Characteristics of the Urinary Proteome in Women with Overactive Bladder Syndrome: A Case-Control Study

Marianne Koch 1, *, Pavel Lyatoshinsky 2, Goran Mitulovic 3, Barbara Bodner-Adler 1,4, Sören Lange 1, Engiebert Hanzal 1 and Wolfgang Umek 1,4

1 Department of Obstetrics and Gynecology, Medical University of Vienna, 1090 Vienna, Austria; barbara.bodner-adler@meduniwien.ac.at (B.B.-A.); soeren.lange@meduniwien.ac.at (S.L.); engelbert.hanzal@meduniwien.ac.at (E.H.); wolfgang.umek@meduniwien.ac.at (W.U.)
2 Department of Urology, Cantonal Hospital St Gallen, 9007 St Gallen, Switzerland; p.liatochinski@gmail.com
3 Clinical Institute of Laboratory Medicine and Proteomics Core Facility, Medical University of Vienna, 1090 Vienna, Austria; goran.mitulovic@meduniwien.ac.at
4 Karl Landsteiner Society, Institute for Special Gynecology and Obstetrics, 3100 St. Pölten, Austria
* Correspondence: marianne.koch@meduniwien.ac.at; Tel.: +43-1-404-002-9150

Abstract: Despite an estimated prevalence of 13% in women, the exact etiology of non-neurogenic overactive bladder syndrome is unclear. The aim of our study was to gain a better understanding of the pathophysiology of female overactive bladder syndrome by mapping the urinary proteomic profile. We collected urine samples of 20 patients with overactive bladder syndrome and 20 controls. We used mass spectrometric analysis for label-free quantitation, Swissprot human database for data search, Scaffold for data allocation and the Reactome Knowledgebase for final pathway enrichment analysis. We identified 1897 proteins at a false discovery rate of 1% and significance level \( p < 0.001 \). Thirty-seven significant proteins of the case group and 53 of the control group met the criteria for further pathway analysis \( (p < 0.0003 \text{ and } \log_2 \text{ (fold change)} > 2) \). Significant proteins of the overactive bladder group were, according to the 25 most relevant pathways, mainly involved in cellular response to stress and apoptosis. In the control group, significant pathways mainly concerned immunological, microbial-protective processes and tissue-elasticity processes. These findings may suggest a loss of protective factors as well as increased cellular response to stress and apoptosis in overactive bladder syndrome.

Keywords: overactive bladder syndrome; mass spectrometry; proteomics; urine; pathophysiology; antimicrobial; apoptosis; cellular response to stress; protein pathway

1. Introduction

Non-neurogenic overactive bladder syndrome (OAB), defined as “urgency, with or without urge incontinence, usually with frequency and nocturia” is a very common disease in women [1]. Its estimated life-time prevalence is 13%, which increases up to 48% if associated with obesity. In women over 70 years of age, OAB reaches a peak-prevalence of 70% [2,3]. OAB causes a large socio-economic burden with high annual health care costs, but also a social impact on affected women including social isolation, loss of confidence and depression [4,5]. The exact causes and pathophysiological mechanisms of OAB are still poorly understood, leaving us with less than optimal treatment options. Neuronal, myogenic and inflammatory processes have previously been discussed regarding their influence on OAB, with, however, limited results [6,7]. Inflammatory proteins, in particular neuronal growth factor, brain-derived neurotrophic factor, prostaglandins, cytokines, adipokines, monocyte chemoattractant protein-1 and C-reactive protein, have been described as significantly higher abundant in OAB patients compared to controls [8–15]. However, these inflammatory proteins are unspecific and elevated in other conditions as well, such as acute cystitis or urinary tract calculi. Moreover, the applied techniques for identification of
these markers, such as enzyme-linked immunosorbent assay, require the use of specific and known antibodies for pre-specified proteins. Thus, only proteins already suspected to be involved in OAB will be detected, and the majority of proteins will not be investigated at all, although they might have a role in the development of OAB. In contrast, the proteomic technique employs chromatographic separation and mass spectrometry, and ultimately identifies and quantifies all proteins of a sample. This method provides a greater overview of the protein turnover in women with OAB in comparison to healthy controls. We have successfully used this approach previously, and were able to identify a urinary proteomic pattern in women with stress urinary incontinence [16,17]. Preliminary findings from a urinary proteomic study on OAB describe vascular cell adhesion protein (VCAM-1) in significantly different abundance in OAB patients compared to controls, but this study included only 16 samples [18]. Identification and comparison of the urinary proteome in patients with OAB should contribute to a more profound understanding of the etiology and could provide a basis for new treatment options. The objective of this study was therefore to identify a urinary proteomic pattern characteristic for OAB.

