Review

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The non-invasive biopsy—will urinary proteomics make the renal tissue biopsy redundant?

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Summary

Proteomics is a rapidly advancing technique which gives functional insight into gene expression in living organisms. Urine is an ideal medium for study as it is readily available, easily obtained and less complex than other bodily fluids. Considerable progress has been made over the last 5 years in the study of urinary proteomics as a diagnostic tool for renal disease. Advantages over the traditional renal biopsy include accessibility, safety, the possibility of serial sampling and the potential for non-invasive prognostic and diagnostic monitoring of disease and an individual's response to treatment. Urinary proteomics is now moving from a discovery phase in small studies to a validation phase in much larger numbers of patients with renal disease. Whilst there are still some limitations in methodology, which are assessed in this review, the possibility of urinary proteomics replacing the invasive tissue biopsy for diagnosis of renal disease is becoming an increasingly realistic option.

Introduction

Bodily fluids in all organisms contain thousands of proteins and peptides, which undergo disease specific changes. A deeper knowledge of the pattern of change and the functional relevance of individual proteins and peptides which reflect the disease process will inevitably better define the 'normal' proteome and provide insight into mechanisms underlying disease. Considerable advances have recently been made in proteomics, which allows the study of protein expression in a tissue or bodily fluid, and the use of urine as a source of candidate biomarkers and potential therapeutic targets is rapidly developing. In relation to renal disease, discovery of specific peptides in the urine of patients with renal impairment could allow the clinician to make a diagnosis without the requirement for an invasive renal biopsy. This is the ultimate goal of urinary proteomics and is the subject of this review article.

A specific diagnosis of renal disease may be straightforward in the presence of pathognomonic clinical or laboratory features; however, there are usually several differential diagnoses and the renal biopsy is currently the definitive investigation for determining pathology. The diagnostic yield from a renal biopsy has improved considerably over the last two decades due to the use of ultrasound and automated guns,[1–3] but the procedure still carries a low but not negligible mortality rate, is associated with significant risk of haemorrhage[4] and may cause considerable discomfort. Patients are also required to remain on the ward for at least 6 h, and usually overnight.

Several factors related to the nature of the disease and patient status can directly impact on the effectiveness of renal biopsy. Some conditions do not

affect the kidney uniformly and the biopsy sample may not be representative of the disease involvement. The use of larger core biopsy needles and multiple samples can circumvent this to a degree,[5,6] but there is a high-complication rates[7] and the biopsy may still yield insufficient sample to enact appropriate clinical decisions.

Preparations for the biopsy such as blood pressure control and correction of coagulopathies may delay the procedure and therefore diagnosis. Where bleeding tendency precludes a conventional method a transjugular approach may be required, which has comparable yield and safety profile to the real-time ultrasound guided automated gun technique[8] but requires personnel with expertise. In some circumstances such as uncontrollable blood pressure, body habitus or the presence of other confounding factors e.g. pregnancy, the biopsy may be contraindicated.

Some information about prognosis may be gained from the clinical severity of the disease or degree of chronic damage in the biopsy[9] but this may not always be a reliable estimate of the rate of deterioration or outcome particularly if the specimen is poor or non-representative. The response to treatment can only be predicted by knowledge of the disease process and the presence or absence of known risk factors. An accurate individual assessment cannot be made on the basis of the renal biopsy alone.

A single urine specimen, sent from the ward or clinic to the laboratory for tests which provide sensitive and specific information on the disease process and prognosis, and also predict the response to treatment at an early stage of disease would provide the ideal. Urine is easy to collect and simple to store and urinary proteins are derived from both blood and kidney.[10] The role of urinary proteomics in renal disease is now being actively explored and the potential of this method as an alternative to the invasive renal biopsy, together with the current obstacles still faced, form the basis of this review.

Background

Renal handling of protein

Human urinary protein excretion is ~150 mg/day protein in healthy normal individuals;[11] of the constituent proteins 70% are derived from the kidney and 30% originate in the plasma.[12,13] Proteins of molecular weight <40 kDa are freely filtered by the glomerulus and almost all are reabsorbed in the early proximal tubule by active transport pathways involving receptor-mediated endocytosis.[14]

When the glomerular filtration barrier is disrupted in glomerular diseases, larger proteins appear in the urine in considerable quantities giving rise to glomerular proteinuria.

Tubular epithelial cells secrete low molecular weight soluble proteins, including Tamm-Horsfall proteins and ß2 microglobulin. When damaged, this process is disrupted (e.g. interstitial nephritis, acute tubular necrosis, Fanconi syndrome), and concentrations of these proteins increase in the urine, together with reduced uptake of filtered low molecular weight proteins. 'Tubular proteinuria' usually produces less total urinary protein than glomerular disease (<2 g/day) due to lower molecular masses of the proteins affected. In healthy individuals tubular proteins make up ~19% of total urinary protein.[15,16]

Urinary sediment proteins account for 48% of total urinary protein from sloughed epithelial cells,[15,16] including podocytes, tubular cells and lower urinary tract cells which are shed into the urine, especially in first void specimens. These increase in inflammatory conditions or in association with malignancy of the urinary tract. The remaining 3% of urinary protein consists of exosomes originating from cells from the tubular epithelial and the urogenital tract, which can be isolated by ultracentrifugation as they remain in the supernatant.[15,16]

Over-flow proteinuria may occur in disease states associated with enhanced synthesis of new small proteins that are freely filtered and overload tubular reabsorptive capacity. Examples include light chain proteinuria, haemoglobinuria in haemolysis, and rhabdomyolysis. Saturation of tubular reabsorption also occurs in glomerular disease, due to the disruption of the glomerular filtration barrier, when the plasma proteins which gain access to the proximal tubule overwhelm reuptake mechanisms.

Available techniques for detecting or quantifying total urinary protein content, together with other standard urine assays for proteins are shown in Table 1. None of these routine tests (other than that for immunoglobulin light chains) identifies or quantifies individual proteins within urine.

