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Application of Proteomic Analysis to Renal Disease in the Clinic

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Chronic kidney disease afflicts millions of persons worldwide, and accounts for a sizeable percentage of the national health-care budgets in many countries, especially those underwriting the costs of dialysis or transplantation. In this article we present our view as to how proteomic analysis may improve the current situation, and outline of some of the most urgent clinical needs and requirements.

Renal damage as a complication of diabetes mellitus is the most common cause of chronic kidney disease and is due to injury to the intrarenal vasculature and glomeruli as an organ-specific consequence of a systemic abnormality of glucose control. For reasons not yet fully clarified but likely genetic in nature, less than about half of all patients with type 1 diabetes mellitus ever develop diabetic nephropathy [1,2]. The second most frequent category of chronic kidney disease is a host of disorders grouped under the label of glomerulonephritis. In these entities, the inciting event initiates inflammatory pathways in the glomeruli, leading to a wide range of histological findings. Unlike the basis of renal injury in patients with diabetes mellitus, the mechanisms responsible for the onset of disease in patients with many forms of glomerulonephritis remain uncertain.

Assessment of the severity of the kidney injury and gauging the response to therapy cannot be determined by routine urinary testing and currently requires a renal biopsy for examination of the histological features. This procedure entails a risk of morbidity due to bleeding complications (0.3–2.7% risk of transfusion and 0.1–1.0% risk for an invasive procedure to stop the bleeding) [3–6], and processing of the tissue to extract the maximal information by light-, immunofluorescence- and electron-microscopy studies is costly (USD 3,500–4,000). Therefore, many patients with early glomerulus-based renal disease are reluctant to undergo renal biopsy. Unfortunately, waiting for renal injury to become overtly manifest forfeits the opportunity to intervene during the course of disease when the greatest benefit from treatment would be anticipated. Furthermore, renal biopsy is performed relatively rarely for patients with low-molecular-weight proteinuria suggestive of tubular or interstitial injury because the cause is often evident clinically and the histological features are usually non-specific.

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In many countries, including the United States, periodic screening for renal disease is not routinely performed. Testing for renal disease by standard assays, including dipstick urinalysis and microscopic examination of the sediment, quantitative measurement of albumin or total protein as a ratio to urinary creatinine, or assessment of urinary protein components by electrophoretic patterns, may not detect early stages of renal injury. Proteomics has the potential to greatly improve the sensitivity of screening tests, as has been shown for adults with diabetic nephropathy [7] or IgA nephropathy [8–11] and children with obstructive uropathy [12–14]. Furthermore, the applicability of this approach extends beyond diseases arising in the kidney or urinary tract, such as patients with coronary disease [15–17]. However, as discussed below, major hurdles must be overcome for urinary proteomics to be of practical use in the clinic.

The information gleaned from the urine by proteomic studies will likely have an important impact on the care of patients with renal diseases. In contrast with genetic testing to determine whether a person has an allele (variant) that confers an increased likelihood to develop a disorder at some point in the future, urinary proteomics has the potential to reveal the presence of renal injury in the initial phases of the disease. Table 1 lists potential applications in nephrology that, in our opinion, seem most suitable for a urinary proteomics-based approach. Renal diseases that start with inflammation in the glomerulus have distinctive histology patterns by light-, immunofluorescence-, and electron-microscopy. Based on this fact, we should expect distinguishing biomarkers, or a panel of markers, in the urine. By the time that renal injury is detected by conventional laboratory tests, such as urinalysis or quantitative urinary protein measurement, the patient has often incurred substantial irreversible damage. Biomarkers that enable physicians to screen for early evidence of renal disease ideally would facilitate intervention in the beginning of the disease process and lead to better outcomes, even in the absence of a disease-specific approach. For example, suppression of the scarring effects of angiotensin II with angiotensin converting-enzyme inhibitors and angiotensin receptor type 1 blockers has been shown to slow the decline in renal function [18]. Therefore, detection of changes in the urinary proteome, followed by appropriate therapy, could well postpone the need for expensive renal replacement therapy with dialysis or transplantation.

