Clinical Chemistry 58:2
353–365 (2012)

Reviews

Searching for New Biomarkers of Renal Diseases through Proteomics

Ana Konvalinka,[1] James W. Scholey,[1] and Eleftherios P. Diamandis[2*]

BACKGROUND: Technological advances have resulted in a renaissance of proteomic studies directed at finding markers of disease progression, diagnosis, or responsiveness to therapy. Renal diseases are ideally suited for such research, given that urine is an easily accessible biofluid and its protein content is derived mainly from the kidney. Current renal prognostic markers have limited value, and renal biopsy remains the sole method for establishing a diagnosis. Mass spectrometry instruments, which can detect thousands of proteins at nanomolar (or even femtomolar) concentrations, may be expected to allow the discovery of improved markers of progression, diagnosis, or treatment responsiveness.

CONTENT: In this review we describe the strengths and limitations of proteomic methods and the drawbacks of existing biomarkers, and provide an overview of opportunities in the field. We also highlight several proteomic studies of biomarkers of renal diseases selected from the plethora of studies performed.

SUMMARY: It is clear that the field of proteomics has not yet fulfilled its promise. However, ongoing efforts to standardize sample collection and preparation, improve study designs, perform multicenter validations, and create joint industry–regulatory bodies offer promise for the recognition of novel molecules that could change clinical nephrology forever.

The effectiveness of treatment for renal diseases is limited by the lack of diagnostic, prognostic, and therapeutic markers. A tissue biopsy is often necessary to establish a diagnosis, particularly in the case of glomerular diseases (GDs).[3] Biopsy is considered the gold standard diagnostic test, but it carries risks, including hemorrhage, pain, and death *(1)*. The most widely used biochemical indicators of prognosis include serum creatinine (SCr) and urine protein. SCr is a late marker of renal dysfunction and its use has additional drawbacks. Urine protein is the best prognostic marker currently available, despite its shortcomings; it is nonspecific, and the levels associated with risk vary across different diseases. For example, 1 g/day of proteinuria portends a poor prognosis in IgA nephropathy (IgAN), but seems to be favorable in membranous nephropathy (MN) *(2, 3)*. Treatment that reduces proteinuria has a favorable effect on prognosis *(4–7)*, but it remains unclear to what degree proteinuria should be suppressed and when to initiate treatment. Knowledge of more specific protein perturbations might better inform the management of renal diseases. The field of proteomics is expanding daily, and it is of interest to clinicians and scientists to understand its fundamentals. In this review we describe studies in which renal disease biomarkers were investigated by use of mass spectrometry (MS) and outline a scheme for understanding the basics of proteomics.

Proteomics: Promises and Limitations

Proteomics is a large-scale study of proteins and their function and structure. Proteome composition is constantly changing and varies with physiological changes. Although proteins are products of genes, multiple distinct protein isoforms can be created from the same gene. Proteins are further susceptible to posttranslational modifications (PTMs). Isoforms and PTMs are

[1] Department of Medicine, Division of Nephrology, and [2] Department of Laboratory Medicine and Pathobiology, Division of Clinical Biochemistry, University of Toronto, Toronto, Ontario, Canada.
* Address correspondence to this author at: Department of Laboratory Medicine and Pathobiology, Mount Sinai Hospital, 60 Murray St., Suite 6-201, Box 32, Toronto, ON, M5T 3L9, Canada. Fax +416-619-5521; e-mail EDiamandis@mtsinai.on.ca.
Received March 18, 2011; accepted July 8, 2011.
Previously published online at DOI: 10.1373/clinchem.2011.165969

[3] Nonstandard abbreviations: GD, glomerular disease; SCr, serum creatinine; IgAN, IgA nephropathy; MN, membranous nephropathy; MS, mass spectrometry; PTM, posttranslational modification; MRM, multiple-reaction monitoring; IF/TA-NOS, interstitial fibrosis and tubular atrophy not otherwise specified; TG, transplant glomerulopathy; AKI, acute kidney injury; DN, diabetic nephropathy; FSGS, focal and segmental glomerulosclerosis; GFR, glomerular filtration rate; RAS, renin–angiotensin–aldosterone system; CKD, chronic kidney disease; kim-1, kidney injury molecule-1; MS/MS, tandem MS; LN, lupus nephritis; IMN, idiopathic MN; PLA2R, phospholipase A2 receptor; SOD2, manganese-superoxide dismutase; MPGN, membranoproliferative glomerulonephritis; MPGN-II, MPGN-type II; LC, liquid chromatography; ESI, electrospray ionization; CE, capillary electrophoresis; ACEI, angiotensin-converting enzyme inhibitor; AR, acute rejection.

detectable only by studying the proteins directly and can be indicative of diverse protein functions. Furthermore, there is evidence that only one third of proteins with altered expression display a concomitant change in mRNA expression *(8)*. Thus proteomics seems to reflect the actual cellular processes more accurately than do genomics or transcriptomics.

Proteomic studies can be discovery based or targeted. Discovery approaches, which predominate in renal literature, can be either broad, when 2 types of samples are compared in a qualitative or quantitative sense, or focused, such as with investigations of protein–protein interactions. Broad approaches can involve taking on a global examination of the entire proteome, or evaluation of a specific subproteome, such as all proteins with a particular PTM. Numerous renal biomarker studies are global; a concrete example of the latter is a renal transplantation study that compared plasma of patients with acute rejection to controls with stable graft function *(9)*. The entire proteomes of individuals in each group were compared, and 18 proteins were found to be differentially expressed between the 2 groups. Three of these 18 proteins were subsequently used in a longitudinal study and proved to be predictors of early acute rejection. In an example of a "subproteome" study, which was both qualitative and quantitative, Rinschen and colleagues examined the phosphoproteome of V2-receptor–mediated response in cultured collecting-duct cells *(10)*. The Rinschen et al. study revealed some important signaling molecules and processes that take place early after V2-receptor stimulation. Broad approaches typically require extensive fractionation of proteins and peptides, and the use of high-accuracy, high-sensitivity MS analyzers. The study of V2-receptor signaling, for example, involved the use of phosphoprotein enrichment and an LTQ-Orbitrap MS analyzer *(10)*.

In a targeted approach the investigator has previous knowledge of the protein candidates of interest and evaluates them in different types of samples using single-reaction or multiple-reaction monitoring (MRM). In this approach, MS analyzers with high sensitivity and high dynamic range are required. As an example, Quintana and colleagues searched for markers of interstitial fibrosis and tubular atrophy not otherwise specified (IF/TA-NOS) *(11)*. In this particular study no enrichment was performed for low molecular weight peptides. The authors compared the urine peptidomes of patients with IF/TA-NOS, patients with transplant glomerulopathy (TG), and controls, and discovered peptides of uromodulin and kininogen that enabled them to differentiate cases from controls. The most promising peptides for distinguishing cases from controls had molecular weights of 1890.09, 2569.2, and 2582.44 Da. Knowledge of these candidates allowed subsequent use of MRM technology for verification of uromodulin and kininogen peptides in a new patient cohort.

Raw data generated by MS are processed by sophisticated computer algorithms leading to peptide and protein identification and quantification. Owing to the nature of peptide and protein assignment by database searching, additional methodologies are used to verify the candidates of interest. Verified candidates should be subjected to testing in a new cohort of patients. Large-scale validations involving prospective studies are the ultimate tests of biomarker adequacy.

