

# Proteomic Analysis in Pediatric Renal Disease

Avram Z. Traum, MD,\*<sup>‡</sup> and Asher D. Schachter, MD, MMSc, MS<sup>†,‡</sup>

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**Summary:** Children with renal disease have tremendous potential for recovery, particularly when disease processes are detected early in the disease course. However, invasive diagnostic maneuvers can be challenging, especially in younger children who may require general anesthesia. Urine proteomics technologies present an opportunity to discover noninvasive yet informative diagnostic and prognostic markers of renal disease in children. In this article we review current concepts regarding the normal urine proteome, followed by an overview of urine proteomics as applied to nephrotic syndrome, and conclude with a discussion of some of the challenges of performing proteomic profiling on nephrotic urine, with its inherent abundance of proteins.

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The pediatric population presents several challenges to clinicians and investigators. Pediatric renal disease comprises a diverse range of disorders that present as a limited number of often-subtle clinical scenarios. Moreover, there is a valuable window of opportunity to prevent or even reverse renal pathophysiology to a greater extent than possible with adult renal disease patients. However, children are less amenable to multiple invasive diagnostic procedures. Smaller children in particular may require general anesthesia and intubation for imaging or biopsy procedures, for which older children and adults could tolerate mild sedation. Even routine blood work, although performed daily at children's hospitals, can be upsetting to a younger child. Therefore, noninvasive biomarkers, especially urine markers, are extremely desirable in the field of pediatrics

and particularly in nephrology where much can be learned from molecular profiling of urine.

In this article we review the current state of the art of urine proteomics, beginning with a focus on what may be considered the normal urine proteome, followed by a review of urine proteomic profiling as applied to pediatric nephrotic syndrome (NS). We conclude with a discussion of some of the challenges and limitations inherent in the use of nephrotic urine for protein biomarker discovery. The application of urine proteomics to renal transplant rejection is reviewed elsewhere.<sup>1</sup> Dr. Devarajan's article in this issue of *Seminars in Nephrology* addresses urine proteomic markers of acute kidney injury.

## THE URINE PROTEOME

Large-scale genomics and proteomics investigations that aim to distinguish disease state from healthy require clear and distinctly defined control groups. In the proteomics domain, the definition of the *normal proteome* is elusive because of the dynamic nature of protein expression even in healthy individuals. However, the normal range is known for many serum proteins such as albumin, immunoglobulins, protein hormones, and binding proteins. The definition of the normal urine proteome is

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\*Pediatric Nephrology Unit, Massachusetts General Hospital, Boston, MA.

†Division of Nephrology, Children's Hospital Boston, Children's Hospital Informatics Program at Harvard-MIT Division of Health Sciences and Technology, Boston, MA.

‡Harvard Medical School, Boston, MA.

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Address reprint requests to Avram Traum, MD, Massachusetts General Hospital, Pediatric Nephrology Unit, 55 Fruit St, Yawkey 6C, Boston, MA 02114. E-mail: atraum@partners.org

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a bigger challenge. Urine proteins reflect the processes that maintain plasma protein homeostasis within very narrow ranges. Appropriate renal responses to changes in the circulation may require excretion and/or reabsorption of large amounts of a given set of proteins. Therefore, the urine proteome may be defined more appropriately as *appropriate* versus *inappropriate* for a given set of normal states or variables such as volume status, blood pressure, body mass index, sex, physical activity, posture (upright versus recumbent), and even the time of day. Complicating the construction of a benchmark data set of control urine or serum samples is the inability to define the healthy state with certainty. An individual who is clinically healthy at the time of sample collection may have early disease processes that are observable only at some molecular level.

Several groups have attempted to delineate the normal human urine proteome by characterizing all proteins present in urine from healthy individuals by using the following: (1) an undisclosed number of samples of "normal male urine from a commercial pooled source,"<sup>2,3</sup> or (2) urine from an undisclosed number of healthy volunteers,<sup>4</sup> or (3) urine from 20 young, healthy male and female subjects,<sup>5</sup> or (4) multiple samples of urine from a healthy male volunteer and a healthy female volunteer.<sup>6</sup> The number of proteins identified in these studies ranged from 47 to 150. Weissinger et al<sup>7</sup> profiled spot urine samples from 57 healthy individuals and from 34 individuals with various nephrotic glomerulopathies (including 16 with minimal change disease [MCD] and 10 with focal segmental glomerulosclerosis [FSGS]) and found 173 polypeptides present in 90% of the healthy control samples. By using a combination of 7 surfaced-enhanced laser desorption/ionization (SELDI) chip types to analyze a urine sample from a single healthy volunteer, Rolofsen et al<sup>8</sup> were able to detect a total of 425 peaks, of which 136 were unique peaks.

