Proteomic Methods for Biomarker Discovery in Urine

Daniel W. Wilkey[*,†] *and Michael L. Merchant, PhD*[*,†]

Summary: Many challenges exist with disease-state biomarker identification. These challenges include sample heterogeneity, poorly designed sample sets, insufficient numbers of samples, as well as inconvenient workflows, inadequate methodology, and development of false-positive markers resulting from protein degradation during sample handling. Yet despite these difficulties, substantial progress has been achieved with the application of proteomic methods toward biomarker discovery in renal disease. Significant advances have occurred in the past decade with electrophoretic, chromatographic, and mass spectrometric methods for discerning biomarkers of disease. Recent applications of proteomics to the study of renal disease have identified new mechanisms in renal disease progression and established protein expression profiles for complex renal diseases including glomerular and tubular pathologies. In some cases these protein profiles have proven successful with guiding patient treatment and markers for pharmacologic therapies. Proteomic analysis only recently has been applied to the study of renal disease, yet it has shown substantial potential for future successes.
Semin Nephrol 27:584-596
Keywords: *Mass spectrometry, MALDI, electrophoresis, liquid chromatography*

Medicine has long relied on physical examination and clinical blood and urine chemistries to diagnose or stage disease. Early historical references to the diagnosis of renal disease are contained in the Ebers papyrus written in approximately 1552 BCE, with references to a condition of polyuria; considered by many as the first reference to a clinical description of diabetes. A millennium later (4th-5th century BCE), diabetes was diagnosed by ancient Indian physicians Charak and Susrutha on observations of ants collecting around the urine of certain individuals. These physicians termed the condition *Madhumeha* or *honey-like urine*. The modern day search for diagnostic markers of disease focuses on the discovery of prognostic markers that allow for intervention before the development of substantial pathophysiology.

*Kidney Disease Program, University of Louisville, Louisville, KY.
†Clinical Proteomics Center, University of Louisville, Louisville, KY.
Address reprint requests to Michael L. Merchant, Room 209, Donald Baxter Research Building, 570 S. Preston St, Louisville, KY 40202. E-mail: mlmerc02@louisville.edu
0270-9295/07/$ - see front matter
 doi:10.1016/j.semnephrol.2007.09.001

The explosion of science leading from the genomic and proteomic revolutions has redefined the potential of clinical chemistries to better personalize medical care[1,2] for such disease complications as diabetic nephropathy (DN).[2,3] The personalization of medical care is predicated on the existence of discrete marker compounds (ie, biomarkers) whose individual or collective abundance is diagnostic of disease. The National Institutes of Health Biomarker Definitions Working Group has provided a consensus definition for biomarker as ". . . cellular, biochemical, and molecular (genetic and epigenetic) alterations by which a normal or abnormal biologic process can be recognized or monitored. Biomarkers are measurable in biological media, such as in tissues, cells, or fluids."[4] Theoretic applications of biomarkers in research are assessment of pharmaceutical efficacy and safety (in vitro as well as in vivo studies) and in medical care for screening or staging progression of disease states, and also for evaluating the response to treatment. These applications hint at half the hope for biomarkers. In addition to performing as a prognostic metric, biomarkers hopefully will provide mechanistic insight into

the molecular and cellular pathways that have been disrupted in the disease state. Thus, biomarkers can guide treatment as well as provide direction for future research on intervention.

CHALLENGES IN BIOMARKER DISCOVERY

General Considerations

The development of biomarkers for routine clinical applications has 3 distinct phases that can be described generically as biomarker discovery, validation, and implementation.[5] Preferably the discovery or preclinical phase is performed in an unbiased fashion with a sufficient number of well-matched samples to power the analysis.[6,7] Here genomic and proteomics methods each lend themselves toward an unbiased analysis of the sample. Optimally, the sample type(s) (ie, fluid, tissue, or casts) and analytic method(s) used for biomarker discovery should be easily translatable into the second phase of validation studies. Hence, easily obtainable body fluids such as urine or blood are desirable for discovery studies. Tissue samples such as biopsy material are not ideal but may be used for biomarker discovery purposes, keeping in mind the end goal of noninvasive diagnosis or staging of disease. Still, substantial progress has been made regarding the extraction of information (proteolytic peptides) from formalin-fixed biopsy material.[8–11] The difficulties of biomarker-lead development, likened to searching for a needle in a haystack, are dissimilar to the difficulties of validation. The principal difficulty of the validation phase is establishing the appropriate validation sample set and using a sensitive, high-throughput method for biomarker quantification. The subject of the third phase of biomarker development, biomarker implementation, is outside the scope of this article and is not addressed here.

A discernable commonality to most recent successes in biomarker discovery for renal diseases is the incorporation of a non–hypothesis-driven approach (genomic and proteomic) for selecting early sentinel/discriminatory disease biomarkers. Several proteins including urinary kidney injury molecule-1 and urinary or plasma neutrophil gelatinase–associated lipocalin, suggested to perform as sensitive markers of acute kidney injury (AKI), first were identified by genomic analysis of kidney tissue isolated from animal models of AKI.[12,13] Expressional differences of each of these protein biomarkers have been confirmed recently at the protein level using immunochemical methods in urine. Other protein biomarkers of acute and chronic renal diseases are suggested to be derived from common plasma proteins having unique patterns of post-translational modification or are differentially compartmentalized into urinary exosomes.[7,14–19] Further, peptide biomarkers or peptide expression patterns diagnostic of renal disease (ie, peptide profiles) have been identified using a more direct approach with tandem technologies of capillary electrophoresis–mass spectrometry (CE-MS) or liquid chromatography–mass spectrometry (LC-MS).[20–24] The success of these biomarker developments is based in part on proteomic advances in high-resolution electrophoretic or LC methods, more robust computational platforms (software and hardware), and, lastly, high-throughput, high-sensitivity MS analyses of peptides.

The Curse of Proteomic Dimensionality in Biomarker Discovery

Biomarker discovery efforts both benefit and suffer from the proteomic approach. A substantial benefit of the proteomic approach is the unbiased nature of the proteomic paradigm. A substantial detriment of the proteomic approach can be inferred from a quote taken from Richard Bellman, "the curse of dimensionality.[25]" The curse of dimensionality is a significant obstacle in elucidating trends within the complex data sets. Statistical analyses of these types of data sets suffer from the paucity of samples compared with number of observations (ie, data points, gel spots, observed peptide ions) per sample. Although a few examples of high-resolution 2-dimensional electrophoresis (2DE) proteomics data sets have been reported in the literature, individual urinary proteomic 2DE data sets typically are defined by a few hundred protein spots. Urinary MS data sets typically are defined by hundreds to thousands of identified proteins based on thousands of tryptic peptides. Because of the increased sensitivity of modern mass spectrometers over con-

ventional protein stains used in 2DE experiments, more data points are observed in an individual LC-MS proteomic experiment compared with an individual 2DE proteomic experiment. But careful design of the sample sets and implementation of biostatistical controls can abate, but not eliminate, the dimensionality curse.