Despite its promise, the translation of proteomic biomarkers to the clinic from a plethora of studies has been slow. Why? First, there is a lack of a unifying method for sample collection or analysis. This is true of all biosamples including urine, for which composition is influenced by diet *(12)*, timing of collection *(13, 14)*, exercise *(15)*, sex *(14)*, and age *(16)*. Generation of a universal protocol for urine collection is the focus of international organizations *(17, 18)*. Urine is a dilute biofluid that requires protein concentration, particularly in gel-based proteomics studies *(19)*. Different methods for urine concentration and protein isolation will yield distinct proteins. Numerous methods have been used for urine concentration, including precipitation with organic solvent, centrifugal filtration, ultracentrifugation, and lyophilization *(12, 20)*. Furthermore, it remains unclear which approaches are most effective: addition of protease inhibitors, depletion of high-abundance proteins, and collection of spot urine vs collection of urine for a longer time period. Urine spot collections can be dilute or concentrated depending on their water content, thus requiring adjustment of biomarker concentrations. The most common adjustment factor is urine creatinine, although specific gravity also has been used. Urine creatinine has been validated as a normalization factor for urine albumin and total protein. Specific gravity is a measure of the weight of the solution compared to that of an equal volume of distilled water and is determined by both the number and size of particles in the solution. Specific gravity can be problematic, particularly when larger molecules are present in urine *(21)*. Only a few studies have compared biomarker normalization with either urine creatinine or specific gravity *(22–25)*, and their conclusions have varied. Depending on the biomarker tested either normalization method may be adequate. If protein or peptide ratios are evaluated as biomarkers, normalization may not be necessary. In acute kidney injury (AKI), in which the steady state is lost, longer collections may be preferred *(26)* over spot samples.

There are additional unresolved technical aspects of proteomics. Blood and urine proteomic studies are challenged by a wide dynamic range of protein concen-

trations, spanning 5–10 orders of magnitude *(27, 28)*. This wide concentration range makes it impossible to characterize all proteins in any given sample by any technique available. Another problem relates to the expense and time of conducting large-scale biomarker studies, and to biological variability of proteins uncovered. Nagaraj and Mann addressed this problem by assessing urine proteomes of healthy individuals to find reference values for the most abundant proteins using label-free quantification in a manner that overcomes the need for exhaustive fractionation and can be translated to the clinic *(29)*. Other efforts to simplify the workflow and address technical aspects are emerging *(30, 31)*. Urine, despite its shortcomings, seems to be an ideal source of biomarkers because it is collected noninvasively, and only a few organs contribute to its proteome.

Given the sensitivity of the proteome to a variety of factors, design of proteomic studies is critically important. It is virtually impossible to define a homogeneous patient population for study. Even small variations in patient selection, such as medications or age, may result in proteome differences that do not reflect the disease state, thus making it difficult to arrive at general conclusions or translate candidate biomarkers into clinical practice. Not surprisingly, validation studies are lacking. To our knowledge, there have been no large-scale efforts to validate markers of interest. This situation is beginning to change, and we are now witnessing first efforts to "qualify" markers of drug-induced nephrotoxicity *(32)*. A consortium of industries, nonprofit institutions, and regulators (Predictive Safety Testing Consortium) represents a collaborative effort to move markers from discovery to clinical practice.

Urine Proteome Composition

Under normal physiologic conditions, urine contains <20 mg of albumin and <150 mg of total protein per day. Approximately 30% of the protein content derives from plasma, whereas 70% is produced by the kidney and lower urinary tract *(33)*. Normal urine contains at least 1500 proteins, most of which are extracellular and membrane bound *(34)*. To appear in urine, proteins or their fragments must be filtered at the glomerulus and bypass or otherwise avoid tubular reabsorption. Alternatively, they must be secreted by the kidney or lower urinary tract directly into urine. During plasma filtration in the glomeruli, the glomerular capillary wall discriminates among molecules of different size, charge, and configuration. The filtration barrier consists of the fenestrated endothelium, glomerular basement membrane, and slit diaphragm. The latter 2 structures limit the passage of macromolecules, and they contain glycosaminoglycans that are highly negatively charged. Small and positively charged molecules are thus most likely to find their way into filtrate. Typically, proteins <20 kDa are freely filtered, whereas those >60 kDa are almost completely restricted. Despite this filtering, the most abundant urine protein is albumin, a negatively charged molecule with a molecular weight slightly more than 66.4 kDa. It is thought that the relative abundance of albumin in urine is a result of the presence of large pores able to filter albumin, immunoglobulins, and other macromolecules *(35)*. The actual amount of albumin filtration is unknown, but is thought to be in the order of 2–4 g/day. Renal hemodynamics and serum concentration of a particular protein also determine the extent of filtration. For example, hyperfiltration states such as pregnancy result in increased filtration of most proteins. Overproduction diseases such as multiple myeloma result in filtration of large amounts of immunoglobulin light chains. Injury to any of the structures involved in the filtration barrier results in leakage of large and negatively charged proteins, and their increased presence in urine. For example, in diabetic nephropathy (DN) and focal and segmental glomerulosclerosis (FSGS), there is evidence of decreased filtration of small molecules and increased filtration of macromolecules, presumably due to formation of an increased number of large pores *(36)*.

Tubules reabsorb most of the filtered proteins. Proximal tubules also catabolize proteins and excrete their peptides in urine. Albumin is reabsorbed via endocytosis mediated by megalin and cubilin receptors in proximal tubules. Tubules secrete proteins directly into urine as a result of the normal maintenance process or in response to injury. Tubular injury could result in decreased reabsorption or catabolism of the filtered proteins and in secretion of tubular proteins in response to the injury.

In addition to being an important source of proteins, urine appears to be enriched with peptides of <750 Da. A study of the normal urine peptidome evaluated polypeptides in multiple fractions segregated on the basis of size *(37)*. Using 2 separate approaches, the authors demonstrated that in both normal urine and urine from patients with Fanconi syndrome, a prototypical disease with preserved filtration and impaired proximal tubular reabsorption, there was more than 100-fold enrichment for components <10 kDa. Furthermore, these authors proposed that most peptides originated from renal-derived proteins, whereas plasma proteins were excreted unchanged. Their study demonstrated that certain peptides of <5 Da may be specific to Fanconi syndrome, a finding that may be useful as a tool to inform diagnosis and disease pathophysiology. A study by the same group similarly demonstrated high concentrations of urine polypeptides

and chemokines that could account for the mechanisms of injury in Fanconi syndrome *(38)*, further supporting the usefulness of exploration of the urine peptidome.

In addition to soluble proteins and their peptides, urine contains exosomes, specialized vesicles shed by renal epithelia directly into urine *(39, 40)*. Finally, distal organs of the lower urinary tract also contribute to the urine proteome.

RENAL PROTEOMIC STUDIES

Currently used markers of renal dysfunction lack both specificity and sensitivity. SCr is the most widely used marker of renal dysfunction. However, it is a late marker and highly nonspecific to the site or type of injury. SCr can be used to predict glomerular filtration rate (GFR) only in a steady state, varies with diet and muscle mass, and tends to lead to overestimates of GFR in advanced disease, in which creatinine undergoes increased tubular secretion. Serum urea [blood urea nitrogen (BUN)] is another marker of renal dysfunction that varies inversely with GFR. BUN is an inferior marker to SCr because its production is not constant and its variations could be a result of diseases unrelated to kidney. BUN also undergoes extensive reabsorption by the proximal tubules. Its main utility is in diagnosis of prerenal insufficiency, in which the ratio of BUN to SCr is increased, owing to the passive reabsorption of BUN with sodium and water. A more promising marker under current investigation is cystatin C. Cystatin C is produced by all nucleated cells, and is freely filtered at the glomerulus and completely metabolized by the tubules. Cystatin C does not appear to be affected by diet or muscle mass, although results of some studies have demonstrated that cystatin C is affected by factors independent of GFR, such as inflammation and diabetes *(41, 42)*. When compared to a gold standard measurement of GFR, cystatin C performed just as well as SCr for estimating GFR adjusted for age, sex, and race *(43)*. Cystatin C may have a special place in populations with lower creatinine production such as elderly individuals, children, and patients with cirrhosis *(44, 45)*.

Urine albumin and urine total protein are additional markers of renal disease and prognosis. Albuminuria is defined as >30 mg/day of albumin excretion, and is the first sign of diabetic nephropathy or cardiovascular dysfunction. Suppression of albuminuria with renin–angiotensin–aldosterone system (RAS) blockers is linked to delayed progression to nephropathy *(46–48)*. As mentioned above, proteinuria is the best prognostic marker of renal disease, and its reduction leads to more favorable outcomes. However, it is a nonspecific marker, not informative with respect to timing of treatment initiation or degree of reduction needed.

