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**Characterizing Patients with Recurrent Urinary Tract Infections in Vesicoureteral Reflux:  
A Pilot Study of the Urinary Proteome**

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## Abstract

Recurrent urinary tract infections (UTIs) pose a significant burden on the health care system. Underlying mechanisms predisposing children to UTIs and associated changes in the urinary proteome are not well understood. We aimed to investigate the urinary proteome of a subset of children who have vesicoureteral reflux (VUR) and recurrent UTIs because of their risk of developing infection-related renal damage. Improving diagnostic modalities to identify UTI risk factors would significantly alter the clinical management of children with VUR. We profiled the urinary proteomes of 22 VUR patients with low grade VUR (1-3 out of 5), a history of recurrent UTIs, and renal scarring, comparing them to those obtained from 22 age-matched controls. Urinary proteins were analyzed by mass spectrometry followed by protein quantitation based on spectral counting. Of the 2,551 proteins identified across both cohorts, 964 were robustly quantified, as defined by meeting criteria with spectral count (SC)  $\geq 2$  in at least 7 patients in either VUR or control cohort based on optimization of signal-to-noise ratio. Eighty proteins had differential expression between the two cohorts, with 44 proteins significantly upregulated and 36 downregulated ( $q < 0.075$ ,  $|FC| \geq 1.2$ ). Urinary proteins involved in inflammation, acute phase response (APR), modulation of extracellular matrix (ECM), and carbohydrate metabolism were overrepresented among the study cohort.

## Introduction

UTIs pose a significant burden on the health care system, with annual societal cost of \$3.5 billion in the United States.<sup>1</sup> In select populations, UTIs can lead to pyelonephritis (kidney infections), causing renal scarring and long-term renal damage, the latter being exacerbated with repeated infections. Molecular mechanisms characterizing patients predisposed to recurrent UTIs have not been completely elucidated. Since the urinary proteome is a source of several host defense proteins, the analysis of the urinary proteome in these patients may provide insights into better understanding the etiology of recurrent UTI.

The urinary tract has both mechanical and molecular host defense mechanisms.<sup>2-3</sup> Molecular host defense encompasses innate and adaptive immune responses involving immune cell activity and soluble factors secreted into urine such as uromodulin. Mechanical defense include physical or anatomic barriers, such as the glycosaminoglycan layer lining the urothelium, complete voiding to eliminate residual urine with bacteria, and valvular mechanisms which isolate the bladder from the kidneys during micturition. Vesicoureteral reflux (VUR) is a congenital condition where failure of this lattermost anatomic mechanical host defense causes backwards flow of urine to the kidneys from the bladder and contributes to incomplete urinary clearance. It is a common condition that often resolves without symptoms or intervention. However, during a UTI, VUR propagates the translocation of pathogenic bacteria from the bladder to the kidney, which can then lead to pyelonephritis and long-term sequelae such as renal scarring.<sup>4-5</sup> Patients with VUR and a predisposition for recurrent UTIs are more likely to be at greater risk for long-term kidney damage and may merit earlier medical or surgical intervention.<sup>4-6</sup> While VUR itself is accurately diagnosed by current, albeit invasive, modalities, it is impossible to identify these patients who are at higher risk for UTI.

Due to the inability to determine the need for intervention, VUR patients are often overmanaged and overtreated both medically and surgically.<sup>6</sup> Patients are placed on long-term daily antibiotic prophylaxis that can lead to antimicrobial resistance and associated side effects.<sup>6-10</sup> Serial assessment for continued presence of VUR exposes patients to invasive catheterization and ionizing radiation. Surgical correction is undertaken for patients in whom the condition persists or for those who experience recurrent breakthrough UTIs despite antibiotic suppression.<sup>4-6</sup> The availability of minimally invasive endoscopic intervention has increased the number of patients who undergo surgery, suggesting a trend of overtreatment without clear indication or need.<sup>11</sup> Beyond the economic impact, these potentially unnecessary interventions present risks of over exposure to antibiotics, surgery and anesthesia, all of which can impact pediatric growth and development.<sup>6-10, 12-13</sup>

We hypothesized that there may be differences in host molecular mechanisms that distinguish VUR patients with recurrent UTIs and renal damage. If these differences become clinically relevant the management of this population would dramatically change. To this aim, we performed a first-step pilot study comparing the urinary proteome of patients who underwent surgery for VUR with a history of recurrent UTI and renal scarring to that of healthy controls to determine if these cohorts can be distinguished by the urinary proteome. To categorize proteins that may be more characteristic of VUR and recurrent infection from those more likely indicative of renal damage, we compared proteomes to those from our previous study on patients with kidney damage resulting from ureteropelvic junction obstruction (UPJO). Furthermore, unlike prior studies, to reduce the potential bias of congenital kidney dysplasia often seen in higher grades of VUR, we focused only on children with lower grades of VUR.<sup>14-19</sup>

By concentrating on a well-defined urologic cohort, this pilot study sought to gain insight into recurrent UTI to serve as a foundation for future prospective, longitudinal studies that will involve children with VUR but no history of UTI. Differences identified in this population could ultimately provide guidance in improving the management of patients with VUR, particularly towards the development of better non-invasive diagnostic tools. Additionally, this work may contribute broader impact to elucidating mechanisms of recurrent UTI in other affected populations.

## Experimental Methods

### *Experimental Design and Statistical Rationale*

All patients were identified from an Institutional Review Board (IRB)-approved protocol from the Boston Children's Hospital Pediatric Urinary Proteome Program Initiative (PUPPI) repository. VUR is graded from 1 to 5 (with 5 being the most severe), and we selected a cohort of 22 patients who underwent open surgical correction for low grade of VUR (grades 1-3), history of recurrent febrile UTIs, and radiographic renal damage. Patients with more severe grades (grades 4-5) were excluded due to the potential confounding factor of congenital renal dysplasia and kidney maldevelopment as the etiology of abnormal renal imaging.<sup>20</sup> VUR grade was determined by voiding cystourethrogram (VCUG), and renal damage was defined as presence of renal cortical scarring on dimercaptosuccinic acid (DMSA) nuclear renal scan. Sample acquisition from VUR patients was by bladder catheterization via standard sterile technique on the day of surgery. None of the VUR patients had active infection as determined by urine culture.

