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EDITORIAL

The Quest for Renal Disease Proteomic Signatures: Where Should We Look?

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Abstract Renal diseases are prevalent and important. However, despite significant strides in medicine, clinical nephrology still relies on nonspecific and inadequate markers such as serum creatinine and total urine protein for monitoring and diagnosis of renal disease. In case of glomerular renal diseases, biopsy is often necessary to establish the diagnosis. With new developments in proteomics technology, numerous studies have emerged, searching for better markers of kidney disease diagnosis and/or prognosis. Blood, urine, and renal biopsy tissue have been explored as potential sources of biomarkers. Some interesting individual or multiparametric biomarkers have been found; however, none have yet been validated or entered clinical practice. This review focuses on some studies of biomarkers of glomerular renal diseases, as well as addresses the question of which sample type(s) might be most promising in preliminary discovery phases of candidate proteins.

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Abbreviations

CE	Capillary electrophoresis
1D-SDS-PAGE	One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
2-DE	Two-dimensional electrophoresis
2DE-DIGE	Two-dimensional fluorescence difference gel electrophoresis
ESI	Electrospray ionization
FSGS	Focal segmental glomerulosclerosis
FT-ICR	Fourier transform ion cyclotron resonance
HPLC	High-performance liquid chromatography
IMN	Idiopathic membranous nephropathy
IT	Ion trap
MALDI	Matrix-assisted laser desorption/ionization
MCD	Minimal-change disease
MPGN-II	Membranoproliferative glomerulonephritis II
MS	Mass spectrometer
SELDI	Surface-enhanced laser desorption/ionization
SLE	Systemic lupus erythematosus
TOF	Time of flight
Q	Quadrupole

Introduction

Chronic kidney disease (CKD) is a worldwide health problem. Based on the data from the USA, the prevalence of end-stage renal disease increased from 86,354 in 1983 to 506,256 in 2006 [1]. The prevalence of CKD when using the

definition of glomerular filtration rate (GFR) <60 ml/min per 1.73 m^2 is 4.7% or 8.3 million in the USA [2]. Renal diseases are thus highly prevalent and important; however, there are few diagnostic markers of kidney disease, and in many cases one must rely on a renal biopsy, particularly in the case of glomerular renal diseases. While biopsy is considered the gold standard diagnostic test, it is associated with multiple problems. First and foremost, it carries risks to the patient including hemorrhage, pain, and even death [3, 4]. It is costly due to postbiopsy monitoring or additional procedures required if complications occur. Biopsy may be contraindicated in uncontrolled hypertension, coagulopathy, pregnancy, or in patients with a single kidney. Finally, biopsy may not be adequate or representative of the disease in the rest of the kidney. Furthermore, there are few clinical or biochemical parameters to guide treatment or to inform prognosis. Main markers used in clinical practice are serum creatinine and total urine protein. Serum creatinine is a late marker of renal injury and suffers from several other drawbacks including the following: it can be used to estimate GFR only in steady state, it varies with diet and muscle mass, and creatinine undergoes increased tubular secretion in advanced stages of kidney disease. Urine protein is one of the best predictors of progression of renal disease, but it is nonspecific, and the levels that predict increased risk are different in distinct diseases (e.g., 1 g/day of proteinuria in IgA nephropathy carries higher risk of progression than the same degree of proteinuria in membranous nephropathy) [5, 6]. In other words, novel markers of renal disease diagnosis or progression are desperately needed.

Biological fluids such as urine and blood, as well as renal tissues, contain thousands of proteins that undergo changes in response to disease. The emergence of proteomics (a large-scale study of proteins and their function and structure) and development of methods to simultaneously analyze hundreds or thousands of proteins have opened the door to potentially capturing these disease or state-specific changes. However, after several years of research and numerous papers in this field, only a few proteins have emerged as potential biomarkers, and none have influenced clinical practice. This review will highlight some basic concepts in mass spectrometry and describe some studies with particular focus on glomerular diseases, as well as tackle the question of which type of sample may be most promising in uncovering candidate biomarkers in kidney diseases.