Given all the described limitations of current markers of renal disease, including a complete absence of diagnostic markers, there is hope that novel markers will be discovered that can be used for: (*a*) earlier diagnosis of renal dysfunction; (*b*) differentiation between distinct pathologic entities, thus obviating the need for biopsy; (*c*) selection of patients who would benefit from immunosuppressive therapies; (*d*) selection of patients who need more RAS blockade; and (*e*) determination of the degree of proteinuria reduction needed in a particular patient.

Studies performed to search for novel renal biomarkers are universally susceptible to some unresolved issues. The large dynamic range of protein concentrations is a major impediment in urine and blood proteomics, considering that low-abundance proteins are the main contenders for promising biomarkers *(49)*. High-abundance proteins are still the majority of biomarkers discovered in studies to this point, and most of these proteins are not specific to one condition, which suggests they are products of systemic disturbances. Recurrent appearance of similar species in various disorders may suggest activation of a universal process; for example, the RAS, known to be activated in most chronic kidney diseases (CKDs). If this is true, directed studies aimed at these systemic disturbances may have higher yields of useful biomarkers. Nevertheless, studies to date have uncovered some promising biomarkers, which are presented in Table 1.

Acute and Drug-Induced Kidney Injury

Improved markers of early kidney injury would allow identification of individuals at risk for kidney disease, timely diagnosis of AKI, avoidance of nephrotoxic drugs, and better understanding of the nephron segment(s) involved. Ability to predict nephrotoxicity would be useful in the process of drug development. These important goals motivated the development of the Nephrotoxicity Working Group of the Predictive Safety Testing Consortium *(50, 51)*, which selected 23 previously discovered urinary biomarkers and evaluated them in rat models of AKI. Sensitivity and specificity testing was accomplished by histologic scoring and analysis employing area under the ROC curve. Seven markers were selected for further preclinical studies, including: kidney injury molecule-1 (kim-1), albumin, total protein, β_2-microglobulin, cystatin C, clusterin, and trefoil factor-3. Clusterin was better than SCr and BUN for detection of proximal tubular injury, and total protein, cystatin C, and β_2-microglobulin each outperformed SCr and BUN in detecting glomerular injury *(52)*. Urine albumin was superior to either

Table 1. Biomarkers of potential diagnostic utility.[a]

Biomarker	Renal condition	Characteristics	Reference interval	Stability	Comments
Serum cystatin C	GFR estimate	MW[b] 13 kDa; produced by all nucleated cells, freely filtered, not secreted, and completely metabolized by tubules.	0.53–0.95 mg/L [Shlipak et al. *(98)*].	Stable with intraindividual CV 7.7% [Shlipak et al. *(98)*].	Promising marker for diagnosis of early dysfunction in children, elderly, patients with cirrhosis, or patients with renal transplant.
Urine albumin	Most tubulointerstitial and glomerular diseases, AKI, cardiovascular dysfunction	MW 66.4 kDa; in urine as a result of plasma filtration; increased urine content due to glomerular injury or incomplete reabsorption by proximal tubules.	<2.5 mg/mmol creatinine in men; <3.5 mg/mmol creatinine in women.	Stable for at least 1 week at 4–20 °C. Freezing at −20 °C causes fragmentation. Freezing at −80 °C preserves integrity].	The most commonly used urine marker of renal dysfunction. It is nonspecific.
Urine cystatin C	AKI	In urine as a result of plasma filtration by glomeruli; increased urine content due to glomerular injury or incomplete reabsorption/catabolism by proximal tubules.	0.03–0.18 mg/L	Stable regardless of pH, storage temperature and freeze-thaw cycles [Herget-Rosenthal et al. *(99)*].	Marker of glomerular and proximal tubular injury [Dieterle et al. *(52)*].
Urine clusterin	AKI	MW 80 kDa, glycosylated with PTMs. Thought to play a role in cell protection, antiapoptosis, lipid recycling [Rosenberg and Silkensen *(100)*].	85.2–628 ng/L	Stable	Marker of tubular injury *(52)*; may be glycosylated with PTMs. Useful in differentiating tubular from glomerular proteinuria.
Urine kim-1	AKI	Type-I cell membrane glycoprotein with an immunoglobulin-like domain and mucin-rich extracellular region; receptor on proximal tubular cells directing apoptotic cells to lysosomes; shed into urine after injury.	59–2146 ng/L	Stable at different temperatures and for up to 3 freeze-thaw cycles [Chaturvedi *(101)*].	Marker of proximal tubular injury Vaidya et al. *(54)*; outperformed traditional markers in multiple nephrotoxic drug screens [Vaidya et al. *(54)*].
Urine β2-microglobulin	AKI	MW 12 kDa; light chain of class I MHC molecule; filtered by glomeruli and catabolized by tubules.	<0.2 mg/L	Unstable in urine at low pH	Marker of glomerular and proximal tubular damage [Dieterle et al. *(52)*].
Serum antibody to PLA2R	MN	IgG4-type antibody to 185-kDa glycoprotein (podocyte receptor) was recovered from sera of >70% patients with idiopathic forms of disease and not those with secondary forms [Beck Jr *(61)*].	Unknown	Unknown	Differentiates IMN from secondary forms and other glomerular diseases; level correlated with disease activity [Hofstra et al. *(102)*], thus could potentially be used to monitor patients.

Continued on page 358

Table 1. Biomarkers of potential diagnostic utility.[a] *(Continued from page 357)*

Biomarker	Renal condition	Characteristics	Reference interval	Stability	Comments
Serum antibody to AR	MN	IgG4 antibody against glomerular AR, in sera and glomeruli of patients with idiopathic disease only. AR was shown to be upregulated de novo in podocytes.	Unknown	Unknown	Appears to differentiate IMN from multiple other glomerular pathologies [Liu et al. *(63)*].
Serum antibody to SOD2	MN	IgG4 antibody against SOD2 in sera and glomeruli of patients with IMN; oxidative stress in vitro induced SOD2 expression in podocyte membrane.	Unknown	Unknown	Appears to differentiate IMN from multiple other glomerular pathologies [Liu et al. *(63)*].
Serum albumin sulfonation on Cys34 residue	FSGS	Cys34 is albumin residue most susceptible to adducts. It was extensively sulfonated upon oxidative stress in patients with active FSGS [Musante et al *(70)*].	Unknown	Sulfonic group at Cys34 generated upon oxidation is a stable end product but may undergo rearrangements during alkylation.	Cys34 sulfonation could differentiate FSGS from other pathologies; it paralleled disease activity, and was a marker of posttransplant recurrence.
Urine kininogen/ serum kininogen	IgAN, acute rejection in renal transplantation, IF/TA-NOS	Kininogen is a 120-kDa protein essential in coagulation and as part of the kallikrein–kinin system, where it is converted to bradykinin, which results in vasodilatation and modulates renal injury. ACE inhibitors prolong bradykinin half-life.	55–90 mg/L (in plasma); abundant protein in urine representing 4% of total urine protein content according to a recent study [Nagaraj and Mann *(29)*].	Stable	Kininogen and its peptides may differentiate IgAN from healthy controls [Kaneshiro et al. *(71)*], or its high urine concentrations may predict responsiveness to RAS blockade [Rocchetti et al. *(73)*]. Urine kininogen distinguished between IF/TA- NOS and TG cases [Quintana et al. *(11)*]. Plasma kininogen was a predictor of acute rejection [Freue et al. *(9)*].
Urine hepcidin	LN	Hepcidin is a 2.8-kD protein produced by the liver and involved in iron metabolism. It is upregulated during infection, inflammation, iron excess. Isoforms 20 and 25 differentiated patients with LN flares and controls [Suzuki et al. *(76)*].	Unknown	Stable for months when stored at −80 °C.	Hepcidin 20 could predict renal flares; hepcidin 25 might predict recovery from flares.
Urine collagen-I	DN, acute rejection in renal transplant.	Collagen-I is an extracellular matrix protein involved in healing and fibrosis. Its presence in urine is likely renal in origin.	Unknown	Unknown	Collagen fragments could differentiate DN patients from normoalbuminuric diabetics [Rossing et al. *(82)*. Urine fragments of collagen predicted acute rejection onset with high specificity and sensitivity [Ling et al. *(87)*].

