Chapter 15
The Application of Urinary Proteomics for the Detection of Biomarkers of Kidney Diseases

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Abstract Urine is a biological material that can be easily obtained in the clinic. The identification of proteins excreted in urine provides useful biological information about the kidney as well as a unique opportunity to examine physiological and pathological changes in the kidney in a noninvasive manner. Recent technological advances in urinary proteomic profiling have provided the foundation for a number of urinary proteomic studies directed at identifying markers of kidney disease diagnosis, prognosis, or responsiveness to therapy. In this review, we describe the strengths of different urinary proteomic methods for the discovery of potential biomarkers of kidney diseases. We also highlight the limitations and future goals of these approaches.

Keywords Urinary proteomics · Biomarker · Kidney diseases

15.1 The Urinary Protein Profile

Under normal physiological conditions, a person's daily urine output contains <20 mg of albumin and <150 mg of total protein. Approximately 30 % of this protein content is derived from plasma, whereas 70 % is produced by the kidney and the lower urinary tract [32]. Normal urine contains at least 1,500 proteins, most of which are extracellular and membrane bound [1]. To be present in the urine, proteins or their fragments must pass through filters at the glomerulus and bypass or otherwise avoid tubular reabsorption. Alternatively, proteins can be secreted by the kidney or lower urinary tract directly into urine. During plasma filtration in the glomeruli, the glomerular capillary walls discriminate among molecules of different sizes, charges, and configurations. The glomerular basement membrane and the slit diaphragm of the filtration barrier limit the passage of macromolecules containing negatively charged glycosaminoglycans. Small, positively charged molecules could be filtrated

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in different ways. Typically, proteins <20 kDa can move freely, whereas proteins >60 kDa are almost completely restricted in their movement between compartments. Despite this filtering, the most abundant urine protein is albumin, a negatively charged molecule with a molecular weight of approximately 66.4 kDa. The relative abundance of albumin in the urine may be due to the presence of large pores through which albumin, immunoglobulins, and other macromolecules can pass [11].

Injury to any of the filtration barrier structures results in the leakage of large, negatively charged proteins, thereby increasing the presence of these proteins in urine; for example, in diabetic nephropathy (DN) and focal and segmental glomerulosclerosis (FSGS) [39], tubules reabsorb most of the filtered proteins. Proximal tubules also catabolize proteins and excrete the resulting peptides into the urine. Tubules secrete proteins directly into urine during normal maintenance processes or in response to injury. Tubular injury may result in the decreased reabsorption or catabolism of the filtered proteins and in the secretion of tubular proteins in response to the injury. In addition to soluble proteins and their peptides, urine also contains exosomes, which are specialized vesicles that are shed by the renal epithelia directly into the urine [21, 33]. The distal organs of the lower urinary tract also contribute to the urinary proteome.

15.2 Urinary Proteomics Approaches

Typically, proteomic biomarker studies consist of 2 main stages: a discovery phase and a validation phase. The discovery phase can be divided into 3 main steps: (1) sample preparation, (2) mass spectrometry analysis, and (3) data analysis. In the discovery phase, a variety of proteomic methods have been used to identify biomarkers of kidney disease, including liquid chromatography mass spectrometry (LC-MS), two-dimensional electrophoresis mass spectrometry (2DE-MS), surface-enhanced laser desorption ionization mass spectrometry (SELDI-MS), and capillary electrophoresis combined mass spectrometry (CE-MS) [14, 23, 34, 36]. Traditional biochemical methods such as enzyme-linked immunosorbent assays (ELISAs) and Western blotting (WB) are widely used in the validation stage.

15.3 The Study of Kidney Disease Through Urinary Proteomics

15.3.1 Diabetic Nephropathy

DN is a complication of diabetes that affects up to 40 % of patients with diabetes. In the Western world, DN is the leading cause of end-stage renal disease (ESRD) [37]. Given the increasing incidence of diabetes [50], DN has already become a major cause of chronic kidney disease in China.

