for individual peptides and the value was log2 transformed.
(TIF)

S1 Table. Overview on the reporter ion isotopic distribution for iTRAQ (8-plex).
(DOCX)

S2 Table. Trascriptomics data for pT2+ vs. pTa generated by the GEO2R based on the data deposited in Gene Expression Omnibus (ID: GSE3167) [33].
(XLSX)

S3 Table. Evaluation of the modification prevalence as calculated by Preview software. The percentage and the fraction (number of peptides containing modifications vs. number of peptides that could possibly contain modification) are reported for the iTRAQ (non-fractionated/ fractionated) and the LFQ experiments. In the case of the label-based approach supported with fractionation, the values are presented separately for each fraction); whereas for the label free experiment three randomly selected samples were evaluated.
(XLSX)

S4 Table. List of identified peptides and proteins in both iTRAQ experiments and LFQ. Quantification values and expression trends as being obtained by using the label-free and iTRAQ approach are presented. In addition, in the case of the quantification of the results obtained after fractionation of iTRAQ sample, the ratios calculated based on the two protocols i.e. a) averaging of the peptide intensities belonging to protein, which was initially applied as well as b) alternative approach based on the calculation of protein ratios as an average of the peptide ratios.
(XLSX)

S5 Table. Comparison of the expression trend for differentially expressed proteins found among overlapping identifications between LFQ and iTRAQ approach.
(XLSX)

S6 Table. Evaluation of reliability and relevance of identified changes in the iTRAQ and LFQ approach. The biological reliability of protein identifications was evaluated based on the data collected in proteomic (Human Protein Atlas [30], ProteomicsDB [31]) and gene expression (Bgee Database [32]) databases. Additionally, credibility of the detected changes was

assessed through the comparison of mRNA expression in non-muscle invasive (pTa) vs. muscle invasive BC (pTa) [33], BCa cancer vs. normal tissue [35] and high grade vs. low grade BCa cancer [36]. (-) This protein-coding gene was not found as differentially expressed in presented studies. (‡) Proteins characterized by the similar expression trend with the transcriptomic study.
(XLSX)

S7 Table. Evaluation of the number of proteins identified by using various fragmentation methods (HCD and CID). Results represent the total number of protein identifications from duplicate runs for selected sample. The presented lists were exported from Proteome Discoverer using following peptide filters: high confidence, ΔM<5ppm and rank 1. Further evaluation, apart exclusion of keratins, was not anticipated for the purpose of this comparison.
(XLSX)

S8 Table. List of proteins identified in duplicate runs of the unfractionated iTRAQ sample. Common and unique proteins for each run are presented. The presented list of peptides were exported from Proteome Discoverer using following peptide filters: high confidence, ΔM<5ppm and rank 1. Further evaluation, apart exclusion of keratins, was not anticipated for the purpose of this comparison.
(XLSX)

Author Contributions

Conceived and designed the experiments: AV HM VJ AL. Performed the experiments: AL WM KV MM MA. Analyzed the data: AL KV JK. Wrote the paper: AL AV HM VJ JK JZ. Designed software used for evaluation of label-free data: JK HM. Collection of the clinical samples: ASM MA KS IK.

References

1. Ong SE, Mann M. Mass spectrometry-based proteomics turns quantitative. Nat Chem Biol. 2005; 1:252–262. PMID: 16408053
2. Ralhan R, Desouza LV, Matta A, Chandra Tripathi S, Ghanny S, Datta Gupta S, et al. Discovery and verification of head-and-neck cancer biomarkers by differential protein expression analysis using iTRAQ labeling, multidimensional liquid chromatography, and tandem mass spectrometry. Mol Cell Proteomics. 2008; 7:1162–1173. doi: 10.1074/mcp.M700500-MCP200 PMID: 18339795
3. Schiess R, Wollscheid B, Aebersold R. Targeted proteomic strategy for clinical biomarker discovery. Mol Oncol. 2009; 3:33–44. doi: 10.1016/j.molonc.2008.12.001 PMID: 19383365
4. Eriksson H, Lengqvist J, Hedlund J, Uhlen K, Orre LM, Bjellqvist B, et al. Quantitative membrane proteomics applying narrow range peptide isoelectric focusing for studies of small cell lung cancer resistance mechanisms. Proteomics. 2008; 8:3008–3018. doi: 10.1002/pmic.200800174 PMID: 18654985
5. Qattan AT, Mulvey C, Crawford M, Natale DA, Godovac-Zimmermann J. Quantitative organelle proteomics of MCF-7 breast cancer cells reveals multiple subcellular locations for proteins in cellular functional processes. J Proteome Res. 2010; 9:495–508. doi: 10.1021/pr9008332 PMID: 19911851
6. Baker CL, Kettenbach AN, Loros JJ, Gerber SA, Dunlap JC. Quantitative proteomics reveals a dynamic interactome and phase-specific phosphorylation in the Neurospora circadian clock. Mol Cell. 2009; 34:354–363. doi: 10.1016/j.molcel.2009.04.023 PMID: 19450533
7. Hubner NC, Bird AW, Cox J, Splettstoesser B, Bandilla P, Poser I, et al. Quantitative proteomics combined with BAC TransgeneOmics reveals in vivo protein interactions. J Cell Biol. 2010; 189:739–754. doi: 10.1083/jcb.200911091 PMID: 20479470
8. Husi H, Sanchez-Nino MD, Delles C, Mullen W, Vlahou A, Ortiz A, et al. A combinatorial approach of Proteomics and Systems Biology in unravelling the mechanisms of acute kidney injury (AKI): involvement of NMDA receptor GRIN1 in murine AKI. BMC Syst Biol. 2013; 7:110. doi: 10.1186/1752-0509-7-110 PMID: 24172336