Another setting in which detection of early renal injury would be immediately helpful is renal transplantation. Acute rejection of the allograft may develop through T cell-mediated mechanisms or circulating antibodies against human leukocyte antigen. Because the treatment of these two types of rejection differs greatly and the change in serum creatinine concentration is a notoriously late marker, biopsy of the single kidney is usually done to define the basis of the renal dysfunction. A means to detect and distinguish these processes by noninvasive protocols before significant injury has occurred will be a major advance in the care of transplant recipients. Another cause for dysfunction of transplanted kidneys is the recurrence of the disease that damaged the native kidneys. For most forms of glomerulonephritis, this process clinically manifests months to years after engraftment, although for a few diseases proteinuria may resume within hours. In addition, urinary proteomics may improve the process of screening persons for living-donor nephrectomy. Such individuals are often relatives of the prospective recipient and may be at increased risk for developing renal injury, depending on the specific type of renal disease. While some genetically determined kidney diseases may be detected by established noninvasive techniques (*e.g.*, ultrasound or genetic testing), glomerular disorders require histological assessment. The biopsy procedure is not medically justifiable in individuals without overt evidence of renal disease. Unfortunately, the unintended use of an allograft from a donor with subclinical glomerulonephritis, in the absence of evidence of renal disease by conventional testing, has been documented [19]. In that instance, the glomerular abnormalities cleared from the transplanted kidney within a few weeks, but the donor was left with decreased renal reserve to deal with a life-long kidney disease. In the era of ever increasing demand for renal transplantation, the search for potential donors has been widened to include

persons without a biological relationship to the recipient, such as spouses and friends. Although generally in excellent health, these persons are at risk for the random affliction with renal disease [20–22]. With validated reliable proteomic markers, clinicians could protect such individuals by precluding nephrectomy and starting treatment to retard the renal injury.

After the diagnosis of renal disease has been established, it is important to monitor the activity of the renal disease to initiate or modify treatment. In patients with glomerulonephritis, the best current method is serial renal biopsies. Therefore, diagnostic tests based on urinary proteomics in this setting would be a welcomed improvement. One study has raised expectations that such monitoring will be feasible. Treatment with candesartan, an angiotensin receptor blocker, altered the urinary proteome from the disease-specific pattern toward the pattern of healthy controls [23]. Similar results were recently obtained with irbesartan (H. Mischak, personal communication). Full validation of the merit of urinary proteomics in this setting will require confirmation of the urinary changes by comparing them with the histological features (for glomerular disease) or accepted imaging criteria (for cystic or obstructive disorders).

Investigation of the urinary proteome has the potential to unlock some of the mysteries of the pathogenesis of various renal diseases. In the analysis of such data, it is paramount to remember that the histological phenotype of some diseases may represent the endpoint of unrelated pathophysiological processes. Thus, the findings in the urinary proteome may be similar in the late/terminal stages of the disease process, but differ in the early stages [24]. It is, therefore, crucial to have access to expert clinicians to ensure well-defined phenotypes and to assist in the interpretation of the data. As disease-associated modifications/alterations in the urinary proteome are established, understanding the basis for these changes may provide a mechanism-based opportunity for intervention as treatment. Further, as therapy becomes more disease-specific, the need to have a precise diagnosis early in the clinical course takes on ever-greater value.

From a clinical perspective, a wide range of variables must be defined to ensure the usefulness and comparability of the results of an analysis of the urinary proteome. It is critical to adopt uniform collection and processing protocols to address issues that superficially seem to be mundane but in reality are vital for interpretation of the results, such as time of day for sampling, in rest or after physical activity, early- *versus* mid-stream, removal of cells and debris, addition of preservatives and long-term storage [25]. Clinical proteinuria is influenced by intensity of physical activity, dietary intake of protein and salt, systemic blood pressure, pubertal status, age, gender, and time of collection (diurnal variation). Medications may substantially alter protein excretion, independent of any effect on the above variables. For example, suppression of the renal effects of angiotensin II by an angiotensin converting-enzyme inhibitor or an angiotensin receptor type 1 blocker has been shown to reduce proteinuria in patients with a variety of primary glomerular renal diseases, diabetic nephropathy, or dysfunctional renal allograft [26,27]. The effect of calcium-channel blockers on proteinuria differs between its two classes. It seems plausible to expect that these changes in protein excretion will not be proportional across the full spectrum of serum proteins because some medications (*e.g.*, angiotensin converting-enzyme inhibitors) alter the permselectivity of the filtration pores in the glomerular basement membrane complex [28]. Thus, many clinical parameters must be taken into account in a detailed analysis of urinary proteins to permit a practical application in the clinic and a meaningful comparison of studies from separate centers.