It should be appreciated, that in addition to the abundance of proteins identified in the urine, analysis is complicated by post-translational modification e.g. advanced glycation in diabetes[17] and splice variants, which may nonetheless provide valuable aids to disease diagnosis and prognosis prediction. These are detectable by a difference in protein mass, and are identifiable by mass spectrometry. So far more than 1500 different proteins have been characterized in normal healthy individuals' urine.[13] Separation, differentiation and quantitation of constituent proteins can be achieved by proteomics.

Table 1 Current methods for assessing proteinuria

Test	Sensitivity	Method	Advantages	Disadvantages
Urine Dip	>250 mg/l	Binding of urine protein to tetrabromophenol blue	Quick and cheap	Lower limit of sensitivity, some proteins do not bind e.g. Bence–Jones protein
24 h urine collection	30 mg/24 h	Biuret method	Current gold-standard	Inaccurate due to incomplete collection, inconvenient and time consuming
Protein:creatinine ratio	0.02 mg/μmol	Corrects for variation in urinary concentration due to hydration	Simple, cheap and quick, replacing 24 h urine collection	Only measures total protein
Immunoglobulin light chains	0.04 g/l	Urine protein electro-phoresis and immuno-fixation or immunoassay-based free light chain assay	Able to detect at low levels	Expensive and disease specific

An overview of current proteomic techniques

The proteome is defined as the profile of the proteins within a bodily fluid, tissue or cell and the peptidome refers to the lower molecular weight peptides in a sample. Whilst this review focuses on the urinary proteome similar principles also apply to the urinary peptidome, although sample preparation steps may vary. Figure 1 shows a general approach to urinary proteomic methods. Currently there is no universal approach for urine collection and preparation for proteomic analysis and this is the focus of the Human Kidney and Proteome Project (http://www.hkupp.org/)/World Human Proteome Organisation (http://www.hupo.org/) and European Kidney and Urinary Proteomics (http://www.eurokup.com).

Sample collection

The volume, protein composition and protein concentration of urine shows considerable diurnal variation. No ideal time of day to collect urine specimens for proteomics has been identified, but it is preferable to obtain specimens after controlled bed rest or at least at the same time of day to minimize variation from position or effects of exercise.[18] First void urine tends to be more concentrated with a higher yield of proteins and two-dimensional electrophoresis (2-DE) proteomic analysis of samples from healthy individuals has shown all proteins found in urine collected over a 24-hour period to be present in first void urine,[19] although contamination in first void samples from cells from the lower urinary tract and bacteria introduces another variable;[20] and, in women, proteins are present in the early morning which are not found at other times of day.[21] The current consensus is to use random midstream urine specimens, other than the first morning void, to mitigate contamination by bacteria harboured in the urinary tract.

Some intra-patient variability in the urine proteome has been previously observed,[22] but other investigations have identified only minor variations in samples collected for up to a year.[19,23] Individual fluctuations seem to be minimally affected by diet and exercise.[24] The relative influence of exogenous and endogenous factors needs further exploration, but should be considered when planning protocols and in interpretation of data.

Sample storage

An advantage of urinary proteomics is that the analytical reproducibility of the urine proteomic profile is unaffected by long term freezing,[25] remaining stable for several years, even when stored at −20°C.[26,27] Examination of the role of centrifugation before and after freezing, the impact of freeze-thaw cycles, the effect of temperature and the use of protease inhibitors[28,29] has suggested that samples should ideally be centrifuged prior to storage at −80°C to reduce contamination by proteins leaking cellular debris and bacteria.[16] Most laboratories have not adhered to this standard and its advantages
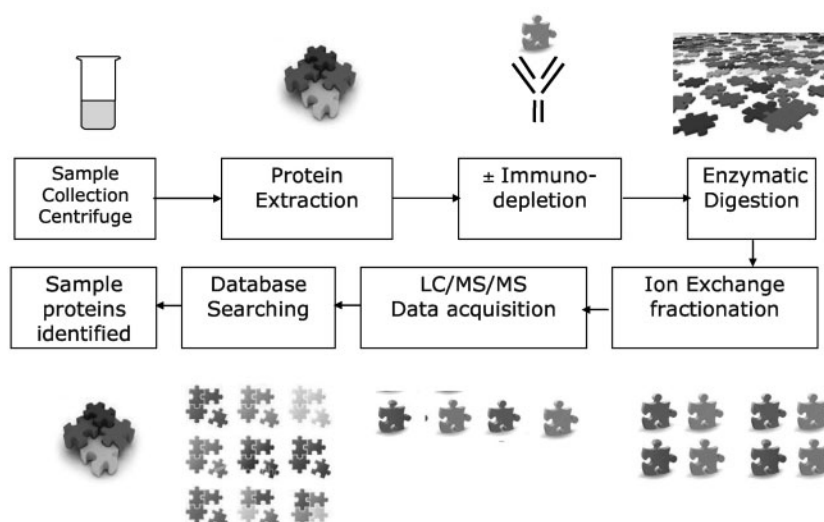
Figure 1. Overview of urinary proteomics: examples of simple workflow for protein identification. Proteins are extracted from urine, optionally immunodepleted of some abundant proteins including albumin and immunoglobulins, and enzymatically digested (usually with trypsin). The resulting peptides are fractionated by string ion exchange before liquid chromatography and tandem mass spectrometry (LC-MS/MS); precursor peptides and fragment ion masses are anlysed by complex search programmes to identify proteins from genomic databases.

remain to be proven. In addition, centrifugation may result in the loss of urinary protein,[30] but this may be an acceptable 'sacrifice' to obviate sample contamination. With or without centrifugation, samples should be stored in multiple aliquots and freeze-thaw cycles minimized to four or less to preserve protein stability[25] (e.g. IgG and alpha-1 antitrypsin[31]). Reproducibility is maintained in samples stored for four to six hours at room temperature,[23,32] or for up to three days at 4°C.[25] Boric acid (2–20 mM) or sodium azide (0.1–1 mM) can be added to retard bacterial overgrowth, with higher concentrations recommended for 24 h collections.[28] Without preservatives, bacterial overgrowth can occur within non-centrifuged samples after eight hours at room temperature, or 20 h at 4°C.[29] Protease inhibitors were previously recommended for preservation[12] but may interfere with mass spectrometry analysis and small molecular weight inhibitors can bind to peptides altering the isoelectric point.[33,34] Urine with low protein concentration contains only modest amounts of protease activity which is unlikely to affect sample stability. However, the abundance and effects of proteases in urine with higher protein concentrations needs further investigation and could have implication for the study of the proteome in patients with significant proteinuria.