Continued on page 359

Table 1. Biomarkers of potential diagnostic utility.[a] *(Continued from page 358)*

Biomarker	Renal condition	Characteristics	Reference interval	Stability	Comments
Urine CD44	Acute rejection in renal transplant.	CD44 is a glycoprotein involved in adhesion, migration, cell–cell interaction. It is implicated in lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis.	Unknown	Unknown	CD44 could distinguish acute rejection from other transplant pathologies and healthy controls [Sigdel et al. *(85)*].

[a] A list of the most promising markers, selected from this review, with potential utility in diagnosis or follow-up of patients with renal diseases.
[b] MW, molecular weight; MHC, major histocompatibility complex; AR = aldolase reductase.

traditional marker, and trefoil factor-3 complemented them in detecting tubular damage *(53)*. Changes in kim-1 outperformed changes in SCr, BUN or N-acetyl-β-D-glucosaminidase for detecting kidney injury due to multiple nephrotoxins *(54)*. In addition, an extended panel of urinary biomarkers *(55)* was found to be useful for monitoring recovery from nephrotoxins.

Additional markers uncovered by direct proteomic studies deserve attention. Urine markers in children undergoing cardiopulmonary bypass were evaluated by use of SELDI-TOF-MS to predict AKI *(56)*. After surgery 3 peaks changed significantly in patients with AKI. Tandem MS (MS/MS) of gel bands containing proteins of interest identified α_1-microglobulin, α_1-acid-glycoprotein, and albumin *(57)*. These candidates were subsequently measured by nephelometry in 365 children. All 3 biomarkers peaked earlier than SCr, and their concentrations correlated with severity of AKI. All 3 proteins are abundant in human urine *(29)*, so it is plausible that total protein would have been just as good. The selected study population makes this finding relevant to only a few clinical settings.

In another study in which the same technology was used, urine markers of AKI in adults undergoing cardiopulmonary bypass were evaluated *(58)*. The investigators discovered hepcidin-25 in patients who were not developing AKI. Hepcidin is involved in iron sequestration and was recognized as a biomarker of lupus nephritis (LN) *(59)*, suggesting a common underlying mechanism. Fetuin-A (also known as α_2-HS-glycoprotein) was discovered and subsequently verified as a marker of AKI in rats given cisplatin and in critically ill patients *(60)*. Fetuin-A may be a novel biomarker, but its usefulness must be confirmed in additional studies.

Glomerular Diseases

GDs are challenging to diagnose and treat, and have poorly defined etiology. Within each histopathologic entity there is great heterogeneity. Introduction of novel biomarkers to assist with diagnosis or inform prognosis and select patients likely to benefit from treatment would revolutionize the field.

Several focused proteomic studies have led to new insights. Beck et al. *(61)* blotted normal glomeruli with sera from patients with idiopathic MN (IMN) and isolated a band, which was subjected to MS analysis and identified as M-type phospholipase A2 receptor (PLA2R). This receptor is expressed in podocytes and was bound by an antibody from sera of 70% of patients with IMN, but none with secondary MN, FSGS, or DN or healthy controls. An independent group subsequently suggested that genetic polymorphisms that af-

fect PLA2R may be the underlying cause of IMN *(62)*. In an analogous study antibodies to aldolase reductase and manganese-superoxide dismutase (SOD2) were found in sera of patients with IMN *(63)*. High titers of anti–aldolase reductase and anti-SOD2 antibodies were present in glomeruli of IMN patients but not in patients with LN or membranoproliferative glomerulonephritis (MPGN). Oxidative stress was linked with upregulation of SOD2 in podocytes. These studies uncovered novel biomarkers, which appear to differentiate IMN from other GDs, and led to new insights into pathophysiology of MN.

Sethi et al. *(64)* took advantage of focused proteomics in the analysis of biopsy tissue *(65)*. These investigators isolated glomeruli of patients with MPGN-type II (MPGN-II). These glomeruli were analyzed by liquid chromatography (LC)-MS/MS (LTQ-Orbitrap) and compared to glomeruli from patients with immune-complex–mediated MPGN and from healthy controls. Surprisingly, MPGN-II deposits contained terminal complement pathway components and apolipoprotein-E. The former finding indicates likely excessive fluid-phase complement production. Apolipoprotein-E may indicate a novel biomarker of MPGN-II, although it was described in association with DN *(66, 67)*. These well-designed experiments demonstrate how focused proteomic studies can yield insights into previously unappreciated proteins.

Urine of patients with GDs is difficult to study because it contains massively increased numbers of highly abundant proteins, which overwhelm the spectrum. The problem of highly abundant proteins was evident in a study in which investigators compared sera of children with genetic and idiopathic FSGS *(68)*. Sample pooling and 2-dimensional electrophoresis followed by MALDI-TOF or electrospray ionization (ESI)-MS/MS were employed. Albumin fragments differentiated the 2 conditions, suggesting a distinct form of albumin in FSGS or, more likely, that these fragments are artifacts of endoproteolytic/exoproteolytic serum activity *(69)*. In another study investigators took advantage of albumin abundance and used LC-ESI-MS/MS to compare plasma albumin from patients with FSGS to plasma albumin from patients with IMN and healthy controls *(70)*. Massive albumin oxidation was linked to active FSGS but not to any of the other conditions; thus the investigators identified a biomarker to follow disease activity and assist with diagnosis.

Enrichment for low-abundance peptides was explored in a discovery study of sera in which IgAN patients were compared with controls *(71)*. The authors used magnetic-bead–assisted peptide capture coupled to MALDI-TOF/TOF. Five peptides (fragments of fibrinogen, C3f, and kininogen-1 light chain) discriminated between untreated IgAN patients and healthy controls.

In another discovery study capillary electrophoresis (CE)-MS was used to identify urinary biomarkers of IgAN *(72)*. Twenty-two polypeptides distinguished IgAN from healthy controls with 100% sensitivity and 90% specificity, and 28 polypeptides differentiated IgAN and MN with 77% sensitivity and 100% specificity. Promising results were also seen when IgAN was compared to FSGS, DN, or minimal-change disease. Unfortunately, the polypeptides were not characterized.

Finally, in an ambitious study investigators examined urine markers of responsiveness to angiotensin-converting enzyme inhibitor (ACEI) in IgAN *(73)*. Urine of IgAN patients on ACEIs was subjected to 2-dimensional PAGE coupled to nano-HPLC-ESI-MS/MS. Three proteins (kininogen, transthyretin, and inter-α-trypsin-inhibitor heavy chain-4) differentiated responders from nonresponders to ACEI. Responsiveness was defined as $\geq$50% reduction in proteinuria and stable GFR. Decreased kininogen concentration predicted nonresponsiveness 6 months after ACEI introduction in a new IgAN cohort. Kininogen is converted by tissue kallikrein to bradykinin, the half-life of which is prolonged by ACEI. Bradykinin results in vasodilatation and regulates renal injury *(74, 75)*. RAS blockade forms the basis of treatment for all GDs. Studies to define responders and to determine how much blockade to use and with which agents would guide therapeutic decisions.

LN is a group of renal diseases for which treatment would be improved by the availability of noninvasive predictors of impending relapse, relapse severity, and recovery. The urine proteome of LN patients at different stages of relapse was analyzed by use of a 30-kDa cutoff filter, to focus on low molecular weight proteins, followed by SELDI-TOF-MS *(59)*. Of the 27 proteins differentially expressed between flare intervals, 2 isoforms of hepcidin predicted flare onset and recovery. As noted above, hepcidin is not disease specific and may indicate inflammation. In another study of the urine proteome of children with LN *(76)*, investigators used SELDI-TOF-MS and identified 8 peaks that enabled them to differentiate patients with active nephritis from remitters and controls. These peaks had an area under the ROC of $\geq$0.9 for diagnosis of active nephritis; thus this approach appeared promising for this particular group of patients.