Microalbuminuria (MA) was wildly used as an early diagnostic marker of DN. However, long-term longitudinal studies have shown that only a subset of patients with MA progress to proteinuria [13, 28, 29]; indeed, many individuals with Type 1 diabetes have already experienced early renal function decline before or coincidental with the onset of MA [30, 31]. These data suggest that MA may be an inadequate early diagnostic biomarker of DN, spurring an intense search for new biomarkers of DN using proteomic techniques.

To identify more sensitive and specific biomarkers of DN Rossing et al. [38], designed a proteomic panel capable of distinguishing diabetes from DN in 305 individuals using CE-MS. The sensitivity and specificity of this panel was 97 %. Further study has shown that this panel has predictive value for the progression of MA toward overt DN over a 3-year follow-up period. To validate this result, a multicenter study involving 145 patients with Type 2 diabetes was initiated [2]. In this independent cohort, the diagnostic panel for DN displayed 93.8 % sensitivity and 91.4 % specificity, with an AUC of 0.948. Statistical analysis demonstrated that the DN diagnostic model score was well correlated with clinical parameters such as presence of albuminuria and the estimated glomerular filtration rate (GFR).

To further explore the underlying pathogenesis of renal function decline in DN with MA proteinuria and to identify a discriminating biomarker, Merchant et al. [23] used LC-MALDI-TOF-MS to analyze the urinary peptidome of a long-term longitudinal DN cohort with MA. In the urine of patients with early renal function decline, 3 peptides with decreased content and 3 peptides with increased content were identified. Of the 3 peptides with increased content, high levels of 2 were observed in renal biopsy tissue from Type I diabetes patients suffering from early nephropathy. This result indicates that these peptides have potential for use as early diagnostic biomarkers, although their sensitivity and specificity remain to be validated in clinical practice.

ITRAQ-labeled LC-MS has recently become a popular proteomic technology. This method was used to identify urinary proteomic biomarkers that may enable the diagnosis of DN in a group of Type 2 diabetes patients with or without MA [18]. Some differentially excreted proteins were verified by multi-reaction mass spectrometry (MRM) analysis of urine collected from 9 individual normoalbuminuric and 14 individual microalbuminuric patients. α-1-Antitrypsin, α-1-acid glycoprotein 1, and prostate stem cell antigen all yielded excellent AUC values (0.849, 0.873, and 0.825, respectively).

15.3.2 IgA Nephropathy

IgA nephropathy (IgAN) is the most common glomerular disease worldwide. The prevalence of this disease is highest in Asian populations, intermediate in European populations, and lowest in African populations. The clinical presentation of IgAN is variable and includes isolated hematuria, rapidly progressive loss of renal function, or full nephrotic syndrome. Similarly, the histological features of IgAN range from

mesangial proliferation to glomerular extracapillary proliferation with crescent formation. In current clinical practice, the clinical and morphological features of IgAN are inadequate to precisely classify its molecular mechanisms or predict the disease outcome or responsiveness to therapeutic intervention.

Several studies have examined the urinary proteome to explore novel biomarkers. He's group identified a panel of 10 urinary proteins (of which 8 were upregulated and 2 were downregulated); the expression of which differed in patients with IgAN and healthy individuals. Moreover, this panel distinguished patients with severe IgAN from those with mild IgAN with 90.48 % sensitivity and 96.77 % specificity [15]. Brigitte et al. analyzed the urinary proteomes of patients with IgAN and healthy individuals using 2 DE-MS and demonstrated that the laminin G-like 3 (LG3) fragment of endorepellin was decreased in the IgAN group [41]. This finding was subsequently validated in 43 IgAN patients and their corresponding controls by ELISA. Statistical analysis indicated a significant inverse correlation between LG3 levels and the glomerular filtration rate of IgAN that was not observed in 65 patients with other glomerular diseases. These results suggest that the LG3 fragment of endorepellin is a potential biomarker of IgAN severity.

