Published in final edited form as:

Adv Chronic Kidney Dis. 2010 November ; 17(6): 480–486. doi:10.1053/j.ackd.2010.09.001.

Proteomic Discovery of Diabetic Nephropathy Biomarkers

Michael L. Merchant, PhD^{1,2} and Jon B. Klein, MD, PhD^{1,2,3,*}

¹Renal Division, University of Louisville, Louisville, KY

²Center for Environmental Genomics and Integrative Biology, University of Louisville, Louisville, KY

³Veterans Affairs Medical Center, Louisville, KY

Abstract

Diabetes mellitus (DM) is a complex systemic disease, with complications that result from both genetic predisposition and dysregulated metabolic pathways. DM is a highly prevalent disease with current estimates that there are 17.5 million diagnosed and 6.6 million un-diagnosed diabetics in the United States. DM and its complications impose a significant societal and economic burden. The medical costs of the most common microvascular complications of uncontrolled DM, diabetic nephropathy (DN) and diabetic retinopathy, account for 29% and 15% respectively of the \$116 billion expenditures associated with diabetes. A substantial gap in our knowledge exists of the understanding these complications. In order to advance therapy and decrease the societal burden of DM there is a clear need for biomarkers that can diagnose DN early and predict its course. Proteomics has evolved into a high-throughput, analytical discipline used to analyze complex biological datasets. These open-ended, hypothesis generating approaches, when appropriately designed and interpreted, are well suited to the study of the pathogenic mechanisms of diabetic microvascular disease and the identification of biomarkers of DN. We will review here the evolving role that proteomics has played in expanding our understanding of the diagnosis and pathogenesis of DN.

Keywords

urine; serum; plasma; peptidome; proteome; targeted proteomics

Introduction

Diabetic nephropathy (DN) is a microvascular complication that occurs in some, but not all, individuals with diabetes. Our understanding of diabetic complications is based to a great extent on our perception that empirically-based risk factors such as hyperglycemia, hyperlipidemia, and hypertension play the dominant role and not from insights into molecular mechanisms that determine cellular or tissue fate. Given the role of proteins as regulators of cellular responses, unbiased methods that provide qualitative and quantitative information of protein abundance could be useful for understanding the pathogenesis of diabetic complications. The proteomic method of protein analysis permits a rapid assessment of the proteome – the complete inventory of proteins expressed within a biological sample. With this method, biological samples such

© 2010 The National Kidney Foundation, Inc. Published by Elsevier Inc. All rights reserved.

*Corresponding author, Room 209, Donald Baxter Research Building, 570 S. Preston Street, Louisville, KY 40202, (502) 852-0245 (office), (502) 852-4384 (fax), mlmerc02@louisville.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

as urine, plasma, or serum can be systematically analyzed with the goal of identifying, quantifying and discerning the function of all observable proteins. The application of the proteomic method for comparison of disease and control samples allows for the rapid development of a hypothesis used to understand or explain aspects of disease biology such as disease initiation, progression or remission.¹ Recent mass spectrometric and bioinformatic advances have produced a set of core techniques including methods to separate complex protein and peptide mixtures², soft ionization approaches used to characterize biological molecules by mass spectrometry^{3–5} and robust computer-assisted data analyses that can cope with highly complex data sets⁶. Ideally, the protein identities of putative biomarkers should provide insight into the pathogenesis or evolution of a disease. While the proteomic approach to discovery is a relatively recent development for medicine and science, its limited application to the study of renal disease has yielded notable results⁷.

The need for the application of proteomic biomarker discovery to DN is made more evident by the fact that our paradigm of DN is in flux. The first models of diabetic nephropathy centered on hyperglycemia and hemodynamic alterations. It was posited that the metabolic insult of hyperglycemia, caused increased activation of protein kinase C, polyol pathway intermediates, advanced glycation end-products and reactive oxygen species. In T1DM, these metabolic changes were associated eventually with glomerular damage and the development of microalbuminuria (MA) and subsequent macroalbuminuria. Based on the finding of increased risk of progressive renal dysfunction in patients with MA, the quantitation of urine protein was accepted as a predictive biomarker of diabetic kidney disease^{8, 9}. These findings were soon extended in part to patients with T2DM. However, recent findings have called into question the correlation of MA with future renal dysfunction in diabetics. In T1DM long-term longitudinal studies have shown that only approximately 20% of patients with MA progress to proteinuria¹⁰, many patients with MA revert to normoalbuminuria^{11–13}; and many individuals with T1D have already experienced early renal function decline (ERFD) before or coincidental with MA onset^{14, 15}. Considered together, these findings suggest that MA may be an inadequate predictive biomarker of T1DM renal disease progression and response to therapy. This perspective has spurred an intense search by investigators to find new biomarkers of DN using proteomic techniques.