To identify urinary proteomic markers for early stages of renal diseases, we favor international collaboration to establish a large sample bank and database with prospectively collected urine samples and clinical data, and long-term observation to identify patients with newly manifested renal disease. However, a prospective randomised clinical trial with serial kidney biopsies

(tissue proteomic) correlated with urinary proteomic markers and comparing various treatment options might be ideal. This population could include persons at high risk for developing renal disease: first-degree relatives of patients with types of primary glomerulonephritis that have shown familial patterns, such as focal segmental glomerulosclerosis or IgA nephropathy [29–32] or renal transplant recipients monitored serially. These diagnoses currently require histological examination of renal tissue and renal biopsy will still be necessary to establish the unequivocal identification of the disease process. Persons with other forms of renal disease, such as diabetic nephropathy or autosomal-dominant polycystic disease, will not require biopsy for diagnosis. The prospectively collected urine samples would then be assayed for markers diagnostic of the disease and those associated with its manifestations and outcome. Ideally, the database would be sufficiently large to allow validation of the markers in a separate cohort of patients. Depending on the specific renal disease and the rate of its progression, this process may require several years to accomplish.

Before currently available approaches for urinary proteomics can be expected to be applied in the clinic, several confounding factors must be addressed (Table 2). This refinement process will require the coordinated input from investigators in the realms of basic science and clinical medicine. At present, it is difficult, even imprudent, to guesstimate the range of per-assay cost that would be acceptable for clinical urinary-proteomics-based testing. Clinical factors extend far beyond simply the expenses of performing the assay. These aspects include the specificity and sensitivity of the assay and the degree to which these exceed that of current tests, number of disorders for which the results are applicable, severity of the disorder identified by the assay, usefulness in monitoring spontaneous fluctuations in pathological activity and response to therapy. Furthermore, the per-assay cost must be balanced against the potential savings realized due to a more specific and earlier diagnosis. For example, if the patient will not require a renal biopsy, the costs of the procedure are set aside. Moreover, an early diagnosis that leads to a therapeutic intervention may reduce the severity of the long-term complications and thereby further lessen health-care expenses. For a urinary proteomics-based assay to gain acceptance in the clinic, the processing of the urine sample must be as simple as possible. For example, handling the sample at room temperature is more convenient than freezing, especially if the sample is to be mailed to a reference laboratory. For most of the conditions listed in Table 1, a turn-around time of several days will suffice. However, in the settings of acute injury due to rejection of an allograft, renal dysfunction in an intensive-care unit, or an aggressive variation of any of several types of glomerulonephritis, an interval of less than 24 hours would be much preferable.

Mass spectrometric techniques currently used for analysis of urinary biomarkers are listed in Table 2 of the accompanying review (Julian BA *et. al.*). In terms of reproducibility, capillary electrophoresis separation with on-line mass spectrometric detection performed best for urinary peptides in the range of 1–5 KDa [10,13,14,33–38]. To improve throughput, future assays may encompass an immunoprecipitation or immunocapture step followed by MALDI-TOF mass spectrometric analysis. In this process, two differentially labeled reference standards could be added for quantification purposes, one for an internal marker and the second for the marker of interest [39]. It seems unlikely that an enzyme-linked immunosorbent assay (ELISA) will soon be developed because the urinary marker(s) of interest are often unique fragments of common proteins [36]. There are, however, ELISA-based approaches that can measure peptides as small as 1 KDa [40]. Development and validation of such assay would involve significant amount of time and effort, as a specific monoclonal antibody, its fragment(s), or pairs of monoclonal antibodies must be produced and characterized [41]. From a statistical perspective, a useful assay must be developed in a cohort large enough to avoid β -type errors whereby the significance is simply a matter of chance. The discovery must then be validated in a separate sufficiently large cohort. The results of the assay must be expressed in a manner by which a clinician not intimately familiar with the technical aspects can appreciate the level