DN does not often display a predictable course from microalbuminuria to macroalbuminuria and renal failure, and markers are needed that can be used to predict disease progression. In a study of patients with type I diabetes mellitus who had various degrees of nephropathy, plasma and SELDI-TOF-MS were used to

define 4 differentially expressed proteins between groups: apolipoprotein-A1, apolipoprotein-C1, transthyretin, and cystatin C *(77)*. In a reanalysis that used LC-MS/MS by LTQ-Orbitrap and iTRAQ for quantification *(78)*, upregulation of several apolipoproteins differentiated macroalbuminuric from normoalbuminuric patients. Apolipoprotein measurement with multiplex immunoassays failed to differentiate between rapid and slow progressors.

A nested case-control study had a 10-year follow-up of patients with type II diabetes *(79)*. The authors of this study performed SELDI-TOF-MS and uncovered a 12-peak urine proteomic signature that enabled them to differentiate DN patients from other diabetes patients and controls. With the use of this signature the investigators correctly predicted DN in 74% of the validation set.

In a study in which investigators employed CE-MS, 112 urine polypeptides segregating macroalbuminuric from normo- and microalbuminuric patients were identified *(80)*. Importantly, 15 polypeptides normalized following treatment with candesartan. Not surprisingly, some of these polypeptides were albumin fragments, which decreased with the use of candesartan. An unpredicted finding was that some uromodulin fragments increased following treatment, mimicking conditions found in healthy controls.

Urine E-cadherin, implicated in epithelial-to-mesenchymal transformation, distinguished diabetics with albuminuria from diabetics with normoalbuminuria and from healthy controls *(81)*. Another extracellular matrix marker, collagen-I, differentiated DN, diabetes type II without nephropathy, and nondiabetic CKD *(82)*. The urine proteome was evaluated by CE-MS. Candidates that enabled investigators to distinguish DN from other groups achieved sensitivity and specificity of approximately 90%. Urine fragments of collagen-I appeared to be decreased in DN, suggesting that decreased clearance might lead to their accumulation. This study served as the basis for a multicenter pilot validation *(83)*, for which recruited study participants included 148 patients with DN and normoalbuminuric patients with diabetes. Sixty-five biomarkers were selected, and 60 of 65 significantly differentiated cases from controls and correlated this finding with albuminuria and creatinine clearance. Collagen fragments were identified as top biomarker candidates. The shortcomings of the study included lack of biopsy-confirmed diagnosis of DN and focus on high-abundance molecules. Nonetheless, this investigation was an important effort to translate biomarkers to clinical practice.

Transplantation

Noninvasive biomarkers useful for identifying causes of acute and chronic allograft dysfunction in transplantation are also being sought. Given the frequent evidence of subclinical rejection in routine allograft biopsies *(84)*, biomarkers indicative of early rejection are needed. In a recent study investigators used LC-MS/MS and spectral counting to compare urine proteomes of patients with kidney transplantation with biopsy-proven rejection [acute rejection (AR)] with patients with normal protocol biopsy results, patients with nonspecific proteinuria, and healthy controls *(85)*. Of 1446 identified proteins, some were specifically altered in AR. Uromodulin, SERPINF1 (serpin peptidase inhibitor, clade F, α-2 antiplasmin, pigment epithelium derived factor, member 1), and CD44 were verified in an independent cohort and shown to differentiate patients with AR from other groups.

Plasma biomarkers of early AR were evaluated by iTRAQ (isobaric tags for relative and absolute quantification)-MALDI-TOF/TOF by comparison of meticulously selected patients with biopsy-proven AR and stable controls *(9)*. Plasma was depleted of 14 highly abundant proteins, and 18 proteins differentiated the groups, although with a $<$2-fold change in each case. Longitudinal monitoring of titin, kininogen-1, and lipopolysaccharide-binding protein distinguished cohorts at the time of AR.

SELDI-TOF-MS of urine differentiated patients with biopsy-confirmed AR from healthy controls and patients with several other pathologies *(14)*. Major peaks that were found to differ between groups belonged to β_2-microglobulin, likely indicating tubular or glomerular dysfunction. Subsequent study of β_2-microglobulin and several other markers measured by ELISA failed to distinguish between patients with and without subclinical rejection *(86)*.

In a comprehensive study investigators performed urine peptidomic analysis to define biomarkers of AR *(87)*. Peptides of collagen, uromodulin, and extracellular matrix components predicted AR with high sensitivity and specificity. Increased expression of corresponding genes was confirmed, and results were verified by MRM.

IF/TA-NOS is a common cause of graft loss, but this process, characterized by renal allograft dysfunction in the absence of an identifiable cause, is poorly understood. Given the absence of diagnostic criteria and incomplete understanding of the phenotype, it is impossible to define a homogenous population ideally suited to proteomic comparisons. Nonetheless, proteomic studies of IF/TA-NOS are emerging *(11, 87–90)*. Quintana and colleagues *(11)* differentiated between patients with IF/TA-NOS, controls, and patients

with TG. These investigators performed LC-MS/MS and extracted ion chromatograms of urinary peptides, and they discovered that peptides of uromodulin and kininogen could differentiate cases from controls. Two other peptides differentiated IF/TA-NOS from TG. Hierarchical clustering segregated patients according to their histopathology. Finally, MRM verified candidates in a separate patient-set.

LC-MS/MS was used in a proteogenomic study to compare allograft biopsy samples of different stages of IF/TA-NOS with samples from healthy controls *(88)*. Proteins differentiating stages of IF/TA-NOS were verified by using single-reaction monitoring. Gene and protein expression showed low correlation. Pathway analysis uncovered new mechanistic insights, including alternative complement activation and actin-cytoskeleton regulation.

CKD and Dialysis

Encouraging initiatives to standardize urine proteomics and arrive at universal biomarkers of CKD are emerging. A multicenter study was performed to define the naturally occurring urine peptidome *(91)* and biomarkers of CKD. Urine peptides ranging from 800 to 17 000 Da were analyzed by using CE-TOF-MS, followed by verification of a subset of samples with CE-Fourier transform ion cyclotron resonance MS. All results were deposited in a public database to serve as potential classifiers. These data were then used for comparison with the CKD urine peptidome, in which diverse CKD etiologies were taken into account. Disease-specific biomarkers were defined with 85% sensitivity and 100% specificity in an independent patient-set. Once again, biomarkers of CKD were mainly comprised of high-abundance proteins. A new technology based on aptamers, single-stranded oligonucleotides that fold into diverse molecular structures that bind proteins, peptides, and small molecules with high affinity and specificity *(92)*, allowed binding and identification/quantification of any protein target with a low detection limit, high dynamic range, and high reproducibility. Authors applied this technology to identification of biomarkers of CKD. Sixty plasma biomarkers were found to distinguish early and late stages of CKD. Many were low-abundance molecules, and 4 had previously been associated with CKD. Aptamers coupled to proteomics is a promising new technology for biomarker discovery.

The main focus of proteomics in dialysis has been identification of proteins involved in uremia. As an example, SELDI-TOF-MS was used to compare high- and low-flux filters in hemodialysis patients, and differences were found in the proteins removed *(93)*. Many of these proteins had been recognized as toxic middle-molecules. Vanholder and colleagues have published an in-depth discussion on uremic toxins that accumulate in patients with renal failure *(94)*. In patients undergoing peritoneal dialysis, it is unclear whether the difference in peritoneal membrane transport characteristics is associated with different proteins cleared. This question was addressed by comparing dialysis effluent among different transporters *(95)*. Two-dimensional PAGE coupled to MALDI-TOF-MS and MS/MS were employed. Five proteins differentiated the transporter types. Apolipoprotein-A1, a major constituent of HDL, was higher in high-transporters, which suggests that its loss in effluent may contribute to atherosclerosis in high-transporters *(96)*. High concentrations of immunoglobulin κ-light chains predicted development of peritonitis in the year following the study. For complete discussion of dialysis-related proteomic studies readers are directed to a recent review *(97)*.