Proteomic Methods to Identify Candidate Biomarkers of Diabetic Nephropathy

Normal urine and urine from MA diabetic patients represents a dilute solution containing proteins derived from a number of sources including plasma proteins and to a lesser extent renal cells¹⁶. Urine from macroalbuminuric and nephrotic patients is a concentrated protein solution dominated by a few high abundant proteins primarily albumin and IgG. In as much as most urinary biomarker investigation focuses on high molecular weight proteins, it is also important to note that urine contains a complex mixture of proteins and protein fragments (peptides). To understand the diabetic phenotype and identify candidate biomarkers for DN, a complete understanding of all protein identities that are sourced into the urine is vital. Therefore methods such as electrophoresis and liquid chromatography (LC) have been used to reduce the analytic complexity (proteins and peptides) of the sample prior to protein identification using mass spectrometry (MS) based methodology. Significant work has been achieved using these two separation methods, however in the present review we will focus on the LC and capillary electrophoresis (CE) peptide separation methods. These approaches can be used to conduct top-down analysis (analysis of intact proteins and peptides) or bottom-up analysis (analysis of trypsinized proteins or native protein fragments) approach. These MS methods can be conducted using multi-dimensional chromatography, require lower mass loads given the high sensitivity ion counting detectors, and direct analysis of the analyte. A significant benefit of

methods such as capillary electrophoresis (CE)-MS or LCMS, that couple the sample separation directly with MS, is that larger data sets can be acquired in shorter periods of time.

In the past five years unlabeled peptide quantitative methods have emerged that simplify proteomic analysis. The basis for this approach to proteomics is a spectral counting methodology used to identify proteins from tryptic digests. The MS/MS data are analyzed by matching the experimentally acquired MS/MS spectra to a database of theoretical peptide spectra derived from known protein sequences. The proteins are identified by correlating deduced peptide sequences to known protein sequences. Several studies have previously demonstrated a correlation between both peptide hits and numbers of observed spectra per peptide. Therefore, in these label-free quantitative MS approaches, each spectra assigned with high confidence to a particular peptide can be assigned a relative expression level following normalization to total numbers of spectra acquired in each LCMS experiment. The corresponding peptides are matched across multiple LCMS experiments. The number of times a discriminatory peptide spectrum (counts) has been observed in different biological samples is normalized across all measured peptides for each protein quantified¹⁷. Following this normalization, the same peptide data or protein data can be compared across sample analyses and changes in expression levels inferred.

Accounting for Age Effects on the Urine Proteome

A pre-requisite to all biomarker discovery studies is to achieve equipoise in the sample sets from cases and controls. To be successful, candidate biomarkers for DN, should be specific to the exclusion of the effects of aging. Given these points, two recent studies Züribig *et al.*¹⁸ and Rossing *et al.*¹⁹ are noteworthy. Züribig utilized CE-MS methods to identify patterns of prevalent urinary polypeptides in 324 normal individuals aged 2–73. The relative urinary abundance of 325 of more than 5000 peptides or approximately 6% of the urinary peptidome was regulated with age. The largest component of change was attributed to alterations in the urinary peptidome of subjects between 11 and 18 years of age. The data for 218 patients with an age range of 19–73 years was reanalyzed. Using the same statistical approach, a grouping of 49 peptides was found to be specific to the aging process in adults. A generalized trend in the data was a decrease in observed peptides with advanced age. MS/MS methods identified fragments of collagen I- α -I, collagen III- α -I, fibrinogen β -chain, and psoriasis susceptibility-1 candidate gene-2 protein that had been associated with different renal diseases in previous studies by this group. Those previous studies had established urinary peptide patterns associated with DN, IgA nephropathy, focal segmental glomerular sclerosis, membranous glomerular nephritis, vasculitis, and minimal change disease. The observed panel of chronic kidney disease peptide panels overlapped with 73.5% of aging specific peptides. These findings lead to the re-analysis of the original data considering the patients in age cohorts of 19–30 years old and 51–73 years old to discern markers of biological renal age. Thirteen peptides were significantly correlated with renal age. The targeted analysis of these peptides in the urinary peptidome data sets for diabetic patients found that the urine of patients with DN resembled that seen in aging patients' urine. Using CEMS methods, Rossing *et al.* conducted biomarker discovery experiments to detect differences in the urinary peptidome of type-1 diabetic patients with normal urine protein excretion, MA and macroalbuminuria with a cohort of age-matched controls. The question raised and addressed by Rossing *et al.* was could the urinary peptidome define candidate biomarkers for 1) early diabetes, 2) early diabetic nephropathy, and 3) diabetic nephropathy in the face of chronic kidney disease? A first review of the CE-MS data by Rossing *et al.* noted that discrete differences were not observed between urinary peptidome profile derived from normals, normal albuminuric patients with DM and MA patients. However, large differences were observed in the urinary peptidome of macroalbuminuric T1DN patients as compared to the other groups. These observations then support the findings of Züribig *et al.* that the urinary peptidome of macroalbuminuric patients

