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Chapter

Stress Urinary Incontinence: A Proteomics Overview

Goran Mitulović, Thomas Mohr and Marianne Koch

Abstract

Proteomics research offers one strategy to elucidate the etiology of stress urinary incontinence (SUI) by identification of a significant and sufficient number of proteins, which provides the ability to avoid a preselection of candidate proteins for a possible early detection of the SUI. SUI represents both a psychological as well as an economic burden, and prevalence rates are expected to increase in the future, due to increasing of life expectancy. The classical epidemiology of SUI is well understood, with many environmental and lifestyle risk factors identified, including age, obesity, parity, vaginal delivery, and family history. Despite this, much of the etiology of SUI remains unclear, and it is difficult to predict which women are at risk. This chapter shows some results based on proteomic analysis of the urine proteome, which might give the answer to the question on pathways activated in SUI. Besides proteins originating from the blood, urine contains proteins secreted from the inner wall of the bladder and the urethra, and these proteins might explain the processes involved in genesis of SUI.

Keywords: stress urinary incontinence, urinary proteome, proteomics

1. Introduction

Stress urinary incontinence (SUI) is a disorder observed with the female population with widely varying prevalence, which is estimated to be 15–80%. The condition represents both a psychological and an economic burden, and it is expected that prevalence rates shall increase in the future, mainly due to increasing of life expectancy.

While the classical epidemiology of SUI is understood quite well, many environmental and lifestyle risk factors leading to the condition have been identified. Among others, these are age, obesity, parity, vaginal delivery, and family history of SUI. Despite this much of the etiology of SUI remains unclear, and it is difficult to predict which women are at risk.

Proteomic research offers one strategy to elucidate the etiology of SUI by identification of a significant and sufficient number of proteins, which provides the ability to avoid a preselection of candidate proteins. Many different serum, urine, and/or tissue protein markers have been investigated in the context of SUI. Almost all studies have targeted specific proteins as putative biomarkers, but with typically negative results. Prior studies have investigated a role for serum C-reactive protein, serum relaxin, and serum estradiol, without finding significant associations with symptoms.

2. Materials and methods

2.1 Samples

In order to generate valid data and exclude possible false-positive and false-negative samples being analyzed, urinary and serum samples from patients affected by stress incontinence cases and a proven history of symptoms of SUI for at least 3 months were obtained. This included a specific history of complaint of involuntary leakage on effort or exertion or on sneezing or coughing, a positive provocation stress test, which was defined as an observed transurethral loss of urine simultaneous with a cough or Valsalva maneuver at a bladder volume of minimum 300 ml. Furthermore, negative urine dipstick testing was necessary; all patients were older than 18 years and capable of independent toileting and having at least one previous vaginal delivery. In accordance with rules of the Medical University of Vienna, written informed consent was obtained from all participants. We excluded patients who had previous treatment for SUI (either surgical or pharmacological), a history of overactive bladder symptoms, and/or urinary incontinence other than SUI (tested using the ICIQ-UI Short Form questionnaire) [1]. In addition, a history of neurological disorders potentially affecting the urinary tract system, such as multiple sclerosis, Parkinson's disease, pelvic organ prolapse stage ≥ II (International Continence Society classification), clinically significant bladder outlet obstruction, and/or post-void residual volume > 100 ml, was also exclusion criteria. A series of other criteria were also observed, as described in Koch et al. [2]: the history of acute urinary retention or history of repeated catheterizations; history of bladder cancer or previous operation on the urinary tract; acute or recurrent urinary tract infection and/or hematuria; history of urinary tract stones, renal insufficiency, and hepatic disease; history of alcohol and/or drug abuse; pregnancy or lactation; and finally any patient with a serious medical condition.