The findings from the studies summarized here are intriguing because the majority of filtered proteins in the normal kidney are processed within the renal parenchyma and largely are reabsorbed. Urine proteins undergo intrarenal processing, which can result in urine pro-

tein profiles very different from those seen in serum. However, it is now almost certain that normal urine likely contains more protein information than was believed previously, although much remains unknown about these normal urine profiles. Notably, several of these studies used pooled samples and therefore were not able to determine interindividual protein variations within control subjects. As well, none of these studies accounted for important baseline physiologic, anthropomorphic, and environmental states that can affect urinary protein excretion. It also is impossible to determine which subjects who are healthy at the time of evaluation will develop proteinuric renal disease in the future.

### PODOCYTE PROTEOMICS AND STEROID THERAPY

Ransom et al<sup>9</sup> reported the results of differential proteomic analysis of proteins induced by steroid therapy in cultured murine podocytes. They reported 6 proteins that show differential expression in vitro in response to steroid treatment in comparison with vehicle-treated controls. Five of the proteins showed increased expression in the steroid-treated group, and the investigators were able to use Western blots to confirm their findings in 2 of the 5 up-regulated proteins: ciliary neurotrophic factor (CNTF) and  $\alpha$  B crystallin. However, they acknowledged that their disease model was limited by the challenges involved in isolating significant numbers of murine podocytes for primary culture. Furthermore, there is as yet no known animal model for many forms of NS, and even cultured human podocytes do not reliably form slit diaphragms in vitro. The difficulty in obtaining sufficient numbers of podocytes from mice underscores the difficulty inherent in obtaining sufficient sample material from patients, and underscores the value of a noninvasive source, such as urine.

### PROTEOMICS IN IDIOPATHIC NS

The selectivity index is based on the findings of more selective proteinuria in steroid-sensitive nephrotic syndrome (SSNS), although some of the steroid-resistant nephrotic syndrome (SRNS) pa-

tients show generalized proteinuria. Ramjee et al<sup>10</sup> studied 57 children with NS and compared the selectivity index with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) to distinguish low-molecular-weight (LMW) from high-molecular-weight proteinuria. Although those with selective proteinuria all had SSNS, nonselective proteinuria was found in both groups. In contrast, SDS-PAGE and IEF correctly categorized all subjects into SSNS and SRNS/FSGS based on the selectivity of proteins seen on the gels. The positive and negative predictive values for SDS-PAGE and IEF were 100% for both. Although this study is exciting in its application of a noninvasive method of distinguishing these 2 groups, no training or test sets were used in these patients to apply this model to a larger cohort. Furthermore, no information was provided on the viral status in this cohort of African children, which may lead to secondary forms of SRNS/FSGS.

Varghese et al<sup>11</sup> recently reported that 2-dimensional gel electrophoresis and mass spectrometry (MS) analysis of urine may distinguish patients with varied causes of primary and secondary nephrotic syndrome. Two recent reports have addressed using proteomics to distinguish SSNS and SRNS, both using SELDI-time of flight (TOF) MS.<sup>12,13</sup> Woroniecki et al<sup>13</sup> collected urine samples from a cross-section of 25 children with NS referred for biopsy, all of whom previously had been treated with steroids. Seventeen control samples also were used and included healthy children, and 3 each with asthma and orthostatic proteinuria. SSNS patients were in remission at the time of sample collection; some had immune complex-mediated forms of NS, such as membranoproliferative glomerulonephritis and immunoglobulin A nephropathy. Samples were depleted of albumin and spotted in duplicate onto 4 chip types (Q10, CM10, H50, and IMAC30). Spectra were analyzed using 2 approaches within a proprietary package (Biomarker Pattern Software; Ciphergen, Fremont, CA). First, a tree-based classification algorithm was applied based on the highest intensity peaks, followed by a boosting algorithm, by categorizing the strength of various peaks as classifiers. This approach identi-

fied a single mass-to-charge ( $m/z$ ) peak at 4144 on the Q10 chip as the strongest predictor of SRNS, followed by a series of other peaks on the CM10 chip. Unfortunately, none of these peaks were identified further, but the approach was successful in distinguishing the 2 groups.