is significantly different from that from healthier kidneys. To address candidate biomarkers of early diabetes, the urinary peptidomes of healthy controls and T1DM with persistent normoalbuminuria were compared by maxT testings (a statistical t-test approach that corrects for large numbers of simultaneous comparisons). A total of 40 peptides were observed at p-values less than 0.05. These same T1DM patients compared to T1DM patients with macroalbuminuria using maxT testing yielded 102 statistically significant peptides. An abbreviated list of peptides was found to classify the normo- versus macro- albuminuric T1DM patients with 93% sensitivity and 97% specificity at cross validation. Twenty-four of the original 102 peptides were identified as fragments of extracellular matrix proteins (collagens I and III), serum proteins (albumin, α -1 anti-trypsin, transthyretin, α -2 HS glycoprotein, serpin peptidase inhibitor, fibrinogen β -chain), uromodulin, β 2-microglobulin, psoriasis susceptibility-1 candidate gene-2 protein, and membrane associated progesterone receptor component 1. To compare candidate biomarkers of DN to that of other CKD causes, the biomarker panel developed previously was used to evaluate urine samples from biopsy-proven IgA nephropathy, focal segmental glomerular sclerosis, membranous glomerular nephritis, and minimal change disease. More than two thirds of the chronic kidney disease patients scored positively for diabetic nephropathy. This indicates that the patterns of diabetic kidney disease detected throughout the study may largely reflect chronic renal damage. To address this, the study then evaluated the non-diabetic renal disease urine samples against urine samples of T1DM patients with macroalbuminuria applying similar statistical methods support vector machine-based model, SVM-BM). A total of 17 peptides were identified that correctly identified 42 of 44 diabetic samples and 98 of 104 non-diabetic renal disease samples. These data might suggest that there are common pathways of renal damage but that small differences in the urinary peptidome imply unique mechanisms in the progression of the disease.

Urinary Peptides as Candidate Biomarkers of Early Progressive Renal Function Decline in Type-1 Diabetic Nephropathy

While biomarker discovery studies have traditionally focused on intact proteins, there is increasing interest in the low molecular weight fraction (<5 kDa) fraction of urine and plasma as a source of candidate disease markers^{20, 21}. This low molecular weight fraction may be composed of intact low molecular weight proteins such as growth factors, as well as fragments of intact proteins. There is increasing evidence that peptide fragments are conditionally unique and reflect disease-induced changes in cellular proteases²². Analysis of the urinary peptidome for biomarkers of renal disease has provided insights into a number of diseases.

In general, the success of biomarker discovery can be a function of the quality of specimens analyzed. The foresight of some groups that initiated prospective specimen collection and biorepository construction has been key to the discovery of DN biomarkers. Many of these samples, such as those from the First and Second Joslin Studies on the Natural History of Microalbuminuria and Diabetes, have provided the opportunity to use proteomic methods to compare urine, plasma, and serum samples from patients years or decades before the development of renal insufficiency. In addition to diagnosis of existing disease, we and other groups have sought to identify biomarkers that might be prognostic of disease. This goal has been greatly aided by the analysis of the well-characterized, curated patient samples of the First Joslin Study on the Natural History of Microalbuminuria and Type-1 Diabetes. Using samples from the Joslin study, we recently reported a bottom-up LC-MALDI-TOF MS approach to identified components of the urinary peptidome whose abundance correlated with future renal function decline in T1DM patients with microalbuminuria²³. Furthermore, these peptides, used as guides to select renal proteins for follow-up immunohistochemical and confocal microscopic analysis, suggested specific roles for the cellular stress response pathway in development of renal function decline.