Proteomics Techniques

Whichever sample type is analyzed, complex protein mixtures must be fractionated to increase detection of low-abundance proteins [7]. Protein separations can be achieved by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (1D-SDS-PAGE), two-dimensional electrophoresis (2-DE), or capillary electrophoresis (CE). 1D-SDS-PAGE separates proteins based on mass, CE separates them based on isoelectric point, and 2-DE separates them in two dimensions using both mass and isoelectric point. The resolution of 2-DE is poor. CE has a much higher resolution, being able to resolve 400–2,000 peptides per run. CE can be coupled to the MS and has been used extensively in proteomic studies of renal disease. Unfortunately, this technique misses small peptides [8]. A variation of two-dimensional separation has been developed which is called two-dimensional fluorescence difference gel electrophoresis (2DE-DIGE) and which relies on fluorescent dyes for more accurate quantification. Peptide separation can be achieved using high-performance liquid chromatography (HPLC), which can also be coupled directly to the mass spectrometer (MS). HPLC separates peptides based on charge and/or hydrophobicity.

Mass Spectrometry

A mass spectrometer contains an ion source, an analyzer, and a detector. An ionization source may be matrix-assisted laser desorption/ionization (MALDI), surface-enhanced laser desorption/ionization (SELDI), or electrospray ionization (ESI). MALDI and SELDI are soft ionization techniques used in mass spectrometry to analyze biomolecules that tend to be fragile and fragment when ionized by more conventional ionization methods. They require an organic matrix to allow analyte ionization by laser pulses. Mostly singly charged ions are generated in this way, and they are easier to work with, but both techniques have been criticized for lack of reproducibility and sensitivity to sample collection and processing procedures [9]. ESI and MALDI were awarded the Nobel Prize in Chemistry in 2002. In the case of ESI, the end of a liquid chromatography column or a metal needle is held at a high electrical potential (several kilovolts) with respect to the entrance of the mass spectrometer. The liquid effluent containing the peptides that are eluting from the chromatography column is thereby electrostatically dispersed. This generates highly charged droplets, which are normally positively charged in proteomics experiments. Once the droplets are airborne, the solvent evaporates, which increases the charge density of the droplets. Desolvated ions are generated by desorption of analyte ions from the droplet surface due to repetitive droplet fission until each droplet contains only one analyte ion. Mass analyzers include: time of flight (TOF), quadrupole (Q), ion trap (IT), and Fourier transform ion cyclotron resonance. Hybrids of these devices can be employed in sequential combination, and this is known as MS/MS (or

tandem mass spectrometry). Examples of such include: Q-Q-Q, Q-TOF, TOF-TOF, etc. In this case, the precursor ion selected from the first MS scan is fragmented by collision-induced dissociation with an inert gas, and subsequent product ions are detected by the second mass analyzer. MALDI is typically coupled to TOF due to its pulsatile generation of ions which can enter the drift tube and travel to the detector. ESI is coupled with Q or Q-TOF, although the newer linear ion traps (LTQ) coupled to the Orbitrap analyzer offer superior resolution and high mass accuracy.

MS analysis generates data which need to be processed by sophisticated computer algorithms and which will ultimately lead to peptide identification. There are two main approaches used in peptide identification: de novo sequencing and database searching. De novo sequencing is an older sequencing method applied when there is no knowledge of sample at hand. This method depends critically on the quality of the data generated and was associated with multiple problems. The most commonly used identification method nowadays is database searching. The reason for its success is that only an infinitesimal fraction of the possible peptide amino acid sequences actually occur in nature [10]. A peptide fragment spectrum might not have all the information to unambiguously derive the entire amino acid sequence, but it might have enough information to match it to a peptide sequence in database based on observed and expected fragment patterns. Several searching algorithms to be used with MS/MS spectral data have been developed and they include: Mascot, Sequest, PeptideSearch, X!Tandem, etc. The list of proteins generated is accompanied by a score based on the peptide number and confidence in identified peptides.

Renal Proteomic Studies

The quest for biomarkers of renal disease diagnosis or progression has been expanded to different sample types including: blood, urine, and tissue from renal biopsies. Which of these sample types should be explored in preliminary experiments, and which one holds most promise as a source of biomarkers? Should researchers use more than one sample type in arriving at the answer? We present some facts and opinions based on the published literature, in an attempt to address these questions. Table 1 summarizes the most important glomerular disease biomarkers mentioned in this review.