2. Materials and Methods

This prospective case-control study was conducted at the Department of Obstetrics and Gynecology in cooperation with the Core Facility Proteomics and the Clinical Department of Laboratory Medicine at the Medical University of Vienna between 2015 and 2020. The Institutional Review Board approval was obtained from the Ethics Committee of the Medical University of Vienna (no. 1376/2014). Written permission from all participants has been obtained. The inclusion criteria for the OAB group were: Females ≥18 years of age with symptoms of overactive bladder syndrome for ≥3 months (Zitat ICS). Exclusion criteria were: previous medical therapy for OAB within the previous 3 months; pregnancy, lactation, any neurologic conditions, pelvic organ prolapse (stage > 2); lower urinary tract surgery within the previous six months; history of interstitial cystitis or pain associated with OAB; recurrent urinary tract infections defined as >3/year; acute urinary tract infection; urinary retention (with or without required self-catheterization); stress urinary incontinence or mixed urinary incontinence; bladder cancer; renal failure and urinary tract stones. Participants in the control group had to be continent and free of any OAB symptoms, all other exclusion criteria applied as well. Cases were recruited at the urogynecology outpatient clinic at the Department of Obstetrics and Gynecology (Medical University of Vienna). Controls were recruited among outpatients and inpatients of the Department of Obstetrics and Gynecology. Controls were matched for age (+/− 5 years) and body mass index. The outcome of the study was the sequence coverage, abundance, and quantity of identified urinary proteins. Included participants were subjected to a detailed patient history, the ICIQ-OAB (International Consultation on Incontinence Questionnaire on OAB) questionnaire (OAB and control group) and a three-day bladder diary for documentation of fluid intake, micturition, frequency, incontinence episodes and nocturia (only OAB group). Clinical evaluation of participants with OAB symptoms included a standard gynecologic examination, cystometry and clinical stress test. We obtained one mid-stream, clean-catch urine sample from each participant at a single time point between 8 a.m. and 2 p.m., which was followed by immediate urine dip stick testing to exclude acute urinary tract infection. All urine samples were subsequently processed within 15 min after collection to avoid protein decomposition and autolysis. For protein precipitation, 2 mL urine of each sample were extracted and protein precipitation was conducted according to the internally modified Wessel-Flüge method [19] and all solvents were kept at −20 °C. All working steps were performed on ice and centrifugation in a cooled centrifuge at +4° Celsius. After careful removal of the resulting supernatant, the remaining protein pellet was dried in the air and later on dissolved in 200 µL of 50 mM triethylammonium bicarbonate at pH 8.5 (TEAB). Protein concentration within the solution was determined using the DeNovix nanodrop photometer. Subsequent protein digestion was performed according to Mitulovic et al. [20]. Upon tryptic digest, 30 µL of tryptic peptides were diluted with 20 µL of aqueous 0.1%
TFA and 1 µg of digested protein was injected. Each sample was injected three times and peptides were separated using the nanoRSLC Ulitmate 3000 HPLC system by ThermoFisher (Bremen, Germany) coupled to the Q Exactive Orbitrap mass spectrometer equipped with the nanospray electrospray source (Thermo Fisher Scientific, Germering, Germany). The separation was performed using mobile phases and the separation gradient as described in Koch et al. [16]. Every sample injection was followed by two blank runs with injections of TFE for removal of the possible sample remains in the injector or on the trap column, and prevention of carryover in the separation system. Mass spectrometry (MS) analysis was performed using the Q Exactive Plus mass spectrometer (Thermo Fisher Scientific) and the “top 20” method for MS/MS experiment; that is, the 20 most intensive ions from the MS scan were selected for tandem MS (MS/MS), single-charged ions were excluded from fragmentation, and detected ions were excluded for further fragmentation for 2 min after initial MS/MS fragmentation had been performed. The mass resolution of 70,000 was selected for MS at AGC set to 3E6, MS/MS resolution was set to 35,000 and AGC set to 1E5 scans. Fragmentation was performed using the HCD approach at a normalized collision energy of 30 eV. Additionally, before the MS, UV peptide detection at 214 nm was also performed, which also served as quality control for HPLC separation. Raw MS/MS files were analyzed using Proteome Discoverer 2.3 (ThermoFisher Scientific, Bremen, Germany) and searching the Swissprot human database using the following parameters: taxonomy: Homo sapiens; modifications: carbamidomethyl on C as fixed, carboxymethylation on M as a variable; peptide tolerance was set to 10 ppm and the MS/MS tolerance to 0.05 Da; trypsin was selected as the enzyme used and two missed cleavages were allowed; False discovery rate (FDR) was set to 1% and the decoy database search was used for estimating the FDR. Scaffold was used for data allocation and The Reactome Pathway Knowledgebase for final pathway enrichment analysis. The sample size calculation was based on the approach of Jung et al. [21] for two-sided paired t-tests with 20 individuals per group. With these sample numbers, an effect size of 0.9 standard deviations can be detected with 80% power when controlling the false discovery rate at 5%. For the sample size calculation, the proportion of differentially abundant proteins was assumed to be 5%. We applied independent t-test or chi-square test for demographic data analysis, considered a p-value of <0.001 as statistically significant and conducted all analyses using the SPSS Statistics software (IBM, Armonk, NY, USA, Version 25). For analysis of relative protein abundance and comparison between groups, the Fisher’s exact test with Benjamini-Hochberg multiple test correction at 1% false discovery rate and p-value < 0.001 was applied. This manuscript was structured according to the “Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) guideline”.