A control cohort of 22 patients was additionally selected from PUPPI. Individuals in this group had no history of urologic conditions (VUR, UTI, or renal damage), or other comorbid conditions. Patients identified as healthy controls were undergoing procedures such as circumcision, hypospadias repair, lysis of labial adhesions removal, or inguinal hernia/hydrocele repair. Urine samples were obtained by voiding or crede maneuver. Due to the male predominance for these surgical procedures, there are a greater number of male samples available in the repository. Controls were selected to provide a similar age distribution to the VUR cohort, but gender and race distribution could not be achieved given limitations on available samples in the PUPPI repository. A pooled internal standard (PIS) was created from combining urine samples from 8 separate healthy controls. Aliquots of PIS were utilized as technical replicates for quality

control of signal normalization between different MS acquisitions and of bioinformatics processing. Demographic categorical and continuous variables were compared using Fisher's exact test and Wilcoxon test, respectively, between the VUR and control groups for each cohort and between the VUR groups for the discovery versus validation cohorts (Table 1).

### *Urinary Protein Extraction*

Samples were analyzed by urinalysis using a Siemens CLINITEK® status automated analyzer (Siemens, Tarrytown, NY) and confirmed there was no potential blood contamination or evidence of proteinuria. All samples were centrifuged at  $4,500 \times g$  for 20 min to remove cellular debris, aliquoted, and frozen at  $-80^{\circ}\text{C}$  prior to protein purification compliant with downstream MS analysis.<sup>21</sup> Urine aliquots were subsequently thawed, desalted, and concentrated on 10 kDa MW cutoff filters. Samples were reduced in 10 mM dithiothreitol (DTT, Sigma Aldrich, St. Louis, MO) and alkylated in 25 mM iodoacetamide (IAA, Sigma Aldrich). Extracted urinary proteins were quantified by micro BCA assay following manufacturer's instruction (Thermo Fisher Scientific, Waltham, MA).

### *Peptide Isolation*

Extracted urinary proteins from each individual were processed separately via solid-phase reversible sample-preparation (SRS) beads, as previously described.<sup>22</sup> Incubation was performed in a benchtop thermomixer (ThermoMixer C, Eppendorf, Germany) simultaneously shaking at 1,400 rpm with collection of supernatants after centrifugation at 5,000 rpm for 30 seconds (Minispin, Eppendorf). Briefly, 10 mg of dry SRS beads ( $\sim 25 \mu\text{L}$ ) was incubated with 0.01 M



NaOH (150  $\mu$ L) for 3 min at room temperature (RT) and then rinsed twice with 150  $\mu$ L phosphate-buffered saline (PBS). Urinary proteins (80  $\mu$ g) were dissolved in 100  $\mu$ L of 0.5% SDS in PBS and incubated with the washed beads. Acetonitrile (100%, ACN, Thermo Fischer Scientific) was immediately added to reach a final concentration of 85% ACN to enable effective binding of proteins, as previously published.<sup>22</sup> This mixture was incubated for 30 min at 1,400 rpm at RT. Samples were centrifuged to remove unbound proteins, and 8 M urea in PBS (150  $\mu$ L) was added. Excess reagent was removed by 8 M urea in PBS (150  $\mu$ L) and 80% ACN in PBS (150  $\mu$ L) (3 washes each). The beads were washed twice with 0.1 M ammonium bicarbonate (ABC, Sigma Aldrich) in heavy water ( $H_2^{18}O$ ) to label the glycosites for a separate prospective study and then placed in 150  $\mu$ L of 0.1 M ABC in  $H_2^{18}O$  for N-deglycosylation.

N-deglycosylation was performed by adding 2  $\mu$ L of peptide-N-glycosidase F (PNGase F, New England Biolabs, Ipswich, MA) to each sample in 2.5:100 enzyme-to-protein ratio and incubated overnight (20h) at 37°C. Released N-glycans were collected in supernatant after centrifugation and in two subsequent washes with 0.1% formic acid (150  $\mu$ L, FA, Sigma-Aldrich) for additional analysis not assessed in this study. Proteins bound to the beads were resuspended in 150  $\mu$ L of 50 mM ABC in unlabeled  $H_2O$ . Trypsin (2  $\mu$ g, Promega, Madison, WI) was added to beads in 1:40 enzyme-to-protein ratio, with a second 2  $\mu$ g added after 4h, and incubated at 37°C for 7h in total. Resulting peptides were collected in the supernatant after centrifugation. Samples were washed with 20% ACN (150  $\mu$ L) and centrifuged, followed by wash and centrifugation with 70% ACN (150  $\mu$ L). All supernatants were pooled for each sample and stored at -20°C prior to MS analysis.

## Mass Spectrometry Analysis and Database Search

Dried peptides were reconstituted with 400  $\mu$ L 5% FA/5% ACN. An aliquot of each sample was transferred into HPLC vial and placed in the autosampler at 4°C. Samples were individually analyzed on a Q-Exactive (ThermoFisher) coupled to a nanoflow UPLC system (Eksigent, Dublin, CA). The LC columns were packed in-house using Magic C18, 5  $\mu$ m, 100 Å (Michrom BioResources/Bruker, Billerica, MA) into PicoTips (15 cm x 100  $\mu$ m ID; New Objective, Woburn, MA). Peptides were separated utilizing a linear LC gradient from 95% solvent A (0.1% FA in water) and 5% solvent B (0.1% FA in ACN) to 70% solvent A and 30% solvent B over 120 min, followed by a steeper gradient to reach 5% solvent A and 95% solvent B over 7 min.

Raw files were searched against Uniprot *Homo sapiens* and common contaminant databases (reviewed September 2015) using Proteome Discoverer 1.4 (ThermoFisher Scientific) and combination of Sequest and Mascot algorithms for database search (42,184 total entries searched). Tryptic peptides with a default charge states of 2+, 3+, and 4+ and up to one miscleaved site were considered. Mono-isotopic precursor mass tolerance was 10 ppm, and 0.02 Da search tolerance was permitted for fragment ion identification. Precursor and neutral loss peaks were removed in MS2 spectra. Carbamidomethylation of cysteine residues was set as fixed modification. Variable modifications were deamidation (N, N(<sup>18</sup>O)), and Q) and oxidation (M). All data were searched against a combined target and decoy database. Confident peptide identifications were filtered at 1% false discovery rate (FDR) and identified proteins were matched by 2 or more spectra. Raw spectral data and Proteome Discoverer-generated MSF files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD010469.<sup>23</sup>