As noted above, for this study the availability of well-characterized curated and longitudinal specimens was key. To determine onset and levels of MA patients were followed from 1991 to 2007 and the albumin excretion rate (in micrograms per minute) was estimated from the albumin-to-creatinine ratio in random urine samples. Within four years after the initial evaluation of the cohort in 1991, new onset MA developed in 109 of the 943 patients with normoalbuminuria. Eighty six patients were followed until 2007 and 61 of these patients met the following criteria and were included in the analysis: 1) greater than 8 years of biennial follow-up examinations after the onset of MA until 2007 in order to measure serial estimates of the glomerular filtration rate; 2) sufficient stored urine specimens (at least one 6 ml aliquot of urine per examination per patient) for analysis of peptide components taken within five years of the onset of MA. For all patients the earliest available urine sample after the documentation of MA onset was used for isolation of the urinary peptidome. Two cohorts of patient samples were analyzed and included T1DM patients with MA who had stable or age-appropriate renal function loss (referred to as controls or non-decliners) and T1DM patients who had early progressive renal function loss (referred to as cases or decliners). Assignment to these cohorts was based on serum cystatin C estimates of the glomerular filtration rate. The glomerular filtration rate in ml/min was approximated numerically by the reciprocal of cystatin C (in mg/L) multiplied by 100 (cC-GFR) and a regression slope fitted to serial measurements of cC-GFR over several years was used to assess the trend in renal function over time. Data available from the Baltimore Aging Study were used to establish the reference distribution for evaluating whether deterioration in renal function qualified as an abnormal rate of decline (designated 'early renal function decline' or 'ERFD'). Based on the Baltimore Aging Study, the acceptable rate of renal function decline in controls was set at < 3.4%/per year. For this study the rate of renal function decline in controls ranged from +1.9 to -3.2% per year and in cases -3.3 to -16.1% per year.

To prevent the development of systematic bias, the order of sample handling during peptide isolation and MALDI-TOF MS data acquisition was randomized. Peptides were isolated from the urine using ultrafiltration that isolated the < 10 kDa peptidome fraction, followed by desalting with solid phase extraction (SPE) methods. The urine peptidome isolate was fractionated into 45 components based on hydrophobicity using reversed-phase capillary scale HPLC column. Each fraction was then analyzed by MALDI-TOF MS. The statistical analysis of LC-MALDI-TOF MS sets presents several challenges. At the expense of eliminating true positives, but to decrease the number of false positive associations between peptide expressions and early renal function decline, we eliminated 3364 peptides that were detected in less than 20% of the specimens. Next, using a modification of the approach of Rossing et al., discussed previously, we analyzed only those MS peaks with at least a 50% difference in the frequency of a peptide between case patients and control subjects and that this difference be statistically significant; reducing the peptidome from 825 to six. For these six peptides, we compared their urinary abundances using the peptide peak's characteristics. The peak characteristics were defined from the integrated signal area for the peptide isotopic series. Three peptides were present more frequently in urine of case patients in comparison with urine of control subjects, and three were present less frequently in urine of case patients in comparison with urine of control subjects. The association of these peptides with early renal function decline was studied further using logistic regression analysis controlling for the effects of other covariates such as HbA1c and albumin excretion rates. Urinary presence of peptides 983.534, 1190.638, and 1838.851 m/z was strongly and independently associated with presence of ERFD. The adjusted odds ratios varied from 4.4 to 4.9 (95% confidence interval [CI] 1.2 to 20.0). Conversely, urinary presence of peptides (i.e., 1841.811, 2195.965, and 2315.018 m/z) was protective against ERFD. Analysis of contemporaneous plasma samples from the same patients by similar methods established that the observed differences in these peptides were specific to the kidney and not derived from filtered, differentially abundant plasma peptides. Therefore these peptides

were now considered to be candidate biomarkers for early renal function decline in T1DM patients with MA.

We undertook MS/MS studies to identify the amino acid sequences of the six peptides. The three more abundant peptides were fragments of the cadherin-like protein FAT tumor suppressor 2, zona occludens-3 (ZO-3), and inositol pentakisphosphate-2 kinase (IPP2K). The three peptides decreased in the early renal function decline specimens were fragments of extracellular matrix proteins- tenascin- X, α -I (IV) collagen, and α -I (V) collagen. The analysis of the MS/MS data for the 1838.851 m/z peptide, assigned to IPP2K, was consistent with a glycyl-glycyl posttranslational modification to the epsilon amino group of the internal lysine, which would be presumed to result from ubiquitination of the parent protein IPP2K.