The dimensionality curse starts with the sample used for analysis. Urine has been criticized as a biomarker reservoir because of the high degree of variability in the protein source and in the variability of the urinary protein concentration.[26] Urine contains soluble proteins from multiple sources including filtered plasma proteins and proteins secreted by genitourinary cells including renal glomerular and tubular cells. Urine also contains insoluble or sedimentary components such as cell casts, sloughed cells (ie, podocytes), excreted vesicular particles (ie, exosomes), and even renal calculi. In addition to high-molecular-weight proteins, urine contains low-molecular-weight polypeptide components (peptides) present at an equivalent mass amount with equivalent complexity to the higher molecular weight urinary proteins. The varied physical nature of urine composition can be capitalized on and urinary subproteomes fractioned, thus increasing the sensitivity or dynamic range for detection of low abundant species.[18] From several studies performed independently, we can draw conclusions regarding the relative abundance of proteins and peptides in urine. Zhou et al[27] determined the protein composition of normal urine and discerned it to be 49% soluble, 48% sedimentary, and 3% exosomal protein. Adachi et al[28] determined that the human urinary proteome contains more than 1,500 proteins, many of which are membrane (exosomal) proteins. The estimate of urinary polypeptide composition by Zhou et al[27] was to the exclusion of low-molecular-weight species, such as urinary peptides. Norden et al[29] concluded normal human urine (excluding Tamm-Horsfall protein [THP]) contains 66% protein (defined as polypeptide >10 kd) and 33% peptide (content in range 750 d to 10 kd), with the numbers of individual peptides observed in normal urine rivaling that of observed proteins.[23,30] In addition, the complexity of the urinary proteome is increased by posttranslational protein processing events. This is evidenced from the fact that a substantial number of urinary proteins detected in the urine are derived from the same gene product with alternative degrees of posttranslational modifications such as proteolysis or glycosylation. The dimensionality curse can be broken only when the number of samples exceeds the number of observations, not just the total number of gene products. It is unlikely the biomarker-lead discovery processes will ever be designed to include thousands of samples per study arm. Therefore, most biomarker-lead development studies are destined to be underpowered.

Concerns With Quantitative Proteomic Methods Used for Biomarker Discovery

A general concern with the detection of unique markers of renal pathophysiology should be natural abundance of the marker species. Analysis for biomarkers in urine is facilitated by the ease of urine sample collection. Larger volumes of urine can be collected and fractionation schemes can be scaled up for purposes of detecting low abundant marker species. Analysis of renal organ or tissue is more problematic. In these cases, tissue or surrogate tissue marker compounds can be used. Methods used to develop surrogate marker compounds to address sensitivity can rely on fractionation of cellular and subcellular proteomes. The fractionation of subcellular organelles acts to enrich for low abundant species. These data are then useful for comparison with genomic data to identify with altered protein expression differences, to identify novel gene transcripts with or without novel post-translational modifications, to identify affected metabolic pathways and for comparison to profile changes in the urine proteome over the disease course.[31]

Integral steps to all biomarker discovery approaches include sample preparation, sample separation, protein or peptide quantification, and peptide/protein identification. Data quality by way of variation in the data can arise as a result of the sample source (urine versus surrogate cell culture models versus tissue), or methods for sample separation (electrophoretic or chromatographic), quantification (noncovalent

staining, covalent labeling, or label-free methods), and identification (mass spectrometric, immunochemical, and bioinformatic analysis platforms). Multiple approaches exist for the analysis of renal pathophysiology and elucidation of biomarkers of renal disease. Two main proteomic separation platforms upstream of mass spectrometers are used in lead-biomarker discovery efforts. These sometimes multidimensional separation methods are based on electrophoretic or liquid chromatographic separation of proteins and/or peptides. Recent reviews from the application of electrophoresis and liquid chromatography in protein separation are available[32–41] and will not be expanded on here. Quantification of proteins or peptides is achieved after high-resolution sample separation. Electrophoresis invariably involves protein staining: either covalent or noncovalent fluorescent stains. Quantification of proteins downstream of the chromatographic separation is achieved using MS and by measurement of the individual ion or total ion current. Recent work by the Association of Biomedical Resource Facilities (Proteomics Research Group PRG2006) on Relative Quantification methods has suggested quite strongly that label-free mass spectrometric quantification of proteins has a lower variability in the observed data as compared with all other methods except radioactive labeling of proteins. Our work provided here supports this position of the benefit of label-free MS quantification of proteins. We highlight this perspective with a few examples of variation in proteomic data sets that then can be used in statistical considerations during biomarker discovery efforts.

2DE

Sample source–derived data variation is intrinsically a problem of contaminating species. To illustrate this concept, representative 2DE images are provided for analyses of cell culture lysates and for normal and diabetic urinary proteomes (Figs. 1-3). A murine mesangial cell proteome reference map was created using 60 μg cell lysate protein, 3 to 10 non-linear immobilized pH gradient (IPG) strips, 4% to 12% NuPAGE (Invitrogen, Carlsbad, CA) slab gels, and SYPRO Ruby staining (Molecular Probes, Invitrogen, Carlsbad, CA), and is presented in Figure 1. Thirty protein spots across the high-to-low isoelectric point (pI) and molecular mass (Mr) ranges were selected as seed spots against which a total of 18 gel images (5 gels per two 2-h treatment conditions and 4 gels per two 24-h treatment conditions) were matched using Progenesis Discovery software (Nonlinear Dynamics, Newcastle upon Tyne, UK). An analysis of variance was calculated for seed spot-volumes within glucose treatments. The coefficient of variance (CVs) for the replicate matched gels were 0.17 (n = 5), 0.17 (n = 5), 0.20 (n = 4), and 0.21 (n = 4). The grand average of all CVs was calculated across all 30 seed spots of the 4 gel sets and determined to be 0.19. The grand CV was taken to represent the sum of all variations and is derived from both the biology of the sample and the 2DE technique.

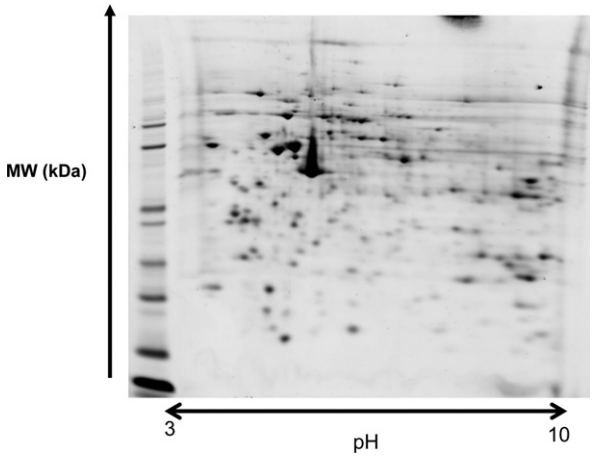

Figure 1. Representative 2DE gel image of murine mesangial cell lysate separated using 7-cm 3 to 10 NL IPG strips and 4% to 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) slab gels. Thirty protein spots across the high-to-low pI and Mr ranges were selected as seed spots against which a total of 18 gel images were matched using Progenesis Discovery software. An analysis of variance was calculated for seed-spot volumes within glucose treatments and a grand CV was calculated equaling 0.19.

