Proteomics and Diabetic Nephropathy

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Summary: Proteomic methods have found broad applications in kidney disease research and more specifically in diabetic nephropathy (DN) research. Proteomic methods such as 2-dimensional gel electrophoresis have been used to gain insight into glomerular and tubular nephropathies including DN. At the protein level, differences in high-abundant proteins in DN have been shown to reflect primarily differentially posttranslationally modified plasma proteins. Higher-sensitivity proteomic methods (eg, liquid chromatography–mass spectrometry) have pushed the boundaries on the known urinary proteome to include more than 1,500 proteins. These same high-sensitivity methods have been applied toward profiling urinary peptides, which has resulted in methods to diagnostically screen urine to differentiate between type-2 diabetes mellitus and type-1 diabetes mellitus urine, normal versus microalbuminuria, or by angiotensin II receptor blocker treatment. Proteomic methods are being used to show response to insulin gene therapy in an animal model or alterations in the renal cortex mitochondrial proteome with the development of the diabetic phenotype. Proteomic methods continue to aid in the discovery of new mechanisms of diabetic pathology and understanding of the etiology of diabetic complications.
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Diabetes is not a new disease despite its epidemic proportions.[1-4] Early historical reference to the diabetic condition is contained in the Ebers papyrus written in approximately 1552 BCE. The condition referenced described 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 the ancient Indian physicians Charak and Susrutha, who had observed ants collecting around the urine of certain individuals. They termed the condition *Madhumeha* or *honey-like urine*. We now know diabetes to be a disease of mixed etiology with both genetic and environmental inputs. In addition, the complications of diabetes are incompletely understood and many of the previously held paradigms are being reversed.[5-7] Given the varied nature of diabetes and our evolving understanding of diabetic complications, an unbiased approach used to develop subsequent hypotheses for testing should be valuable and necessary in diabetes research. Proteomics represents an approach to rapidly develop a set of testable hypotheses from a set of well-controlled unbiased scientific experiments.

The proteomic method is a method whereby biological samples are analyzed systematically with the intent of identifying, quantifying, and discerning the function of conditionally unique proteins or peptides.[8] Although the underlying methods used in proteomics have existed for decades, if not centuries in some cases, recent proteomic advances have been driven by developments in a set of core technologies including separation sciences, mass spectrometry, and computer-assisted data analyses (ie, bioinformatics) that can accommodate extremely large information sets. Recent reviews of proteomic methods and advantages or disadvantages of individual methods are available.[9-12] Specific categorization or review of these

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methods is outside the scope of this article. The insight gained from proteomics should allow for a better understanding in the initiation or propagation of disease state(s). Significant efforts in many laboratories are building to capitalize on identifying proteins or peptides unique to the disease state and using these quantifiable features in comparison with contemporaneous controls as metrics to overtly gauge the disease state progression. These features are referred to as *biomarkers*.[13-15] In addition to allowing for diagnosis of the disease state, the hope of the biomarker is that it will allow for a more clear mechanistic understanding of the disease state such that rational design of interventional methods are feasible.

Despite the current knowledge of the natural history of diabetic nephropathy (DN), renal microvascular disease can occur with insulin-dependent (type 1) and non–insulin-dependent (type 2) diabetes mellitus (T1DM and T2DM, respectively). The overall incidence of end-stage renal disease with T1DM is higher than with T2DM and is approximately 4% to 17% at 20 years from time of initial diagnosis. Early pathologic abnormalities include glomerular mesangial cell expansion progressing to glomerular basement membrane thickening and glomerular sclerosis, which often is associated with deposition of plasma proteins such as fibrin, immunoglobulin, and complement proteins. Later nonglomerular pathologic abnormalities include but are not limited to the loss of visceral epithelial cell (podocyte) foot processes, tubular basement membrane thickening, interstitial fibrosis, and, controversially, tubular epithelial to mesenchymal cell transitions. Several animal models recapitulating some of the DN phenotype have been used in recent proteomic investigations including the OVE26, alloxan, and streptozotocin models of T1DM and the *db/db*, *ob/ob*, Zucker fatty rat, KK mouse, and Ostuka-Evans Tokushima Fatty rats. Although none of these models recapitulate the diabetic phenotype perfectly, a better understanding of DN has been achieved through the proteomic analysis of an individual diabetic model.