Participants in the control group met identical criteria, but with no symptomatic SUI (ICIQ-short form score equal to 0) and negative cough stress test. Urine samples were obtained once only without requirement for a specific time of day. Participants were given a sterile urine cup (maximum 50 ml) and asked to deliver the first-void urine. In addition we retrieved blood samples from peripheral veins of all participants to determine their creatinine, transaminase, and bilirubin status. Urine samples were stored in the refrigerator at 4°C for a maximum of 1 hour before they were taken to the Clinical Institute of Laboratory Medicine (Proteomics Core Facility) for immediate processing.

2.2 Proteomics sample preparation

Trypsin for protein digestion was purchased from Promega Inc. (Vienna, Austria). Solvents for high-performance liquid chromatography (HPLC)—methanol (MeOH), acetonitrile (AcN), 2,2,2-trifluoroethanol (TFE), formic acid (FA), heptafluorobutyric acid (HFBA), iodoacetamide (IAA), triethyl bicarbonate (TEAB), and dithiothreitol—were purchased from Sigma-Aldrich (Vienna, Austria).

Protein precipitation from urine was performed according to the internally modified Wessel-Fluege method for protein precipitation, and all solvents were kept at −20°C. All working steps were performed on ice and centrifugation in a cooled centrifuge at +4°C. A sample volume of 2 ml of each urine sample was mixed with 6 ml methanol and 2 ml dichloromethane in a 50 ml Falcon tube, and samples were vigorously vortexed. After adding 6 ml of water to each sample, solutions were

vortexed another time. Samples were subsequently stored at −20°C for a minimum of 20 minutes for enhancement of protein precipitation. Phase separation was carried out by subsequent centrifugation for 5 minutes at 4500 rounds per minute (rpm). The upper layer of the solution was then carefully discarded while keeping the interphase and lower layer, and additional 6 ml of methanol were added prior to vigorous vortexing. Final centrifugation was performed for 5 minutes. The resulting supernatant was carefully removed, and the remaining protein pellet was dried on air. The dried protein pellet was later dissolved in 200 μl of 50 mM triethylammonium bicarbonate (TEAB) at pH 8.5. In cases where the protein pellet could not be properly dissolved in 200 μl of 50 mM TEAB, additional 50–1000 μl 50 mM TEAB were added, and the sample was sonicated by using the ultrasonic cell disruptor (Ultrasonic Cell Disruptor, Branson 5200, Dietzenbach, Germany).

Blood samples were prepared as described by Koch et al. [3]. Briefly, samples were immediately centrifuged to separate serum from blood cells and then frozen in separate vials at −20°C until further processing and an in-solution enzymatic digestion of all proteins extracted from both urinary and serum samples were achieved by applying a combination of Glu-C and trypsin (Promega, Vienna, Austria). This combination was selected in order to achieve improved sequence coverage for proteins. All steps for sample preparation were performed using previously published protocols [4–6].

2.3 Chromatographic separation and detection

Peptide separation was achieved using nano-high-performance liquid chromatography on a nano-RSLC Ultimate 3000 system (ThermoFisher Scientific, Vienna, Austria) using the PepMap C18 column (75 μm ID × 50 cm length, 3 μm ID, 100 Å pore size, ThermoFisher Scientific, Vienna, Austria). The separation column was mounted in a column oven and operated at 60°C. Prior to the separation on the nano-separation column, peptides were loaded onto a trap column (300 μm ID × 5 mm length, PepMap 300 Å pore size, ThermoFisher Scientific, Vienna, Austria). The analysis of biological samples bears the risk of carry-over and contamination of subsequent runs in cases where injected samples contained high amounts of peptides. Therefore, separation system was flushed between sample injections using the method developed earlier and described by Mitulovic et al. [7]. Optimization of loading conditions have been described in a number of other publications; however, we have used the conditions described in a paper by Schöbinger et al. where loading mobile phase was cooled to 3°C in order to enable improved peptide trapping on the trap column, which was operated at 60°C.

Details on separation gradient formation and mobile phases used are described in publications by Koch et al. [2, 3].