Figures 2 and 3 are illustrative of 2DE gel images using 25 μg normal pediatric urine protein and new-onset (pediatric; urine collection <6 mo since time of diagnosis) type 1 diabetes mellitus (DM) urine protein. CVs for individual gel spot volumes were calculated for 20 protein seed spot volumes found in normal adult urine gels (n = 5). The grand average for these CVs was found to be 0.299. The 2DE CV was calcu-

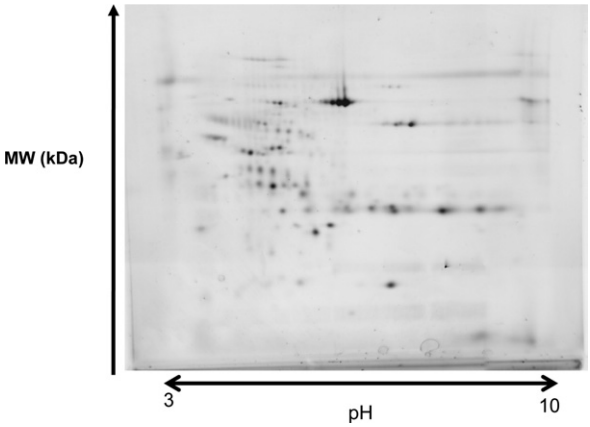

Figure 2. Representative 2DE gel image of 25μg normal pediatric urine protein desalted by trichloroacetic acid (TCA) precipitation and proteins resolved on 7-cm 3–10 non-linear immobilized pH gradient (IPG) strips and 4–12% SDS PAGE slab gel. Twenty protein spots across the high-to-low isoelectric (pI) and molecular mass (Mr) ranges were selected as seed spots against which a total of 5 gel images were matched using Progenesis Discovery software. An analysis of variance was calculated for seed spot-volumes and a grand coefficient of variance calculated equaling 0.299.

lated for 20 matched protein spots found within the type 1 DM urine 2DE gel images (n = 5) and the grand CV was calculated to be 0.49.

Two conclusions can be drawn from these studies. First, cell cultures or other more genetically homogenous protein sources inherently will provide a more reproducible result. Second, despite having high variability, properly designed experiments using power analyses based on these urine gel spot-volume CV calculations increase the likelihood of avoiding type II errors. The calculated 2DE CV data were used in power calculations. Power calculations are prospective and are predictive of the number of sample replicates needed if one wishes to avoid a statistical type II error. A power calculation requires an estimate of the variability intrinsic to the method being used, the desired statistical significance level, expected measurement in both arms of the experiment, and the minimum observed change. Assuming a normal distribution of measured values and equal variances in the measured values, a replicate gel set of 4 gels per group of cell culture lysate ensure that an observed change of 50% in the prototypical protein spot volume (2,010 $\pm$ 382 pixel units) at the 95% confidence interval provides for a power of 0.869. The computed power estimate (0.869) is greater than 0.80, which is considered a minimum benchmark for statistical soundness. However, increasing the number of replicate gels of cell culture lysate to 10 per group provides a power of 0.999, which is more than sufficient for purposes of discovery science and establishing proteomic candidates for validation by other means.

Matrix-Assisted Laser Desorption Ionization–Time of Flight MS

Recent advances in liquid chromatography and robotics have provided for the automated spotting of nanoliter volumes of peptide or protein samples onto matrix-assisted laser desorption ionization (MALDI) target plates. Efficient ionization of peptides and proteins requires the presence of an ultraviolet-absorbing organic molecule (the matrix) such as cinnamic acid or a cinnamic acid derivative. MALDI–time of flight (TOF) MS analysis of peptides historically has been viewed as semiquantitative. In many ways,

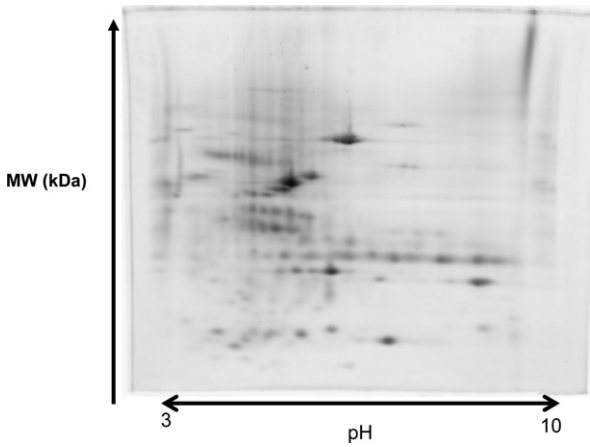

Figure 3. 2DE image of 25μg urine protein from new-onset (<6 mo from diagnosis) T1DM urine sample desalted by trichloroacetic acid (TCA) precipitation and proteins resolved on 7-cm 3-10 non-linear immobilized pH gradient (IPG) strips and 4–12% SDS PAGE slab gel. Twenty protein spots across the high-to-low isoelectric (pI) and molecular mass (Mr) ranges were selected as seed spots against which a total of 5 gel images and to the normal urine gel images (n=5) were matched using Progenesis Discovery software. An analysis of variance was calculated for seed spot-volumes and a grand coefficient of variance calculated equaling 0.49.

this approach offers much in terms of throughput and accuracy to investigate urine protein and peptide expression. This is particularly the case when the MALDI-MS instrument possesses MS/MS capability that allows peptide sequence identification.

Assessing Variability in Clinical Proteomic Studies and the Impact on Power Analysis

An all too often neglected aspect of MS-based biomarker analysis is the assessment of biological and instrument variability. These variables ultimately control the reproducibility of the analytic method. To determine the reproducibility of the MALDI-TOF MS method, we collected data in triplicate (ie, samples were spotted on the plate 3 times) using 2 separate standard peptide mixtures, with each peptide present at the 100-fmol level. These experiments were repeated a total of 3 times. The analysis of standards was based on (1) the order of sample application or (2) the MALDI matrix selected as the co-crystal. To simulate a complex peptide mixture, a commercially available trypsin digest of β-galactosidase (β-gal), was chosen to benchmark variance within the acquired data. A second peptide mixture was composed of 2 peptides: a hydrophilic peptide, angiotensin II, a hydrophobic peptide, $P_{14}R$, and was used to benchmark the variation in the data as a result of physiochemical parameters. Peptide standards were spotted at random plate locations and in 3 sample application techniques: (1) co-spotted with MALDI matrix, (2) sample spotted first and allowed to dry, and (3) sample spotted onto dried matrix. Peptide standards were spotted using the prototypical matrix, α-cyano-4-hydroxycinnamic acid (α-CN), and with 2 salts derived from α-CN: (1) n-butylammonium α-CN and (2) diethylammonium α-cyano-4-hydroxycinnamate (DEA α-CN). These salts are reported as ionic liquids and in theory ensure a more homogenous distribution of the peptide within the forming crystalline matrix. The resulting homogeneity should therefore provide for a lower incidence of observed variation in the acquired data set. Under the conditions used in this study, the samples behaved as solids and were applied as solutions in acetonitrile.