Distinct urinary protein profiles distinguishing healthy individuals and patients with IgAN have also been identified [27, 52], although these findings have not been applied in a clinical setting to confirm the clinical utility of urinary protein profiling.

Urinary proteomic methods have also yielded potential predictive markers of the response of IgAN to intervention. For example, the urinary proteomic profile of patients with IgAN predicted their response to angiotensin-converting enzyme (ACE) inhibitors and urine levels of kininogen-1, inter-α-trypsin inhibitor heavy chain H4, and transthyretin differed significantly between ACE inhibitor therapy responders and nonresponders [36]. Very low urinary levels of kininogen-1 were correlated with a poor response to this treatment. Studies with large sample sizes will be needed to evaluate the clinical applicability of these urinary protein markers.

15.3.3 Membranous Nephropathy

Membranous nephropathy (MN) is a common type of primary glomerulonephritis in North America, Europe, and Asia. In severe cases, MN can produce ESRD [22]. This antibody-mediated autoimmune glomerular disease is characterized by the presence of immune deposits on the epithelial side of the glomerular capillary wall. Our understanding of the pathogenesis of membranous nephropathy is mostly derived from studies in rats with passive Heymann nephritis (PHN), a glomerular disease that closely resembles human membranous nephropathy. In rats, PHN can be induced by a single injection of heterologous antiserum or IgG against renal tubular cell antigens [19].

The discovery of the anti-phospholipase A2 receptor (PLA2R) antibody greatly improved our understanding of the molecular mechanisms of MN. Serum levels of the anti-PLA2R antibody are used to guide diagnosis, monitor disease activity, and

assess the response to treatment in patients with membranous nephropathy [5, 6, 35]. However, PLA2R is inadequate for the management of these patients, and nephrologists are seeking to identify additional biomarkers with clinical utility [8].

A serial analysis of the urinary proteomic profile of rats based on urine samples collected at days 0, 10, 20, 30, 40, and 50 after PHN induction [26] revealed that 37 proteins were differentially expressed across these time points. The differentially expressed proteins were classified into several categories: proteins that decreased after PHN induction; proteins that increased after PHN induction; proteins that increased during the early phases of PHN but returned to basal levels in later phases; proteins that were undetectable during PHN; and proteins that were detectable only during PHN. Most of the differentially expressed proteins are related to signaling pathways, protein trafficking, and the regulation of glomerular permeability.

Urinary proteomics studies addressing MN are rare due to limitations in the technology used to detect protein profiles in mass proteinuria. However, kidney and podocyte proteomic studies of human MN are ongoing [47]. Comparative studies of kidney or podocyte proteomes and urinary proteomes will likely represent a breakthrough in this field.

15.3.4 Focal Segmental Glomeruloscelerosis

Focal segmental glomeruloscelerosis (FSGS) is a major cause of proteinuria and renal failure [20]. This disease comprises a number of clinical and pathological syndromes that share a common glomerular lesion, including primary (or idiopathic) FSGS, secondary FSGS (mediated by glomerular hypertension and hyperfiltration), and genetic, virus-associated, and drug-induced forms of the disease [10]. Histologically, FSGS is classified into several subtypes, including tip variant, perihilar variant, cellular variant, collapsing variant, and "not otherwise specified" FSGS [9, 43].

The critical clinical feature of FSGS is proteinuria. To distinguish FSGS-induced proteinuria from other proteinuria diseases based on proteins present in the urine, Sanju et al. [44] used 2-DE to compare urine samples from 32 patients with proteinuria-causing diseases including FSGS, lupus nephritis (LN), MN, and DN. Differentiated spots from 16 patients were used to train an artificial neural network to create a prediction model, which then was validated in the remaining 16 patients. The model achieved sensitivities of between 75 and 86 % and specificities of between 67 and 92 %.