9. Bantscheff M, Schirle M, Sweetman G, Rick J, Kuster B. Quantitative mass spectrometry in proteomics: a critical review. Anal Bioanal Chem. 2007; 389:1017–1031. PMID: 17668192
10. Pottiez G, Wiederin J, Fox HS, Ciborowski P. Comparison of 4-plex to 8-plex iTRAQ quantitative measurements of proteins in human plasma samples. J Proteome Res. 2012; 11:3774–3781. doi: 10.1021/pr300414z PMID: 22594965
11. Werner T, Sweetman G, Savitski MF, Mathieson T, Bantscheff M, Savitski MM. Ion coalescence of neutron encoded TMT 10-plex reporter ions. Anal Chem. 2014; 86:3594–3601. doi: 10.1021/ac500140s PMID: 24579773
12. Li Z, Adams RM, Chourey K, Hurst GB, Hettich RL, Pan C. Systematic comparison of label-free, metabolic labeling, and isobaric chemical labeling for quantitative proteomics on LTQ Orbitrap Velos. J Proteome Res. 2012; 11:1582–1590. doi: 10.1021/pr200748h PMID: 22188275
13. Patel VJ, Thalassinos K, Slade SE, Connolly JB, Crombie A, Murrell JC, et al. A comparison of labeling and label-free mass spectrometry-based proteomics approaches. J Proteome Res. 2009; 8:3752–3759. doi: 10.1021/pr900080y PMID: 19435289
14. Sjodin MO, Wetterhall M, Kultima K, Artemenko K. Comparative study of label and label-free techniques using shotgun proteomics for relative protein quantification. J Chromatogr B Analyt Technol Biomed Life Sci. 2013; 928:83–92. doi: 10.1016/j.jchromb.2013.03.027 PMID: 23608324
15. Trinh HV, Grossmann J, Gehrig P, Roschitzki B, Schlapbach R, Greber UF, et al. iTRAQ-Based and Label-Free Proteomics Approaches for Studies of Human Adenovirus Infections. Int J Proteomics. 2013; 2013:581862. doi: 10.1155/2013/581862 PMID: 23555056
16. Wang H, Alvarez S, Hicks LM. Comprehensive comparison of iTRAQ and label-free LC-based quantitative proteomics approaches using two Chlamydomonas reinhardtii strains of interest for biofuels engineering. J Proteome Res. 2012; 11:487–501. doi: 10.1021/pr2008225 PMID: 22059437
17. Jain R, Kulkarni P, Dhali S, Rapole S, Srivastava S. Quantitative proteomic analysis of global effect of LLL12 on U87 cell's proteome: An insight into the molecular mechanism of LLL12. J Proteomics. 2015; 113:127–142. doi: 10.1016/j.jprot.2014.09.020 PMID: 25286751
18. Sharma S, Ray S, Moiyadi A, Sridhar E, Srivastava S. Quantitative proteomic analysis of meningiomas for the identification of surrogate protein markers. Sci Rep. 2014; 4:7140. doi: 10.1038/srep07140 PMID: 25413266
19. Sobin L, Gospodarowicz K, Wittekind C. TNM Classification of Malignant Tumours, 7th edition. UICC International Union Against Cancer Wiley-Blackwell. 2009.
20. Wisniewski JR, Zougman A, Nagaraj N, Mann M. Universal sample preparation method for proteome analysis. Nat Methods. 2009; 6:359–362. doi: 10.1038/nmeth.1322 PMID: 19377485
21. Bairoch A, Apweiler R. The SWISS-PROT protein sequence database and its supplement TrEMBL in 2000. Nucleic Acids Res. 2000; 28:45–48. PMID: 10592178
22. UniProt C. UniProt: a hub for protein information. Nucleic Acids Res. 2015; 43:D204–212. doi: 10.1093/nar/gku989 PMID: 25348405
23. Eng JK, McCormack AL, Yates JR. An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. J Am Soc Mass Spectrom. 1994; 5:976–989. doi: 10.1016/1044-0305(94)80016-2 PMID: 24226387
24. Kall L, Canterbury JD, Weston J, Noble WS, MacCoss MJ. Semi-supervised learning for peptide identification from shotgun proteomics datasets. Nat Methods. 2007; 4:923–925. PMID: 17952086
25. Kil YJ, Becker C, Sandoval W, Goldberg D, Bern M. Preview: a program for surveying shotgun proteomics tandem mass spectrometry data. Anal Chem. 2011; 83:5259–5267. doi: 10.1021/ac200609a PMID: 21619057
26. Vizcaino JA, Cote RG, Csordas A, Dianes JA, Fabregat A, Foster JM, et al. The PRoteomics IDEntifications (PRIDE) database and associated tools: status in 2013. Nucleic Acids Res. 2013; 41:D1063–1069. doi: 10.1093/nar/gks1262 PMID: 23203882
27. Serang O, Noble W. A review of statistical methods for protein identification using tandem mass spectrometry. Stat Interface. 2012; 5:3–20. PMID: 22833779
28. Sandberg A, Branca RM, Lehtio J, Forshed J. Quantitative accuracy in mass spectrometry based proteomics of complex samples: the impact of labeling and precursor interference. J Proteomics. 2014; 96:133–144. doi: 10.1016/j.jprot.2013.10.035 PMID: 24211767
29. Deutsch EW, Mendoza L, Shteynberg D, Farrah T, Lam H, Tasman N, et al. A guided tour of the Trans-Proteomic Pipeline. Proteomics. 2010; 10:1150–1159. doi: 10.1002/pmic.200900375 PMID: 20101611
30. Uhlen M, Bjorling E, Agaton C, Szigyarto CA, Amini B, Andersen E, et al. A human protein atlas for normal and cancer tissues based on antibody proteomics. Mol Cell Proteomics. 2005; 4:1920–1932. PMID: 16127175