Conclusions

Nephrology is in need of improved diagnostic and therapeutic markers. The most promising biomarkers seem to be found in well-designed studies guided by specific research questions. Discovery-based approaches from biofluids may also be useful once sample-preparation methods become standardized and more reliable techniques are available for examining the low-abundance proteome. Other indirect approaches, such as cell cultures, may be more effective for the discovery of potential biomarker candidates that could be subsequently targeted in biofluids. Technological improvements and the use of validation studies promise to define a place for proteomics in clinical practice.

Author Contributions: *All authors confirmed they have contributed to the intellectual content of this paper and have met the following 3 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; and (c) final approval of the published article.*

Authors' Disclosures or Potential Conflicts of Interest: *Upon manuscript submission, all authors completed the Disclosures of Potential Conflict of Interest form. Potential conflicts of interest:*

Employment or Leadership: None declared.
Consultant or Advisory Role: None declared.
Stock Ownership: None declared.
Honoraria: J.W. Scholey, AMGEN Canada Inc.
Research Funding: J.W. Scholey, AMGEN Canada Inc.
Expert Testimony: None declared.

References

1. Manno C, Strippoli GF, Arnesano L, Bonifati C, Campobasso N, Gesualdo L, Schena FP. Predictors of bleeding complications in percutaneous ultrasound-guided renal biopsy. Kidney Int 2004;66:1570–7.
2. Cattran D. Predicting outcome in the idiopathic glomerulopathies. J Nephrol 1998;11:57–60.
3. Reich HN, Troyanov S, Scholey JW, Cattran DC. Remission of proteinuria improves prognosis in IgA nephropathy. J Am Soc Nephrol 2007;18: 3177–83.
4. de Zeeuw D, Remuzzi G, Parving HH, Keane WF, Zhang Z, Shahinfar S, et al. Proteinuria, a target for renoprotection in patients with type 2 diabetic nephropathy: lessons from RENAAL. Kidney Int 2004;65:2309–20.
5. Mann JF, Gerstein HC, Pogue J, Bosch J, Yusuf S. Renal insufficiency as a predictor of cardiovascular outcomes and the impact of ramipril: the HOPE randomized trial. Ann Intern Med 2001; 134:629–36.
6. Norris KC, Greene T, Kopple J, Lea J, Lewis J, Lipkowitz M, et al. Baseline predictors of renal disease progression in the African American Study of Hypertension and Kidney Disease. J Am Soc Nephrol 2006;17:2928–36.
7. Eijkelkamp WB, Zhang Z, Remuzzi G, Parving HH, Cooper ME, Keane WF, et al. Albuminuria is a target for renoprotective therapy independent from blood pressure in patients with type 2 diabetic nephropathy: post hoc analysis from the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) trial. J Am Soc Nephrol 2007;18:1540–6.
8. Cagney G, Park S, Chung C, Tong B, O'Dushlaine C, Shields DC, Emili A. Human tissue profiling with multidimensional protein identification technology. J Proteome Res 2005;4:1757–67.
9. Freue GV, Sasaki M, Meredith A, Gunther OP, Bergman A, Takhar M, et al. Proteomic signatures in plasma during early acute renal allograft rejection. Mol Cell Proteomics 2010;9: 1954–67.
10. Rinschen MM, Yu MJ, Wang G, Boja ES, Hoffert JD, Pisitkun T, Knepper MA. Quantitative phosphoproteomic analysis reveals vasopressin V2-receptor-dependent signaling pathways in renal collecting duct cells. Proc Natl Acad Sci U S A 2010;107:3882–7.
11. Quintana LF, Campistol JM, Alcolea MP, Banon-Maneus E, Sol-Gonzalez A, Cutillas PR. Application of label-free quantitative peptidomics for the identification of urinary biomarkers of kidney chronic allograft dysfunction. Mol Cell Proteomics 2009;8:1658–73.
12. Thongboonkerd V. Practical points in urinary proteomics. J Proteome Res 2007;6:3881–90.
13. Lafitte D, Dussol B, Andersen S, Vazi A, Dupuy P, Jensen ON, et al. Optimized preparation of urine samples for two-dimensional electrophoresis and initial application to patient samples. Clin Biochem 2002;35:581–9.
14. Schaub S, Rush D, Wilkins J, Gibson IW, Weiler T, Sangster K, et al. Proteomic-based detection of urine proteins associated with acute renal allograft rejection. J Am Soc Nephrol 2004;15: 219–27.
15. Hortin GL, Sviridov D. Diagnostic potential for urinary proteomics. Pharmacogenomics 2007;8: 237–55.
16. Zurbig P, Decramer S, Dakna M, Jantos J, Good DM, Coon JJ, et al. The human urinary proteome reveals high similarity between kidney aging and chronic kidney disease. Proteomics 2009;9: 2108–17.
17. Yamamoto T. The 4th Human Kidney and Urine Proteome project (HKUPP) workshop. 26 September 2009, Toronto, Canada. Proteomics 2010;10:2069–70.
18. Vlahou A, Schanstra J, Frokiaer J, El Nahas M, Spasovski G, Mischak H, et al. Establishment of a European network for urine and kidney proteomics. J Proteomics 2008;71:490–2.
19. Thongboonkerd V, Klein E, Klein JB. Sample preparation for 2-D proteomic analysis. Contrib Nephrol 2004;141:11–24.
20. Thongboonkerd V, Chutipongtanate S, Kanlaya R. Systematic evaluation of sample preparation methods for gel-based human urinary proteomics: quantity, quality, and variability. J Proteome Res 2006;5:183–91.
21. Zwelling LA, Balow JE. Hypersthenuria in high-dose carbenicillin therapy. Ann Intern Med 1978;89:225–6.
22. Gaines LG, Fent KW, Flack SL, Thomasen JM, Ball LM, Zhou H, et al. Effect of creatinine and specific gravity normalization on urinary biomarker 1,6-hexamethylene diamine. J Environ Monit 2010;12:591–9.
23. Moriguchi J, Inoue Y, Kamiyama S, Horiguchi M, Murata K, Sakuragi S, et al. N-acetyl-beta-d-glucosaminidase (NAG) as the most sensitive marker of tubular dysfunction for monitoring residents in non-polluted areas. Toxicol Lett 2009;190:1–8.
24. Andersson L, Haraldsson B, Johansson C, Barregard L. Methodological issues on the use of urinary alpha-1-microglobuline in epidemiological studies. Nephrol Dial Transplant 2008;23: 1252–6.
25. Heavner DL, Morgan WT, Sears SB, Richardson JD, Byrd GD, Ogden MW. Effect of creatinine and specific gravity normalization techniques on xenobiotic biomarkers in smokers' spot and 24-h urines. J Pharm Biomed Anal 2006;40: 928–42.
26. Waikar SS, Sabbisetti VS, Bonventre JV. Normalization of urinary biomarkers to creatinine during changes in glomerular filtration rate. Kidney Int;78:486–94.
27. Anderson NL, Anderson NG. The human plasma proteome: history, character, and diagnostic prospects. Mol Cell Proteomics 2002;1:845–67.
28. Anderson NL, Polanski M, Pieper R, Gatlin T, Tirumalai RS, Conrads TP, et al. The human plasma proteome: a nonredundant list developed by combination of four separate sources. Mol Cell Proteomics 2004;3:311–26.
29. Nagaraj N, Mann M. Quantitative analysis of the intra- and inter-individual variability of the normal urinary proteome. J Proteome Res 2011; 10:637–45.
30. Afkarian M, Bhasin M, Dillon ST, Guerrero MC, Nelson RG, Knowler WC, et al. Optimizing a proteomics platform for urine biomarker discovery. Mol Cell Proteomics 2010;9:2195–204.
31. Vaezzadeh AR, Briscoe AC, Steen H, Lee RS. One-step sample concentration, purification, and albumin depletion method for urinary proteomics. J Proteome Res 2010;9:6082–9.
32. Biomarkers on a roll. Nat Biotechnol 2010;28: 431.
33. Pieper R, Gatlin CL, McGrath AM, Makusky AJ, Mondal M, Seonarain M, et al. Characterization of the human urinary proteome: a method for high-resolution display of urinary proteins on two-dimensional electrophoresis gels with a yield of nearly 1400 distinct protein spots. Proteomics 2004;4:1159–74.
34. Adachi J, Kumar C, Zhang Y, Olsen JV, Mann M. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol 2006;7: R80.
35. Deen WM, Bridges CR, Brenner BM, Myers BD. Heteroporous model of glomerular size selectivity: application to normal and nephrotic humans. Am J Physiol 1985;249:F374–89.
36. Shemesh O, Ross JC, Deen WM, Grant GW, Myers BD. Nature of the glomerular capillary injury in human membranous glomerulopathy. J Clin Invest 1986;77:868–77.
37. Norden AG, Sharratt P, Cutillas PR, Cramer R, Gardner SC, Unwin RJ. Quantitative amino acid and proteomic analysis: very low excretion of polypeptides >750 Da in normal urine. Kidney Int 2004;66:1994–2003.
38. Norden AG, Lapsley M, Lee PJ, Pusey CD, Scheinman SJ, Tam FW, et al. Glomerular protein sieving and implications for renal failure in Fanconi syndrome. Kidney Int 2001;60:1885–92.
39. Pisitkun T, Johnstone R, Knepper MA. Discovery of urinary biomarkers. Mol Cell Proteomics 2006;5:1760–71.
40. Gonzales PA, Pisitkun T, Hoffert JD, Tchapyjnikov D, Star RA, Kleta R, et al. Large-scale proteomics and phosphoproteomics of urinary exosomes. J Am Soc Nephrol 2009;20:363–79.
41. Knight EL, Verhave JC, Spiegelman D, Hillege HL, de Zeeuw D, Curhan GC, de Jong PE. Factors influencing serum cystatin C levels other than renal function and the impact on renal function measurement. Kidney Int 2004;65:1416–21.
42. Stevens LA, Schmid CH, Greene T, Li L, Beck GJ, Joffe MM, et al. Factors other than glomerular filtration rate affect serum cystatin C levels. Kidney Int 2009;75:652–60.
43. Stevens LA, Coresh J, Schmid CH, Feldman HI, Froissart M, Kusek J, et al. Estimating GFR using serum cystatin C alone and in combination with serum creatinine: a pooled analysis of 3,418 individuals with CKD. Am J Kidney Dis 2008;51: 395–406.
44. Poge U, Gerhardt T, Stoffel-Wagner B, Klehr HU, Sauerbruch T, Woitas RP. Calculation of glomerular filtration rate based on cystatin C in cirrhotic patients. Nephrol Dial Transplant 2006; 21:660–4.
45. Rule AD, Bergstralh EJ, Slezak JM, Bergert J, Larson TS. Glomerular filtration rate estimated