## *Protein Quantification and Statistical Analysis*

The SpectR Spectral Analysis R Package was developed in-house to provide comprehensive quantitative analysis of data based on spectral counts (SC). The package in R is provided as supplemental file. MSF files were re-searched to assign a SC score to each peptide and only unique peptides were extracted for quantitation. The detection threshold was defined as raw SC  $\geq 2$ , as non-zero values in this range were indistinguishable from zero. The latter values were assigned as missing and replaced with *NA* until after normalization. For non-missing raw SC data, normalization was performed using square root sum. In brief, the square root was normalized to the sum of all square root data and then squared, as shown below for  $n$  non-zero spectral count values among all samples analyzed.

$$\left[ \frac{\sqrt{SC_i}}{\sum_{i=1}^n \sqrt{SC_i}} \right]^2$$

Reproducibility of the processing was assessed using the 1,327 total proteins identified and quantified across the 5 technical replicates of PIS. Consistency of results was assessed based on pairwise comparisons of protein quantifications on peptides that were SC  $\geq 2$  (Supplemental Table 1 and Supplemental Figure 1). Proteins that were identified with SC  $\geq 2$  in at least 7 patients in either VUR or control cohort were defined as reliably detected based on optimization of signal-to-noise ratio. This list of proteins was then subjected to permutation-based FDR to further filter the candidate protein lists for comparison. Non-parametric Wilcoxon testing was utilized to compare urinary protein abundances between VUR and control cohorts, and the resulting p-values for each protein ID were adjusted for multiple testing via Benjamini-Hochberg correction. Positive false discovery rate (q-value) was controlled at 7.5%. Hierarchical clustering was performed utilizing proteins meeting the above threshold. Fold change (FC) for each protein was determined using the

ratio of the mean value in the VUR cohort to that in the control cohort. For ratios  $<1$  where the mean value VUR was less than that of control, FC is expressed as  $-1/\text{ratio}$  to reflect downregulation in VUR. The relationship of FC and q-value for each protein was visualized by volcano plot (Figure 1).

### *Functional Pathway Analysis*

In total, 80 proteins were differentially-regulated in the VUR patients compared to the control cohort (q-value  $<0.075$ ;  $|\text{FC}| \geq 1.2$ ); 44 proteins showed increase in abundance while 36 had decreased. These differentially-abundant urinary proteins were assessed via Functional Enrichment Analysis Tool (FunRich version 3.0).<sup>26</sup>

### *Comparison of the Urinary Proteome of the VUR and UPJO Patients*

VUR data was compared to our previously published urinary proteome of pediatric ureteropelvic junction obstruction (UPJO) patients (750 proteins) and list of the candidate biomarkers for UPJO-associated renal damage (76 proteins) but unrelated to UTI.<sup>27</sup> The comparison of VUR with UPJO data was performed to attempt segregating proteins that are more likely to be indicative of UTI from those that are associated with renal damage.

*Validation of MS Results via Immunoaffinity-based Quantitation in Discovery and Independent Patient Cohorts*

Alpha-1-acid glycoprotein (orosomuroid, ORM1) was selected for validation by enzyme-linked immunosorbent assay (ELISA R&D Systems, DAGP00, Minneapolis, MN). Initial verification was performed on remaining available urine from the original MS discovery cohort which included 19 VUR patients and 16 age-matched controls. A separate validation cohort of 20 VUR patients with similar disease phenotype and 20 age-matched controls was then tested (Table 1). Urine aliquots (1 ml) were thawed and centrifuged at  $4,000 \times g$  for 10 min to remove insoluble particles. Samples were diluted 1:10 in HPLC grade water (Thermo Fisher) and analyzed in replicates following manufacturer's instructions. Optical density was measured at 450 nm with a correction at 540 nm using a FilterMax F3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). Readouts were normalized to total urinary protein amount as determined by Bradford assay (Biorad, Hercules, CA) on a Beckman DU600 Spectrophotometer as previously described.<sup>28-31</sup> Significant changes between ORM1 levels in VUR and control samples were assessed by Mann-Whitney-Wilcoxon test.

## Results

### *Altered Urinary Proteome in VUR Patients with UTI and Renal Scarring*

Demographics and clinical characteristics of the VUR and control groups in both the MS discovery and validation cohorts were not significantly different, as presented in Table 1. In total 2,551 proteins were detected in the urine of our VUR patients and healthy individuals. To strengthen the comparative analysis, only proteins that were identified with SC  $\geq 2$  in more than 30% of samples from disease and control cohort were considered, resulting in 964 proteins. The threshold settings were determined based on analysis of the technical PIS replicates and optimization of signal-to-noise ratio, as mentioned above. Criteria of q-value  $< 7.5\%$  and  $|FC| \geq 1.2$  were applied which resulted in 80 candidate protein biomarkers that may characterize the VUR patients with UTI and renal scarring based on urinary protein profiling (Figure 1 and Supplemental Table 2). Hierarchical clustering utilizing these 80 proteins indicated near complete segregation of the two cohorts supporting a different expression profile of the proteomes in the VUR and healthy urine (Figure 2).

### *Functional Characterization of Differentially Expressed Proteins in VUR Patients*

Significantly-altered proteins between VUR patient and control cohort were functionally assessed via FunRich. The proteins eligible for FunRich's functional enrichment are listed within Supplemental Tables 3-5. Regulation of complement cascade and hemostasis were the most significantly altered pathways in the VUR cohort compared to controls (Figure 3a, Supplemental Table 3). Over-representation of proteins involved in formation of the fibrin clot and activation of the platelet signaling was also observed. Extracellular matrix (ECM) proteins involved in integrin

family cell surface interaction and EphrinB-EPHB pathway were significantly affected in disease cohort (Figure 3a). Interestingly, many of acute phase response proteins that are known inflammatory plasma protein markers were significantly altered in the urine from the VUR patients compared to control. Proteins that were increased or decreased in abundance had distinctive molecular and biological functions (Figure 3b). In particular, we observed an increase in proteins involved in complement binding/activation (C3, C4A, C9, CFH and CFB) and in immune defense mechanism (ORM1 and ORM2) (Supplemental Table 4). On the contrary, proteins with hydrolase activity, cysteine-type peptidase (CP), and structural constituent of the ECM were downregulated (Supplemental Table 5). Additionally, certain protease inhibitors (PIs) were specifically down- or upregulated in the VUR patients. In particular, serine protease inhibitors (serpins) SERPINB3 and SERPINB4 were downregulated while SERPINA1, SERPINC1 and SERPIND1 were upregulated. Upregulation of two additional PIs, alpha-2-macroglobulin (A2M) and inter- $\alpha$  trypsin inhibitor heavy chain 2 (ITIH2) was also observed. Four glycoprotein-binding proteins were significantly regulated: complement factor H (CFH) and histidine-rich glycoprotein (HRG) were upregulated, while insulin-like growth factor 2 receptor (IGF2R) and SERPINA1 were downregulated.