3. Results

We included 40 urine samples in the analysis (n = 20 cases and n = 20 controls). Demographic data were similar in both groups except for the ICIQ sum score (Table 1). We identified 1897 proteins in analyzed urine samples at a false discovery rate of 1% and the significance level of p < 0.001. Out of these, 297 proteins were found in significantly higher abundance in women with OAB as compared to controls (p < 0.003, Benjamini-Hochberg multiple test correction), of which 108 proteins were detected in the OAB group only. Furthermore, we found 269 proteins in significantly higher abundance in the control group (p < 0.0003, Benjamini-Hochberg multiple test correction), of which 111 proteins were exclusively detected among controls (Figures 1 and 2). The remaining 1331 identified proteins were found in similar and non-significant abundance in both groups. Only proteins, which met the assigned criteria (significance level p < 0.0003 and Log2 (FC) > 2) were selected for pathway analysis, which resulted in 37 proteins of the OAB group (Table 2) and 53 proteins of the control group (Table 3). Pathway analysis was conducted using the Reactome Pathway Knowledgebase Enrichment Analysis (generated probability factor (p-value); Benjamini-Hochberg multiple test correction (FDR)). According to the resulting p-values, a ranking of the 25 most relevant signal cascades was generated for each
group. Ten of the 37 most relevant proteins of the OAB group had to be excluded during pathway analysis as these could not be assigned to any pathway of the Reactome Pathway Knowledgebase. Similarly, 18 of the 53 most relevant proteins of the control group had to be excluded during pathway analysis. For the included 27 most relevant proteins of the OAB group, 386 different pathways were identified, each including a minimum of one identified protein. Similarly, for the included 35 most relevant proteins of the control group, 116 different pathways were identified, each also including a minimum of one identified protein. Tables 4 and 5 show the 25 most relevant pathways of the OAB group and control group, respectively. A genome-wide overview of pathway analysis results is displayed in Figures 1 and 2. Color codes indicate a hit in the respective pathway.

Table 1. Demographic data.

|                        | OAB (N = 20) | Control (N = 20) | p-Value |
|------------------------|-------------|-----------------|---------|
| (Mean ± SD)            |             |                 |         |
| Age (years)            | 57.5 (±16)  | 55.3 (±17)      | 0.904   |
| BMI (kg/m²)            | 27.7 (±5)   | 25.5 (±5)       | 0.740   |
| Vaginal deliveries (n) | 1.15        | 0.99            | 0.484   |
| Chronic diseases * (n) | 12/20 (60%) | 8/20 (40%)      | 0.206   |
| Menopausal status (n)  |             |                 | 0.744   |
| premenopausal          | 7/20 (35%)  | 8/20 (40%)      |         |
| postmenopausal         | 13/20 (65%) | 12/20 (60%)     |         |
| Smoking (yes)          | 2/20 (10%)  | 3/20 (15%)      | 0.633   |
| Caffeine consumption   | 17/20 (85%) | 16/20 (80%)     | 0.677   |
| ICIQ sum score         | 31          | 0               |         |

* at least one of the following: hypertension, coronary heart disease, bronchitis, glaucoma, depression, irritable bowel syndrome, COPD, DM type 2, adipositas, hyperlipidemia, hypercholesterolemia.

Figure 1. Pathway overview of the 27 most relevant proteins of the OAB group. Color codes indicate a hit in the respective pathway. Higher color intensity (yellow) means a higher significance of one or more proteins within this signal cascade (FDR). Reactome Pathway database, open access open source [22].
Figure 2. Pathway overview of the 35 most relevant proteins of the control group. Color codes (yellow) indicate a hit in the respective pathway. Higher color intensity (yellow) means a higher significance of one or more proteins within this signal cascade (FDR). Reactome Pathway database, open access open source [22].

Table 2. Significant proteins detected in the overactive bladder syndrome group.