Glucocorticoids are the main intervention for FSGS; however, not all patients respond to glucocorticoid treatment. Nuntawan et al. compared the urinary proteomic profile of steroid-resistant nephrotic syndrome (SRNS) with that of steroid-sensitive nephrotic syndrome (SSNS) using SELDI-TOF-MS [34]. A 13.8-kDa-fragment of α-1-β glycoprotein was significantly differentially excreted between these 2 groups. The results of the validation study demonstrated that this peptide was present in 7 of

the 19 SRNS patients but absent in all SSNS patients ($n = 15$) and controls ($n = 10$). The detection of this small molecular fragment in the urine may help nephrologists make better choices in the future treatment of FSGS patients.

15.3.5 Lupus Nephritis

LN is a common complication of systemic lupus erythematosus (SLE). In SLE, renal involvement occurs in between 15 and 75 % of patients; histological evidence of renal involvement is found in most biopsy specimens [7]. Proteomics approaches have been employed to explore noninvasive predictors of the impending relapse, relapse severity, and recovery from LN.

Zhang et al. [53] profiled the urinary proteome of LN patients in different stages of relapse using a 30-kDa cutoff filter to focus on low molecular weight proteins. Among the 27 proteins that were differentially expressed between flare intervals, 2 isoforms of hepcidin were able to predict flare onset and recovery. However, further research indicated that hepcidin was not disease specific or associated with inflammation. Somparn et al. [40] used 2-DE to profile urine samples from 5 active and 5 inactive LN patients. Two differentially excreted proteins (ZA2G and PGDS) were validated by ELISA in samples from an independent set of 78 subjects, including 30 active LN cases, 26 inactive LN cases, and 14 non-LN glomerular disease cases. ZA2G levels were elevated in the urine of patients with active LN and non-LN glomerular diseases, whereas PGDS levels were elevated only in urine from the active LN group. Urinary PGDS, not ZA2G, may thus serve as a biomarker for active LN.

In another study of the urinary proteome of children with LN [42], investigators used SELDI-TOF-MS to identify 8 peaks that differentiated patients with active nephritis from remitters and controls. These peaks had an area under the AUC of ≥0.9 for the diagnosis of active nephritis; thus, this approach appears promising for this particular group of patients.

Wu et al. [48] screened the levels of ~280 molecules in urine samples from 3 healthy individuals and 5 patients with SLE. Elevated angiostatin levels were observed and validated in an independent cohort of SLE patients ($n = 100$) by ELISA. Urine angiostatin was significantly increased in active SLE compared to inactive SLE, as was further confirmed by an ROC curve analysis with an AUC value of 0.83. However, correlation analysis of the urine angiostatin levels and renal morphological changes indicated that urine angiostatin was strongly associated with the renal pathology chronicity index but not with the activity index.

These urinary proteomics studies have revealed the potential of a urine protein panel as a noninvasive biomarker panel for distinguishing the disease activity of LN. However, the specificity and sensitivity of these markers remain inferior to that of traditional markers (such as complementary levels) and require further study, optimization, and modification.

Table 15.1 lists some urinary proteomics studies in chronic kidney diseases.

Table 15.1 Urinary proteomics studies in chronic kidney diseases

Authors	Type of disease	Participants	Method	Identified proteins
Rossing et al. [38]	DN	305 individuals	CE-MS	A model that included 65 regulated genes correctly identified diabetic nephropathy with 97 % sensitivity and specificity
Alkhalaf et al. [2]	DN	148 DM patients with albuminuria 83 DM patients without albuminruai	CE-MS	The "DN model"[a] for DN showed 93.8 % sensitivity and 91.4 % specificity, with an AUC of 0.948 (95 % CI 0.898–0.978)
Jin et al. [18]	DN	43 diabetes patients with microalbuminuria 43 diabetes patients without microalbuminuria	iTRAQ and 2DE/ Western blot/MRM	alpha-1-antitrypsin, alpha-1-acid glycoprotein 1 precursor, and prostate stem cell antigen, which had AUC values >0.8, are good biomarker candidates, and the AUC value was improved to 0.921 on combining the 3 proteins
Park et al. [27]	IgAN	13 patients with IgAN 12 healthy controls	2-GE	59 proteins were differentially expressed
Yokota et al. [52]	IgAN	17 patients with IgAN 10 healthy controls	2-D DIGE	10 proteins (albumin, transferrin, α1-antitrypsin, β-globin, α1-globin, carbonic anhydrase I, cystatin C, retinol-binding protein 4 and 1-microglobulin) were differentially expressed[b]
He et al. [15]	IgAN	56 patients with IgAN[c] 14 healthy controls	MALDI-TOF-MS	21 peaks distinguished mild and severe groups[d] 50 peaks distinguished mild and normal groups[e] 50 peaks distinguished severe and normal groups[f]