Mass spectrometric detection of digested peptides was performed using the maXis Impact time-of-flight (qToF) MS (Bruker, Bremen, Germany) equipped with the Captive Spray nano-electrospray source and operated at 1.6 kV; source temperature was set to 180°C for effective desolvatization of the analytes introduced from LC. Peptide masses were scanned in the range of m/z 300–m/z 2000, and 20 most intense signals were selected for MS/MS fragmentation. Fragmentation was performed by using collision-induced dissociation with nitrogen in the CID cell. Single-charged ions were excluded from MS/MS fragmentation, and those carrying charges of +2 to +4 were fragmented. In order to ensure fragmentation of a maximum number of ions, already fragmented masses were excluded from further fragmentation for 60 seconds but were allowed if the following MS/MS intensity was three times higher as compared to the previous MS/MS peak intensity. All

measurements were performed in triplicate to provide corrections for technical variability of chromatographic separation and the ionization.

2.4 Data analysis

In order to identify proteins in analyzed sample, database search of mass spectrometric data was performed using the Human Swiss-Prot Database in its actual version at the time of analysis. Details of data search are described by Koch et al. [3]. Briefly, all searches were performed using Mascot v. 2.51 (http://www.matrixscience.com/). For the database search, trypsin and Glu-C were selected as enzymes with carbamidomethyl on Cys as fixed modification and oxidation on Met, phosphorylation on Ser, Thr, and Tyr as variable modifications.

Protein abundance was estimated by using peptide counts normalized to counts per million (cpm). Log2-fold change was estimated based on variance stabilized average log2 cpm values using the package edgeR. Resulting p values were corrected for multiple testing according to Burden et al. [8].

3. Results

Only proteins identified with at least two detected and identified peptides were selected for further analysis.

3.1 Proteins identified in urinary samples

The total number of identified individual proteins in the case group was 1459 and 2148 in the control group. The median number of identified proteins per urine sample was 377 (range 1167) in the case group and 417 (range 1197) in the control group.

Only 6 of the 828 proteins showed a significant difference in abundance in urine samples. This difference between SUI and controls was observed with a q-value <0.25. Out of these six identifications, three known proteins showed a higher abundance in SUI samples compared to controls: plasma serine protease inhibitor (logFC 1.11), leucine-rich alpha-2-glycoprotein (logFC 3.91), and lysosomal alpha-glucosidase (logFC 1.24). From three uncharacterized proteins, one protein (gene symbol: PPIA) also showed higher abundance in SUI samples (logFC 1.96), whereas the other two uncharacterized proteins (gene symbol, UMOD; gene symbol, KIAA0586) presented a lower abundance in SUI samples than controls (logFC-4.87; logFC-1.99, respectively). **Table 1** shows the proteins identified in urinary samples with significant difference between the control and the case group.

Protein	Gene symbol	LogFC	q-value
Plasma serine protease inhibitor	SERPINA5	1.111	0.029
Leucine-rich alpha-2-glycoprotein	LRG1	3.909	0.019
Lysosomal alpha-glycosidase	GAA	1.237	0.062
Uromodulin	UMOD	−4.867	0.002
Peptidyl-prolyl cis-trans isomerase A	PPIA	1.962	0.227
TALPID3 (KIAA0586)	TALPID3 (KIAA0586)	−1.992	0.227

Table 1.
Proteins identified with a significantly different abundance in urine of patients with stress urinary incontinence (SUI) compared to control samples.

4. Discussion

Current study is not the first one describing the urinary proteome [9–13]. However, this study was the first one to address specific clinical problem of SUI. The methodology used for both sample preparation and sample analysis was kept as simple as possible so that it can be easily reproduced in any proteomic laboratory without adaptations of existing hardware.

Figure 1 shows the typical chromatogram for separation of tryptic peptides from a patient's urinary sample. The large number of peaks in the chromatogram indicates the presence of a large number of peptides. Database search revealed that in almost all cases of urinary proteomic analysis, the major proteins being identified are serum albumin and uromodulin. This is physiologically normal and common, although a common knowledge implies that no proteins or, at least, very low number of proteins shall be present in the urine.