Figure 4 depicts the comparison of β-gal MALDI-TOF spectra for the various sample spotting methods. From these data, it was determined that application of the sample followed by overlay of α-CN onto the dried sample afforded the best reproducibility (CV), but with slightly fewer peptide ions than the peptide/matrix co-mixed spot. Figures 5 to 7 illustrate the peptide ion signal intensities for the detection of the 2-peptide mixture of angiotensin II (ATII) and $P_{14}R$. These data suggest that the ionic liquids (salts) (n-butylammonium α-CN and DEA α-CN) perform best when applied as a sample underlay. However, the ionization efficiency of the 2-peptide mixture for the cinnamate salts is not reproduced with complex peptide mixtures of the β-gal digest and therefore cannot be taken to function as the optimum MALDI-TOF matrix. Therefore, in consideration of the total number of observed peptides and the variation in observed peptide ionization, it is suggested that the matrix spotted onto the dried sample to be the best method to reproducibly observe the maximum number of peptide ions with a calculated CV of 0.28. These values of variation in the MALDI-TOF–acquired data approximate those CVs calculated for LC-ESI-MS methods, which are reported to be 10% for CV and ±15% for standard error.[42–44]

The calculated MALDI-TOF MS CV data are essential to determine the statistical power needed in a clinical proteomics study. Assuming a normal distribution and equal variances, a replicate peptide set of 4 samples per group ensure that an observed change of 50% in a peptide ion intensity (2,000 ± 560) at the 95% confidence interval is calculated to be greater than 0.99. The computed value (0.997) is sufficiently powered to greater than 0.80, which is considered a minimum benchmark for statistical soundness. However, increasing the number of replicates to 10 per group calculates a power of greater than 0.999, which is more than sufficient for purposes of discovery science and establishing proteomic candidates for validation by other means.

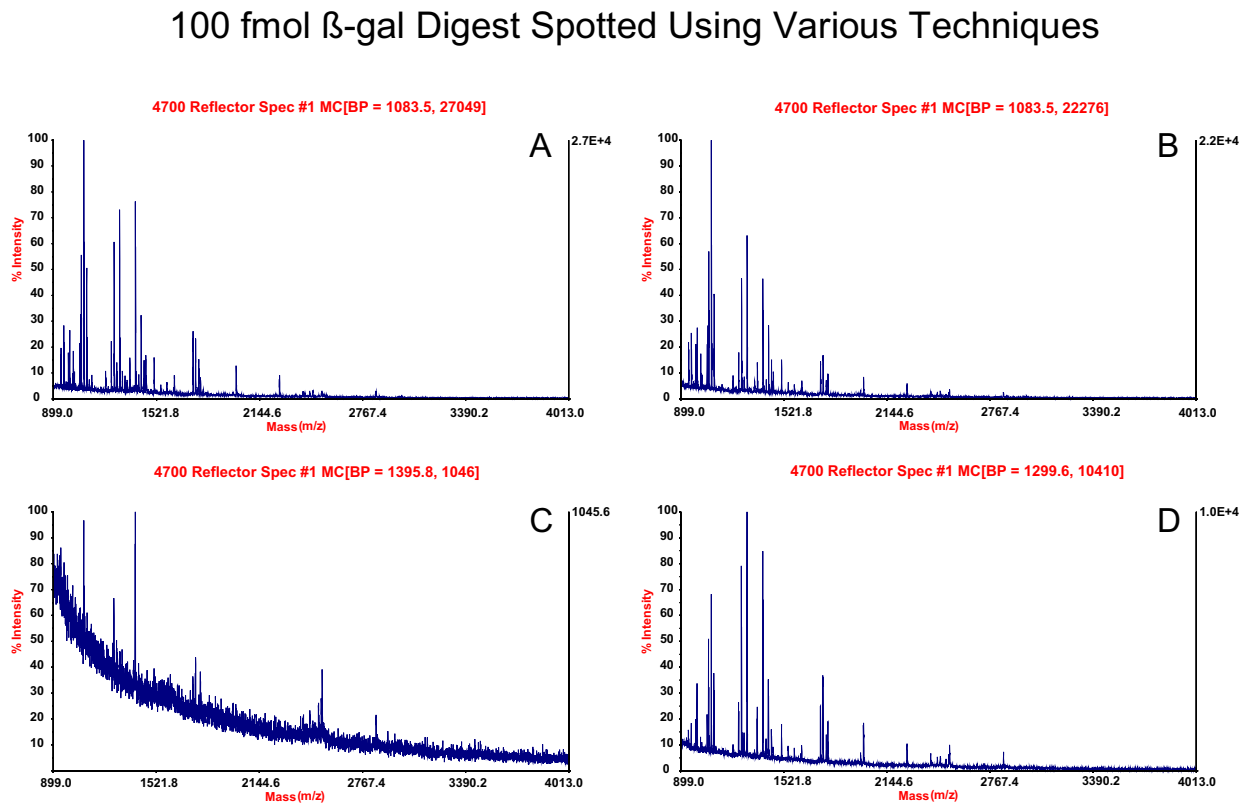

Figure 4. Comparison of MALDI-TOF MS spectra reproducibility and effects of sample application techniques using a complex peptide mixture (β-galactosidase digest). (A) β-gal digest pre-spotted with α-CN matrix overlay, (B) β-gal digest co-spotted with α-CN matrix, (C) β-gal digest applied in 30% acetonitrile to pre-spotted α-CN matrix, and (D) β-gal digest applied in 50% acetonitrile to pre-spotted α-CN matrix. Note the application of the β-gal peptide mixture in 30% acetonitrile results in poor peptide ionization. The percentage acetonitrile is insufficient to dissolve the α-CN matrix and allow for the production of the required peptide matrix co-crystal.

RECENT FINDINGS IN URINARY BIOMARKER DISCOVERY

Glomerular Nephropathies

Chronic renal diseases such as DN, focal segmental glomerular sclerosis (FSGS), lupus nephritis, membranous nephropathy (MN), and IgA nephropathy (IgAN) are associated with proteinuric states. Proteinuric states can develop from failure of the glomerular filtration barrier, the proximal tubule endocytic protein scavenging system, or both. In many instances the pathology of the disease is restricted to specific nephron units.[45–48] The gold standard for diagnosing many or most of these states is with a biopsy examination. Biopsy examinations have inherent risks such as the invasive nature of the procedure. Another risk is the underassessment of the diseased kidney owing to the limited area of the biopsy versus the total kidney surface area. Therefore, discriminatory urinary biomarkers should be more advantageous toward screening for glomerular disease. Significant progress has been made in the application of 2DE and MS profiling of urine for establishing protein and peptide profiles that can define disease versus nondisease states.