The pH of urine, which can vary from 5 to 8, influences enzyme activity and therefore protein cleavage.[10] In a study of renal transplant rejection the urine pH was lower with active rejection, playing a permissive role in the action of endogenous proteases in cleavage of ß2-microglobulin, leading to greater abundance of different ß2-microglobulin fractions.[35] The effect of pH on posttranslational modification of urinary proteins is poorly understood. It is likely to be significant and should routinely be reported in urinary proteomics studies.

Sample preparation

Appropriate preparation of samples is strongly dependent on the proteomic techniques to be used and should be factored into the study design. Some of the more common approaches are discussed below.

Protein extraction and salt removal from urine samples

Isolating or concentrating urinary proteins may be essential in low concentration specimens, particularly for less sophisticated gel-based studies. Numerous methods have been compared including precipitation with organic solvents, centrifugal filtration, lyophilization and ultrafiltration but with varying results. In one study lyophilization afforded the greatest quantitative protein yield, but lowest qualitative yield, whereas acetonitrile precipitation gave the best qualitative yield.[22] In contrast, others found

that centrifugal filtration was superior in terms of ease of analysis and consistency of protein yield compared with dialysis/lyophilization and organic salt precipitation.[36] Reverse phase extraction has also been shown to be effective in specimen concentration as well as for desalting urinary peptides and segregating lower-molecular weight proteins.[18] Different proteins appear to be lost with each of the preparative techniques and therefore a combination approach is most likely to give the complete proteome. These methods simultaneously remove sodium, potassium and urea from the sample, which otherwise could affect the efficiency of enzymatic proteolysis, for example trypsin activity.

Albumin removal

The dynamic range of plasma proteins in the urine is estimated to be of 12 orders of magnitude of concentration, ranging from high-abundance albumin (in renal disease) and uromodulin to low-abundance cytokines.[37] Even in urine from healthy individuals there are many free amino acids and many low molecular weight peptides (<250 Da).[38] The concentration of albumin is a particular problem in nephrotic syndrome when it may be ten-fold higher than normal. The most advanced mass spectrometer (MS) can probe through proteomic depths of about 8 orders of magnitude, but to distinguish lower abundance proteins specific sample enrichment is required. No single approach can yet reveal the entire urinary proteome but complementary application of different protein separation and MS techniques can provide a more comprehensive analysis.[23]

The removal of albumin by immunoprecipitation, affinity capture or protein size fraction improves the identification of low abundance proteins.[13,39–41] However, albumin depletion is not necessarily desirable as albumin itself or other abundant proteins may reveal invaluable information about the disease process. For example, in nephrotic patients a characteristic repetitive fragmentation pattern of urinary albumin has been identified,[42] and a disease specific albumin fragment has been isolated in Type 2 diabetics with low-level microalbuminuria who may be at risk of disease progression.[43] Furthermore, lower abundant proteins may be bound to albumin and co-depleted. Ultracentrifugation with detergents and chaotrophic agents (e.g. urea and SDS) can be employed to disrupt protein–protein interactions, but these interfere with MS and 2-DE methods and necessitate additional sample clean-up.[44]

Concentration and normalization

The variable dilution of urine needs to be factored into proteomic analysis to enable accurate quantification.[45,46] Some laboratories standardize specimen concentration on the basis of creatinine (e.g. 50 nmol/100 µl)[47,48] which is excreted at a relatively constant rate,[48] except in renal disease, which could obviously confound interpretation. If the specimen is concentrated proportionally to creatinine different amounts of proteins are analysed which will affect the profile identified, but this method does allow absolute quantification and therefore direct comparison of samples. Another approach is to study identical total protein loads, and therefore only relative amounts of individual proteins can be compared. Jantos-Siwy *et al.* have recently defined a series of 'house-keeping' collagen fragments that allow relative quantification of biomarker peptides between samples in patients with chronic kidney disease (CKD). These endogenous peptides were superior to exogenous isotope-labeled peptide standards for biomarker quantification.[49]

Trypsin digestion

For the purposes of mass spectrometry, proteins are typically digested by the endoprotease trypsin to generate proteolytic peptides. Trypsin cleaves proteins after the basic arginine and lysine residues, and the resulting tryptic peptides generally possess a positively charged C-terminal arginine or lysine residue. The presence and localization of the positively charged residue facilitates ionization and fragmentation of the peptide during mass spectrometry; thus trypsin is the enzyme of choice for most proteomic applications and is favoured over proteases that cleave at other residues. To improve efficacy of digestion and to achieve total protein lysis, proteins are usually first reduced, to cleave disulfide bridges, and free cysteines are alkylated in order to prevent the reformation of disulfides, or other undesired reactions.

Protein/peptide separation

The enormous differences in protein content and abundances in biological matrices such as urine and plasma renders it impossible to analyse the proteome comprehensively by MS, without prior fractionation.[10] Fractionation can be performed at the protein level or the peptide level, or both, depending on sample complexity. Protein separation is commonly achieved using 1D-SDS–PAGE, 2-DE, or capillary electrophoresis (CE); 1D-SDS–PAGE separates proteins according to molecular mass,

CE by differences in isoelectric point and 2-DE by both molecular mass and isoelectric point. These methods have the advantage of separating many lower abundance proteins from higher abundance proteins to improve proteomic coverage and depth. Peptide separation after proteolytic digestion is typically performed using reverse phase-high performance liquid chromatography (RP-HPLC) and/or strong cation exchange (SCX), which separate peptides based on hydrophobicity and charge state, respectively. Both methods can be linked online to MS processing. Achieved by whatever means, fractionation provides enriched sub-populations of peptides for introduction to the MS.