4.1 Proteins identified

Uromodulin, being the major urinary protein, was a major hit following serum albumin.

The study identified six different, putative, probably SUI-specific urinary proteins for the first time.

The abundance of all these proteins was found to be higher in SUI samples, and these are plasma serine protease inhibitor (SERPINA5), leucine-rich alpha-2-glycoprotein (LRG1), and lysosomal alpha-glucosidase (GAA).

The results showing the enrichment of mentioned proteins based on KEGG pathway analysis are shown in **Figure 2**.

SERPINA5 is usually present in urine in very low concentrations and serves, among other functions, as a pro-inflammatory factor, which might be an explanation for it overexpression in samples of patients with SUI [1, 14–20]. Furthermore, SERPINA5 was recently mentioned in a number of publications addressing diverse medical conditions, including pediatric leukemia, breast cancer, HIV infection, and hepatocellular carcinoma, which have identified a role played by SERPINA5 during disease development [21–24].

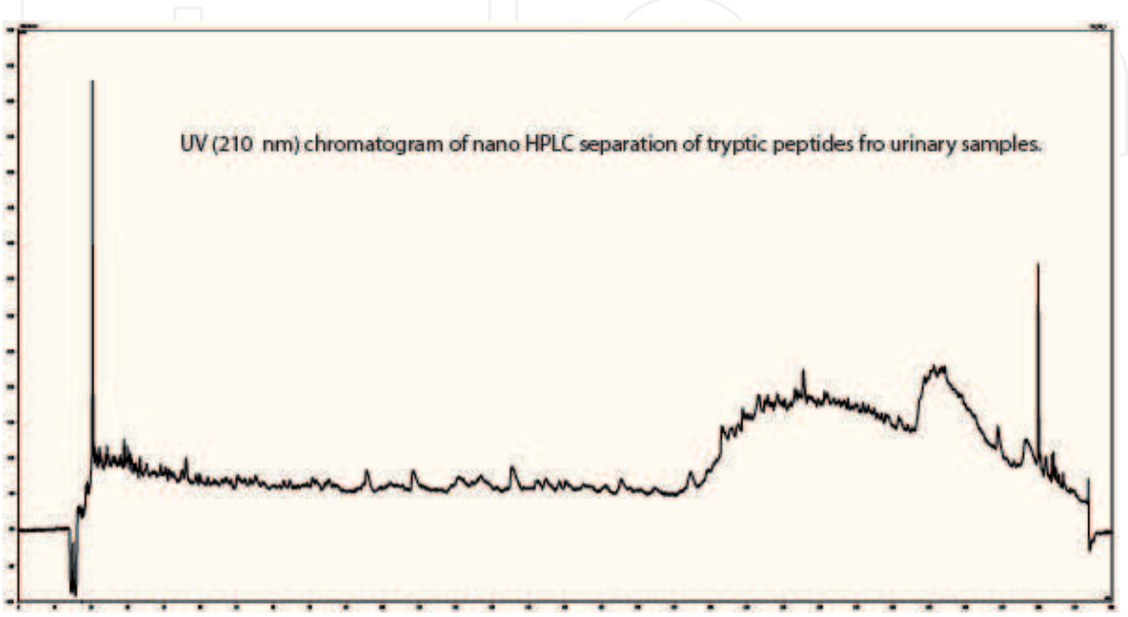

Figure 1.
UV Chromatogram trace at 210 nm showing the separation of tryptic peptides from urinary sample.