#### *Comparison of VUR and UPJO Urinary Proteomes Demonstrates Limited Numbers of Shared Varying Proteins*

In a previous study, we focused on the discovery of putative urinary biomarkers of patients with renal damage caused by ureteropelvic junction obstruction (UPJO).<sup>27</sup> We hypothesized that overlap of proteins related to both VUR and UPJO cohorts will reveal biomarkers indicative of renal damage commonly associated with injury. Proteins specifically regulated in one of the examined patient cohort may be considered indicative of disease-specific clinical traits.

In the case of the UPJO patients, 76 proteins were significantly altered compared to the control cohort.<sup>27</sup> This dataset was intersected with 80 proteins that were significantly changed in urine of the VUR patients. Eighteen overlapping biomarkers that were altered in both UPJO and VUR patients suggest there is a shared host response to inflammation and tissue repair resulting from either obstructive or UTI-related kidney damage. The remaining 62 proteins were distinct candidate biomarkers which may be characteristic of the VUR group and potentially of recurrent infection. Disease-specific alteration of the proteins that belong to the same protein class was observed. For example, inter-alpha-trypsin inhibitor heavy chain 1 (ITIH1) and 2 (ITIH2) were specifically altered in the urine of the UPJO and VUR patients, respectively. Altered expression of S100P was solely observed in UPJO, while changes in S100A7 was VUR specific. Upregulated serine protease inhibitors (SERPINA1, SERPINC1 and SERPIND1) in the VUR cohort were similarly regulated in UPJO patients. However, downregulated SERPINS (SERPINB3 and SERPINB4) were VUR specific.

#### *ELISA Confirmation of ORM1 Levels in Discovery and Validation Cohorts*

Levels of ORM1 were initially cross-validated by ELISA in 19 VUR and 16 controls of the original MS cohort ( $p < 0.01$ , Supplemental Figure 2). With this confirmation, an independent validation cohort of 20 VUR and 20 control patients were tested for ORM1. ELISA results confirmed significant upregulation of ORM1 in the independent cohort of VUR patients compared to controls ( $p < 0.05$ , Figure 4). This independent immunoaffinity assays mirrored the quantification in the MS-based proteomic approach, supporting reliability of our spectral count-based protein quantitation.



## Discussion

We performed a comparative analysis of the urinary proteome in 22 healthy and 22 VUR patients with recurrent UTIs and renal scarring and identified 80 urinary proteins that were significantly altered in abundance. Unique to our study, we focused on a clinical cohort of children with low grade VUR (grade 1-3), history of recurrent UTIs, and radiographic confirmation of renal damage. Focusing on patients with low grade VUR favors discovery of proteins related to UTI and infection-induced renal damage as opposed to congenital renal dysplasia which is often observed in high grade VUR. This cohort enabled the identification of urinary proteins that may be related specifically to the VUR cohort (62 proteins) and a minor subpopulation of proteins that may be indicative of generalized renal damage (18 proteins). Functionally, these urinary proteins appeared to be involved in host defense mechanism and inflammation, including acute phase response and ECM remodeling, which are critical for clearing the pathogen and restoring homeostasis after resolving infection. With additional testing these candidate biomarkers may represent a pool of urinary proteins critical for distinguishing patients who are at higher risk for UTI and subsequent UTI-related renal scarring. Our discussion focuses on proteins that are known to directly or indirectly mediate inflammatory response and their putative role within the urine in the context of UTI.

Immunomodulatory acute phase proteins (APP) are mainly secreted from the liver and their serum concentration increases (positive APPs) or decreases (negative APPs) by at least 25% in response to bacterial infection.<sup>32-33</sup> Release of APPs is a host defense response that mediates pathogen clearance by recruitment of neutrophils and macrophages and sequestering serum iron critical for bacterial growth, while it also limits tissue injury during inflammation and mediates restoration of homeostasis post infection.<sup>33-34</sup> Serum alteration of APPs, such as C-reactive protein

(CRP) or serum amyloid A (SAA) are rapid and large,<sup>32, 35-36</sup> but the majority of APPs have a slower and moderate fluctuation occurring over days to weeks post infection. In our cohort numerous APPs that are altered in plasma during inflammation were also found altered in the urine of the VUR cohort. We investigated significantly-regulated urinary APPs, including those with moderate to low fold change.

Upregulated urinary APPs are part of the complement system cascade (C3, C4-A, C4-B), involved in coagulation and fibrinolytic system (fibrinogen beta and gamma chains), protease inhibition (alpha-1-antitrypsin/SERPINA1)<sup>37</sup> and protein transport (hemopexin), or have more complex systemic function (ORM1, ORM2 and alpha-2-macroglobulin/A2M).<sup>38</sup> Notably, we did not detect significant alteration in urinary CRP and SAA, as their concentration peaks within the first couple of days of infection and normalizes after 1-2 weeks.<sup>36</sup> The urine from our cohort was taken at a much later date when the children were not actively infected. A rapid increase in CRP levels activates the complement system<sup>34, 39</sup> and thus increase of complement system proteins in the urine from the VUR cohort suggests that CRP was elevated during the initial phase of UTI. Surprisingly, several APPs that have been reported as downregulated in serum after infection (*i.e.*, negative APPs) were significantly increased in our VUR cohort urine, such as transthyretin (*i.e.*, prealbumin), antithrombin-III (SERPINC1) and coagulation factor XII. Future longitudinal studies will be needed to confirm how these negative APPs change in the urine along the course of infection and resolution phase.

Using ELISA, we validated the quantitative MS upregulation of ORM1 in a separate cohort of patients. ORM1 is a 44 kDa glycoprotein synthesized in the liver and secreted in plasma during stress conditions. As an APP ORM1 has broad immunomodulatory and anti-inflammatory function, but its role in systemic inflammatory response during UTI is less defined.<sup>40-42</sup> ORM1

inhibits macrophage and neutrophil infiltration into injured tissue which may be reason for increased survival rates observed during systemic inflammation and septic shock.<sup>43</sup> The immunosuppression triggered by ORM1, however, leads to increased susceptibility for opportunistic infections.<sup>44-45</sup> Although, ORM1 is limited as a standalone marker of susceptibility to UTI, it could serve as a marker for detection and progression of subclinical renal damage. Diagnostic potential of urinary ORM1 in the future may be extended for evaluating a risk from recurrent UTI in the VUR patients as a preceding, early-stage event that can eventually lead to UTI-associated renal damage.