Two-dimensional electrophoresis, which has been in use for several decades, requires isolation of protein spots and physical dissection before enzymatic digestion and entry into the MS for ionization. It is labour intensive and can analyse only 70–420 protein spots per gel, compared to 400–2000 polypeptides in a single run of CE–MS,[50–52] but does allow the detection of some large proteins which may not elute in a defined peak or which precipitate during HPLC. More recently, 2D difference gel electrophoresis (2D-DIGE) has been developed and used successfully in urine.[53] It utilizes fluorescent dyes and internal standards for more accurate quantification and comparison of two samples, as well improved reproducibility. Smaller peptides (<10 Da), however, are not detectable.[26,54]

Mass spectrometry

Each mass spectrometer consists of an ion source, a mass analyser that measures the mass-to-charge ratio (m/z) of the ionized analytes and a detector that registers the number of ions at each m/z value. Different ionization sources include matrix assisted laser desorption/ionization (MALDI) and its variant surface enhanced laser desorption/ionization (SELDI), which employ an organic acid matrix to sublimate and ionize analytes with laser pulses, and electron-spray ionization (ESI) which involves spraying voltage charged analyte solutions to desolvate and ionize the analytes. Different mass analysers include ion trap (IT), time-of-flight (TOF), quadrupole (Q) and Fourier transform ion cyclotron resonance (FT-ICR) devices. Sequential combination of two mass analysers, known as MS/MS or tandem mass spectrometry, can also be performed, for example using QQQ, Q-TOF and TOF/TOF configurations. Initial selection of a precursor peptide ion of interest by the first mass analyser is immediately followed by the fragmentation of the precursor ion, most often by collision induced dissociation with an inert gas such as argon or nitrogen, and subsequent

detection of the resulting daughter fragment ions by the second mass analyser. MS/MS experiments for individual precursor ions occur on millisecond time-scales and rapidly generate peptide sequence information to complement the mass of the selected precursor peptide. The different mass analyser configurations can be alternately combined with the ionization sources although most commonly MALDI is coupled with TOF (MALDI-TOF and MALDI-TOF/TOF) and ESI with Q (ESI-QQQ and ESI-QTOF). More recent instruments include linear ion trap instruments which have expanded capability over traditional QQQ analysers, including MS/MS/MS functionality and superior sensitivity. Additional coupling of the linear ion trap to the recent Orbitrap mass analyser further enables the simultaneous acquisition of selected ions with extremely high resolution and mass accuracy. The different MS platforms employed in proteomics have been comprehensively reviewed by others.[26,55]

Bioinformatics and data analysis

Proteins may be identified after MS analysis by two common methods. The first and older method of peptide mass fingerprinting (PMF) involves matching the observed experimental peptide masses against theoretical peptide masses calculated from *in silico* digestion of protein databases e.g. for MALDI-TOF. The confidence in protein identifications obtained by PMF is strongly dependent on the mass accuracy of the observed peptide ions and the complexity of the analyte. Although it is possible to identify proteins confidently in simple mixtures of a few proteins, the additional peptides observed after digesting more complex samples compromises confidence in protein identification. The advent of tandem mass spectrometry enabled a second method for confident identification of peptides and proteins based on the peptide mass combined with fragment ions that elucidate sequence information. The precursor peptide and fragment ion masses are analysed by complex search programs, such as Mascot (http://www.matrixscience.com) and Sequest (http://www.thermo.com), to identify and score the observations against theoretical results generated by *in silico* digestion and fragmentation of proteins derived from genomic databases.[56,57] A probability scored protein 'hit list' can then be constructed based on the number and confidence of the identified peptides. The success of the database interrogation is dependent on the accuracy of the database to reflect the true biology, and the input of search parameters to account for appropriate post-translational modifications. For instance, search results can be adversely affected if
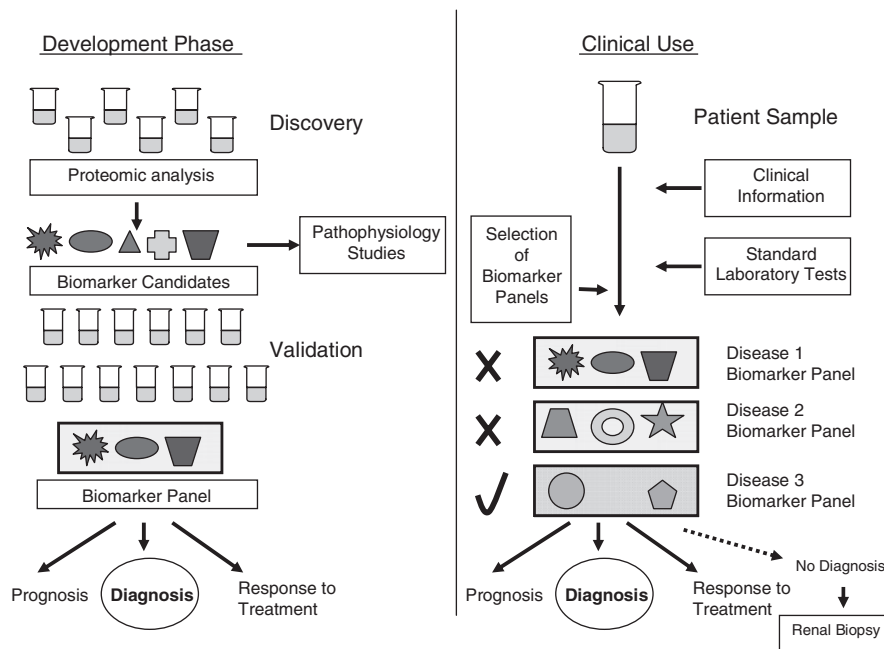
Figure 2. Schema of translation of urinary proteomics into clinical practice.

polymorphisms or mutations are not included in the database sequence, and in the latter case peptides modified by phosphorylation, alternative splicing or endogenous proteolytic degradation will be overlooked if unaccounted for. However, recent advances in bioinformatics and computing power are beginning to address some of these issues by enabling *de novo* search strategies to account for unanticipated polymorphisms, proteolysis or other sequence modifications.[58]

The importance of bioinformatics in studies of urinary proteomics cannot be underestimated and with the creation of substantive proteomic databases, sophisticated computer hardware and software are required. Individual MS runs can identify hundreds to thousands of proteins, any of which could be potential biomarkers. Analysis generally focuses on small subsets of the data exhibiting obvious differential changes between control and case samples rather than evaluation of the entire dataset.[59] A general approach is to develop an algorithm for analysis in a 'training set', which incorporates the most prominent differentially expressed proteins for validation against a 'validation set' for diagnostic utility; in some cases validation may involve measuring the new candidate biomarkers using a conventional technique such as ELISA or immunohistochemistry[60] or the development of MS based multiple reaction monitoring (MRM) assays for high throughput analysis.