by cystatin C among different clinical presentations. Kidney Int 2006;69:399–405.
46. Parving HH, Lehnert H, Brochner-Mortensen J, Gomis R, Andersen S, Arner P. The effect of irbesartan on the development of diabetic nephropathy in patients with type 2 diabetes. N Engl J Med 2001;345:870–8.
47. Ravid M, Lang R, Rachmani R, Lishner M. Long-term renoprotective effect of angiotensin-converting enzyme inhibition in non-insulin-dependent diabetes mellitus. A 7-year follow-up study. Arch Intern Med 1996;156:286–9.
48. Lebovitz HE, Wiegmann TB, Cnaan A, Shahinfar S, Sica DA, Broadstone V, et al. Renal protective effects of enalapril in hypertensive NIDDM: role of baseline albuminuria. Kidney Int Suppl 1994; 45:S150–5.
49. Kulasingam V, Diamandis EP. Strategies for discovering novel cancer biomarkers through utilization of emerging technologies. Nat Clin Pract Oncol 2008;5:588–99.
50. Mattes WB, Walker EG, Abadie E, Sistare FD, Vonderscher J, Woodcock J, Woosley RL. Research at the interface of industry, academia and regulatory science. Nat Biotechnol 2010;28: 432–3.
51. Warnock DG, Peck CC. A roadmap for biomarker qualification. Nat Biotechnol 2010;28:444–5.
52. Dieterle F, Perentes E, Cordier A, Roth DR, Verdes P, Grenet O, et al. Urinary clusterin, cystatin C, beta2-microglobulin and total protein as markers to detect drug-induced kidney injury. Nat Biotechnol 2010;28:463–9.
53. Yu Y, Jin H, Holder D, Ozer JS, Villarreal S, Shughrue P, et al. Urinary biomarkers trefoil factor 3 and albumin enable early detection of kidney tubular injury. Nat Biotechnol 2010;28: 470–7.
54. Vaidya VS, Ozer JS, Dieterle F, Collings FB, Ramirez V, Troth S, et al. Kidney injury molecule-1 outperforms traditional biomarkers of kidney injury in preclinical biomarker qualification studies. Nat Biotechnol 2010;28:478–85.
55. Ozer JS, Dieterle F, Troth S, Perentes E, Cordier A, Verdes P, et al. A panel of urinary biomarkers to monitor reversibility of renal injury and a serum marker with improved potential to assess renal function. Nat Biotechnol 2010;28:486–94.
56. Nguyen MT, Ross GF, Dent CL, Devarajan P. Early prediction of acute renal injury using urinary proteomics. Am J Nephrol 2005;25:318–26.
57. Devarajan P, Krawczeski CD, Nguyen MT, Kathman T, Wang Z, Parikh CR. Proteomic identification of early biomarkers of acute kidney injury after cardiac surgery in children. Am J Kidney Dis 2010;56:632–42.
58. Ho J, Lucy M, Krokhin O, Hayglass K, Pascoe E, Darroch G, et al. Mass spectrometry-based proteomic analysis of urine in acute kidney injury following cardiopulmonary bypass: a nested case-control study. Am J Kidney Dis 2009;53: 584–95.
59. Zhang X, Jin M, Wu H, Nadasdy T, Nadasdy G, Harris N, et al. Biomarkers of lupus nephritis determined by serial urine proteomics. Kidney Int 2008;74:799–807.
60. Zhou H, Pisitkun T, Aponte A, Yuen PS, Hoffert JD, Yasuda H, et al. Exosomal fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int 2006; 70:1847–57.
61. Beck LH Jr, Bonegio RG, Lambeau G, Beck DM, Powell DW, Cummins TD, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. N Engl J Med 2009;361:11–21.
62. Liu YH, Chen CH, Chen SY, Lin YJ, Liao WL, Tsai CH, et al. Association of phospholipase A2 receptor 1 polymorphisms with idiopathic membranous nephropathy in Chinese patients in Taiwan. J Biomed Sci 2010;17:81.
63. Prunotto M, Carnevali ML, Candiano G, Murtas C, Bruschi M, Corradini E, et al. Autoimmunity in membranous nephropathy targets aldose reductase and SOD2. J Am Soc Nephrol 2010;21:507–19.
64. Sethi S, Gamez JD, Vrana JA, Theis JD, Bergen HR 3rd, Zipfel PF, et al. Glomeruli of dense deposit disease contain components of the alternative and terminal complement pathway. Kidney Int 2009;75:952–60.
65. Hood BL, Conrads TP, Veenstra TD. Unravelling the proteome of formalin-fixed paraffin-embedded tissue. Brief Funct Genomic Proteomic 2006;5:169–75.
66. Onuma T, Laffel LM, Angelico MC, Krolewski AS. Apolipoprotein E genotypes and risk of diabetic nephropathy. J Am Soc Nephrol 1996;7: 1075–8.
67. Araki S, Moczulski DK, Hanna L, Scott LJ, Warram JH, Krolewski AS. ApoE polymorphisms and the development of diabetic nephropathy in type 1 diabetes: results of case-control and family-based studies. Diabetes 2000;49: 2190–5.
68. Hellin JL, Bech-Serra JJ, Moctezuma EL, Chocron S, Santin S, Madrid A, et al. Very low-molecular-mass fragments of albumin in the plasma of patients with focal segmental glomerulosclerosis. Am J Kidney Dis 2009;54:871–80.
69. Koomen JM, Li D, Xiao LC, Liu TC, Coombes KR, Abbruzzese J, Kobayashi R. Direct tandem mass spectrometry reveals limitations in protein profiling experiments for plasma biomarker discovery. J Proteome Res 2005;4:972–81.
70. Musante L, Candiano G, Petretto A, Bruschi M, Dimasi N, Caridi G, et al. Active focal segmental glomerulosclerosis is associated with massive oxidation of plasma albumin. J Am Soc Nephrol 2007;18:799–810.
71. Kaneshiro N, Xiang Y, Nagai K, Kurokawa MS, Okamoto K, Arito M, et al. Comprehensive analysis of short peptides in sera from patients with IgA nephropathy. Rapid Commun Mass Spectrom 2009;23:3720–8.
72. Haubitz M, Wittke S, Weissinger EM, Walden M, Rupprecht HD, Floege J, et al. Urine protein patterns can serve as diagnostic tools in patients with IgA nephropathy. Kidney Int 2005; 67:2313–20.
73. Rocchetti MT, Centra M, Papale M, Bortone G, Palermo C, Centonze D, et al. Urine protein profile of IgA nephropathy patients may predict the response to ACE-inhibitor therapy. Proteomics 2008;8:206–16.
74. Kakoki M, Smithies O. The kallikrein-kinin system in health and in diseases of the kidney. Kidney Int 2009;75:1019–30.
75. Liu K, Li QZ, Delgado-Vega AM, Abelson AK, Sanchez E, Kelly JA, et al. Kallikrein genes are associated with lupus and glomerular basement membrane-specific antibody-induced nephritis in mice and humans. J Clin Invest 2009;119: 911–23.
76. Suzuki M, Ross GF, Wiers K, Nelson S, Bennett M, Passo MH, et al. Identification of a urinary proteomic signature for lupus nephritis in children. Pediatr Nephrol 2007;22:2047–57.
77. Overgaard AJ, Hansen HG, Lajer M, Pedersen L, Tarnow L, Rossing P, et al. Plasma proteome analysis of patients with type 1 diabetes with diabetic nephropathy. Proteome Sci 2010;8:4.
78. Overgaard AJ, Thingholm TE, Larsen MR, Tarnow L, Rossing P, McGuire JN, Pociot F. Quantitative iTRAQ-based proteomic identification of candidate biomarkers for diabetic nephropathy in plasma of type 1 diabetic patients. Clin Proteomics 2010;6:105–14.
79. Otu HH, Can H, Spentzos D, Nelson RG, Hanson RL, Looker HC, et al. Prediction of diabetic nephropathy using urine proteomic profiling 10 years prior to development of nephropathy. Diabetes Care 2007;30:638–43.
80. Rossing K, Mischak H, Parving HH, Christensen PK, Walden M, Hillmann M, Kaiser T. Impact of diabetic nephropathy and angiotensin II receptor blockade on urinary polypeptide patterns. Kidney Int 2005;68:193–205.
81. Jiang H, Guan G, Zhang R, Liu G, Cheng J, Hou X, Cui Y. Identification of urinary soluble E-cadherin as a novel biomarker for diabetic nephropathy. Diabetes Metab Res Rev 2009;25: 232–41.
82. Rossing K, Mischak H, Dakna M, Zurbig P, Novak J, Julian BA, et al. Urinary proteomics in diabetes and CKD. J Am Soc Nephrol 2008;19: 1283–90.
83. Alkhalaf A, Zurbig P, Bakker SJ, Bilo HJ, Cerna M, Fischer C, et al. Multicentric validation of proteomic biomarkers in urine specific for diabetic nephropathy. PLoS One 2010;5:e13421.
84. Rush DN, Henry SF, Jeffery JR, Schroeder TJ, Gough J. Histological findings in early routine biopsies of stable renal allograft recipients. Transplantation 1994;57:208–11.
85. Sigdel TK, Kaushal A, Gritsenko M, Norbeck AD, Qian WJ, Xiao W, et al. Shotgun proteomics identifies proteins specific for acute renal transplant rejection. Proteomics Clin Appl 2010;4: 32–47.
86. Schaub S, Mayr M, Honger G, Bestland J, Steiger J, Regeniter A, et al. Detection of subclinical tubular injury after renal transplantation: comparison of urine protein analysis with allograft histopathology. Transplantation 2007;84:104–12.
87. Ling XB, Sigdel TK, Lau K, Ying L, Lau I, Schilling J, Sarwal MM. Integrative urinary peptidomics in renal transplantation identifies biomarkers for acute rejection. J Am Soc Nephrol 2010;21:646–53.
88. Nakorchevsky A, Hewel JA, Kurian SM, Mondala TS, Campbell D, Head SR, et al. Molecular mechanisms of chronic kidney transplant rejection via large-scale proteogenomic analysis of tissue biopsies. J Am Soc Nephrol 2010;21:362–73.
89. Quintana LF, Sole-Gonzalez A, Kalko SG, Banon-Maneus E, Sole M, Diekmann F, et al. Urine proteomics to detect biomarkers for chronic al-