| Protein | Accession | Gene Symbol |
|---------|-----------|-------------|
| 26S proteasome non-ATPase regulatory subunit 11 | O00231 | PSD11 |
| 26S proteasome regulatory subunit 10B | A0A087 × 2I1 | A0A087 × 2I1 |
| 3-hydroxyacyl-CoA dehydrogenase type-2 | Q99714 | HCD2 |
| 60S ribosomal protein L15 | P61313 | RL15 |
| Alpha-centractin | A0A087 × 2I1 | A0A087 × 2I1 |
| Alpha-mannosidase 2 | Q16706 | MA2A1 |
| Asparagine–tRNA ligase, cytoplasmic | O43776 | SYNC |
| Beta/gamma crystallin domain-containing protein 1 | A0A0J9YWL0 | A0A0J9YWL0 |
| Bleomycin hydrolase | Q13867 | BLMH |
| Coatomer subunit beta | P53618 | COPB |
| Cytoplasmic dynein 1 heavy chain 1 | Q14204 | DYHC1 |
| Cytosolic phospholipase A2 delta | Q86XP0 | PA24D |
| Electron transfer flavoprotein subunit alpha, mitochondrial | P13804 | ETFA |
| Eukaryotic translation initiation factor 1 | P05198 | IF2A |
| Eukaryotic translation initiation factor 1 | P14152 | EIF3A |
| Heat shock 70 kDa protein 4L | Q95757 | HSP40 |
| Isoform 2 of 26S proteasome regulatory subunit 6B | P43686-2 | PRS6B |
| Isoform 2 of Alpha-S1-casein | Q9Y2A7-2 | NCKP1 |
| Isoform 2 of Glutamine–tRNA ligase | P47897-2 | SYQ |
| Isoform 2 of Nck-associated protein 1 | Q9Y2A7-2 | NCKP1 |
| Isoform 2 of Serine/threonine-protein phosphatase 2A 55 kDa regulatory subunit B alpha isoform | P63151-2 | 2ABA |
| Isoform 2 of Threonine–tRNA ligase, cytoplasmic | P26639-2 | SYTC |
| Kappa-casein | P07498 | CASK |
| Mitogen-activated protein kinase 1 | P28482 | MK01 |
| Mucin-4 | Q99102 | MUC4 |
| Peroxisomal acyl-CoA oxidase 1 | Q15067 | ACOX1 |
| Polypyrimidine tract-binding protein 1 | A0A0U1RRM4 | A0A0U1RRM4 |
| Prenylcysteine oxidase 1 | Q9UHG3 | PCYOX |
| Proteasome subunit alpha type-2 | P25787 | PSA2 |
| Protein flightless-1 homolog | Q13045 | FLL1 |
| Sptctin beta chain, non-erythrocytic 2 | O15200 | SPTN2 |
| Spliceosome RNA helicase DDX39B | Q13838 | DDX39B |
| Tripartite motif-containing protein 16 | Q95361 | TRIM16 |
| Tropomodulin-3 | Q9NYL9 | TMOD3 |
| UDP-glucose 4-epimerase | Q14376 | GALE |
| Vesicle-fusing ATPase | P46459 | NSF |
| X-ray repair complementing protein 5 | P13010 | XRCC5 |
Table 3. Significant proteins detected in the control group.

| Protein                                      | Accession | Gene Symbol |
|----------------------------------------------|-----------|-------------|
| Trefoil factor 3                             | X6R3S7    | X6R3S7      |
| Latent-transforming growth factor beta-binding protein 2 | G3V3 × 5  | G3V3 × 5    |
| Meprin A subunit                             | B7ZL91    | B7ZL91      |
| Testican-1                                   | A0A0A0MQX7| A0A0A0MQX7  |
| IgGFc-binding protein                        | Q9Y6R7    | FCGB        |
| Frizzled-4                                   | Q9ULV1    | FZD4        |
| Mucin-5B                                     | Q9HC84    | MUC5B       |
| Growth/differentiation factor 15             | Q99988    | GDF15       |
| Isoform 2 of Phosphoinositide-3-kinase-interacting protein 1 | Q96FE7-2 | P3IP1      |
| BPI fold-containing family B member 1        | Q8TDL5    | BPIB1       |
| CD99 antigen-like protein 2                 | Q8TCZ2    | CD99        |
| Adhesion G protein-coupled receptor F5       | Q81ZF6    | AGFR5       |
| Protein SU00-A7A                             | Q86SC5    | SIA7A       |
| Elongation factor Tu                         | Q83ES6    | EFTU        |
| Protocadherin Fat 4                          | Q6V0I7    | FAT4        |
| Na(+) / H(+) exchange regulatory cofactor NHE-RF3 | Q5T2W1   | NHRF3       |
| FRAS1-related extracellular matrix protein 2 | Q5SZK8    | FREM2       |
| Trefoil factor 2                             | Q03403    | TFF2        |
| Fibulin-2                                   | P98095    | FBLN2       |
| Hepcidin                                    | P81172    | HEPC        |
| Brain acid soluble protein 1                 | P80273    | BASP1       |
| Hemoglobin subunit gamma-2                   | P98992    | HBG2        |
| NPC intracellular cholesterol transporter 2  | P61916    | NPC2        |
| Beta-defense 1                               | P60022    | DEFB1       |
| Secreted Ly-6/uPAR-related protein 1         | P55000    | SLUR1       |
| Isoform 2 of Cysteine-rich secretory protein 3 | P54108-2 | CRIS3       |
| Afamin                                       | P43652    | AFAM        |
| Fibrillin-1                                  | P35555    | FBN1        |
| Isoform 2 of Syndecan-4                      | P31431-2  | SDC4        |
| Granulins                                    | P28799    | GRN         |
| Serum paraoxonase/arylesterase 1             | P27169    | PON1        |
| CD27 antigen                                 | P26842    | CD27        |
| Fibulin-1                                    | P23142    | FBLN1       |
| Small proline-rich protein 2D                | P22532    | SPR2D       |
| Cornitin-B                                   | P22528    | SPR1B       |
| Azurocidin                                   | P20160    | CAP7        |
| Elafin                                       | P19957    | ELAF        |
| Inter-alpha-trypsin inhibitor heavy chain H2 | P19823    | ITIH2       |
| Complement component C7                      | P10643    | CO7         |
| Matrilysin                                   | P09237    | MMP7        |
| Tumor necrosis factor receptor superfamily member 16 | P08138   | TNR16       |
| Calbindin                                    | P05937    | CALB1       |
| Heparin cofactor 2                           | P05546    | HEP2        |
| Major prion protein                          | P04156    | PRO         |
| Trefoil factor 1                             | P04155    | TFF1        |
| Antileukoproteinase                          | P03973    | SLPI        |
| Cystatin-A                                   | P01040    | CYTA        |
| Complement C5                                | P01031    | CO5         |
| Plasminogen                                  | P00747    | PLMN        |
| Superoxide dismutase [Cu-Zn]                 | P00441    | SODC        |
| CD5 antigen-like                             | O43866    | CD5L        |
| Ribonuclease T2                              | O00584    | RNT2        |
| Isoform 2 of Deoxyriboonuclease-2-alpha      | O00115-2  | DNS2A       |
Table 4. Pathway analysis of most relevant proteins of the OAB group (ranked according to the \( p \)-value of the enrichment analysis of the Reactome Pathway Analysis of the OAB group; modified after Fabregat et al. [23]).