Secretion of APPs is often accompanied by changes in immunomodulatory peptides.<sup>46</sup> These peptides can be directly involved in inflammatory response, or their constitutive expression may be required for maintaining a homeostatic environment. In particular we closely investigated host-derived antimicrobial peptides (AMPs) and identified several AMPs in the urine. AMPs such as beta-defensin 1 (DEFB1), cathelicidin antimicrobial peptide (CAMP), and lysozyme (LYZ) were unaltered between cohorts, solely S100A7 (psoriasin) was found to be significantly downregulated in VUR patients compared to controls. S100A7 is the only member of S100 family that is regulated in our VUR cohort, and it has been identified as a major host-defense protein in vaginal fluid that protects from *E. coli* infection.<sup>47</sup> This suggests that downregulation of S100A7 in the urine of VUR patients may be indicative of less effective host innate immunity and higher risk for UTI. As both ORM1 and S100A7 have been involved in modulating immune response to infection and host-defense mechanism,<sup>44, 48-50</sup> there may be a functional dependence between S100A7 down- and ORM1/2- upregulation that merits further investigation.

Inflammation is a tightly regulated process which coincides with complex changes of the cell extracellular matrix (ECM).<sup>51-52</sup> ECM composition and associated changes are critical for

pathogen attachment to the cell, as well as recruitment of immune cells to the infected site.<sup>51, 53-54</sup> Overrepresentation of regulated structural components of extracellular matrix (ECM), extracellular proteases (ECP) and inhibitors (ECPIs) was observed in VUR cohort (Figure 3). ECPs are promiscuous enzymes with complex substrate specificity and poorly defined sequence cleavage preference.<sup>55</sup> Nevertheless, they are critical components of ECM that maintain a healthy tissue environment and modulate signaling events during disease such as inflammation.<sup>56-57</sup> We found significant alteration of protein-based ECPIs in the urine of VUR patients (Figure 3b, Supplemental Table 4 and 5). The change in abundance of these inhibitory proteins was not uniform, but rather isoform specific. In particular, serpin peptidase inhibitors SERPINB3 and SERPINB4 were downregulated, while SERPINA1, SERPINC1 and SERPIND1 were upregulated in the urine of VUR patients. This pattern is intriguingly correlated with the tissue-specific expression of up- and downregulated SERPINs. According to the RNA expression database ([www.proteinatlas.org](http://www.proteinatlas.org)), upregulated SERPINs are almost exclusively synthesized in the liver and gallbladder, whereas downregulated SERPINs originate from gastrointestinal tract, with a modest contribution from kidney and urinary bladder. At this stage, we are unable to distinguish the specific mechanism for the downregulation of SERPINB3 and SERPINB4, but many children with VUR and/or UTI have concomitant functional constipation and many are on antibiotic therapy and it is unclear if these interactions are responsible for the observed downregulation.

SERPINC1 (Antithrombin III), a negative APP, and SERPIND1 (Heparin cofactor 2) are the two major heparin-dependent thrombin inhibitors in plasma that play a critical role in preventing coagulation and thrombosis.<sup>58</sup> More recent reports also suggest their involvement in inflammation.<sup>59</sup> Interestingly, binding of SERPINs to their targeted proteases increase up to 1000-fold in a presence of glycosaminoglycan structures on the surface of host cells.<sup>60-61</sup> This suggests

a critical interplay of cell-surface glycan structures and ECPIs in regulation and fine-tuning activity of ECPs. As protein glycosylation is an abundant PTM on the cell surface proteins and soluble proteins found in urine,<sup>62-63</sup> alteration in a single protein that binds or modifies glycan structure can have a broader, systemic impact. In our study, we identified two glucosidases that were significantly downregulated in the urine of the VUR patients:  $\alpha$ -fucosidase 2 (FUCA2) and  $\alpha$ -N-acetylgalactosaminidase (NAGA). FUCA2 is a plasma protein that cleaves  $\alpha$ -1,6-linked fucose that is attached to the N-acetylglucosamine of carbohydrate moieties of glycoproteins. Interestingly, deficiency in  $\alpha$ -fucosidase 1 (FUCA1) is a rare autosomal recessive disorder that along with other symptoms, is characterized by recurrent infections.<sup>64</sup> In contrast to FUCA2, FUCA1 is primarily expressed in tissues, and its abundance is unaltered between VUR patient and control urine samples. What remains unclear is whether the observed decrease in FUCA2 in the urine of VUR patients can predispose them to develop UTI, similar to autosomal recessive FUCA1 deficiency disorder. The second glycosidase of interest, NAGA, is a lysosomal enzyme that hydrolyzes  $\alpha$ -N-acetylgalactosamine residues from glycolipids and glycopeptides. Similar to FUCA, NAGA deficiency results in impaired glycan degradation and accumulation of complex glycan structures in tissue as well as their excessive secretion in the urine.<sup>65</sup> Changes in enzymes that regulate glycan metabolism can be correlated to observable changes in glycan structures identified in the urine of VUR patients. Reports on a competitive advantage of pathogenic bacteria that bind fucosylated glycan structures in the gastrointestinal tract urges exploration of this relation in the bladder epithelium.<sup>66-69</sup> In future studies it will be critical to correlate the function of these carbohydrates in the bladder and in the urine to those observed in the gut. Such studies will determine whether these structures provide any advantage for bacterial growth or pathogen

adhesion to the surface of the host cells and may explain an increased susceptibility to infection observed in the VUR patients.

While inflammatory response is beneficial for resolving infection, it damages surrounding tissues. Therefore, the inhibition of prolonged inflammatory response is critical for arresting further damage to the injured site. Proteins known to be involved in regulation of hemostasis and hemolysis were altered in the urine from the VUR patients compared to controls (Figure 3a). Hemostasis is reported to be critical for the balance of host immune defense, being related to both innate and adaptive immune response.<sup>70</sup> In contrast, hemolysis has been correlated with susceptibility to infection in various diseases and mouse models.<sup>71-73</sup> It is well established that hemolysis-mediated release of iron can be beneficial for pathogen growth.<sup>74-75</sup> A recent study additionally suggests that free heme decreases mobility of macrophages and prevents adequate pathogen clearance.<sup>76</sup> To this end, disrupted hemostasis can have two detrimental outcomes which assist bacterial outgrowth: release of iron<sup>77-78</sup> that benefits bacterial replication, along with release of heme that may impair macrophage-mediated phagocytosis of pathogenic bacteria.<sup>76</sup> As urinary samples from VUR patients have been obtained at minimum one month after infection, longitudinal follow up over the longer course of time is needed to further understand if quantitative changes of these urinary proteins are short term or remains altered over prolonged period of time.