31. Wilhelm M, Schlegl J, Hahne H, Moghaddas Gholami A, Lieberenz M, Savitski MM, et al. Mass-spectrometry-based draft of the human proteome. Nature. 2014; 509:582–587. doi: 10.1038/nature13319 PMID: 24870543

32. Bastian F, Parmentier G, Roux J, Moretti S, Laudet V. Bgee: Integrating and Comparing Heterogeneous Transcriptome Data Among Species. In: Bairoch A, Cohen-Boulakia S, Froidevaux C, editors. Data Integration in the Life Sciences. Berlin Heidelberg: Spinger; 2008. p. 124–131.

33. Dyrskjot L, Kruhoffer M, Thykjaer T, Marcussen N, Jensen JL, Moller K, et al. Gene expression in the urinary bladder: a common carcinoma in situ gene expression signature exists disregarding histopathological classification. Cancer Res. 2004; 64:4040–4048. PMID: 15173019

34. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 2002; 30:207–210. PMID: 11752295

35. Kawakami K, Enokida H, Tachiwada T, Gotanda T, Tsuneyoshi K, Kubo H, et al. Identification of differentially expressed genes in human bladder cancer through genome-wide gene expression profiling. Oncol Rep. 2006; 16:521–531. PMID: 16865252

36. Liu Y, Noon AP, Aguiar Cabeza E, Shen J, Kuk C, Ilczynski C, et al. Next-generation RNA Sequencing of Archival Formalin-fixed Paraffin-embedded Urothelial Bladder Cancer. Eur Urol. 2014; 66:982–986. doi: 10.1016/j.eururo.2014.07.045 PMID: 25199720

37. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, et al. NCBI GEO: archive for functional genomics data sets—update. Nucleic Acids Res. 2013; 41:D991–995. doi: 10.1093/nar/gks1193 PMID: 23193258

38. Ruifrok AC, Johnston DA. Quantification of histochemical staining by color deconvolution. Anal Quant Cytol Histol. 2001; 23:291–299. PMID: 11531144