| Pathway                                                                 | Ratio of Identified Proteins | \( p \)-Value | False Discovery Rate |
|------------------------------------------------------------------------|------------------------------|---------------|---------------------|
| COPI-mediated anterograde transport                                   | 5/102                        | 0.00003       | 0.006               |
| Transport to the Golgi and subsequent modification                    | 6/186                        | 0.00004       | 0.007               |
| ER to Golgi Anterograde Transport                                     | 5/155                        | 0.0002        | 0.012               |
| ABC-family proteins mediated transport                                | 4/103                        | 0.0005        | 0.012               |
| Cellular responses to stress                                          | 7/408                        | 0.0005        | 0.012               |
| G2/M Transition                                                       | 5/198                        | 0.0006        | 0.012               |
| Asparagine N-linked glycosylation                                     | 6/305                        | 0.0006        | 0.012               |
| Mitotic G2-G2/M phases                                                | 5/200                        | 0.0007        | 0.012               |
| Regulation of activated PAK-2p34 by proteasome mediated degradation   | 3/50                         | 0.0007        | 0.012               |
| Cross-presentation of soluble exogenous antigens (endosomes)         | 3/50                         | 0.0007        | 0.012               |
| Intra-Golgi and retrograde Golgi-to-ER traffic                        | 5/206                        | 0.0007        | 0.012               |
| Regulation of ornithine decarboxylase (ODC)                          | 3/51                         | 0.0008        | 0.012               |
| Autodegradation of the E3 ubiquitin ligase COP1                       | 3/52                         | 0.0008        | 0.012               |
| Ubiquitin-dependent degradation of Cyclin D1                          | 3/52                         | 0.0008        | 0.012               |
| Ubiquitin-dependent degradation of Cyclin D                            | 3/52                         | 0.0008        | 0.012               |
| Transcriptional regulation by RUNX2                                   | 4/121                        | 0.0009        | 0.012               |
| Vpu mediated degradation of CD4                                       | 3/53                         | 0.0009        | 0.012               |
| Regulation of Apoptosis                                               | 3/53                         | 0.0009        | 0.012               |
| p53-Independent DNA Damage Response                                   | 3/53                         | 0.0009        | 0.012               |
| p53-Independent G1/S DNA damage checkpoint                            | 3/53                         | 0.0009        | 0.012               |
| Ubiquitin Mediated Degradation of Phosphorylated Cdc25A               | 3/53                         | 0.0009        | 0.012               |
| FBXL7 down-regulates AURKA during mitotic entry and in early mitosis  | 3/55                         | 0.0010        | 0.012               |
| SCF-beta-TrCP mediated degradation of Emi1                            | 3/55                         | 0.0010        | 0.012               |
| Degradation of AXIN                                                   | 3/55                         | 0.0010        | 0.012               |
| Negative regulation of NOTCH4 signaling                               | 3/55                         | 0.0010        | 0.012               |

Table 5. Pathway analysis of most relevant proteins of the control group (ranked according to the \( p \)-value of the enrichment analysis of the Reactome Pathway Analysis of the OAB group; modified after Fabregat et al. [23]).