We recognize that this study has limitations, including small sample size, the inherent clinical variability of patient selection and a single urinary proteome profiling after resolution of UTI. Controls were selected to provide a similar age distribution to the VUR cohort. Our VUR cohort was predominantly female, consistent with a known clinical preponderance of VUR, but the availability of female controls to allow for equal distribution was extremely limited. We have used our resources as the largest pediatric urologic department in the world to generate an

extremely well characterized clinical cohort that minimizes variability by controlling for grade of VUR, recurrent UTI and renal scarring. Although no clinical cohort is completely homogeneous, we identified significant changes in proteins that can contribute to susceptibility to UTI and UTI-acquired renal damage in patients with low grade VUR. Whether these changes preceded or resulted from recurrent UTIs will be answered in follow-up longitudinal studies, using the information gained from this pilot study. These longitudinal studies will expand VUR patient cohorts to include asymptomatic VUR patients, and VUR patients with UTI but no renal scarring, as well as well-defined urine sampling over the longer course of recovery from a UTI. Together, this can not only aid in a finer separation of urinary biomarkers for susceptibility to infection from the ones that are indicative of renal damage as a consequence of recurrent UTIs, but also resolve temporal alteration of inflammatory markers after resolution of UTI.

## Conclusions

In order to better characterize VUR patients with a history of recurrent UTIs and renal scarring, we compared their urinary proteome profiles with that of healthy controls. Eighty proteins were differentially expressed in the patients' urine. We identified 62 proteins that were solely altered in our chosen VUR cohort which may represent candidate biomarkers to characterize susceptibility of these patients to develop UTI, and 18 proteins that may more broadly represent renal injury. The former, for instance, include AMPs, protease inhibitors and proteins involved in glycoside catabolism. Further studies will be extended to distinct VUR cohorts in order to separate candidate biomarkers on the basis of susceptibility to UTIs and renal scarring. Ultimately, larger cohorts and longitudinal studies utilizing these findings will improve the diagnosis, delivery and cost-effectiveness of care for VUR patients.



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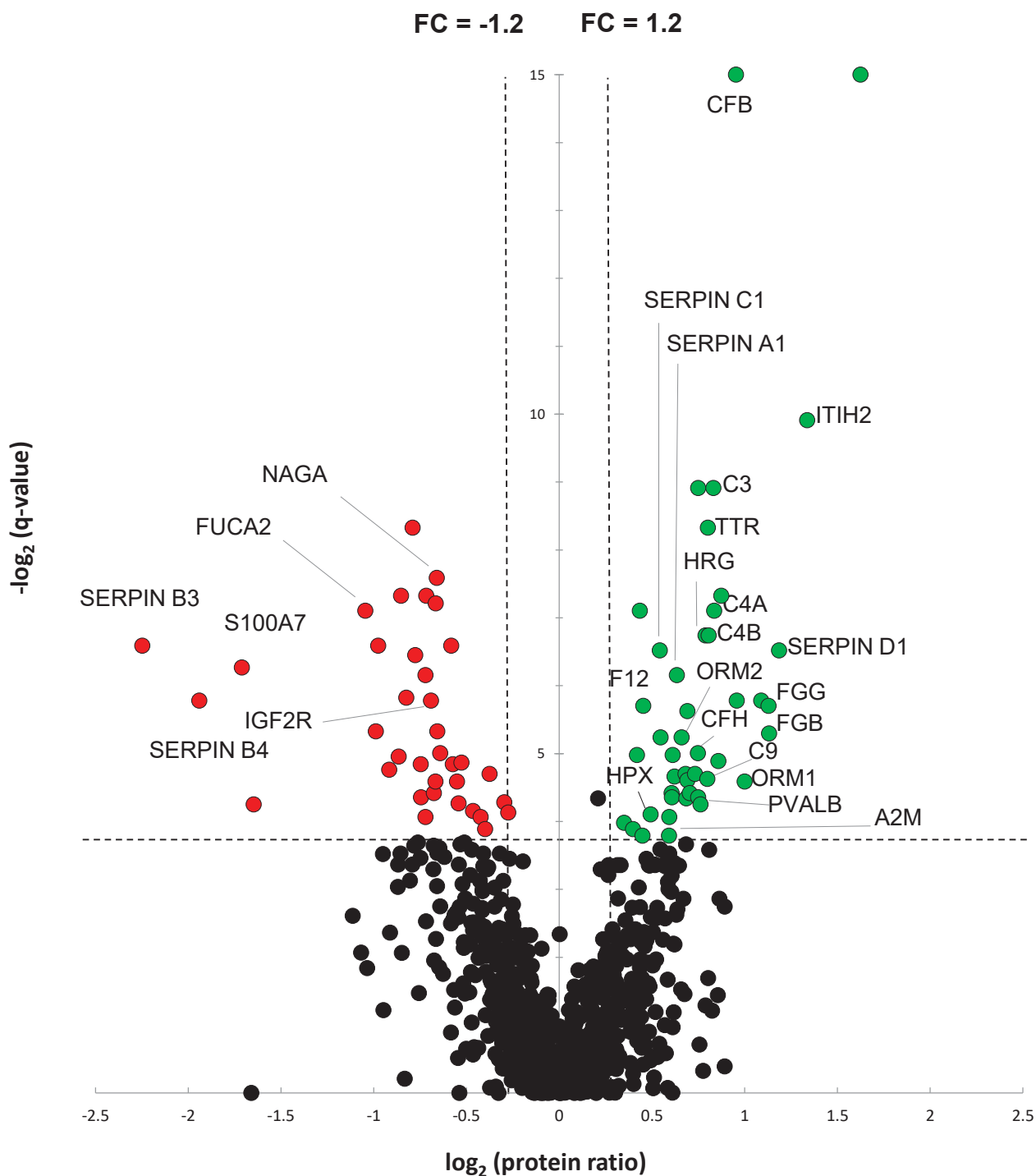
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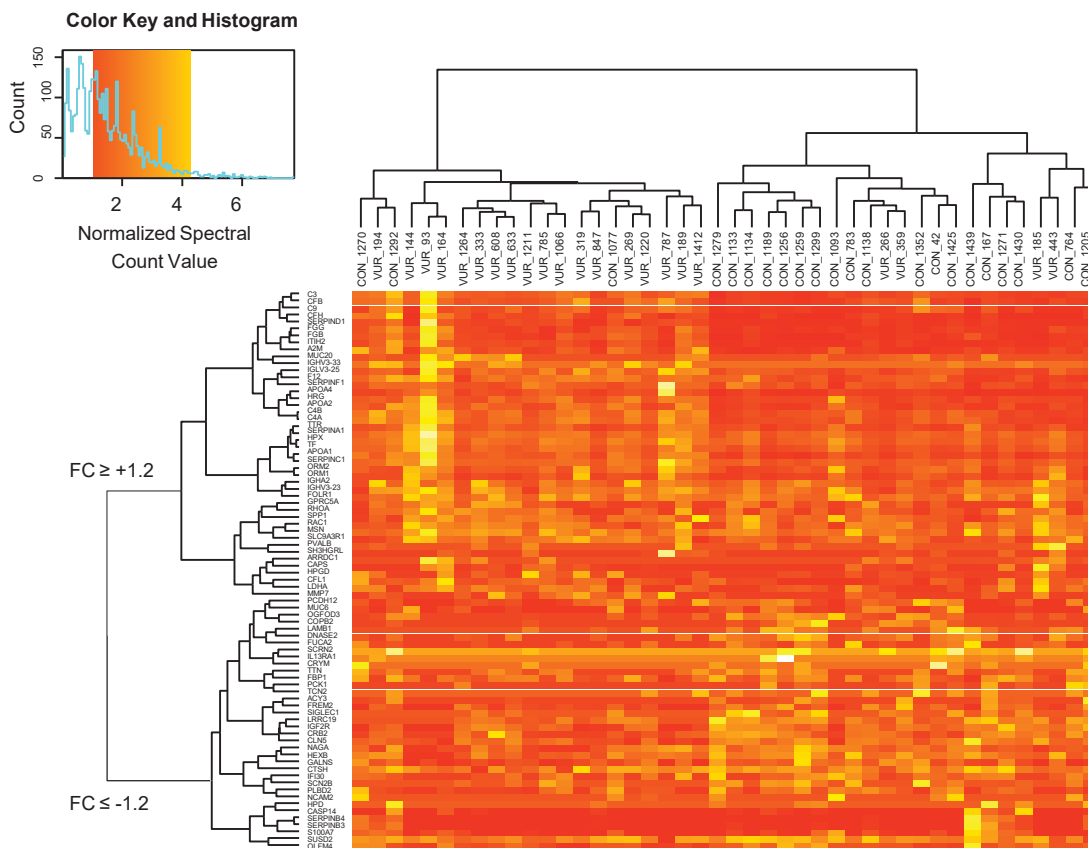