We also sought to determine if these candidate urinary biomarkers might reflect changes in renal parenchymal protein expression and thereby provide insight into the pathophysiology of progressive renal function decline in diabetes. Unlike urine samples, few studies are approved for prospective renal biopsy collection. Therefore using renal biopsies from normal individuals and patients with diabetes, we examined the tissue expression of IPP2K and ZO-3 using immunohistochemistry. To maintain the focus on early renal function decline, the biopsies were from patients with diabetes, minimal albuminuria, and serum creatinine levels of 1.2 to 1.9 mg/dl. Patients with DN had increased IPP2K expression in renal tubules and glomeruli. ZO-3 had increased expression in biopsies from patients with DN as compared with control subject. Furthermore, the ZO-3 staining was less linear, not confined to the cell periphery, and increased in cytoplasm when compared with normal biopsy sections. This staining pattern of ZO-3 is similar to that of another zona occludens protein, ZO-1, whose expression has also recently been shown to be altered in a similar manner in cultured podocytes incubated in high-glucose medium.²⁴ The finding for IPP2K was intriguing. IPP2K has been shown be a constituent of mRNA-containing granules responsible for protein translation arrest in stressed cells.²⁵ These cytoplasmic inclusions, referred to as stress granules, are observed in cells subjected to environmental stress, including heat, irradiation, oxidative conditions, and hyperosmolarity.²⁶ Because we observed increased intact IPP2K in the kidney and IPP2K urinary fragments, we examined whether stress granules are present in DN. To establish the stress granule, we determined the expression of a constitutive stress granule protein, T cell intracellular antigen 1 (TIA1). We observed increased TIA1 staining in DN. TIA1 was primarily localized to the cytoplasm and in a granular pattern, indicative of stress granules. To confirm the presence of stress granules in DN, we determined the extent to which TIA1 and IPP2K co-localize renal tissue from patients with diabetes. Renal biopsies from normal donors and patients with diabetes were stained with TIA1 and IPP2K antibodies. Normal biopsies stained positively for TIA1, the staining was with a cytoplasmic distribution but are far smaller than stress granules. IPP2K staining in DN biopsies was faint. DN renal biopsies stained positively for both IPP2K and TIA1 in large granular structures consistent with the expected diameter and distribution of stress granules.

This study achieved the goals of identifying low molecular weight urinary peptides that predict progressive early renal function decline and establishing an association of the observed urinary peptides with changes in the renal parenchyma. These peptides reflected changes in both tubular and glomerular protein expression that were associated with the formation of stress granules and may define a new cellular mechanism by which DN is initiated or progresses. The usefulness of these discriminating peptides as biomarkers of diabetes-associated renal function decline must be determined in additional rigorous studies in a larger patient population. These results provide the hope that candidate biomarkers can provide insight into the mechanisms of diabetic kidney disease.

Plasma Peptides as Candidate Biomarkers of Renal Function Decline in Type-1 Diabetic Nephropathy

Until recently, there have been very few studies that applied proteomic discovery methods to the study of plasma or serum in order to identify candidate biomarkers of DN. Overgaard et al have reported a proteomic analysis of plasma from a cross-sectional cohort of T1DM patients previously diagnosed as normoalbuminuric, MA or macroalbuminuric²⁷. Plasma samples were fractionated by two different techniques: anion exchange chromatographic Q resin and hexapeptide library beads. The plasma peptide fractions from both preparation techniques were then studied using SELDI-TOF-MS and detected 290 peaks, clusters of which 16 were selected as the most promising biomarker candidates based on statistical performance, including independent component analysis. Four of the peaks that were discovered were identified as transthyretin, apolipoprotein A1, apolipoprotein C1 and cystatin C.