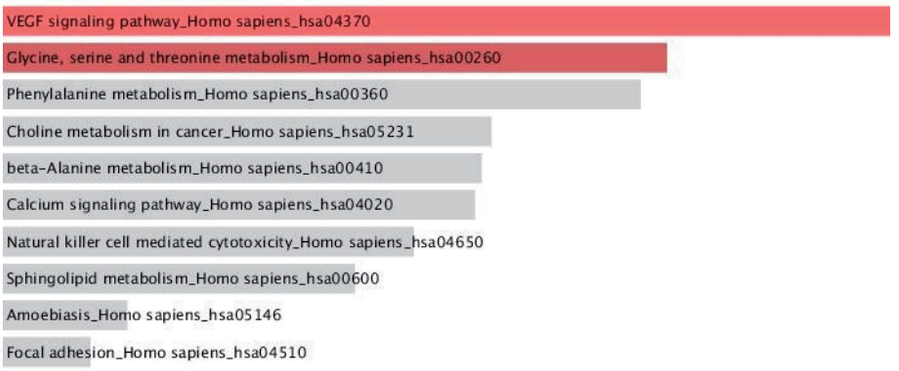

Figure 2.
KEGG pathway for the set of proteins with affected expression in SUI samples.

Another protein, the leucine-rich alpha-2-glycoprotein, was also found to be increased in samples of SUI patients. This protein is secreted and normally present in plasma; however, it was also described to be involved in nonspecific inflammatory and cancer processes [25–28]. It has recently been described in the context of ulcerative colitis activity, pediatric, invasive bladder cancer, biliary tract cancer, lung cancer, pancreatic cancer, heart failure, neutrophilic granulocyte differentiation, and autoimmune diseases [29, 30].

Lysosomal alpha-glucosidase, another protein with increased expression in SUI samples, is essential for the degradation of glycogen to glucose in lysosomes, and it is present in, basically, all cells. Mutations in the respective gene result in Pompe disease, a severe and devastating glycogen storage disease caused by a deficiency in acid α-glucosidase. This condition is characterized by the lack of lysosomal alpha-glucosidase, which leads to intralysosomal accumulation of glycogen, the final consequence of which is the failure of the heart and skeletal muscles. The Pompe disease is being treated by enzyme replacement therapy. However, this is not sufficient, although it helps preventing assisted patients' ventilation and ensuring a ventilation-free survival. GAA is an enzyme that is essential for lysosomal glycogen hydrolysis, and the protein has also been identified as a potential biomarker for gut wall integrity in infants with necrotizing enterocolitis, an inflammatory process involving the intestinal tissue [31]. GAA has not been described as a factor for SUI, but the involvement of GAA in pathologies of smooth muscle [32] suggests that this protein might have an important role for the proper function of the bladder. Niedworok et al. [33] suggested that GAA might be involved in bladder cancer as an endogenous inhibitor of bladder cancer cell proliferation. The authors concluded that GAA is upregulated in response to antiproliferative tyrosine kinase inhibitors. That would mean that high biglycan expression is associated with favorable prognosis for patients with bladder cancer.

Alsaikhan et al. [34] investigated the partial bladder obstruction and the expression, among other factors, of GAA. Authors describe that small leucine-rich proteoglycans, required for collagen fibrillogenesis showed a significant reduction, which was consistent with a pro-fibrotic environment and deregulated collagen assembly. Although this study did not address the matter of incontinence, it showed that leucine-rich proteoglycans have an important role to play for the regulation of bladder function.

A similar observation was made by Appunni et al. [35, 36] for the role of leucine-rich proteoglycans and the bladder cancer. Leucine-rich proteoglycans are not only required in the matrix for structural framework, but they also show to be effective in controlling various physiological functions. Among these functions are also the cell cycle regulation and the leucine-rich proteoglycans which perform the role of the guardians of the cellular matrix.

Upon database search and quantitation, peptidyl-prolyl cis-trans isomerase A (PPIA) was found to be overexpressed. This protein has been described to be involved in inflammatory processes and immunomodulation and induction of interleukin-6 release from macrophages. Recent publications have discussed an involvement in type 2 diabetes mellitus, vascular disease, and gastric adenocarcinoma [31].

Two of the identified uncharacterized proteins, which are encoded by associated with the genes UMOD and KIAA0586, showed lower expression in SUI samples.