Appropriate statistical tests must be applied with clinical *a priori* hypotheses, including the need for suitable corrections for application of multiple

testing (such as Bonferroni tests).[61] Figure 2 shows a general approach to biomarker development and clinical application.

Role of urinary proteomics in a clinical setting

Traditionally approaches towards identifying potential useful urinary biomarkers have evolved from our understanding of pathophysiology of diseases—i.e. they are 'hypothesis driven'. Increasingly, urinary proteomics is used to discover potential biomarkers for both diagnosis and prognosis of renal disease, which indirectly give insight into pathogenic processes—i.e. an approach which is 'hypothesis generating'. The advantages of urinary proteomics for disease diagnosis are described in Table 2.

Diagnosis

Proteomic biomarkers which are to be suitable as non-invasive alternatives to renal biopsy, must exhibit differential detection in the urine of patients with and without disease. They must be specific to the disease in question and ideally should be detected early in disease onset to maximize the benefits of therapeutic intervention. The urinary proteomes of both glomerular and interstitial diseases have been described in small numbers of patients and are now moving into a validation phase.

One of the first proteomic studies in renal disease was reported by Lafitte *et al.* who compared 2-DE

Table 2 Advantages and disadvantages of renal biopsy and urinary proteomics

Renal Biopsy	Urinary Proteomics
Invasive	Easily available
Resampling difficult	Resampling same day possible
Requires admission	Can be used in clinic
Blood pressure and coagulation may delay procedure	Immediately available
Patient habitus may make impossible	Only impossible in anuric patients
Inadequate sampling	Only 10 ml urine required
No guide to treatment	Response to treatment predicted
Minimal guide to prognosis	Prognostic information possible
Cheap processing techniques, but requires hospital admission	Currently expensive until panels of biomarkers are available
Current gold standard	Sensitivity and specificity continually improving

analysis of urine from healthy controls with four patients with incipient diabetic nephropathy, minimal change disease, myelomatous kidney and proximal tubular acidosis. A stable map for healthy controls was established and there were distinct differences between the patients with renal disease.[19]

In a subsequent study, the urine from patients with other proteinuric glomerular diseases including focal segmental glomerulosclerosis (FSGS) ($n = 10$), minimal change ($n = 16$) and membranous nephropathy ($n = 18$) were analysed by CE-MS and compared with control ($n = 57$).[24] One hundred and three proteins were identified in >90% of healthy individuals' urine, which allowed the definition of a 'normal' urinary proteome, and when compared with profiles of patients with disease enabled the correct classification of 92.9% of patients with membranous nephropathy and 71.4% of patients with minimal change or FSGS. Further potential biomarkers for membranous nephropathy have also been identified in the urine using both SELDI and CE-MS approaches.[62]

A similar study identifying diagnostic biomarkers using 2DE and MALDI-TOF-MS with a training set of 16 patients and validation set of 16 patients correctly identified patients with FSGS, lupus nephritis, membranous nephropathy and diabetic nephropathy with sensitivities of 75–86% and specificities of 67–92%.[63] Other candidate biomarkers for diagnosis of FSGS, membranous nephropathy, minimal change disease, IgA nephropathy and diabetic nephropathy have also been identified with CE/ESI-QTOF-MS.[64]

Pooled urine from 13 patients with IgA nephropathy analysed with 2-DE showed differential expression of proteins (82 spots over-expressed and 134 spots under-expressed) compared with 12 normal healthy individuals. Fifty-nine proteins which significantly varied between patients and controls were subsequently identified by MALDI-TOF.[65] Further

definition of the urinary proteome of 45 patients with IgA nephropathy, using a CE-MS approach, had a sensitivity of 100% and specificity of 90% when compared to 57 healthy controls, 100% sensitivity and specificity when compared to patients with diabetes, minimal change disease and FSGS and a sensitivity of 77% and specificity of 100% when compared to patients with membranous nephropathy.[51]

Larger studies are now being performed including a CE-MS analysis of 402 patients with various renal disorders and 207 controls.[66] This has allowed the definition of a characteristic pattern of renal damage and IgA nephropathy. Furthermore, blinded analysis of an additional cohort of patients with different aetiologies of IgA nephropathy allowed the distinction between some subgroups.[66]

Whilst offering exciting potential, the diagnostic accuracy of these techniques is not yet sufficient to replace the renal biopsy. Studies are limited by size and assessment of a potentially restricted component of the urinary proteome. In addition, further analysis needs to be performed using complementary MS techniques to define more comprehensively the proteins that constitute the individual fingerprint of renal pathology for each disease, and to improve the sensitivity and specificity of each battery of tests.

Lupus nephritis
Disease activity in lupus nephritis is a clinical scenario which may require multiple biopsies throughout the disease course to guide the initiation and escalation of treatment. The International Society of Nephrology/Renal Pathology Society (ISN/RPS) class of lupus nephritis may change with time and ideally a test should also be able to determine this as well as activity and progression of the disease.

Mosely *et al.* compared the urinary proteome of 26 patients with active lupus nephritis (defined by renal biopsy, or the presence of predefined rise in creatinine, proteinuria or the presence of new haematuria) and 49 patients with inactive disease using SELDI-TOF.[47] These authors identified differentially expressed proteins. It was possible to classify patients correctly according to disease activity with both a sensitivity and specificity of 92%. On further scrutiny of four wrongly classified inactive patients there was some evidence of incipient disease activity. Similarly, two patients incorrectly classified as active, on post-analysis review were becoming inactive according to biochemical parameters, suggesting that the novel urinary biomarkers may also be capable of detecting early stages of relapse or recovery. Sub-analysis of ISN/RPS classifications was not made.