| Pathway                                                                 | Ratio of Identified Proteins | \( p \)-Value | False Discovery Rate |
|------------------------------------------------------------------------|------------------------------|---------------|---------------------|
| Molecules associated with elastic fibers                               | 4/38                         | 0.00004       | 0.004               |
| Elastic fiber formation                                                | 4/45                         | 0.00007       | 0.004               |
| Terminal pathway of complement                                         | 2/8                          | 0.0007        | 0.026               |
| Antimicrobial peptides                                                 | 4/95                         | 0.0019        | 0.033               |
| Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs) | 4/124                        | 0.003         | 0.054               |
| Extracellular matrix organization                                       | 6/301                        | 0.003         | 0.054               |
| Formation of the cornified envelope                                    | 4/129                        | 0.003         | 0.054               |
| Innate Immune System                                                   | 12/1183                      | 0.009         | 0.122               |
| Activation of Matrix Metalloproteinases                               | 2/33                         | 0.011         | 0.132               |
| NFG and proNGF binds to p75NTR                                         | 1/3                          | 0.014         | 0.132               |
| Ceramide signaling                                                     | 1/3                          | 0.014         | 0.132               |
| Post-translational protein phosphorylation                             | 3/107                        | 0.015         | 0.132               |
| Axonal growth stimulation                                             | 1/4                          | 0.019         | 0.151               |
| Keratinization                                                         | 4/217                        | 0.020         | 0.160               |
| NADE modulates death signaling                                        | 1/6                          | 0.028         | 0.169               |
| p75NTR negatively regulates cell cycle via SC1                         | 1/6                          | 0.028         | 0.169               |
| Metal sequestration by antimicrobial proteins                          | 1/6                          | 0.028         | 0.169               |
| Degradation of the extracellular matrix                               | 3/140                        | 0.029         | 0.176               |
| Activation of C3 and C5                                               | 1/7                          | 0.033         | 0.187               |
| RNF mutants show enhanced WNT signaling and proliferation             | 1/8                          | 0.037         | 0.187               |
| Axonal growth inhibition (RHOA activation)                             | 1/9                          | 0.042         | 0.205               |
| Synthesis of 5-eicosatetraenoic acids                                  | 1/9                          | 0.042         | 0.205               |
| p75NTR regulates axonogenesis                                          | 1/10                         | 0.047         | 0.205               |
| Regulated proteolysis of p75NTR                                       | 1/11                         | 0.051         | 0.205               |
| Dissolution of Fibrin Clot                                             | 1/13                         | 0.06          | 0.224               |
4. Discussion

To our knowledge, this is the largest study aiming to determine a urinary proteomic pattern in female overactive bladder syndrome for a better understanding of the diseases’ pathophysiology. We were able to identify significantly different protein pathways between women with OAB and healthy controls, suggesting a proteomic pattern characteristic for this disease. Several significant proteins of the OAB group could be assigned to the pathways “cellular responses to stress” and “regulation of apoptosis”, which may suggest a disruption of normal homeostasis by external stimuli. The ability of cells to respond to external influences, and to sustain a fine balance between cell survival and apoptosis, is crucial for normal development and homeostasis [24]. Disruption of this balance can contribute to the development of autoimmune diseases, neurodegeneration, and cancer [25]. In contrast, the most relevant pathways of the control group belong to the categories “extracellular matrix organization” and “immune system”, which suggests the presence of a more protective immune system and a generally healthier tissue turnover of the urinary bladder. Our findings may indicate a disruption in the capability of the urinary bladder tissue to adequately respond to external stimuli in OAB patients—possibly due to an altered immune response. One smaller proteomic study on OAB identified the protein VCAM-1 as only significantly different protein when compared to controls [18]. VCAM-1 protein plays a role in immune response and leukocyte emigration and can be located in the pathway categories “extracellular matrix organization” and “immune system”. Even though VCAM-1 was not identified as significant protein in our analysis, several significant protein pathways of our analysis could be assigned to the same categories. In congruence with results from this prior study, we also suggest alterations in the immune system—among others—as relevant in the pathophysiology of OAB. Surprisingly, further significant proteins of the OAB group are involved in neuronal processes (Spectrin beta chain, non-erythrocytic 2; 3-hydroxyacyl-CoA Dehydrogenase type 2; Cytoplasmic dynein 1 heavy chain 1; Isoform 2 of glutamine–tRNA ligase; Isoform 2 of Nck-associated Protein 1). Subsequent pathway analysis substantiated this finding where the Asparagine N-linked glycosylation signal cascade was identified as one of the 25 most relevant pathways in the OAB group. N-linked glycosylation is a form of post-translational modification and most important for proteins synthesized and folded in the endoplasmic reticulum [26]. Mutations in the relevant genes may trigger severe developmental problems which are frequently involving the central nervous system [27]. Our study population was carefully selected to guarantee only non-neurogenic OAB cases. Thus, the detection of significant proteins linked to neurological processes in our OAB group came somewhat unexpected. If confirmed, this could indicate a neurogenic component, even in the pathophysiology of non-neurogenic OAB. Hypersensitivity of the urinary bladder, which clinically shows as frequency and urgency, may be explained by such alterations in neurological processes. Overall, we were able to observe a larger diversity of pathways and their categories in women with OAB compared to controls, which is displayed in Figures 1 and 2. Whereas significant protein pathways of both case and control groups appear in the same category (e.g., the category “immune system”), it seems interesting that the respective pathways do not overlap between the groups. This underlines the uniqueness of the urinary proteome of women with OAB. Strengths of this study are the very strict inclusion criteria of the study population. We included women with pure OAB symptoms only and no other form of incontinence or lower urinary tract symptoms. The observed differences in the urinary proteome can, therefore, be fully attributed to the presence or absence of OAB symptoms. In contrast to previous studies, we were able to identify and compare the complete proteome of urine samples, which allows a more holistic view of the pathophysiology of OAB. While other studies focused on proteins, already imagined of with a role in the development of OAB (mainly inflammatory proteins), we took a step back and looked at the complete proteome, being able to identify previously unsuspected compounds. Using this approach, previously neglected but important molecular processes characteristic for OAB, can be drawn to attention. We also have to acknowledge certain limitations of this study. It can be
assumed that the urinary proteome follows a circadian rhythm as well as changes according to food and drink intake [28]. As we only took one urinary sample per participant at one time point, we are unable to account for this intra-individual proteome turnover. However, as cases and controls equally underlie this circadian proteome rhythm, we assume that our observed differences should nevertheless be valid. Another limitation is the yet incomplete human proteome mapping, meaning that global research has not yet been able to identify and describe all proteins of the human body. Results from our study can therefore only reflect the status quo of proteome knowledge.