**Table 1. Demographics of the VUR and Control Groups.** Percentage values are listed with number in parentheses for the VUR and matched control groups for both the MS discovery (left) and validation (right) cohorts. The p value column reflects comparison between the VUR groups between the discovery and validation cohorts. The p value for comparison between the VUR and control groups within cohorts is listed below the comparison, where applicable. VUR, vesicoureteral reflux.

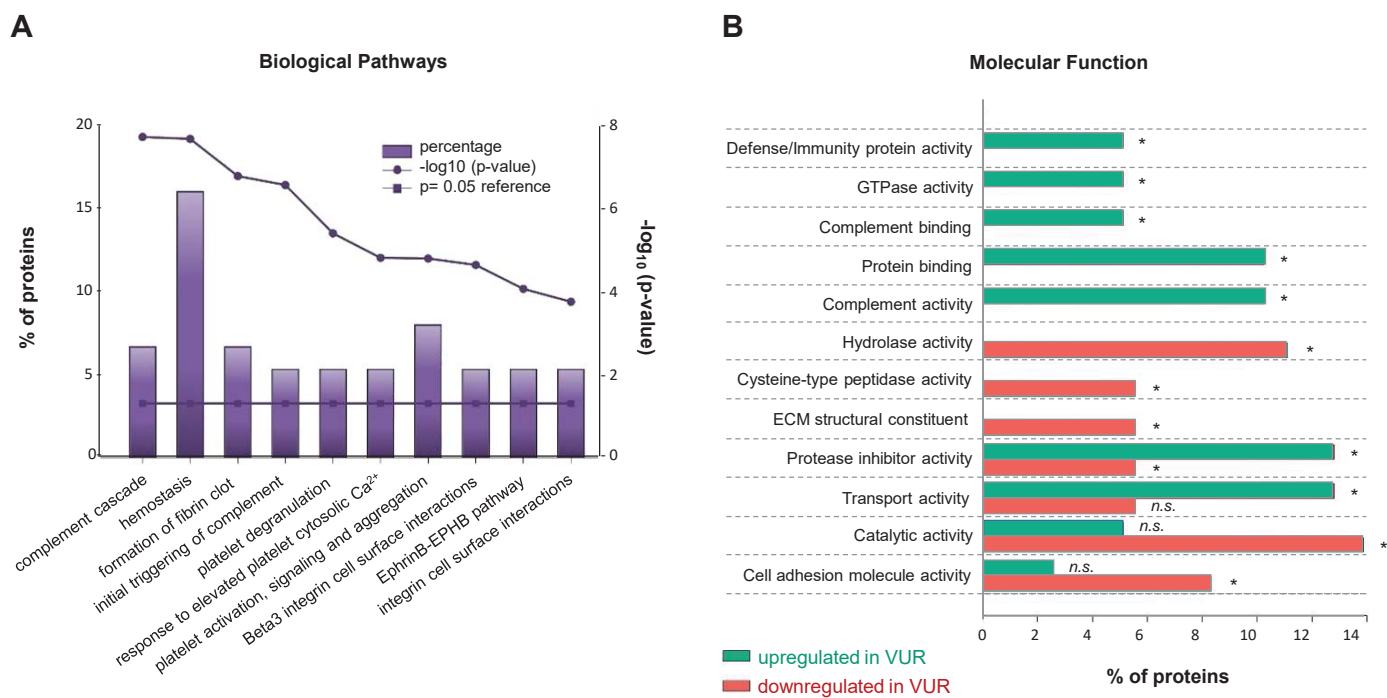
	MS Discovery Cohort		Validation Cohort		p value
	VUR	Control	VUR	Control	
<b>n</b>	22	22	20	20	
<b>Age (years)</b>					
Median	5.3	5.7	6.2	5.4	0.44
Range	1.1-22.2	1.0-22.0	1.2-14.0	0.4-14.0	
	p=1.00		p=0.86		
<b>Gender</b>					
Male	13.6% (3)	59.1% (13)	20% (4)	50% (10)	0.40
Female	86.4% (19)	40.9% (9)	80% (16)	50% (10)	0.40
	p<0.01		p=0.10		
<b>Reflux</b>					1.00
Unilateral	40.9% (9)		45.0% (9)		
Bilateral	59.1% (13)		55.0% (11)		
<b>Reflux Grade (Right)</b>					0.25
None	18.2% (4)		25.0% (4)		
Grade 1	18.2% (4)		0		
Grade 2	36.4% (8)		40% (8)		
Grade 3	27.3% (6)		35% (7)		
<b>Reflux Grade (Left)</b>					0.57
None	22.7% (5)		15.0% (3)		
Grade 1	4.5% (1)		0		
Grade 2	31.8% (7)		45.0% (9)		
Grade 3	40.9% (9)		35.0% (7)		
Grade 4	0		5.0% (1)		
<b>Antibiotic Prophylaxis</b>	95.5% (21)		75% (15)		<0.01
<b>Renal Scarring (DMSA)</b>					1.00
Unilateral	72.7% (16)		75.0% (15)		
Bilateral	27.3% (6)		25.0% (5)		