A study of children with lupus nephritis identified an eight-biomarker proteomic signature for SLE nephritis using SELDI-TOF-MS. The protein expression differed between ISN/RPS classes, but did not reach statistical significance, although the biomarkers were able to identify clinical and biochemical disease activity correctly.[67] One limitation was a median delay of 9 months between biopsy and sample collection, potentially reducing discrimination between classes.

Oates *et al.* collected urine samples immediately prior to biopsy in 20 patients investigated for lupus nephritis, which were analysed with 2-DE. The sensitivity and specificity for the ISN/RPS classes were class II 100%, 100%; III 86%, 100%; IV 100%, 92%; and V 92%, 50% according to a trained artificial neural network.[68] Nine patients had characteristics of more than one class.

Whilst larger studies are needed to develop tests which can be used routinely in the diagnosis and monitoring of lupus nephritis, these preliminary studies hold promise for obviation of serial biopsies in the future.

Allograft rejection

The detection of acute rejection is a common indication for biopsy of a renal transplant in the first year, in which renal tubular damage is a principal feature. Clarke *et al.* examined the urinary proteome of 17 renal transplant patients with biopsy proven acute rejection and 15 with stable allograft function. Using independent statistical analysis they identified five peaks with the greatest discriminatory function (6.5, 6.6, 6.7, 7.1 and 13.4 kDa). Two separate biomarkers (3.4 and 10 kDa) gave sensitivity and specificity of 83% and 100%, respectively.[69]

A similar study including 18 patients with acute allograft rejection, 22 with stable allograft function, five patients with urinary tract infection and 32 normal controls defined two different profiles (5270–5550 and 10 530–11 000 Da) for differentiation between patients with acute rejection and patients with stable function or normal controls. In two patients the profile switched from acute rejection to stable and visa versa.[21] The proteins have subsequently been identified as cleaved forms of β-2 microglobulin, which were present with higher levels of intact β-2 microglobulin and aspartic proteases (which cleave β-2 microglobulin).[35] Oetting *et al.* have subsequently confirmed the use of β-2 microglobulin as a biomarker for rejection.[70]

A further study demonstrated proteins with masses of 4756.3, 25 665.7 and 19 018.8 Da in renal transplant patients with acute rejection, which allowed distinction from patients with stable organ function in >90% of cases with a sensitivity of 90.5–91.3% and a specificity of 77.2–83.3%.[71] Subsequent identification of peaks detected a reduction in beta-defensin-1 and an increase in alpha-1 antichymotrypsin in patients with acute rejection.[72]

Perhaps not surprisingly in view of the complexity of the proteome and the variation in methodology, the profiles were different in the three studies and no two studies have described the same discriminatory peaks or proteins, despite all groups using SELDI-MS. This is likely to represent differences in sample collection, storage and preparation or immunosuppression regime which has been shown to affect the urinary proteome,[73] but is more likely to be due to SELDI laser and detector performance and the use of different chip surfaces. Urinary proteomics have also been able to discriminate between urinary tract infection and acute allograft rejection and between tubular and vascular rejection.[73] These new biomarkers should be validated by the development of appropriate ELISAs to allow reproducibility and quantification, given the current intra-sampling variability of SELDI-MS. Thus there is the potential to create a diagnostic panel with enhanced sensitivity and specificity which would allow a reduction in the need for renal biopsies in the post transplant period.

Quintana *et al.* have examined the urinary proteome of 39 patients with chronic allograft nephropathy (CAN) and 32 controls by label free quantitative LC-MS/MS followed by MRM validation. Specific peptides derived from uromodulin and kininogen were more abundant in controls than patients and differential expression of two ions diagnosed CAN in virtually all cases.[74] They propose that these biomarkers could form the basis of a biopsy-free urine test for the early diagnosis of

CAN and will facilitate a more rapid introduction of targeted and personalized immunosuppressive regimes to improve long-term graft outcome.

Predictors of disease/prognosis

Acute kidney injury

Acute kidney injury (AKI), formally known as acute renal failure, is a clinical problem associated with high mortality and morbidity which has been extensively studied.[75] The current method of diagnosis is to monitor serum creatinine, an unreliable indicator during acute changes in renal disease, as a steady-state equilibrium must be achieved before creatinine can provide a reliable measure of renal function. Creatinine is also influenced by other variables including sex, age, muscle mass and metabolism, drugs and hydration. Other approaches include the detection of casts and fractional excretion of sodium, which are both non-specific and insensitive for early detection of AKI. Consequently many cases are detected late resulting in an unacceptable delay for appropriate therapeutic intervention. The goal for urinary proteomics is to define a panel of tests, which will allow early identification of patients at risk, in order to institute rapid and aggressive treatment.

Nguyen *et al.* identified biomarkers with *m/z* of 6.4, 28.5, 43 and 66 kDa, using SELDI-TOF MS which were elevated in the urine of children with ischemic kidney injury (defined as a rise in creatinine >50%) following cardiopulmonary bypass surgery when compared with preoperative samples. A combination of these three markers predicted the development of AKI at 2 h in 100% of patients, despite serum creatinine not rising for 2–3 days after the procedure.[76]

Using a rat model of sepsis-induced AKI and 2-DE/MALDI-TOF analysis, Molls *et al.* identified urinary peptides that were upregulated in AKI including albumin, aminopeptidase and neutrophil gelatinase associated lipocalin (NGAL) when compared with animals, which did not develop AKI.[77] Other urinary proteins were decreased, including uromodulin (Tamm-Horsfall mucoprotein), serum protease inhibitors and meprin-1-alpha (a brush border enzyme). Meprin-1-alpha was also reduced in the rats with AKI when analysed by western blotting. The administration of actinonin (which inhibits meprin) partially resolved the sepsis-induced AKI, suggesting a potential role for meprin in pathogenesis, and a strategy for therapeutic intervention. This urgently needs to be evaluated in humans and highlights an important role for the urinary proteomics in identifying novel pathophysiological pathways and targets for treatment.