2DE Identification of Urine Biomarkers

Recent work by several groups has suggested that suitable biomarkers for glomerular disease might be based on differential urinary expression of common serum proteins having specific degrees of posttranslational modification.[7,49] In one study, the urinary proteome of 32 individuals comprising FSGS, lupus nephritis, MN, and DN glomerular nephropathies were studied. Protein patterns were developed using 2DE, and features (protein gel spots) diagnostic for

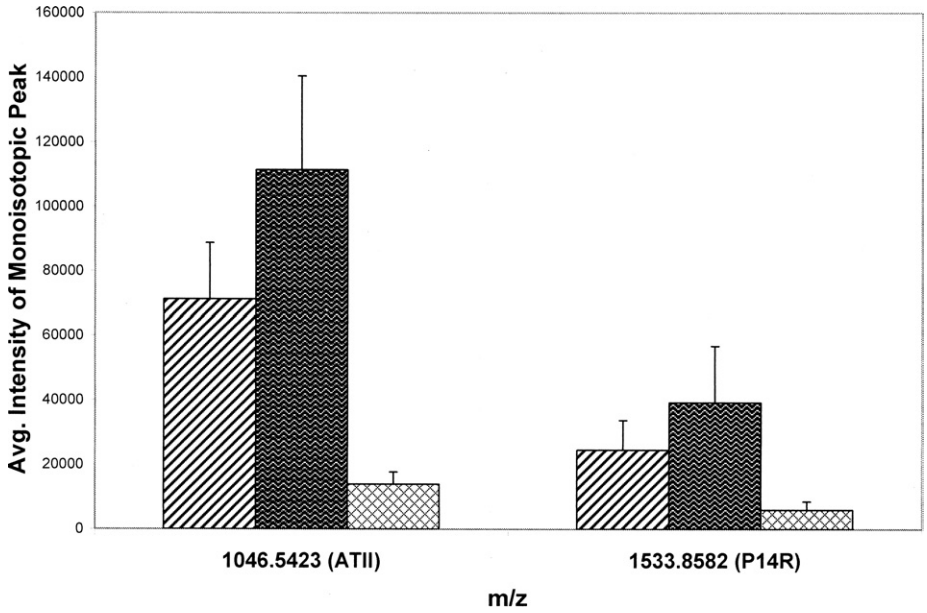

Figure 5. Comparison of sample application technique with hydrophobic versus hydrophilic peptides using α-CN MALDI matrix. Sample application technique was evaluated using ATII and $P_{14}R$ peptides as prototypical hydrophilic and hydrophobic peptides. The sample underlay method resulted in the lowest observed CV for peptide ion intensity measurements. ▨, spot sample, overlay matrix (CV-ATII = 0.25, CV-$P_{14}R$ = 0.37); ▩, mix sample and matrix (CV-ATII = 0.26, CV-$P_{14}R$ = 0.45); ▧, spot matrix, overlay sample (CV-ATII = 0.27, CV-$P_{14}R$ = 0.44).

pathology were evaluated using unsupervised clustering algorithms and also by artificial neural network (ANN) analyses. The unsupervised approach involved the simultaneous clustering of gels and spots by unweighted pair group average. Based on the analyses of all 32 gels, no single gel spot could differentiate all 4 diseases and no significant variation in the gel spot patterns or gel spot intensities could be discerned considering sample collection order, diagnosis, and race, age, or serum creatinine level. Aggregate variation in the data was discerned using a double-cluster analysis of gels and spots. Protein spots were ranked by intensity and parsed into quartiles. The ANN was trained on 50% of the samples randomly selected using ranked protein spot volumes and known values of inflammation markers (interleukin-6, interleukin-8, and interleukin-16) as clinical inputs. Care was taken not to over-fit the data, and the external validation set (the remaining 50% of samples) was retained as a future validation pending identification of discriminatory gel spot features. By using the trained ANN the validation set was analyzed with 64 predictions made (16 patients $\times$ 4 disease states). The ANN made predictions with 83% accuracy. The sensitivity (75-86%) and specificity (67-92%) are comparable or better than many currently used biomarker tests. Twenty-one protein spots comprising at least 12 gene products provided the most sensitive discriminatory features. Six proteins, all plasma proteins, were aggregately responsible for 13 gel spots. These 6 proteins (Zn-α-2 glycoprotein, α-1 antitrypsin, haptoglobin, transferrin, albumin, and α-1 microglobulin) are all known to be glycosylated, thus providing an explanation for multiple protein charge isoforms observed in the 2DE gels. The conclusions from these data and supported by the literature[50,51] are that the glomerulopathy induces a change permeability/selectivity that biases the glomerular filtration of plasma proteins. Here the advantage of 2DE is in the identification of protein isoforms that could have been missed by top-down LC-MS methods.

In a second study, the urine from 43 patients (16 IgAN, 10 normal, and 16 DN) were compared using differential in-gel electrophoresis (DIGE) labeling. DIGE is a 2DE method that relies on covalent labeling of sample proteins

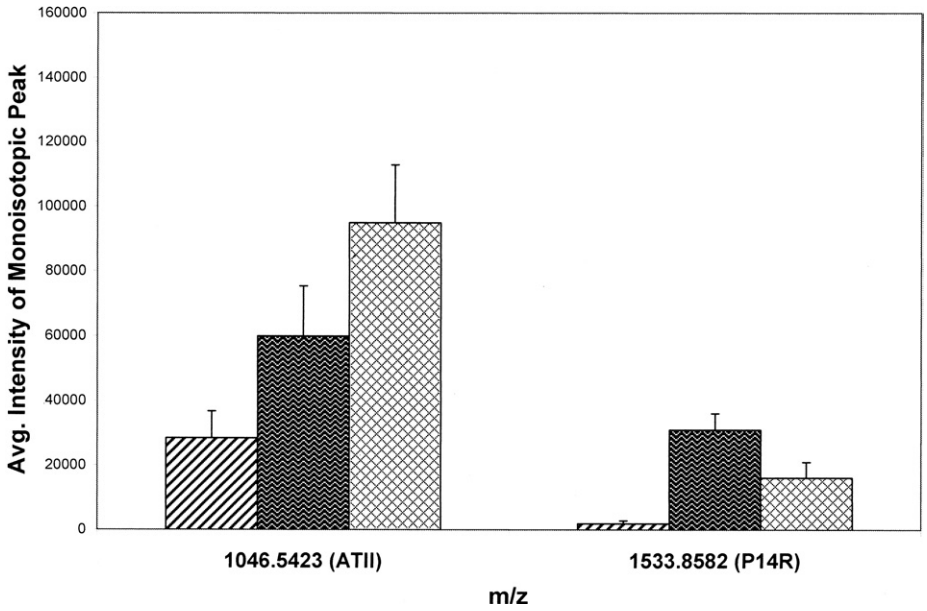

Figure 6. Comparison of sample application technique with hydrophobic versus hydrophilic peptides using DEA α-CN MALDI matrix. Sample application technique was evaluated using ATII and $P_{14}R$ peptides as prototypical hydrophilic and hydrophobic peptides. The sample co-mix method resulted in the lowest observed CV for peptide ion intensity measurements. ▨, spot sample, overlay matrix (CV-ATII = 0.29, CV-$P_{14}R$ = 0.46); ▩, mix sample and matrix (CV-ATII = 0.26, CV-$P_{14}R$ = 0.16); ▧, spot matrix, overlay sample (CV-ATII = 0.19, CV-$P_{14}R$ = 0.30).

with fluorescent dyes derived from cyanine functional groups (eg, Cy-2, Cy-3, and Cy-5). These dyes have varying extents of unsaturation, absorb light at slightly different wavelengths, and fluorescently emit light at slightly different wavelengths. The dyes react with primary amines using a minimal labeling or saturation-labeling strategy. With DIGE labeling it is possible to label individual samples with specific dyes, mix-labeled samples, and conduct simultaneous 2DE experiments within one gel. Identical proteins from separate samples are co-resolved and are quantified differentially using specific fluorescence excitation/emission wavelength combinations. The ability to directly compare and quantify proteins from 2 different samples minimizes the problems of gel-to-gel variation, ensures a more accurate alignment of gel images between replicate gels, and reduces the number of individual gel experiments. Finally, the availability of 3 Cy dyes provides for 2 experimental conditions (case and control) and allows for use of the third dye to label a composite internal standard derived from a mix of all samples.