5. Conclusions

We found a more diverse protein turnover in urine samples of the OAB group compared to controls. Significant pathways describe protein interactions at different time points of the cell cycle and processes of phosphorylation and degradation, as well as regulation of cellular response to stress. Unexpectedly, we found several significant proteins and pathways involved in neurological processes, which may indicate a neurogenic component even in non-neurogenic OAB. Significant protein pathways of the control group mainly concerned immunological, microbial-protective processes, and tissue-elasticity processes. We suggest a combination of increased cellular response to stress and apoptosis, as well as a loss of protective factors (such as antimicrobial and tissue-elasticity) as characteristic for OAB.

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References

1. Haylen, B.T.; de Ridder, D.; Freeman, R.M.; Swift, S.E.; Berghmans, B.; Lee, J.; Monga, A.; Petri, E.; Rizk, D.E.; Sand, P.K.; et al. An International Urogynecological Association (IUGA)/International Continence Society (ICS) joint report on the terminology for female pelvic floor dysfunction. *Int. Urogynecol. J.* 2010, 21, 5–26. [CrossRef] [PubMed]
2. Cheung, W.W.; Khan, N.H.; Choi, K.K.; Bluth, M.H.; Vincent, M.T. Prevalence, evaluation and management of overactive bladder in primary care. *BMC Fam. Pract.* 2009, 10, 8. [CrossRef]
3. Melville, J.L.; Katon, W.; Delaney, K.; Newton, K. Urinary incontinence in US women: A population-based study. *Arch. Intern. Med.* 2005, 165, 537–542. [CrossRef]
4. Irwin, D.E.; Mungapen, L.; Milsom, I.; Kopp, Z.; Reeves, P.; Kelleher, C. The economic impact of overactive bladder syndrome in six Western countries. *BJU Int.* 2009, 103, 202–209. [CrossRef]
5. Corcos, J.; Przydacz, M.; Campenau, L.; Gray, G.; Hickling, D.; Honeine, C.; Radomski, S.B.; Stothers, L.; Wagg, A.; Lond, F. CUA guideline on adult overactive bladder. *Can. Urol. Assoc. J.* L’association Urol. Can. 2017, 11, E142–E173. [CrossRef]
6. Cardozo, L.; Chapple, C.R.; Dmochowski, R.; Fitzgerald, M.P.; Hanno, P.; Michel, M.C.; Staskin, D.; Van Kerrebroeck, P.; Wyndaele, J.J.; Yamaguchi, Ö.; et al. Urinary urgency—Translating the evidence base into daily clinical practice. Int. J. Clin. Pract. 2009, 63, 1675–1682. [CrossRef]

7. Bhide, A.A.; Cartwright, R.; Khullar, V.; Digesu, G.A. Biomarkers in overactive bladder. Int. Urogynecol. J. 2013, 24, 1065–1072. [CrossRef]

8. Tyagi, P.; Tyagi, V.; Qu, X.; Lin, H.T.; Kuo, H.C.; Chuang, Y.C.; Chancellor, M. Association of inflammation (inflammation + aging) with higher prevalence of OAB in elderly population. Int. Urol. Nephrol. 2013. [CrossRef]

9. Liu, H.T.; Jiang, Y.H.; Kuo, H.C. Increased serum adipokines implicate chronic inflammation in the pathogenesis of overactive bladder syndrome refractory to antimuscarinic therapy. PLoS ONE 2013, 8, e76706. [CrossRef]

10. Rachaneni, S.; Arya, P.; Latthe, P. Urinary nerve growth factor: A biomarker of detrusor overactivity? A systematic review. Int. Urogynecol. J. 2013, 24, 1603–1609. [CrossRef]