Blood Proteomics

Blood has emerged as a gold mine for biomarkers in a variety of different fields. It has intimate contact with every tissue in the body, thus carrying subliminal messages in the form of proteins or peptides. Concentrations of plasma proteins span 10 orders of magnitude [11]. This means that no single experimental approach would give insight into all the proteins present in blood. Furthermore, the search for low-abundance proteins, which represent the most plausible source of biomarkers, is challenging [12]. To illustrate this point, we look at several studies reporting albumin fragments as potential biomarkers of disease. One of these studies examined differential protein expression in children with genetic focal segmental glomerulosclerosis (FSGS) versus those with idiopathic FSGS [13]. The authors used 2-DE followed by MALDI-TOF and ESI-MS/MS, and they pooled serum samples from patients in each group. This methodology precluded the discovery of low-abundance

Table 1 The most promising proteomic biomarkers of glomerular renal diseases described in this review

Biomarker	Disease	Technique used	Reference
Blood			
IgG4 Ab to M-phospholipase A2 receptor	IMN	HPLC-MS/MS	[22]
Albumin sulfonation on Cys34 residue	FSGS	LC-ESI-MS/MS	[21]
Kininogen-1-light chain; C3f fragment	IgA nephropathy	Magnetic bead peptide capture; MALDI-TOF; ESI-IT-MS/MS	[17]
Urine			
Collagen I fragments	Diabetic nephropathy	CE-MS	[29]
E-cadherin	Diabetics with proteinuria	2D-DIGE; MALDI-TOF	[31]
Hepcidin-20	Lupus nephritis	Columns ≤30 kDa; SELDI-TOF	[33]
Renal tissue			
Apolipoprotein E	MPGN-II?	Laser microdissection; LTQ-Orbitrap	[36]
C7, C8 fragments	MPGN-II vs IC-MPGN		[36]

Ab antibody, *IMN* idiopathic membranous nephropathy, *FSGS* focal segmental glomerulosclerosis, *MPGN-II* membranoproliferative glomerulonephritis type II, *IC-MPGN* immune-complex-mediated membranoproliferative glomerulonephritis

proteins, and in fact, albumin fragments differentiated genetic FSGS from idiopathic FSGS. While it is plausible that a distinct albumin form exists in blood of these subjects, as the authors speculate, it is tempting to attribute this finding to high albumin abundance alone. Additionally, serum is subject to endoproteolytic and exoproteolytic activity, and this result could be a side effect of this potential artifact [14, 15]. Similarly, albumin fragments are reported in several urine proteomic studies [16], and this is further discussed below. There are several ways of resolving the problem associated with high-abundance proteins. One method used was magnetic bead technology focusing on peptides less than 7 kDa [17]. Magnetic bead-assisted serum peptide capture was followed by MALDI-TOF or ESI-IT MS/MS in patients with IgA nephropathy and healthy controls. Out of 92 peptides, five were able to distinguish the two groups, and three of these were increased in IgA nephropathy and belonged to fibrinogen alpha chain. The remaining two peptides were higher in the control group and were identified as C3f fragment and kininogen-1 light chain. Several other approaches have been developed to deplete abundant proteins by affinity chromatography. Unfortunately, this depletion is not quantitative [18]. Furthermore, elimination of abundant proteins depletes additional, potentially useful molecules, as illustrated by a study where 814 proteins were codepleted with albumin [19]. Instead of trying to eliminate albumin, some investigators took advantage of this highly abundant protein. Albumin had been shown in vitro to be oxidized during oxidative stress [20]. Musante and colleagues used LC-ESI-MS/MS to analyze plasma albumin from patients with active FSGS or other glomerular disorders including IgA nephropathy, idiopathic membranous nephropathy (IMN), or normal controls and determined that albumin oxidation was dramatic only in active FSGS [21]. This was shown by demonstrating sulfonated albumin on residue Cys34. Given that this finding was not only specific for FSGS but was also present during active disease, oxidized albumin may represent a potential biomarker of disease activity as well as diagnosis. Future validation studies of this particular molecule would be of interest.

Besides the “shotgun” (discovery-based) proteomic approaches investigating human sera in relation to renal disease, other more focused questions have yielded important and novel information. Beck and colleagues blotted normal renal glomeruli with sera from patients with IMN [22]. They identified a band and with the help of MS were able to delineate the identity of glomerular antigen that was bound by an antibody from sera of 70% of patients with IMN, but none of the secondary membranous cases, FSGS, diabetic nephropathy, or normal controls. This antigen was M-type phospholipase A2 receptor, which is highly expressed in glomerular podocytes and which was bound by an IgG4 antibody. This discovery has led to new insights into the pathogenesis of this disease and may facilitate development of novel serologic tests for diagnosis of IMN or monitoring disease activity.