lograft dysfunction. J Am Soc Nephrol 2009;20:428–35.

90. Kurian SM, Heilman R, Mondala TS, Nakorchevsky A, Hewel JA, Campbell D, et al. Biomarkers for early and late stage chronic allograft nephropathy by proteogenomic profiling of peripheral blood. PLoS One 2009;4:e6212.
91. Good DM, Zurbig P, Argiles A, Bauer HW, Behrens G, Coon JJ, et al. Naturally occurring human urinary peptides for use in diagnosis of chronic kidney disease. Mol Cell Proteomics 2010;9:2424–37.
92. Gold L, Ayers D, Bertino J, Bock C, Bock A, Brody EN, et al. Aptamer-based multiplexed proteomic technology for biomarker discovery. PLoS One 2010;5:e15004.
93. Dihazi H, Muller CA, Mattes H, Muller GA. Proteomic analysis to improve adequacy of hemo- and peritoneal dialysis: removal of small and high molecular weight proteins with high- and low-flux filters or a peritoneal membrane. Proteomics Clin Appl 2008;2:1167–82.
94. Vanholder R, De Smet R, Glorieux G, Argiles A, Baurmeister U, Brunet P, et al. Review on uremic toxins: classification, concentration, and interindividual variability. Kidney Int 2003;63:1934–43.
95. Sritippayawan S, Chiangjong W, Semangoen T, Aiyasanon N, Jaetanawanitch P, Sinchaikul S, et al. Proteomic analysis of peritoneal dialysate fluid in patients with different types of peritoneal membranes. J Proteome Res 2007;6:4356–62.
96. Sezer S, Tutal E, Arat Z, Akcay A, Celik H, Ozdemir FN, Haberal M. Peritoneal transport status influence on atherosclerosis/inflammation in CAPD patients. J Ren Nutr 2005;15:427–34.
97. Thongboonkerd V. Proteomics in extracorporeal blood purification and peritoneal dialysis. J Proteomics 2010;73:521–6.
98. Shlipak MG, Sarnak MJ, Katz R, Fried LF, Seliger SL, Newman AB, et al. Cystatin C and the risk of death and cardiovascular events among elderly persons. N Engl J Med 2005;352:2049–60.
99. Herget-Rosenthal S, Feldkamp T, Volbracht L, Kribben A. Measurement of urinary cystatin C by particle-enhanced nephelometric immunoassay: precision, interferences, stability and reference range. Ann Clin Biochem 2004;41:111–8.
100. Rosenberg ME, Silkensen J. Clusterin and the kidney. Exp Nephrol 1995;3:9–14.
101. Chaturvedi S, Farmer T, Kapke GF. Assay validation for kim-1: human urinary renal dysfunction biomarker. Int J Biol Sci 2009;5:128–34.
102. Hofstra JM, Beck LH, Jr, Beck DM, Wetzels JF, Salant DJ. Anti-phospholipase A2 receptor antibodies correlate with clinical status in idiopathic membranous nephropathy. Clin J Am Soc Nephrol 2011;6:1286–91.