A Targeted Proteomic Analysis to Discover Candidate Intact Protein Biomarkers of Early Renal Function Decline in Type-1 Diabetic Nephropathy

Many studies have demonstrated a correlation of T2DM its complications and a chronic, low-grade inflammatory state. In addition to the prominent role of the glomerular lesion, this chronic low-grade inflammatory state is postulated to be pathologically involved with the development of complications such as diabetic nephropathy^{28–31}. However, few studies have addressed the association of inflammation and T1DM and its complications. Two recent targeted proteomic studies from the Joslin Diabetes Center looked specifically at the association of serum and urinary markers of inflammation and T1DN. Using clinical data obtained from longitudinal follow-up studies, encompassing many cases followed more than a decade, Wolkow *et al* studied urine sample of three groups of T1DM patients recruited into the First Joslin Study of the Natural History of Microalbuminuria in Type 1 Diabetes. These patient groups were defined as T1DM patients with a) persistent normoalbuminuria and no renal function decline, b) new onset MA and no renal function decline and c) new onset MA and early progressive renal function decline. The cohorts were assembled using longitudinal data but the urine and serum used for analysis was derived from study entry samples. Using multiplexed antibody-based assays, quantitative measures of a targeted set of five urinary inflammatory markers including IL-6, IL-8, monocyte chemoattractant protein-1, interferon-gamma-inducible protein (IP-10), and macrophage inflammatory protein-1 δ were established. The chemokines IL-8, monocyte chemoattractant protein-1, interferon-gamma-inducible protein (IP-10), and macrophage inflammatory protein-1 δ were increased with significance ($p < 0.05$) in the urine of new-onset MA patients who experienced significant future renal function decline. The cytokine IL-6 was also increased in the urine of new-onset MA patients who experience progressive renal function decline but the significance value was less ($p < 0.08$). An analysis of contemporaneous serum samples for IL-8, macrophage inflammatory protein-1 δ , and C-reactive protein did not document any significant differences suggesting that the observed urinary differences were specific to the kidney. These differences were maintained after adjustment for urinary creatinine. A multivariate analysis was used to find association of elevated levels of more than one chemokine with the future development of early renal function decline. This analysis suggested a five-fold increase in the risk to develop early progressive decline of renal function with an elevation of two or more chemokines.

Using the specimens from the 2nd Joslin Kidney Study, Niewczas et al measured serum concentrations of proteins that are members of the TNF and Fas signaling families, as well inflammatory pathway proteins such as sICAM-1, IL-8, IP10, MCP-1, CRP and IL-6³². Of these endpoints, TNF, the sTNFR, sFas, sICAM-1, and sIP10 were associated with early renal function decline in T1DM. Only the TNF receptors and sFas were associated with changes in

GFR estimated using the Cystatin C method when a multivariate analysis was performed. Variation in the concentration of the TNF receptors had a much stronger impact on GFR than clinical covariates such as age and albumin excretion. In these two important studies, a targeted proteomic approach was used to establish a risk association of inflammatory processes in urine samples years well before a measurable loss of renal function. Furthermore, these results support the hypothesis that both renal specific, as well as systemic inflammatory processes contribute to the progression of nephropathy in T1DM.

Proteomic Discovery of DN Biomarkers in T2DM

Despite its greater prevalence, there are fewer reports of proteomic analysis of biospecimens in patients with T2DM and DN. This may have resulted from the fact that many biorepositories were created when there was a lesser emphasis on T2DM research as it was at that time far less common. Regardless, most biomarker studies in T2DM and DN have been performed with the lower resolution and sensitivity SELDI-TOF-MS. Yang et al performed a SELDI-TOF-MS analysis of serum samples from 65 patients with DN and 65 without DN. The identified six m/z values that correlated with DN and confirmed this correlation in samples from a nested group³³. Yang et al did not use other mass spectrometry methods to identify the amino acid sequence of the candidate biomarker peaks. Papale et al also used SELDI-TOF-MS, but in urine, to identify candidate biomarkers of DN in T2DM. The studied 190 patients composed of 20 healthy patients, 20 diabetics with normoalbuminuria and 19 diabetics with MA, 132 with biopsy proven nephropathy (65 with DN, 10 diabetics with other nephropathy, and 57 with non-diabetic CKD). The classification model they developed correctly identified 75% NAD, 87.5% MICRO and 87.5% DN when applied to a blinded testing set. It was also able to reliably differentiate DN from non-diabetic-CKD in both diabetic and non-diabetic patients. Among the best predictors in this classification model were m/z peaks that were identified and validated as two proteins, ubiquitin and ss2-microglobulin.