Other studies utilizing microarrays and ELISAs have identified higher concentrations of Kidney injury molecule 1(KIM-1), IL-18, cystatin C, 1-micro-globulin, Gro-&agr in the urine of patients with incipient AKI[75,78,79] and have confirmed the role of urinary NGAL as a marker of AKI post cardiopulmonary bypass,[80] delayed graft function[81] and as a predictor of severity in haemolytic-uremic syndrome[82] and contrast induced nephropathy.[83] More recently, urinary active hepcidin detected by SELDI-TOF has also been proposed to be an early marker of AKI in patients after cardiopulmonary bypass suggesting a role for iron sequestration in modulating AKI.[84] A combination of these tests with candidate biomarkers from proteomic discovery needs to be applied to a clinical setting in order to define a panel of markers, which when combined in an algorithm would have increased specificity and sensitivity than a single test alone.

Chronic kidney disease

Currently in the US 20-million people suffer from kidney disease, of which 60 000 die due to end stage renal failure (http://www.asn-online.org). Early predictors of CKD are urgently needed to enable appropriate monitoring and timely treatment in order to minimize disease burden especially since the deterioration in renal function can be reduced if aggressive management is instituted early. Serum creatinine and urine protein are the standard methods for detecting progression, but there are no current prognostic biomarkers other than microalbuminuria, which already represents structural renal damage, that might allow preventative treatment to be introduced.

The urine NGAL concentration shown to correlate with future changes in serum creatinine in a pilot study of 78 patients with CKD.[78] Similarly liver-type fatty acid-binding protein (L-FABP) is significantly higher in the urine of CKD patients with progressive disease than those with stable disease.[85] The use of proteomics in this clinical setting would potentially allow the identification of many more biomarkers, which together with clinical indices and pre-existing markers of renal function could give an individual prediction of deterioration in renal disease. Nephrologists could then focus on those patients at highest risk, whereas patients with stable disease could be managed in primary care.

Diabetic nephropathy

Meier *et al.* studied 44 adolescents with type 1 diabetes for more than 5 years compared with age-matched healthy controls and identified a urinary proteome with CE-MS typical for patients with

diabetes without microalbuminuria, which may represent early diabetic vascular dysfunction and subtle changes in the glomerular filtration barrier due to increased protein glycation, as well as 88 polypeptides which were present in differential amounts in patients with albumin:creatinine ratios >35 mg/mmol.[52] The predictive value of individual polypeptides, which are not present in healthy controls, need to be evaluated further.

Unlike type 1 diabetes, the development of proteinuria in type 2 diabetes may not be indicative of diabetic nephropathy and may be due to other causes such as hypertensive nephropathy. The development of biomarkers for both diagnosis and prediction of those at risk of diabetic nephropathy in patients with type 2 diabetes would be invaluable to allow appropriate intervention and reduce the need for a renal biopsy in some individuals. Proteomic analysis of the urine of 112 patients with type 2 diabetes and healthy controls has allowed the definition of a diabetic, non-proteinuric pattern. In addition a distinct pattern has been noted in patients with microalbuminuria (>100 mg/l), in 35% of patients with low levels of microalbuminuria (20–100 mg/l) and in 4% of patients with no albuminuria. Patients in these latter two groups were more likely to suffer retinopathy and therefore statistically have a higher risk of developing nephropathy.[43] The presence of the characteristic polypeptides identified (insulin-like peptide 3, uromodulin and an albumin fragment) may indicate a population at risk of incipient nephropathy who require early intervention to prevent disease progression.

A different group have examined the urine of 100 patients with type 2 diabetes using 2-DE followed by MALDI-TOF. Proteins identified included zinc alpha-2 glycoprotein, alpha-1 acid glycoprotein, alpha-1 microglobulin and IgG, and these could also be used as markers for the early detection of diabetic nephropathy.[86] Furthermore, Rao *et al.* have identified six proteins in addition to zinc alpha-2 glycoprotein which were found to be up-regulated in diabetic nephropathy and four proteins which were progressively down-regulated using 2D-DE and LC/ES-MS/MS.[53]

A nested case–control study of 62 Pima Indians with type 2 diabetes has confirmed a 12-peak urine proteomic profile, which correctly predicted the development of nephropathy over 10 years in 89% in a training set ($n = 14$) and 74% in a validation set ($n = 17$), with sensitivities and specificities of 93% and 86%, and 71% and 76%, respectively.[87] However, the cases and controls could not be matched for HbA1C due to the sample size, which

may have influenced the proteomic signature and hence outcome.

Rossing *et al.* have recently described a panel of 40 biomarkers which identified patients with diabetes from healthy individuals in a large cohort with 89% sensitivity and 91% specificity. They also describe a profile, which accurately diagnosed nephropathy in patients with diabetes with 97% sensitivity and specificity and identified those with microalbuminuria who progressed to overt nephropathy over a 3 year period.[88]

Given the exponential rise in diabetic nephropathy in the last decade, identification of those at risk would allow early intervention and have major implications for health economics. Urinary proteomics clearly has an important role to play in achieving this goal.

Prediction of response to treatment and disease monitoring

The response to steroids is an important predictor of prognosis and determinant of biopsy in paediatric idiopathic nephrotic syndrome. Two studies have examined the urine of children with steroid sensitive and steroid resistant nephrotic syndrome with SELDI-TOF-MS. One group identified ß2 microglobulin as having diagnostic accuracy in 95% of children with steroid-resistant disease, together with five other distinct peaks, which distinguished these children from those with steroid sensitive disease.[89] Another group identified a protein of mass 4144 Da, which reliably classified 25 patients with steroid sensitive and resistant disease.[90] These studies need validating in larger cohorts, and the predictive value of algorithm, a combining these candidate biomarkers should be explored.