(continued)

Table 15.1 (continued)

Authors	Type of disease	Participants	Method	Identified proteins
Rocchetti et al. [36]	IgAN	18 patients with IgAN 20 healthy controls	2-D PAGE and nano-HPLC-ESI-MS/MS	Among patients with IgAN, kininogen, ITI-HC1 and transthyretin levels were different in responders and nonresponders to ACE inhibitors Low levels of urine kininogen predicted inadequate or absent clinical response to ACE inhibitors in 20 patients with biopsy-proven IgAN
Ngai et al. [26]	MN	Control rats Rats with PHN assessed at postinduction days 0,10, 20, 30, 40 and 50[g]	2D-PAGE	37 differentially expressed proteins across all time points
Piyaphanee et al. [2]	SRNS	19 SRNS 15 SSNS 10 controls	SELDI-TOF-MS	The α1-B glycoprotein was only present in 7 of 19 patients with SRNS; but absent in all SSNS and controls and associated with lower GFR.
Varghese et al. [44]	FSGS	32 patients with FSGS, LN, MN, or DN	2-DE	The urine proteins panel could distinguish different proteinuria diseases with sensitivity ranged from 75 to 86 %, and specificity ranged from 92 to 67 %
Zhang et al. [53]	LN	5 class III LN patients 11 class IV LN patients 3 class V LN patients	SELDI-TOF MS	27 protein irons showed significant differential expression between specific flare intervals of LN
Somparn et al. [40]	LN	5 active LN patients 5 inactive LN patients	2-DE	prostaglandin H 2 D-isomerase was only elevated only in the urine of the active LN group

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Table 15.1 (continued)

Authors	Type of disease	Participants	Method	Identified proteins
Suzuki et al. [42]	LN	32 pediatric LN patients 11 juvenile idiopathic arthritis patients as control	SELDI-TOF-MS	8 proteins with peaks at m/z of 2.7, 22, 23, 44, 56, 79, 100, and 133 kDa were changed in the LN patients compared with non-LN patients

[a] The DN module contains 65 genes from Rossing et al. [38] study
[b] All except 1-microglobulin were higher in patients with IgAN than controls
[c] Of whom 23 had a severe and 33 had a mild presentation
[d] For a subgroup of 10 peaks selected as biomarkers, sensitivity was 90.48 % and specificity 96.77 %
[e] For a subgroup of 10 peaks selected as biomarkers, sensitivity was 93.55 % and specificity 85.71 %
[f] For a subgroup of 20 peaks selected as biomarkers, sensitivity was 100 % and specificity 92.86 %
[g] 6 mice per group

Abbreviations *DM* diabetes mellitus; *DN* diabetic nephropathy; *iTRAQ* Isobaric tags for relative and absolute quantification; *MRM*, multiple reaction monitoring; *CE-MS* capillary electrophoresis coupled with mass spectrometry; *FSGS* focal segmental glomerulosclerosis; *MN* membranous nephropathy; *LN* lupus nephritis; *PHN* Heymann nephritis; *SRNS*, steroid-resistant nephrotic syndrome; *SSNS* steroid-sensitive nephrotic syndrome; *GFR* glomerular filtration rate; *ACE* angiotensin-converting enzyme; *DIGE* difference gel electrophoresis; *GE* gel electrophoresis; *HPLC-ESI* high performance liquid chromatography with electrospray ionization; *SELDI* surface-enhanced laser desorption/ionization; *IgAN* IgA nephropathy; *LC*, liquid chromatography; *MALDI-TOF* matrix-assisted laser desorption/ionization time-of-flight; *MS* mass spectrometry; *MS/MS* tandem mass spectrometry; and *PAGE*, polyacrylamide gel electrophoresis