Here, the DIGE labeling resulted in the visualization of 172 protein spots. By using MS and bioinformatics, the majority of the spots were identified as serum proteins and fragments of serum proteins. More than 70% (122 of 172) of all protein spots were identified as fragments of albumin. DIGE labeling indicated a gross increased expression of all proteins in the IgAN urine except for α_1-microglobulin. This expressional change was unexpected and was interpreted as a sample handling issue. The gel images did not indicate the presence of THP. THP is a major, dominant component of almost all urine samples and should be present on the DIGE images. The authors (Yokota et al[49]) pursued the question of down-regulation of urinary α_1-microglobulin in IgAN. α_1-microglobulin also is known as protein HC and is a member of the lipocalin family (which includes neutrophil gelatinase-associated lipocalin or NGAL) and has been reported previously as a marker for glomerular and tubular insults. Typically α_1-microglobulin is up-regulated in the disease condition but notably here is down-regulated. The authors were unable to confirm the down-expression using α_1-microglobulin enzyme-linked immunosorbent assay and included DN urine samples as disease controls. The disparity in

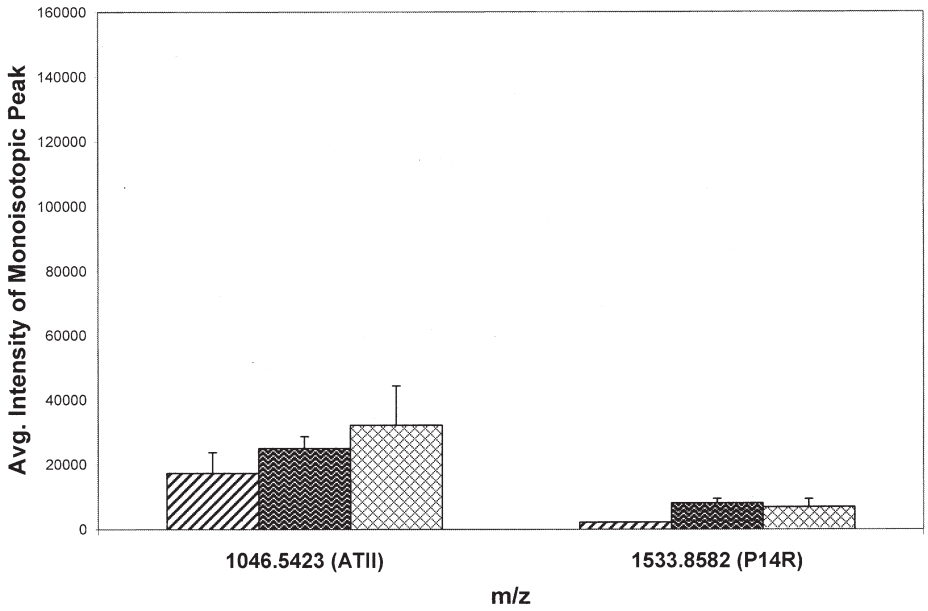

Figure 7. Comparison of sample application technique with hydrophobic versus hydrophilic peptides using α-CN butylamine salt matrix and various spotting techniques. Sample application technique was evaluated using ATII and $P_{14}R$ peptides as prototypical hydrophilic and hydrophobic peptides. The sample co-mix method resulted in the lowest observed CV for peptide ion intensity measurements. ▨, spot sample, overlay matrix (CV-ATII = 0.37, CV-$P_{14}R$ = NA); ▩, mix sample and matrix (CV-ATII = 0.15, CV-$P_{14}R$ = 0.17); ▧, spot matrix, overlay sample (CV-ATII = 0.37, CV-$P_{14}R$ = 0.35).

DIGE and enzyme-linked immunosorbent assay results was not fully explained but suggested that a source of variability could be the result of sample labeling.

MS-Based Discovery of Biomarkers

Substantial effort has been placed into direct analyses of urine proteins by MS methods. Two relatively high-throughput MS approaches, CE-MS and surface enhanced laser desorption ionization, appearing in the recent literature have identified proteomic profiles associated with renal disease states including glomerular diseases such as DN, IgAN, MN, FSGS, and minimal change disease.[20–24,52,53] These 2 MS methods have the advantage of identifying polypeptide masses from the peptidome mass range into the low-molecular-weight proteome mass range. The 2 methods suffer from low mass accuracies, an inability to perform true top-down proteomic analyses, and issues regarding reproducibility with replicate sample analyses. Stringent control of sample handling and extensive development of software to deal with technical variability has allowed CE-MS to become a robust tool for urinary biomarker discovery.[23]

The complexity of a normal human proteome as analyzed by CE-MS is comparable with that by 2DE. For instance, Wittke et al[54] analyzed the urine from 18 healthy individuals and discerned a common pattern of approximately 250 polypeptides present in at least 50% of all samples. Studies by Meier et al[22] showed that the complexity of the urine polypeptidome is such that no one sentinel biomarker or polypeptide feature can discriminate between normals and glomerular diseases such as DN, FSGS, or minimal change disease. However, panels of polypeptides could be used to biostatistically sort the urine samples into defining renal disease states. This work was extended by the same group[22] to show the ability to differentiate type 1 DM from type 2 DM from normal samples as well as defining response to angiotensin II receptor blocker treatment for DN patients.[55] Lastly and significantly, using the CE-MS peptide profiling approach, urinary biomarker studies have produced the fist prognostic biomarker panel using purely proteomic methods. Decramer et al[21] analyzed the urine of infants with ureteropelvic junction obstruction. The CE-MS data then were classified or clustered into groups and correlated with the severity of ure-

teropelvic junction obstruction. In a follow-up, prospective, blinded study, these discerned biomarkers performed with a 94% success rate for identifying which newborn required corrective surgery.

URINARY BIOMARKER DISCOVERY FOR TUBULAR NEPHROPATHIES

Acute kidney injury (AKI), previously referred to as acute renal failure, represents a significant clinical problem with incidence rates as high as 50% among intensive care patients. Positive outcomes for AKI are low as based on reported morbidity and mortality rates from 25% to 70%. The gold standard used to monitor for AKI is serum creatinine, despite the fact that serum creatinine levels are lagging indicators of renal status. Serum creatinine levels vary with a laundry list of factors including lean muscle mass, metabolic state, hydration status, as well as sex and age. Several markers of AKI have been mentioned, with strong candidates being urinary or plasma neutrophil gelatinase–associated lipocalin, and kidney injury molecule-1. Although these markers perform admirably in animal models, they await validation in larger human studies to ensure that they are more specific for acute tubular nephropathies than for chronic nephropathies.