Urine Proteomics

The most promising fluid as a future source of biomarkers of renal disease is urine. What makes urine particularly attractive is that it can be obtained in a noninvasive fashion, it is present in abundance and can be collected as often as needed without any risks to the patient. Furthermore, urine proteomic profile appears to be stable for many years when urine is stored at −80°C [23]. Urine is produced by the kidney, and its final proteome is further influenced by few additional downstream organs. Urine contains ≤150 mg/day of total protein, 70% of which is derived from the kidney, with 30% originating from plasma [24]. Unfortunately, there are disadvantages to using urine which have hindered the discovery of biomarkers. A universal method for urine collection and storage has not yet been developed, although this has been the focus of several international organizations including the Human Kidney and Proteome Project and European Kidney and Urinary Proteomics. Urinary proteome is influenced by exercise [25], diet [23], time of day when urine is collected [26, 27], and sex [27]. Similar to blood, urine has a wide dynamic range of protein concentrations, with albumin being the most abundant protein [28]. This becomes an even greater problem in patients who have higher grades of proteinuria, such as all patients with glomerular diseases. The high-abundance proteins dominate the proteome, with low-abundance ones being potentially more informative as biomarkers of disease. Not surprisingly, some of the same problems associated with blood proteomics can be reiterated here. Candiano and colleagues employed 2-DE and MALDI-TOF to investigate urines of children with FSGS, minimal-change disease, IMN, and normal controls [16]. The main differences detected between cases and controls were fragments of albumin and alpha-1-antitrypsin. While the author's interpretation of an existing novel albumin adduct is plausible and analogous to the case of FSGS described above, both albumin and alpha-1-antitrypsin fragments could be there solely due to higher concentration of these highly abundant proteins in patients with higher than normal proteinuria. Several proteomic studies examining biomarkers in urine of patients with diabetes mellitus have been published. One of the studies used CE-MS to define peptide sets associated with diabetes and diabetic nephropathy, as well as markers that could differentiate diabetic nephropathy from other glomerular renal diseases with sensitivity and specificity

around 81–97% [29]. Several peptides correlated with fragments of collagen I, known to accumulate in kidneys of patients with diabetic nephropathy. These fragments were lower in diabetic patients than those with other renal diseases, inviting a hypothesis that decreased clearance and cleavage of this product might lead to its accumulation in the kidney. Contrary to this, a different group studying urine of patients with diabetic nephropathy, making comparison to other renal diseases or normal controls, used MALDI-TOF/TOF and isolated fragments of collagen-alpha-5 and collagen-alpha-I which were high in the diseased states, compared to normal controls [30]. This difference in findings could be due to sample preparation, variability in the two cohorts studied, or reasons that are not yet apparent. In terms of other markers of extracellular matrix, urine E-cadherin was discovered to be a potential biomarker reliably distinguishing between diabetics with microalbuminuria or macroalbuminuria from healthy controls and diabetics with normoalbuminuria [31]. The authors used 2DE-DIGE followed by MALDI-TOF, and results were confirmed by ELISA and Western blotting. Increased levels of soluble urine E-cadherin could be a sign of epithelial-to-mesenchymal transition, described in the process leading to renal fibrosis [32]. Not surprisingly, among their top hits, these authors also identified albumin, uromodulin, and retinol-binding protein, all highly abundant urine proteins, likely correlating with the degree of proteinuria.

Lupus nephritis represents a conglomeration of glomerular renal diseases caused by systemic lupus erythematosus, which would greatly benefit from the ability to predict flares or monitor therapeutic response. One longitudinal study ambitiously evaluated the urine proteome from 19 patients at many different time points [33]. To uncover lower molecular weight proteins, the authors fractionated urine using columns with cutoff size of 30 kDa. This was followed by SELDI-TOF. The most interesting protein isolated increased 4 months before the flares and was identified as an isoform of hepcidin. Immunohistochemistry of renal tissue confirmed hepcidin staining of infiltrating leukocytes in patients with active disease. This finding provides an interesting molecule that could potentially be evaluated in future studies. Nonetheless, even when using 30-kDa cutoff columns, the authors found albumin and alpha-1-antitrypsin fragments which were significantly elevated during flares. Another study cross-sectionally compared patients with active versus those with inactive lupus nephritis [34]. They identified two peaks that could discriminate between active and inactive disease with 92% sensitivity and specificity and confirmed findings in a separate small cohort of cases. The identities of these two peaks are unknown, and their value in monitoring disease activity has not been validated.