15.3.6 Acute Kidney Injury

Acute kidney injury (AKI) represents a common and devastating problem in clinical medicine. The incidence of AKI varies from 5 % of hospitalized patients to 30–50 % of patients in intensive care units. Despite significant improvements in therapeutics, evidence suggests that the incidence of AKI is increasing at an alarming rate, and the associated mortality and morbidity have remained high despite improvements in clinical care [46, 49, 51]. A major reason for this high mortality and morbidity is the lack of early biomarkers for AKI, resulting in an unacceptable delay in the initiation of therapy. In addition, convenient biomarkers are urgently needed to distinguish between the various etiologies of AKI and to predict its clinical outcomes. Fortunately, the application of proteomics research to human and animal models of AKI has uncovered several novel biomarkers.

Significant efforts have been made to develop an early diagnostic biomarker for AKI in the hope that the early identification of renal injury will enable more effective therapeutic interventions. Ho et al. [16] used SELDI-TOF/MA to determine urinary proteomic profiles at different time points following coronary artery bypass grafting (CABG) operations. The active 25-amino acid form of hepcidin (hepcidin-25) was found to be dominantly elevated in postoperative non-AKI urine samples compared with AKI samples. This biomarker was further validated in an independent cohort of 338 patients [17]. The log10 hepcidin-25/Cr ratio reached a sensitivity of 68 % and a specificity of 68 %, with an AUC of 0.80 for the avoidance of AKI and a negative predictive value 0.96. Areeger et al. [3] collected urine samples from 36 patients after cardiopulmonary bypass surgery. They compared the urinary proteomes of patients with and without AKI on the first postoperative day. After the operation, inflammation-associated (zinc-α-2-glycoprotein, leucine-rich α-2-glycoprotein, mannan-binding lectin serine protease 2, basement membrane-specific heparan sulfate proteoglycan, and immunoglobulin kappa) or tubular dysfunction-associated (retinol-binding protein, adrenomedullin-binding protein, and uromodulin) proteins were found to be differentially regulated. Zinc-α-2-glycoprotein and a fragment of adrenomedullin-binding protein were decreased in patients with AKI. The decreased excretion of zinc-α-2-glycoprotein in patients with AKI was confirmed by Western blot and ELISA in an independent cohort of 22 patients with and 46 patients without AKI. Zinc-α-2-glycoprotein is thus a potentially useful predictive marker for AKI after cardiopulmonary bypass surgery.

In the last 10 years, urine neutrophil gelatinase-associated lipocalin (NGAL, also known as lcn2) has become one of the most important predictive biomarkers of AKI. NGAL is one of the earliest and most robustly induced proteins in the kidney after ischemic or nephrotoxic AKI in animal models. Indeed, the NGAL protein is easily detected in urine soon after AKI [24, 25, 45]. However, NGAL measurements may be influenced by a number of coexisting variables, such as preexisting renal disease and systemic or urinary tract infections [12]. Research to explore more accurate AKI predictive biomarkers is ongoing. Areeger et al. [4] collected urine on the first day of AKI in critically ill patients; 12 patients with an early recovery and

12 matching patients with late/non-recovery were selected, and their proteomes were analyzed by gel electrophoresis and mass spectrometry. A total of 8 prognostic candidates were identified. Subsequent ELISA quantification demonstrated that IGFBP-7 was the most potent predictor of renal recovery. IGFBP-7 and NGAL, a traditional AKI biomarker, were chosen for further analyses in an independent verification group of 28 patients with AKI and 12 control patients without AKI. The comparative analysis indicated that IGFBP-7 and NGAL were significantly upregulated in the urine of AKI patients, which in turn predicted the mortality (IGFBP-7: AUC 0.68; NGAL: AUC 0.81), recovery (IGFBP-7: AUC 0.74; NGAL: AUC 0.70), and severity (IGFBP-7: AUC 0.77; NGAL: AUC 0.69) of AKI. The levels of these proteins were also associated with AKI duration. IGFBP-7 was a more accurate predictor of renal outcome than NGAL. Thus, IGFBP-7 is a novel prognostic urinary marker that warrants further investigation.