of confidence in the findings. Clinical tests rarely exhibit 100% sensitivity and 100% specificity. As is the case for many tests now in clinical practice, new diagnostic assays derived from urinary proteomics will serve as a trigger for more specific testing to confirm the findings. Detection of a disease in its early stages constitutes an opportunity to begin treatment when the best clinical results should be anticipated and the prospects to mitigate long-term expenses are the greatest [24,41].

In summary, recent advances in urinary proteomic analyses have the potential to significantly enhance the care of patients with renal disease, as well as other disorders, and alleviate some of the financial burden on national health care systems. To achieve these goals, cooperation of interdisciplinary teams from centers around the world will be vital in the efforts to standardize the methodology for preparation the sample, performance of the analysis and interpretation of the findings. In this regard, an effort to enhance networking activities in this field has been initiated in Europe. Recently, funded by COST (European COoperation in the field of Scientific and Technical Research: www.cost.esf.org), EuroKUP (European Kidney and Urine Proteomics: www.eurokup.org) multidisciplinary network composed of scientists from 19 European and four non-European countries was established. This will facilitate translational research in kidney diseases in a manner that will allow fruitful interactions, dissemination of expertise and promotion of collaborations between researchers with similar interests as well as the identification of clinical center networks for specific diseases [42]. It is crucial for our patients to seize the opportunity.

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Table 1

Renal disorders amenable to early diagnosis or monitoring of clinical course by urinary biomarkers.

Renal disorders	Specific cause or diagnosis	Clinical application
Intrinsic	Glomerulonephritis	Diagnosis of primary disease Monitoring response to therapy - Immunomodulatory treatment for glomerulonephritis - Suppression of angiotensin II to lessen scarring and reduce proteinuria
	Acute renal injury in hospitalized patient	Drug toxicity - antibiotics, imaging agents, chemotherapy Hypoperfusion - hypotension, vasopressors, or cytokine-induced shunting
	Cystic renal diseases	Identify quickly progressing patients for early treatment that may retard growth of cysts
	Obstructive uropathy in childhood	Identify patients needing early surgical intervention
	Transplantation	
	Acute rejection	
	- T cell-mediated	Occurs in 10–15% of patients within 3 month of engraftment
	- Antibody-mediated (anti-HLA, donor-specific antibodies)	Common cause of progressive renal dysfunction
	Nephrotoxicity of immunosuppression (<i>e.g.</i> , calcineurin inhibitor)	Frequently confused clinically with antibody-mediated rejection
	Recurrence of glomerulonephritis	Common cause of progressive renal dysfunction
	Screening prospective living donors	Likely to increase donor pool for patients with familial renal disease

Table 2

Confounding factors to be addressed for the clinical application of urinary proteomics.

Major factors	Specific aspects	Comments
Cost of the assay	Equipment	Newer generations of mass spectrometers likely to be more affordable
	Reagents	Mostly relevant to separation (HPLC, CE)
Turn-around time	Skill level of technician(s)	Automation will reduce expense
	Goal varies by acuity of clinical situation	Generally, not less than 24 h
	Processing and handling of sample	Preferably, minimal on-site processing
	Reference laboratory <i>versus</i> on-site laboratory	High-volume reference laboratory may be less expensive
	Throughput of analysis	Cost reduced with higher throughput
Statistical aspects and interpretation	Identification of the appropriate biomarker(s)	Biomarker development and validation for translation into clinical use
	- Sensitivity and specificity of the assay	
	- Level of confidence in the result	- Analysis that accounts for repeat testing of multiple variables
		- Cohort size, sufficient to avoid flawed results due to β - type errors
		- Validation in separate cohort
	Interpretation by clinicians not well versed in the technical aspects of assay	The results should be expressed in well defined standardized notations