Future Developments and Applications of Proteomics for Biomarker Discovery

Diabetes is a complex disease. The complexity of the etiology is reflected by the complexity of disease complications such as DN. Proteomic approaches have evolved to deal with disease complexity using such label-free top down LCMS approaches such as we used, labeled methods or targeted proteomic approaches used by Wolkow et al^{23, 34, 35}. We have seen that urine based biomarkers of renal diseases involving proteinuria will likely be composed of complex mixtures of intact proteins or conditionally specific protein fragments derived from urinary-resident serum proteins or renal parenchymal proteins. Further investigation into the enzymatic pathways producing these biomarker patterns can perhaps yield more relevant mechanistic information into renal glomerular and or tubular pathophysiology. The variability of the human urinary proteome has to be addressed or offset before any meaningful advances occur. Nonetheless, as high- resolution MS methods are coupled with improving protein separation and simplified quantitation algorithms in the general proteomic fields we should begin to see these methods successfully applied toward the study of DN.

References

1. Merchant ML, Klein JB. Proteomics and diabetic nephropathy. *Semin Nephrol* 2007 Nov;27(6):627–636. [PubMed: 18061845]
2. Issaq H, Veenstra T. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE): advances and perspectives. *Biotechniques* 2008 Apr;44(5):697–698. 700. [PubMed: 18474047]
3. Zhou M, Veenstra T. Mass spectrometry: m/z 1983–2008. *Biotechniques* 2008 Apr;44(5):667–668. 670. [PubMed: 18474043]

4. Matt P, Fu Z, Fu Q, Van Eyk JE. Biomarker discovery: proteome fractionation and separation in biological samples. *Physiol Genomics* 2008 Mar 14;33(1):12–17. [PubMed: 18162500]
5. Qian WJ, Jacobs JM, Liu T, Camp DG 2nd, Smith RD. Advances and challenges in liquid chromatography-mass spectrometry-based proteomics profiling for clinical applications. *Mol Cell Proteomics* 2006 Oct;5(10):1727–1744. [PubMed: 16887931]
6. Chalkley RJ, Hansen KC, Baldwin MA. Bioinformatic methods to exploit mass spectrometric data for proteomic applications. *Methods Enzymol* 2005;402:289–312. [PubMed: 16401513]
7. Beck LH Jr, Bonegio RG, Lambeau G, et al. M-type phospholipase A2 receptor as target antigen in idiopathic membranous nephropathy. *N Engl J Med* 2009 Jul 2;361(1):11–21. [PubMed: 19571279]
8. Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. *Lancet* 1982 Jun 26;1(8287):1430–1432. [PubMed: 6123720]
9. Parving HH, Oxenboll B, Svendsen PA, Christiansen JS, Andersen AR. Early detection of patients at risk of developing diabetic nephropathy. A longitudinal study of urinary albumin excretion. *Acta Endocrinol (Copenh)* 1982 Aug;100(4):550–555. [PubMed: 6812342]
10. Perkins BA, Krolewski AS. Early nephropathy in type 1 diabetes: a new perspective on who will and who will not progress. *Curr Diab Rep* 2005 Dec;5(6):455–463. [PubMed: 16316598]
11. Giorgino F, Laviola L, Cavallo Perin P, Solnica B, Fuller J, Chaturvedi N. Factors associated with progression to macroalbuminuria in microalbuminuric Type 1 diabetic patients: the EURODIAB Prospective Complications Study. *Diabetologia* 2004 Jun;47(6):1020–1028. [PubMed: 15170497]
12. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. *N.Engl.J.Med* 2003;348(23):2285–2293. [PubMed: 12788992]
13. Araki S, Haneda M, Sugimoto T, et al. Factors associated with frequent remission of microalbuminuria in patients with type 2 diabetes. *Diabetes* 2005 Oct;54(10):2983–2987. [PubMed: 16186402]
14. Perkins BA, Ficociello LH, Ostrander BE, et al. Microalbuminuria and the risk for early progressive renal function decline in type 1 diabetes. *J Am Soc Nephrol* 2007 Apr;18(4):1353–1361. [PubMed: 17329575]
15. Perkins BA, Nelson RG, Ostrander BE, et al. Detection of renal function decline in patients with diabetes and normal or elevated GFR by serial measurements of serum cystatin C concentration: results of a 4-year follow-up study. *J.Am.Soc.Nephrol* 2005;16(5):1404–1412. [PubMed: 15788478]
16. Julian BA, Suzuki H, Suzuki Y, Tomino Y, Spasovski G, Novak J. Sources of urinary proteins and their analysis by urinary proteomics for the detection of biomarkers of disease. *Proteomics Clinical Applicatins* 2009;3(9):1029–1043.
17. Powell DW, Merchant ML, Link AJ. Discovery of regulatory molecular events and biomarkers using 2D capillary chromatography and mass spectrometry. *Expert Rev Proteomics* 2006 Feb;3(1):63–74. [PubMed: 16445351]
18. Zurbig P, Decramer S, Dakna M, et al. The human urinary proteome reveals high similarity between kidney aging and chronic kidney disease. *Proteomics* 2009 Apr;9(8):2108–2117. [PubMed: 19296547]
19. Rossing K, Mischak H, Dakna M, et al. Urinary proteomics in diabetes and CKD. *J Am Soc Nephrol* 2008 Jul;19(17):1283–1290. [PubMed: 18448586]
20. Baggerman G, Verleyen P, Clynen E, Huybrechts J, De Loof A, Schoofs L. Peptidomics. *J Chromatogr.B Analyt.Technol.Biomed.Life Sci* 2004;803(1):3–16.
21. Jurgens M, Appel A, Heine G, et al. Towards characterization of the human urinary peptidome. *Comb Chem High Throughput Screen* 2005 Dec;8(8):757–765. [PubMed: 16464162]
22. Villanueva J, Shaffer DR, Philip J, et al. Differential exoprotease activities confer tumor-specific serum peptidome patterns. *J Clin Invest* 2006 Jan;116(1):271–284. [PubMed: 16395409]
23. Merchant ML, Perkins BA, Boratyn GM, et al. Urinary peptidome may predict renal function decline in type 1 diabetes and microalbuminuria. *J Am Soc Nephrol* 2009 Sep;20(9):2065–2074. [PubMed: 19643930]
24. Sharma K, Ramachandrarao S, Qiu G, et al. Adiponectin regulates albuminuria and podocyte function in mice. *J Clin Invest* 2008 May;118(5):1645–1656. [PubMed: 18431508]
25. Brehm MA, Schenk TM, Zhou X, et al. Intracellular localization of human inositol 1,3,4,5,6-pentakisphosphate 2-kinase. *Biochem J.* 2007 Aug 17;