39. Abdallah C, Sergeant K, Guillier C, Dumas-Gaudot E, Leclercq CC, Renaut J. Optimization of iTRAQ labelling coupled to OFFGEL fractionation as a proteomic workflow to the analysis of microsomal proteins of Medicago truncatula roots. Proteome Sci. 2012; 10:37. doi: 10.1186/1477-5956-10-37 PMID: 22672774

40. McDowell GS, Gaun A, Steen H. iFASP: combining isobaric mass tagging with filter-aided sample preparation. J Proteome Res. 2013; 12:3809–3812. doi: 10.1021/pr400032m PMID: 23692318

41. Zhang SY, Li BY, Li XL, Cheng M, Cai Q, Yu F, et al. Effects of phlorizin on diabetic retinopathy according to isobaric tags for relative and absolute quantification-based proteomics in db/db mice. Mol Vis. 2013; 19:812–821. PMID: 23592918

42. Ross PL, Huang YN, Marchese JN, Williamson B, Parker K, Hattan S, et al. Multiplexed protein quantitation in Saccharomyces cerevisiae using amine-reactive isobaric tagging reagents. Mol Cell Proteomics. 2004; 3:1154–1169. PMID: 15385600

43. Simpson DM, Beynon RJ. Acetone precipitation of proteins and the modification of peptides. J Proteome Res. 2010; 9:444–450. doi: 10.1021/pr900806x PMID: 20000691

44. Thingholm TE, Palmisano G, Kjeldsen F, Larsen MR. Undesirable charge-enhancement of isobaric tagged phosphopeptides leads to reduced identification efficiency. J Proteome Res. 2010; 9:4045–4052. doi: 10.1021/pr100230q PMID: 20515019

45. Pejchinovski M, Klein J, Ramirez-Torres A, Bitsika V, Mermelekas G, Vlahou A, et al. Comparison of higher-energy collisional dissociation and collision-induced dissociation MS/MS sequencing methods for identification of naturally occurring peptides in human urine. Proteomics Clin Appl. 2015.

46. Karp NA, Huber W, Sadowski PG, Charles PD, Hester SV, Lilley KS. Addressing accuracy and precision issues in iTRAQ quantitation. Mol Cell Proteomics. 2010; 9:1885–1897. doi: 10.1074/mcp.M900628-MCP200 PMID: 20382981

47. Ow SY, Salim M, Noirel J, Evans C, Rehman I, Wright PC. iTRAQ underestimation in simple and complex mixtures: "the good, the bad and the ugly". J Proteome Res. 2009; 8:5347–5355. doi: 10.1021/pr900634c PMID: 19754192

48. Drabovich AP, Diamandis EP. Combinatorial peptide libraries facilitate development of multiple reaction monitoring assays for low-abundance proteins. J Proteome Res. 2010; 9:1236–1245. doi: 10.1021/pr900729g PMID: 20070123

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CORRECTION

Correction: Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis

The *PLOS ONE* Staff

Tables 1 and 5 are incorrectly aligned due to errors introduced in the production process. The publisher apologizes for the errors. Please see the corrected Tables 1 and 5 here.

Citation: The *PLOS ONE* Staff (2015) Correction: Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis. PLoS ONE 10(10): e0139268. doi:10.1371/journal.pone.0139268

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Table 1. Overview of the number of peptides and the corresponding proteins as being identified in the individual MS-runs.

Method	Sample ID	# peptide groups	# protein groups
LFQ	3_pTa	5073	1096
	11_pTa	5269	1099
	16_pTa	5725	1185
	19_pTa	3931	937
	Mean (SD)_pTa	**5000 ± 763**	**1079 ± 103**
	9_pT2+	5360	1130
	12_pT2+	5112	1136
	15_pT2+	5516	1169
	17_pT2+	5485	1155
	Mean (SD)_pT2+	**5368 ± 184**	**1148 ± 18**
iTRAQ	10 µg	1859	664
	fractionation	6099	2064

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Table 5. Comparison of the quantification results at the peptide and protein level for identifications with conflicting expression trends between fractionated iTRAQ and LFQ