The prevention of progression of IgA nephropathy is related to control of hypertension and proteinuria. Standard treatment for this condition in the presence of these complications is the use of angiotensin-converting enzyme inhibitors (ACEi) or angiotensin II receptor blockers, but some patients do not respond and alternatives are sought. A study of urine of 18 patients with IgA nephropathy who had been treated with long term ACEi identified three proteins (kininogen, inter-alpha-trypsin-inhibitor heavy chain 4 and transthyretin), using 2-DE then nano-HPLC-ESI-MS/MS analysis, which were excreted differently in responders to ACEi when compared to those who had not responded.[91] A reduction of daily proteinuria >50% and evidence of stable renal function over time were used to classify patients as responders. In a prospective study, kininogen was measured in 20 patients with biopsy-proven IgA nephropathy, before starting any therapy. Very low

levels of kininogen urine excretion were predictive of an inadequate or absent clinical response to ACEi therapy of after 6-month follow-up.[91] The use of proteomics in this capacity would guide the early initiation of individualized treatment, which is not possible from information gained from the routine tissue biopsy.

In a randomized double-blinded study, patients with diabetic nephropathy were given varying doses of candesartan. Proteomic analysis of the urine before and after treatment revealed a change in 15 out of 113 proteins previously found to be specific for diabetic nephropathy,[92] providing additional evidence for the potential role of proteomics in monitoring individual response to disease.

Understanding renal pathophysiology

Exosomes are small membrane vesicles <80 nm diameter that originate within epithelial cells and are secreted in the urine from all cells within the nephron and urinary tract. They contain both cytosolic and membrane proteins and are easily extracted from the urine in the ultracentrifugation supernatant, and are thus a rich source of proteins for proteomic analysis. Two hundred and ninety-five proteins in the exosomes in normal urine have been identified including many disease associated proteins; aquaporin-2, polycystin-1, podocyin, angiotensin-converting enzyme, thiazide-sensitive Na–Cl cotransporter, epithelial sodium channel.[93] Additional proteins have been found to be differentially expressed in urinary exosomes of rats in response to sodium loading, including alpha 2-microglobulin, solute carrier family 3, diphor-1, meprin-1-a, H1-ATPase, and ezrin.[94] This gives great insight into the potential physiological processes at play and with further analysis will lead to better understanding of renal handling of sodium.

Identification of the urinary proteome in Dent's disease (a form of Fanconi syndrome) has led to further insight into mechanisms of the proximal tubule receptor-mediated endocytic pathway including a reduction in vitamin and prosthetic group carriers and an abundance of cytokines and complement components present in the urine, which could potentially lead to the development of directed therapies.[95]

Although many of the sequenced renal disease biomarkers do not immediately provide insight into pathogenic mechanisms e.g. albumin and collagen fragments, they may give information about disease-specific protease activity within the kidney, such as that resulting in ß2 microglobulin cleavage in acute transplant rejection. Many proteins are high abundance plasma proteins and appearance in the urine is simply due to disruption of the glomerular basement membrane, but certain fragments are characteristic of particular diseases e.g. albumin fragments specific for IgA nephropathy,[66] diabetic nephropathy,[88] minimal change disease, FSGS, membranous glomerulonephropathy[42] and autosomal dominant polycystic kidney disease[96] reflecting tubular processing of these proteins. The lack of collagen fragments in diabetic nephropathy has led to the speculation that reduced protease activity *per se* contributes to disease pathology, resulting in excess collagen and extracellular matrix deposition.[88] Indeed the abundance of certain specific collagen fragments has been shown to correlate with matrix metalloprotease activity.[97]

Growth hormone (GH) has been uniquely identified in the urine of four patients with FSGS.[98] Together with the findings of FSGS in transgenic mice overexpressing GH, in patients with acromegaly and with pan-hypopituitism treated with GH this suggests that GH has a role in the pathogenesis in FSGS. Proteomics is likely to be able to contribute towards many missing links in pathophysiological models, especially as advances in technologies are becoming available to detect the functional state of proteins.

Other applications for urinary proteomics in non-renal disease

Malignancies of the urinary tract in which proteins are excreted into the urine have been a large focus of recent study. Markers for prostate,[99] bladder[100] and renal cell tumours[101] have been identified in small studies and larger validation studies are underway to validate use in a clinical setting. Other applications for urinary proteomics include diagnosis of interstital and bacterial cystitis,[102] renal calculi,[103] ureteropelvic junction obstruction,[104] ovarian and lung cancer,[105,106] graft-versus-host disease[27] and coronary artery disease.[107] Undoubtedly proteomics will be applied to many other disease states and the use of the urinary proteome will not be exclusive to the diagnosis of renal pathology.

Limitations

Additional methodological problems to those discussed above include selection of controls, as all quantitative approaches require reliable control samples. In part, this can be addressed by pooling samples from larger number of normal subjects. Accurate information on the confounding effects of factors such as age, gender, and diet on the urinary proteome is not yet available; thus wherever

possible these variables should be matched between cases and controls.[108] A working group has recently concluded that it is not sufficient to compare the disease in question with healthy individuals, given that patients with diseases with similar metabolic, clinical or biochemical profiles may have an indistinguishable urinary proteome from that of the disease studied.[109] This adds further complexities to study design, but this caveat may apply only to non-renal diseases given that it has already been shown that it is recognized already that patients with proteinuric renal disease including membranous nephropathy, FSGS and minimal change have distinct urinary proteomes.[19]

Another potential limitation to proteomics based discovery of urinary biomarkers lies in the associated cost; popularization of this approach will inevitably lead to the need for acquisition of expansive MS and associated instrumentation by clinical laboratories.[108] Ultimately, however, this could also lower manufacturing costs and, especially if reliable, assays of disease biomarkers in urine will lead to a reduction in renal biopsies, their associated complications, and early intervention to reduce disease progression.

Conclusion

Recent developments in proteomic techniques promise exciting insight into normal renal physiology and renal disease processes, allowing precise definition of the disease proteome. In turn this will enable diagnosis, prognosis and prediction of response to treatment of individuals. Considerable developmental work is still required to refine the standardization of urinary proteomics and to validate earlier reports. There is the need for exploration of diagnostic profiles in hitherto unexplored renal diseases and in longitudinal analysis for evaluation of treatment and assessment of prognosis. Expanding knowledge in recent years and the rapidly advancing field of proteomics will facilitate studies of renal physiology and pathophysiology and ultimately should make redundant the traditional renal biopsy.

Funding

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