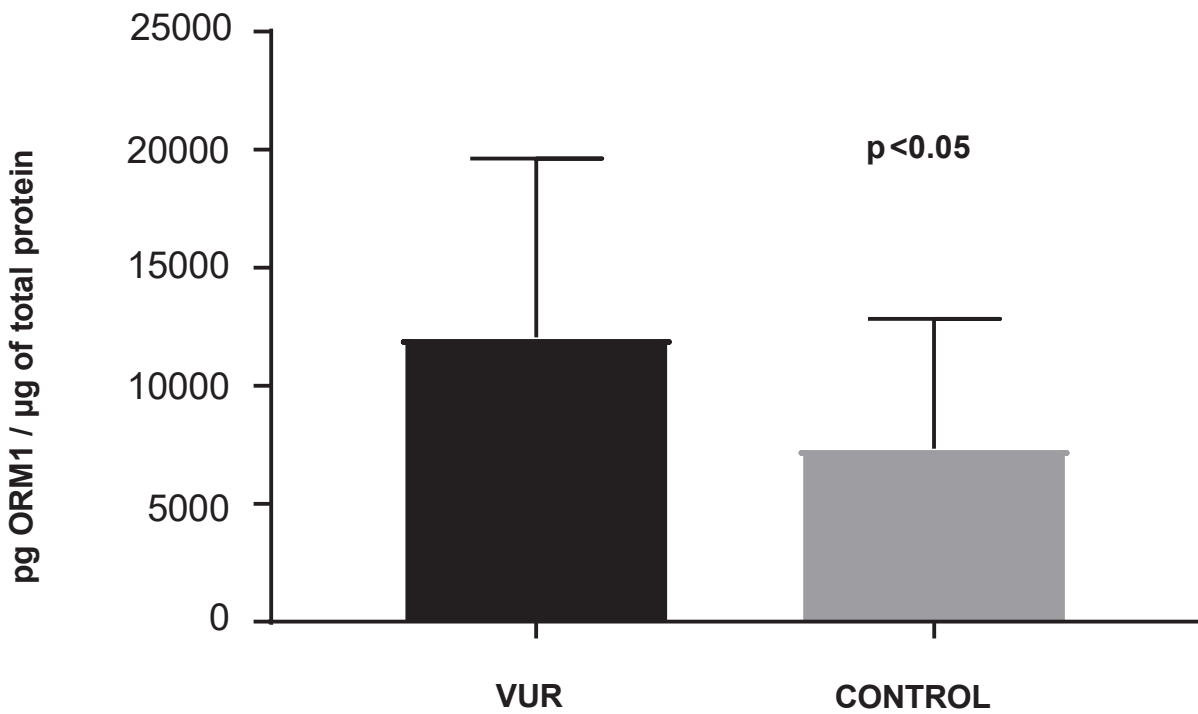
**Figure 1. Volcano Plot of VUR to Control Cohort Ratios for All Quantified Proteins.** Of the 964 robustly identified and quantified proteins in the VUR and control cohorts, 80 proteins were deemed differentially regulated candidate markers by meeting criteria of  $q\text{-value} < 7.5\%$  and  $FC \geq +1.2$  (up-regulated in VUR; green) or  $\leq -1.2$  (down-regulated in VUR; red). Specific candidate proteins of interest as determined by biological network analysis are indicated. FC, fold change.



**Figure 2. Hierarchical Clustering of Differentially Regulated Proteins Demonstrates Segregation of the VUR and Control Cohorts.** Eighty proteins met criteria of corrected q-value  $FDR < 7.5\%$  and  $|FC| \geq 1.2$ . Hierarchical clustering utilized the normalized SC for these proteins in each sample, with color intensity based on SC value. The resulting dendrograms demonstrated segregation and clustering for the majority of the VUR and control samples (horizontal axis). The candidate markers also clustered and segregated based on whether they were up- or down-regulated in the VUR cohort (vertical axis).



**Figure 3. Functional Characterization of VUR-regulated Proteins.** (A) Overrepresented biological pathways from all regulated proteins in the urine of the VUR patients. Percentage of proteins indicates the VUR-altered proteins over the entire proteins in the pathway (bars), while significance of the enrichment is represented by the  $p$ -value (enrichment and reference 0.05  $p$ -value). (B) Representative molecular function was separately assigned to the urinary proteins that were up- (green) or downregulated (red). Asterix represents  $p$ -value  $< 0.05$ . Functional enrichment was performed via FunRich version 3.0. ECM, extracellular matrix; n.s., not significant; VUR, vesicoureteral reflux.



**Figure 4. ELISA Quantification of ORM1 in Validation Cohort.**

A validation cohort consisting of VUR (n=20) and control (n=20) samples separate from the discovery cohort were tested. Mean levels with standard error are shown, demonstrating a statistically significant elevation of ORM1 levels in the VUR cohort by Mann-Whitney-Wilcoxon test. ORM, orosomucoid; VUR, vesicoureteral reflux.