Protein/ Peptide		Peptide Log2 Ratio (pT2 + vs. pTa)		Protein Log2 Ratio (pT2 + vs. pTa)	
Protein Name	**Peptide sequence used for quantification**	**iTRAQ + fractionation**	**LFQ**	**iTRAQ + fractionation**	**LFQ**
Heterochromatin protein 1-binding protein 3	eASYSLIR	-0.24	0.03	**-0,30**	**0.72**
	mDAILTEAIk	-0,37	0.32		
	tIPSWATLSASQLAR	-0.30	0.41		
	SSAVDPEPQVK	-	1.31		
	LEDVLPLAFTR	-	0.24		
	GASGSFVVVQK	-	5.12		
Actin-related protein 2/3 complex subunit 3	aYLQQLR	0.01	0.61	**-0.25**	**0.37**
	IIGNMALLPIR	-0.43	-0.06		
	IIGNmALLPIR	-0.34	-		
Dolichyl-diphosphooligosaccharide—protein glycosyltransferase subunit 2	sIVEEIEDLVAR	0,21	2.46	**-0.06**	**1.23**
	eDQVIQLMNAIFSk	-0.36	-		
	fELDTSER	0.15	-		
	nFESLSEAFSVASAAAVLSHNR	-0.12	-		
	qEIQHLFR	0.09	-		
	yHVPVVVVPEGSASDTHEQAILR	-0.38	0.62		
	LQVTNVLSQPLTQATVK	-	0.33		
	ISTEVGITNVDLSTVDKDQSIAPK	-	1.78		
	NPILWNVADVVIK	-	3.65		
	YIANTVELR	0.06	3.15		
KH domain-containing, RNA-binding, signal transduction-associated protein 1	KDDEENYLDLFSHK	-	0.39	**-0.25**	**0.63**
	ILGPQGNTIK	-	0.55		
	DSLDPSFTHAMQLLTAEIEK	-	2.42		
	SGSMDPSGAHPSVR	0.06	0.68		
	qPPLPHR	-0.56	-		
General vesicular transport factor p115	SSQTSGTNEQSSAIVSAR	-	0.27	**-0.10**	**0.52**
	SQLNSQSVEITK	-	0.25		
	NDGVLLLQALTR	-0,23	2.44		
	eQDLQLEELR	-0.47	-		
	qSEDLGSQFTEIFIk	0.31	-		
	vASSTLLDDRR	-0.00007	-		

doi:10.1371/journal.pone.0139268.t002

Reference

1. Latosinska A, Vougas K, Makridakis M, Klein J, Mullen W, Abbas M, et al. (2015) Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis. PLoS ONE 10 (9): e0137048. doi:10.1371/journal.pone.0137048 PMID: 26331617

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

6.5. List of publications

(i) Original research articles

1) Chatziharalambous D, Lygirou V, Latosinska A, Stravodimos K, Vlahou A, Jankowski V, Zoidakis J. Analytical performance of ELISA assays in urine: one more bottleneck towards biomarker validation and clinical implementation. PLoS One. 2016; 11:e0149471.

2) Jankowska U, Latosinska A, Skupien-Rabian B, Swiderska B, Dziedzicka-Wasylewska M, Kedracka-Krok S. Optimized procedure of extraction, purification and proteomic analysis of nuclear proteins from mouse brain. J Neurosci Methods. 2016; 261:1-9.

3) Latosinska A, Vougas K, Makridakis M, Klein J, Mullen W, Abbas M, Stravodimos K, Katafigiotis I, Merseburger AS, Zoidakis J, Mischak H, Vlahou A, Jankowski V. Comparative Analysis of Label-Free and 8-Plex iTRAQ Approach for Quantitative Tissue Proteomic Analysis. PLoS One. 2015; 10:e0137048.

4) Filip S, Vougas K, Zoidakis J, Latosinska A, Mullen W, Spasovski G, Mischak H, Vlahou A, Jankowski J. Comparison of Depletion Strategies for the Enrichment of Low-Abundance Proteins in Urine. PLoS One. 2015; 10:e0133773.

(ii) Review Articles

5) Frantzi M, Latosinska A, Merseburger AS, Mischak H. Recent progress in urinary proteome analysis for prostate cancer diagnosis and management. Expert Rev Mol Diagn. 2015; 15:1539-54.

6) Frantzi M, Latosinska A, Flühe L, Hupe MC, Critselis E, Kramer MW, Merseburger AS, Mischak H, Vlahou A. Developing proteomic biomarkers for bladder cancer: towards clinical application. Nat Rev Urol. 2015; 12:317-30.

7) Frantzi M, Bhat A, Latosinska A. Clinical proteomic biomarkers: relevant issues on study design & technical considerations in biomarker development. Clin Transl Med. 2014; 3:1-22.

8) Latosinska A, Frantzi M, Vlahou A, Mischak H. Clinical applications of capillary electrophoresis coupled to mass spectrometry in biomarker discovery: Focus on Bladder Cancer. Proteomics Clin Appl. 2013; 7:779-93.

(iii) Book chapters

9) Latosinska A, Frantzi M, Mullen W, Vlahou A, Makridakis M. Targeting the proteome of cellular fractions: Focus on secreted proteins. Methods Mol Biol. 2015;1243:29-41.

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