We recently reported a similar experience in a cross-section of children with idiopathic NS.<sup>12</sup> A total of 44 children were recruited including 14 with SSNS in relapse, 5 with SRNS in relapse, 19 with SSNS in remission, and 6 with orthostatic proteinuria. No depletion protocol was used, and SELDI-TOF MS was used on CM10 and IMAC30 chips with urine samples spotted in duplicate. We then used 2 separate bioinformatics algorithms tailored for this approach. The first technique began with a noise reduction algorithm that determined the nadir between the bimodal distributions of  $m/z$  intensity values of each spectrum and defined the  $m/z$  values below the nadir as noise whereas those above were defined as peaks. This removed potential bias of applying a random signal-to-noise ratio, and used each spot's distribution of  $m/z$  values to determine a distinct, data-driven threshold for each spot. Once we had defined peaks, we then subtracted the peaks found in subjects with SSNS in remission and in subjects with orthostatic proteinuria with the rationale that proteins found in the urine of subjects in those 2 groups were passively filtered and not related to glomerular disease. Finally, we used a genetic algorithm to search for the group of up to 10 peaks that distinguished between SSNS and SRNS with an accuracy of 95% or greater. After 2,000 generations, the genetic algorithm identified a single peak at an  $m/z$  of 11,117.4 that distinguished the 2 groups.

Because any threshold-based algorithm that forces discrete value from continuous data, such as our noise-reduction algorithm, risks losing low-intensity but potentially biologically important information, we used principal component analysis on the normalized, nondiscrete spectra from the relapsed groups. A genetic algorithm then was used to identify the peaks that provided the maximal separation between SSNS and SRNS in the top 3 components (dimensions) of principal component space. Five peaks were identified that included 11,117.4.

Because this peak was found by both methods, it was identified using a series of fractionation steps followed by MALDI-TOF/TOF MS as  $\beta_2$ -microglobulin, and validated by immunodepletion using a monoclonal antibody against  $\beta_2$ -microglobulin. The peak at  $m/z$  11,117.4 likely was not intact  $\beta_2$ -microglobulin, but rather a nontryptic cleavage product caused by tubular injury from chronic kidney disease in the SRNS subjects. Nonetheless, these last 2 studies showed that even lower resolution approaches such as SELDI can be useful for distinguishing SSNS from SRNS. We recently completed collecting urine and blood samples as part of a prospective study of a cohort of children with newly diagnosed NS, and we currently are performing MS/MS analyses to identify urinary and circulating predictive markers of steroid responsiveness.

Candiano et al<sup>14</sup> studied urine protein patterns from 10 children with SSNS and MCD, 7 children with SRNS and SRNS/FSGS, and 6 adolescents and adults with membranous nephropathy. By using 2-dimensional gel electrophoresis, followed by MALDI-TOF MS and peptide mass fingerprinting, they identified fragments of albumin and  $\alpha_1$ -antitrypsin not seen in urine from healthy controls. Interestingly, some of these fragments also were found in plasma, suggesting that fragmentation is not merely owing to urinary proteases. However, they do not distinguish clearly between the categories of NS in this study.

## TECHNICAL ISSUES OF NEPHROTIC URINE

A main limitation imposed by the use of urine is the harsh environment (mostly owing to the extreme range of pH values and concentration of solutes found in normal urine), which likely alters excreted protein characteristics. For example, high-abundance urinary substances such as urea, uric acid, and ammonium can modify proteins in ways and to an extent not seen in the serum. Urea can induce carbamylation,<sup>15,16</sup> phosphorylation,<sup>17</sup> and methylation<sup>18</sup> of specific amino acid residues. Urate may protect against oxidation of lipoproteins, depending on the concurrent concentration of copper,  $\text{Cu}^{2+}$ .<sup>19,20</sup> Ammonia and ammonium are involved in modulating phosphorylation,<sup>21,22</sup> nitration,<sup>22</sup> and oxidation.<sup>23-25</sup> The use of serum-based proteomic methods to profile

urine proteins requires innovative adjustments that take into account the harsh urinary environment.