Numerous other studies of urine proteomics addressing various glomerular and other renal diseases have been published. Unfortunately, none of these have been validated in large clinical trials or have influenced clinical practice. Thus, urine represents an attractive source of biomarkers which may not be ready yet for the prime time.

Renal Tissue Proteomics

Renal tissue represents an important source of potential biomarkers, particularly in the preliminary, exploratory phases. However, this sample type is not ideal for ultimate disease monitoring or diagnosis, since it is difficult to obtain, particularly when repetitive sampling is needed. Patient risks associated with kidney biopsy have been outlined above. Tissue proteomics has been advanced by technical developments allowing analysis of formalin-fixed, paraffin-embedded tissues [35]. In one of the most interesting and original approaches, Sethi and colleagues performed laser microdissection of glomeruli in membranoproliferative glomerulonephritis type II (MPGN-II) [36]. Immune-complex-mediated MPGN cases and implantation biopsies of living donors served as controls. The deposits were subjected to LC-MS/MS using LTQ-Orbitrap. Not surprisingly, proteins identified with the highest confidence comprised the alternative complement cascade, known to be causative in MPGN-II. However, terminal complement components were also identified in MPGN-II glomeruli, suggesting excessive fluid-phase production of the terminal complement complex. Finally, apolipoprotein E was reproducibly present in glomeruli of patients with MPGN-II, and it remains unclear whether this protein is important in the pathogenesis of this disorder. If proven to be important, the search for apolipoprotein E may be warranted in urine. Unfortunately, despite high resolution and accuracy of the techniques used, no quantification was applied, thus making it difficult to draw conclusions regarding relative abundances of these proteins in MPGN-II. Another study of renal graft interstitial fibrosis and tubular atrophy took advantage of the biopsy tissue to derive lists of proteins differentiating various histopathologies associated with interstitial fibrosis and tubular atrophy and formulated hypothesis-generating mechanisms [37]. They used LC-MS (LTQ) followed by single-reaction ion monitoring for verification of top candidates. Renal tissue is useful when used in conjunction with other exploratory platforms to verify findings or in well-designed questions addressing specific disease entity or specific renal compartments.

Lastly, various groups are beginning to use cell cultures in the hope of defining potential biomarkers in a controlled in vitro setting where protein quantification is more readily performed. These systems do not suffer from some of the

shortcomings of fluid proteomics already discussed and could be a preliminary step to finding potential secretory markers of interest that could inform subsequent rigorous validation studies in urine or blood. Additionally, these studies could lead to further hypothesis-generating mechanisms of renal diseases or processes involved in normal renal physiology [38, 39] and that could be subsequently tested in human samples.

Conclusion

Nephrology is in desperate need of biomarkers that could assist in renal disease diagnosis or monitoring of disease activity. Countless markers have emerged from the proteomic pipeline, but none have been validated or become available in clinical practice. It appears that the most promising markers can come from well-designed, specific research questions if using complex fluids or tissues and from use of more than one fluid or tissue for verification. Nonetheless, there is hope for discovery-based approaches from fluids, once the sample preparation and the applications of MS technologies to these complex fluids are better understood and once there are more reliable ways of examining the low-abundance proteome. An alternative approach could be the use of an indirect system, such as cell cultures, to discover plausible candidates that could be subsequently tested in biological fluids. The most promising fluid for ultimate monitoring of renal diseases still appears to be urine. Aspects of proteomic study validation have not been addressed here, but this is one of the fundamental steps in discovering reliable biomarkers.

We conclude by mentioning that we are still struggling to take advantage of MS as a multiparametric, quantitative technique. Intuitively, it is highly likely that a carefully selected panel of urinary or serum proteins should do much better in renal disease diagnosis or prognosis than creatinine or total protein measurements. Hopefully, such a panel should emerge soon.

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