Two recent AKI biomarker studies used rat models of AKI and following changes in the urinary proteome by DIGE.[14,15] In one case, the animals were injected with cisplatin and urinary exosomes isolated by differential centrifugation. Exosomes are small vesicles (50-80 nm) comprising a single-membrane bilayer enriched in membrane proteins. The luminal contents of the exosome contain cytoplasmic protein derived from the cell type that exocytosed the exosome into the urine. Specific marker proteins for cell types found along the length of the nephron have been detected in urinary exosomes. Hence, urinary exosomes represent a valuable source of protein rich with potential biomarkers. In this study almost 1,800 gel spots were detected and 74 had a 1.5-fold statistically significant ($P < .05$) expressional change. Twenty-eight proteins were identified by a combination of MALDI-TOF MS and LC-MS methods. These expressional differences were evaluated by a complementary immunoblotting (IB) method. Nine identified proteins had commercially available antibodies. By IB analysis, the DIGE results for 2 proteins (annexin V and fetuin A) were confirmed. Further investigation of fetuin A was conducted to determine the temporal expression as a function of the cisplatin insult. Urinary exosomal fetuin A had a 2.75-fold expressional increase by 24 hours, peaking at 48 hours at 52.5-fold. The expression of fetuin A remained increased past day 5 after AKI. Fetuin A was shown to be present exclusively in the exosomes and observed in the lumen of detached tubular cells by day 5. Increased expression of exosomal fetuin A was confirmed in 3 human patients with AKI as compared with hospitalized non-AKI patients.

In the second case, the model of AKI is a sepsis model of renal failure in aged animals (mice and rats). Sepsis is a major cause of AKI and despite investigation remains a persistent cause of AKI mortality. Some efforts into treating sepsis as an intervention for AKI mortality have had good results. Holly et al[14] used a DIGE approach to study the urinary proteome of a sepsis model of AKI. In this model sepsis was induced by cecal ligation and puncture (CLP). The contents of the ligated and punctured cecum are extruded into the abdominal cavity. The model requires aged mice or rats (Sprague-Dawley) and used CLP sepsis nonresponders as the control for the CLP sepsis responders. DIGE experiments were performed in triplicate. Protein spots were chosen if there was a change in relative expression with statistical significance ($P < .05$). In all, 97 protein spots were selected for MS identification and 30 were identified. Three groups of proteins were found to have altered expression and included a decrease in circulating proteins such as albumin, a decrease in serine protease inhibitors, and an altered expression in brush-border enzymes (an increase in aminopeptidase and a decrease in meprin-1-α). IB analyses for meprin-1-α did not confirm DIGE results. To test the biologic relevance of the data, an inhibitor of brush-border enzymes (actinonin) was used to treat CLP sepsis responders and evaluate the role of brush-border enzymes in AKI. Mice treated with actinonin had a lower serum creatinine level compared with vehicle-treated mice at 24 hours after CLP. Together these results

suggest the DIGE method can provide lead biomarkers for AKI that can be used to design intervention strategies.

CONCLUSIONS

Proteomics is an adaptive, unbiased methodology that is well suited for lead discovery phases of biomarker development. Proteomic methods, although imperfect, rapidly develop large constrained data sets. The high dimensionality of the proteomic data set can be offset by the comparison of large numbers of samples, comparison of samples derived from phenotypic extremes, and by the use of both unsupervised and supervised data analyses approaches. Despite the recent advances in electrophoretic, chromatographic, and mass spectrometric technologies, less biomarker development work has been performed in the field of renal disease when compared with other fields such as cancer research or cardiology. We can see from the work here that biomarkers likely will be comprised of sets of discriminatory protein features and less likely will be comprised of a single protein. This complexity in the diagnostic process certainly will necessitate more development on the analytic platforms currently in widespread use among hospital clinical chemistry departments. As proteomic methods become more widely integrated into medical research, the ability to discern complex disease patterns will help address the early diagnosis of renal disease.

REFERENCES

1. Kenyon GL, DeMarini DM, Fuchs E, et al. Defining the mandate of proteomics in the post-genomics era: workshop report. Mol Cell Proteomics. 2002;1:763-80.
2. Susztak K, Sharma K, Schiffer M, et al. Genomic strategies for diabetic nephropathy. J Am Soc Nephrol. 2003;14 Suppl:S271-8.
3. Susztak K, Bottinger EP. Diabetic nephropathy: a frontier for personalized medicine. J Am Soc Nephrol. 2006;17:361-7.
4. Atkinson AJ, Colburn WA, DeGruttola VG, et al. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69:89-95.
5. Pisitkun T, Johnstone R, Knepper MA. Discovery of urinary biomarkers. Mol Cell Proteomics. 2006;5:1760-71.
6. Hewitt SM, Dear J, Star RA. Discovery of protein biomarkers for renal diseases. J Am Soc Nephrol. 2004;15:1677-89.
7. Varghese SA, Powell TB, Budisavljevic MN, et al. Urine biomarkers predict the cause of glomerular disease. J Am Soc Nephrol. 2007;18:913-22.
8. Prieto DA, Hood BL, Darfler MM, et al. Liquid tissue: proteomic profiling of formalin-fixed tissues. Biotechniques. 2005;June Suppl:32-5.
9. Hood BL, Conrads TP, Veenstra TD. Mass spectrometric analysis of formalin-fixed paraffin-embedded tissue: unlocking the proteome within. Proteomics. 2006;6:4106-14.
10. Hood BL, Conrads TP, Veenstra TD. Unravelling the proteome of formalin-fixed paraffin-embedded tissue. Brief Funct Genomic Proteomic. 2006;5:169-75.
11. Hood BL, Darfler MM, Guiel TG, et al. Proteomic analysis of formalin-fixed prostate cancer tissue. Mol Cell Proteomics. 2005;4:1741-53.
12. Ichimura T, Bonventre JV, Bailly V, et al. Kidney injury molecule-1 (KIM-1), a putative epithelial cell adhesion molecule containing a novel immunoglobulin domain, is up-regulated in renal cells after injury. J Biol Chem. 1998;273:4135-42.
13. Supavekin S, Zhang W, Kucherlapati R, et al. Differential gene expression following early renal ischemia/reperfusion. Kidney Int. 2003;63:1714-24.
14. Holly MK, Dear JW, Hu X, et al. Biomarker and drug target discovery using proteomics in a new rat model of sepsis-induced acute renal failure. Kidney Int. 2006;70:496-506.
15. Zhou H, Pisitkun T, Aponte A, et al. Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury. Kidney Int. 2006;70:1847-57.
16. Elliot S, Goldsmith P, Knepper M, et al. Urinary excretion of aquaporin-2 in humans: a potential marker of collecting duct responsiveness to vasopressin. J Am Soc Nephrol. 1996;7:403-9.
17. Hoorn EJ, Pisitkun T, Zietse R, et al. Prospects for urinary proteomics: exosomes as a source of urinary biomarkers. Nephrology (Carlton). 2005;10:283-90.
18. Pisitkun T, Shen RF, Knepper MA. Identification and proteomic profiling of exosomes in human urine. Proc Natl Acad Sci U S A. 204;101:13368-73.
19. Oates JC, Varghese S, Bland AM, et al. Prediction of urinary protein markers in lupus nephritis. Kidney Int. 2005;68:2588-92.
20. Chalmers MJ, Mackay CL, Hendrickson CL, et al. Combined top-down and bottom-up mass spectrometric approach to characterization of biomarkers for renal disease. Anal Chem. 2005;77:7163-71.
21. Decramer S, Wittke S, Mischak H, et al. Predicting the clinical outcome of congenital unilateral ureteropelvic junction obstruction in newborn by urinary proteome analysis. Nat Med. 2006;12:398-400.
22. Meier M, Kaiser T, Herrmann A, et al. Identification of urinary protein pattern in type 1 diabetic adolescents with early diabetic nephropathy by a novel combined proteome analysis. J Diabetes Complications. 2005;19:223-32.
23. Schiffer E, Mischak H, Novak J. High resolution proteome/peptidome analysis of body fluids by capillary