Urinary proteomics provide a novel method for identifying early diagnostic and prognostic biomarkers of AKI. This technique can be integrated with and is complementary to traditional hypothesis-driven approaches. Moreover, this technique provides an additional armamentarium for discovery-based biomarker studies and can provide novel insights into the underlying pathophysiology of AKI, which may ultimately lead to the identification of novel therapeutic targets.

15.4 Limitations and Future Perspectives

Kidney disease has been the subject of a number of urinary proteomics studies. This research has greatly improved our understanding of the mechanisms of various kidney diseases and has provided alternative biomarkers for classification, diagnosis, and response prediction. However, several limitations have hampered the development of this approach and the translation of results to clinical applications.

First, there are challenges in the standardization of urine collection, preparation, and storage in urinary proteomics. The quality and quantity of urine proteins are affected by diet and exercise, and thus, sample collection under stable conditions is critical for the reliability and comparability of urinary proteomics results. Moreover, the storage, preparation, and analysis of urine samples may also affect the profiling. Standardization of these techniques is required to obtain more reliable proteomics data. Although an international normal urine collection protocol has been developed by the European Kidney and Urine Proteomics (EuroKUP) group and the Human Kidney and Urine Proteome Project (HKUPP) (http://www.hkupp.org), there are still no globally acceptable guidelines for urine sampling with mass proteinuria [23].

Second, compared with transcriptomic and genetic studies, urinary proteomic data sets for kidney diseases, particularly for glomerular diseases, are scarce, primarily due to the limited technology that is available for this type of study. Proteinuria is a common clinical manifestation of many kidney diseases, but severely high levels of urinary proteins complicate proteomic data collection. Thus, the technology required for pre-MS handling of samples is much more important for

proteinuria proteomics than for normal urine analysis. Unfortunately, the study of pre-MS handling for proteinuria proteomics has received much less attention than for serum proteomics. Strengthening efforts to improve pre-MS handling will benefit future biomarker discovery for kidney diseases.

Third, a lack of knowledge about the molecular mechanisms of kidney diseases poses a major challenge for detecting biomarkers through urinary proteomics. To date, most kidney diseases have been diagnosed by histological changes. Many kidney diseases, such as IgAN and FSGS, are molecularly heterogeneous diseases, which complicates the analysis of the primary data in urinary proteomics studies. Due to the biological variability and complex pathophysiology of kidney disease, urinary proteomics studies that have attempted to identify a single biomarker for kidney disease have all failed.

Furthermore, the sample size of most published studies has been small, which limits the data interpretation and predisposes the analyses to multiple testing biases. To organize a large-scale urinary proteomics study, the development of national and international consortia is required to promote strict disease classification criteria, clear criteria for the recruitment of patients into prospective cohorts, and standardized protocols for the collection of samples and detailed clinical data.

The ultimate aim of the field of urinary proteomics is to further characterize the molecular mechanisms underlying kidney diseases and to facilitate the development of improved biomarkers for the diagnosis and prediction of the therapeutic response of various kidney diseases. This is a systemic approach, and the collaborative efforts of a multidisciplinary team of physicians, molecular biologists, statisticians, and systems biologists with computer science and mathematics backgrounds are therefore needed.

There are >1,500 proteins in normal urine. Changes in these proteins reflect physiological and pathological changes in the kidney. While nephrologists have made excellent clinical diagnostic and prognostic use of albuminuria and many other urinary proteins, it is now time to delve much deeper into the urinary proteome to maximize its incredible diagnostic and prognostic potential.

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