11. Kashyap, M.; Kawamorita, N.; Tyagi, V.; Sugino, Y.; Chancellor, M.; Yoshimura, N.; Tyagi, P. Down-regulation of nerve growth factor expression in the bladder by antisense oligonucleotides as new treatment for overactive bladder. J. Urol. 2013, 190, 757–764. [CrossRef]

12. Seth, J.H.; Sahai, A.; Khan, M.S.; van der Aa, F.; de Ridder, D.; Panicker, J.N.; Dasgupta, P.; Fowler, C.J. Nerve growth factor (NGF): A potential urinary biomarker for overactive bladder syndrome (OAB)? BJU Int. 2013, 111, 372–380. [CrossRef]

13. Antunes-Lopes, T.; Pinto, R.; Barros, S.C.; Botelho, F.; Silva, C.M.; Cruz, C.D.; Cruz, F. Urinary neurotrophic factors in healthy individuals and patients with overactive bladder. J. Urol. 2013, 189, 359–365. [CrossRef]

14. Cho, K.J.; Kim, H.S.; Koh, J.S.; Kim, J.C. Changes in urinary nerve growth factor and prostaglandin E2 in women with overactive bladder after anticholinergics. Int. Urogynecol. J. 2013, 24, 325–330. [CrossRef]

15. Wang, L.W.; Han, X.M.; Chen, C.H.; Ma, Y.; Hai, B. Urinary brain-derived neurotrophic factor: A potential biomarker for objective diagnosis of overactive bladder. Int. Urol. Nephrol. 2013. [CrossRef]

16. Koch, M.; Mitulovic, G.; Hanzal, E.; Umek, W.; Seyfert, S.; Mohr, T.; Koelbl, H.; Laterza, R.M. Urinary proteomic pattern in female stress urinary incontinence: A pilot study. Int. Urogynecol. J. 2016, 27, 1729–1734. [CrossRef]

17. Koch, M.; Umek, W.; Hanzal, E.; Mohr, T.; Seyfert, S.; Koelbl, H.; Mitulović, G. Serum proteomic pattern in female stress urinary incontinence. Electrophoresis 2018, 39, 1071–1078. [CrossRef]

18. Futyma, K.; Nowakowski, Ł.; Ziętek-Strobl, A.; Kamińska, A.; Taoussi, N.; Rechberger, T. Urine Proteomic Study in OAB Patients-Preliminary Report. J. Clin. Med. 2020, 9, 1389. [CrossRef]

19. Wessel, D.; Flügge, U.I. A method for the quantitative recovery of protein in dilute solution in the presence of detergents and lipids. Anal. Biochem. 1984, 138, 141–143. [CrossRef]

20. Mitulović, G.; Stingl, C.; Steinmacher, I.; Hudecz, O.; Hutchins, J.R.; Peters, J.M.; Mechtler, K. Preventing carryover of peptides and proteins in nano LC-MS separations. Anal. Chem. 2009, 81, 5955–5960. [CrossRef]

21. Jung, S.H. Sample size for FDR-control in microarray data analysis. Bioinformatics 2005, 21, 3097–3104. [CrossRef] [PubMed]

22. Jassal, B.; Matthews, L.; Viteri, G.; Gong, C.; Lorente, P.; Fabregat, A.; Sidiropoulos, K.; Cook, J.; Gillespie, M.; Haw, R.; et al. The reactome pathway knowledgebase. Nucleic Acids Res. 2020, 48, D498–D503. [CrossRef]

23. Fabregat, A.; Sidiropoulos, K.; Viteri, G.; Forner, O.; Marin-Garcia, P.; Arnau, V.; D’Eustachio, P.; Stein, L.; Hermjakob, H. Reactome pathway analysis: A high-performance in-memory approach. BMC Bioinform. 2017, 18, 142. [CrossRef]

24. Kültz, D. Molecular and evolutionary basis of the cellular stress response. J. Clin. Med. 2018, 7, 359–365. [CrossRef]

25. Yang, Y.; Yu, X. Regulation of apoptosis: The ubiquitous way. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. 2003, 17, 790–799. [CrossRef]

26. Stanley, P.; Schachter, H.; Taniguchi, N. N-Glycans. In Essentials of Glycobiology; Varik, A., Cummings, R.D., Esko, J.D., Freeze, H.H., Stanley, P., Bertozzi, C.R., Hart, G.W., Etzler, M.E., Eds.; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, USA, 2009.

27. Sparks, S.E.; Krasnewich, D.M. Congenital Disorders of N-Linked Glycosylation and Multiple Pathway Overview. In GeneReviews®; Adam, M.P., Ardinger, H.H., Pagon, R.A., Wallace, S.E., Bean, L.J.H., Mirzaa, G., Amemiya, A., Eds.; University of Washington: Seattle, WA, USA, 1993.

28. Depner, C.M.; Melanson, E.L.; McHill, A.W.; Wright, K.P., Jr. Mistimed food intake and sleep alters 24-hour time-of-day patterns of the human plasma proteome. Proc. Natl. Acad. Sci. USA 2018, 115, E5390–E5399. [CrossRef]