There is little published about the protein content of nephrotic urine. The protein content of plasma and serum has been studied extensively, with the albumin constituting approximately half of the plasma proteome. The next most abundant proteins make up another 40% of plasma proteins, whereas another 12 proteins constitute the next 9%. Thus, 22 proteins make up 99% of the plasma proteome, whereas the remaining 1% constitutes low-abundance proteins that are a potential rich source of biomarkers. A number of approaches have been published to mine these low-abundance proteins. The most commonly used method makes use of columns that deplete high-abundance proteins, such as albumin, immunoglobulin G, transferrin, and others. This approach has gained wide acceptance in plasma proteomics and is highly effective at depletion of high-abundance proteins. However, others have raised concerns about the loss of data that then occurs. Many low-abundance proteins are also of LMW and travel in plasma bound to carrier proteins such as albumin. Thus, depletion methods risk losing these LMW proteins and, along with them, potential biomarkers. A more recent approach successfully has used centrifugal ultrafiltration to enrich the LMW plasma proteome.

We have applied some of these approaches to the study of nephrotic urine. We first performed liquid chromatography (LC)-MS/MS on unfractionated nephrotic urine from 1 patient each with SSNS and SRNS. The proteins with the top 10 highest scores are displayed in Table 1, which shows that nephrotic urine mirrors the protein content of plasma. We then used the approach of Sutton using molecular weight cut-off filters of 30 kd with centrifugal ultrafiltration.<sup>26</sup> The flow-throughs then were subjected to trypsin digestion and LC-MS/MS. Unfortunately, the chromatograms were plagued by contamination with polyethylene glycol (PEG), as manifested by multiple peaks separated by 22 d on multiple scans. We suspected that the source of contamination was from the original urine containers used in the clinic setting be-

**Table 1.** Proteins Identified in Unfractionated Nephrotic Urines in Relapse

N	Steroid Sensitive		Steroid Resistant	
	Score	Protein Name	Score	Protein Name
1	43.06	Albumin	72.89	Albumin
2	37.04	Transferrin	29.05	Transferrin
3	36.57	Ceruloplasmin	15.32	$\alpha$ -1-antitrypsin
4	32.36	$\alpha$ -1-antitrypsin	14.65	Haptoglobin
5	19.38	$\alpha$ -1-antichymotrypsin	13.07	Complement C3
6	14.96	$\alpha$ -1B-glycoprotein	10.21	Apolipoprotein A-I
7	12.37	$\alpha$ -2-glycoprotein 1, zinc	10.2	Immunoglobulin gamma-1 chain C region
8	10.75	Haptoglobin	9.45	Orosomuroid 1
9	8.49	Uromodulin	7.91	$\alpha$ -1B-glycoprotein
10	8.43	Thyroxine-binding globulin	6.85	Ceruloplasmin

NOTE. Proteins shown with top 10 scores from each sample.

fore transfer to MS-friendly cryotubes. This problem would not necessarily have manifested in plasma studies because blood is collected in glass tubes, in which PEG is not a concern.

We next focused on removing the contaminating PEG. We believed that dialysis of the samples would have given inconsistent removal and also was counter to our goal of developing a high-throughput to working with nephrotic urine. Next, we performed 1-dimensional gel electrophoresis on nephrotic urine samples that were controlled for protein mass. Gel lanes were cut below albumin and designated the LMW fraction. After in-gel tryptic digestion, peptides were extracted and underwent LC-MS/MS. The resulting spectra were free of PEG contamination and gave interpretable results with Mascot (Matrix Science, London, England).

## SUMMARY AND FUTURE DIRECTIONS

The noninvasive yet informative nature of urine proteomic profiling suggests that this approach to biomarker discovery will continue to be developed, particularly for diseases that have a relatively large impact in children, such as NS. One important remaining challenge is the establishment of publicly available benchmark data sets for normal or control urine proteomic profiles, preferably including extensive clinical data to allow detailed and comprehensive characterization of the urinary proteome. The high albu-

min content in nephrotic urine is a hurdle that likely will be resolved in the near future.

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