electrophoresis coupled with MS. Proteomics. 2006; 6:5615-27.
24. Wittke S, Haubitz M, Walden M, et al. Detection of acute tubulointerstitial rejection by proteomic analysis of urinary samples in renal transplant recipients. Am J Transplant. 2005;5:2479-88.
25. Bellman R. Adaptive control processes: a guided tour. Princeton University Press, 1961.
26. Norden AG, Rodriguez-Cutillas P, Unwin RJ. Clinical urinary peptidomics: learning to walk before we can run. Clin Chem. 2007;53:375-6.
27. Zhou H, Yuen PS, Pisitkun T, et al. Collection, storage, preservation, and normalization of human urinary exosomes for biomarker discovery. Kidney Int. 2006;69:1471-6.
28. Adachi J, Kumar C, Zhang Y, et al. The human urinary proteome contains more than 1500 proteins, including a large proportion of membrane proteins. Genome Biol. 2006;7:R80.
29. Norden AG, Sharratt P, Cutillas PR, et al. Quantitative amino acid and proteomic analysis: very low excretion of polypeptides > 750 Da in normal urine. Kidney Int. 2004;66:1994-2003.
30. Fiedler GM, Baumann S, Leichtle A, et al. Standardized peptidome profiling of human urine by magnetic bead separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Clin Chem. 2007;53:421-8.
31. Merrick BA, Tomer KB. Toxicoproteomics: a parallel approach to identifying biomarkers. Environ Health Perspec. 2003;111:A578-9.
32. Wittmann-Liebold B, Graack HR, Pohl T. Two-dimensional gel electrophoresis as tool for proteomics studies in combination with protein identification by mass spectrometry. Proteomics. 2006;6:4688-703.
33. Lopez JL. Two-dimensional electrophoresis in proteome expression analysis. J Chromatogr B Analyt Technol Biomed Life Sci. 2007;849:190-202.
34. Smith RD. Future directions for electrospray ionization for biological analysis using mass spectrometry. Biotechniques. 2006;41:147-8.
35. Smith RD, Tang K, Shen Y. Ultra-sensitive and quantitative characterization of proteomes. Mol Biosyst. 2006;2:221-30.
36. Smith RD, Shen Y, Tang K. Ultrasensitive and quantitative analyses from combined separations-mass spectrometry for the characterization of proteomes. Acc Chem Res. 2004;37:269-78.
37. Powell DW, Merchant ML, Link AJ. Discovery of regulatory molecular events and biomarkers using 2D capillary chromatography and mass spectrometry. Expert Rev Proteomics. 2006;3:63-74.
38. McDonald T, Sheng S, Stanley B, et al. Expanding the subproteome of the inner mitochondria using protein separation technologies: one- and two-dimensional liquid chromatography and two-dimensional gel electrophoresis. Mol Cell Proteomics. 2006;5:2392-411.
39. Sheng S, Chen D, Van Eyk JE. Multidimensional liquid chromatography separation of intact proteins by chromatographic focusing and reversed phase of the human serum proteome: optimization and protein database. Mol Cell Proteomics. 2006;5:26-34.
40. Lemley KV. An introduction to biomarkers: applications to chronic kidney disease. Pediatr Nephrol. 2007 DOI number 10.1007/s00467-007-0455-9.
41. Geurts P, Fillet M, de Seny D, et al. Proteomic mass spectra classification using decision tree based ensemble methods. Bioinformatics. 2005;21:3138-45.
42. Silva JC, Denny R, Dorschel C, et al. Simultaneous qualitative and quantitative analysis of the Escherichia coli proteome: a sweet tale. Mol Cell Proteomics. 2006;5:589-607.
43. Silva JC, Gorenstein MV, Li GZ, et al. Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. Mol Cell Proteomics. 2006;5:144-56.
44. Silva JC, Denny R, Dorschel CA, et al. Quantitative proteomic analysis by accurate mass retention time pairs. Anal Chem. 2005;77:2187-200.
45. Russo LM, Sandoval RM, McKee M, et al. The normal kidney filters nephrotic levels of albumin retrieved by proximal tubule cells: retrieval is disrupted in nephritic states. Kidney Int. 2007;71:504-13.
46. Thomas MC, Burns WC, Cooper ME. Tubular changes in early diabetic nephropathy. Adv Chronic Kidney Dis. 2005;12:177-86.
47. van Timmeren MM, Bakker SJ, Vaidya VS, et al. Tubular kidney injury molecule-1 in protein-overload nephropathy. Am J Physiol. 2006;291:F456-64.
48. Theilig F, Kriz W, Jerichow T, et al. Abrogation of protein uptake through megalin-deficient proximal tubules does not safeguard against tubulointerstitial injury. J Am Soc Nephrol. 2007;18:1824-34.
49. Yokota H, Hiramoto M, Okada H, et al. Absence of Increased α1-microglobulin in IgA nephropathy proteinuria. Mol Cell Proteomics. 2007;6:738-44.
50. Myers BD, Okarma TB, Friedman S, et al. Mechanisms of proteinuria in human glomerulonephritis. J Clin Invest. 1982;70:732-46.
51. Machii R, Sakatume M, Kubota R, et al. Examination of the molecular diversity of alpha1 antitrypsin in urine: deficit of an alpha1 globulin fraction on cellulose acetate membrane electrophoresis. J Clin Lab Anal. 2005;19:16-21.
52. Clarke W, Silverman BC, Zhang Z, et al. Characterization of renal allograft rejection by urinary proteomic analysis. Ann Surg. 2003;237:660-4.
53. Fliser D, Novak J, Thongboonkerd V, et al. Advances in urinary proteome analysis and biomarker discovery. J Am Soc Nephrol. 2007;18:1057-71.
54. Wittke S, Fliser D, Haubitz M, et al. Determination of peptides and proteins in human urine with capillary electrophoresis-mass spectrometry, a suitable tool for the establishment of new diagnostic markers. J Chromatogr A. 2003;1013:173-81.
55. Rossing K, Mischak H, Parving HH, et al. Impact of diabetic nephropathy and angiotensin II receptor blockade on urinary polypeptide patterns. Kidney Int. 2005;68:193-205.