26. Anderson P, Kedersha N. RNA granules. *J Cell Biol* 2006 Mar 13;172(6):803–808. [PubMed: 16520386]
27. Overgaard AJ, Hansen HG, Lajer M, et al. Plasma proteome analysis of patients with type 1 diabetes with diabetic nephropathy. *Proteome Sci* 2010;8:4. [PubMed: 20205888]
28. Gilbert RE, Cooper ME. The tubulointerstitium in progressive diabetic kidney disease: more than an aftermath of glomerular injury? *Kidney Int* 1999;56(5):1627–1637. [PubMed: 10571771]
29. Rivero A, Mora C, Muros M, Garcia J, Herrera H, Navarro-Gonzalez JF. Pathogenic perspectives for the role of inflammation in diabetic nephropathy. *Clin Sci (Lond)* 2009 Mar;116(6):479–492. [PubMed: 19200057]
30. Navarro-Gonzalez JF, Jarque A, Muros M, Mora C, Garcia J. Tumor necrosis factor-alpha as a therapeutic target for diabetic nephropathy. *Cytokine Growth Factor Rev* 2009 Apr;20(2):165–173. [PubMed: 19251467]
31. Navarro-Gonzalez JF, Mora-Fernandez C. The role of inflammatory cytokines in diabetic nephropathy. *J Am Soc Nephrol* 2008 Mar;19(3):433–442. [PubMed: 18256353]
32. Niewczas MA, Ficociello LH, Johnson AC, et al. Serum concentrations of markers of TNFalpha and Fas-mediated pathways and renal function in nonproteinuric patients with type 1 diabetes. *Clin J Am Soc Nephrol* 2009 Jan;4(1):62–70. [PubMed: 19073786]
33. Yang YH, Zhang S, Cui JF, et al. Diagnostic potential of serum protein pattern in Type 2 diabetic nephropathy. *Diabet Med* 2007 Dec;24(12):1386–1392. [PubMed: 18042080]
34. Wolkow PP, Niewczas MA, Perkins B, et al. Association of urinary inflammatory markers and renal decline in microalbuminuric type 1 diabetics. *J Am Soc Nephrol* 2008 Apr;19(4):789–797. [PubMed: 18256362]
35. Afkarian M, Bhasin M, Dillon ST, et al. Optimizing a proteomics platform for urine biomarker discovery. *Mol Cell Proteomics*. 2010 May 28;