UMOD encodes for the protein uromodulin, which is, among other functions, involved in the prevention of urinary tract infection, water/electrolyte balance, and kidney innate immunity. Uromodulin is usually highly abundant in the urine of healthy humans, and, as mentioned previously, it is the most abundant protein in normal urine [37]. Interestingly, uromodulin is another glycoprotein identified to have different expression patterns in SUI samples as compared to control samples. UMOD is a GPI-anchored glycoprotein produced by the kidney but not derived from the blood. The function of these proteins is still not well understood, but it is taught to be linked to the water/electrolyte balance and kidney innate immunity. Hypertension in pregnancy was associated with a decrease in the uromodulin's excretion rate [38], and the results of SUI samples also revealed that the level of uromodulin was decreased. Furthermore, UMOD can be used as a predictive factor for preeclampsia [39]. UMOD has been described to prevent the binding of the IgG light chain to their putative receptors [40]. Da Silva et al. described the role of UMOD as an allergen epitope [41] for activation of the allergy-associated T cells in mouse. There is no description of causality in humans; however, the lower expression of this protein in samples of SUI patients might be of importance considering the function of the smooth muscle of the urinary bladder.

KIAA0586 encodes for the protein TALPID3, which is required for ciliogenesis and sonic hedgehog/SHH signaling [42–48]. Fleming et al. [49] described the possible involvement of TALPID3 in kidney damage in patients with Joubert syndrome. Interestingly, all patients enrolled in this study and having a mutation on KIAA05866 gene, which encodes for TALPID3, showed to have significantly better chances of preserving the kidneys, which are, otherwise, affected by the Joubert syndrome. It is still unclear why this protein was identified with a decreased abundancy in samples of SUI patients.

Another protein that was ubiquitous in all samples was keratin. Keratin is commonly identified during proteomics analysis, and it often serves as a quality control of the analysis, if not present in high amounts. However, more often, keratin is considered being a contaminant and something that shall be kept out of the sample by any means.

Therefore, requirements were taken to exclude any possible contamination with keratin, but it was still identified in large amount in all samples. Besides being considered a contaminant for proteomics experiments, keratin is an important part of the urinary proteome which seems to be present in all collected samples.

As for now, no biomarkers have yet been identified for SUI, and it is rather improbable that a single protein will be a marker. A more probable scenario is that a group of proteins with a significantly different abundance in SUI patients compared to controls will be defined as putative markers.

The best chance to identify these proteins will be by investigating the known functions, tissue specificities, and interactions of the specific proteins identified in samples of SUI patients. It is also important to gain a detailed insight into potential mechanisms of the pathophysiology and etiology of SUI, which seem to depend on many factors and might be a complex process depending on more physiological processes taking place in the urinary bladder. Proteins, which were identified with

significantly higher abundance in SUI samples, have been described earlier as active participants in inflammatory processes and cancer development. On the other hand, proteins that were identified and quantified with a significantly lower abundance usually seem to have a protective effect in the urinary tract system although we cannot be explained at the current time.

5. Conclusion

It is important to stress out that one of the most important factors for a successful analysis is the selection of samples to be analyzed. It is very important to include urine samples retrieved from a population with very strict inclusion and exclusion criteria in order to avoid confounding factors. Urine samples must be processed according to a standardized protocol within a short time frame after collection.

Although a thorough map of the human proteome has been described, and made available to researchers [50], this map is still not complete and it is prone to errors and biases. Therefore, the incomplete "humane proteome mapping" is an additional challenge despite efforts of the research community to identify and characterize all human proteins.

By investigating the urinary proteome at one time point only, no conclusion can be made on whether the significantly differently expressed proteins are a consequence of the pathological process or whether they themselves are directly involved in causal processes.

Therefore, due to the characteristics of the identified proteins, it can be said that inflammatory processes may be involved in the etiology of SUI. However, the relevance of these findings regarding the pathogenesis of SUI needs to be broadly investigated, and the results described need to be replicated in a different population and at different time points.

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