PROTEOMIC ANALYSIS OF NORMAL URINARY PROTEINS

Urine represents a dilute solution containing protein derived from a number of sources—predominately plasma proteins and to a lesser extent renal cell types (visceral epithelial, glomerular, and tubular origins). A complete understanding of protein identities that are sourced into the urine in normal individuals is necessary to understand the diabetic phenotype. To that end a number of studies have implemented combinations of electrophoretic (1- or 2-dimensional electrophoresis), chromatographic (affinity capture or chip-based adsorption methods), and mass spectrometric (matrix-assisted laser desorption ionization time-of-flight mass spectrometry [MALDI-TOF MS] or liquid chromatography electrospray ionization mass spectrometry [LC-ESI-MS]) methods to identify prevalent urinary proteins. Wittke et al[16] used capillary electrophoresis-mass spectrometry (CE-MS) to identify a pattern of 247 prevalent urinary polypeptides in normal human urine. Fiedler et al[17] used reversed-phase resin-coated magnetic beads to capture urinary polypeptides with profiling of 427 urinary polypeptides by MALDI-TOF MS. Castagna et al[18] used a similar affinity capture approach to identify 383 unique urinary proteins. A recent application of surface-enhanced laser desorption/ionization (SELDI), a lower-resolution MS method, identified 136 nonredundant urinary polypeptides.[19] Wang et al[20] and Sleat et al[21] both used affinity capture methods to isolate the urinary glycoprotein subproteome and identified 225 and 67 proteins, respectively, including a large number of lysosomal proteins. This is in agreement with earlier work by Thongboonkerd et al[22] and Pisitkun et al[23] on urinary exosomes that identified up to 295 proteins; a large portion of which were membrane proteins derived from the proximal tubular endosomal-lysosomal-exosomal protein catabolic pathway. Adachi et al[24] used analyzed urine protein from an individual and also a pooled urine sample established from 10 individuals. The urine protein analyses was conducted using LC–ion trap MS with and without size prefractionation by 1-dimensional sodium dodecyl sulfate–polyacrylamide gel elec-

trophoresis (PAGE). In their study a large number of tryptic peptides were observed (8,041), a high degree of variability existed between individuals or replicate runs. Less than 10% were observed within 4 replicate runs, 10.6% were observed within 3 replicate runs, and 26.0% were observed in half of all replicate runs. A substantial number of proteins were hydrophobic in nature and a greater number of hydrophobic proteins were identified from samples prefractionated using 1-dimensional sodium dodecyl sulfate–PAGE than from direct analyses of the identical samples. Overall, 1,543 proteins were identified successfully and used to annotate a publicly accessible urine proteomic database.[25]

ELECTROPHORETIC ANALYSIS OF THE DN URINARY PROTEOME

A number of advances have occurred with the study of DN using urinary proteomics and 2-dimensional electrophoresis (2DE). The variability of the urinary proteome and the variability in the diabetic phenotype are such that frequently included within the analyses of the diabetic urinary proteome are control urine samples inclusive of nondiabetic glomerular pathophysiology. Recently, the human urinary proteomes of normal controls, diabetics, and glomerular nephropathy were compared with other disease controls including focal segmental glomerular sclerosis, lupus nephritis, and membranous nephropathy. This analysis used high-resolution 2DE and artificial neural networks to analyze the gel spot patterns.[26] The 2DE urine gel data set was analyzed for gel spots (referred to as *features*) that would distinguish one gel group from other gel groups. By using univariate and multivariate methods, no one feature was able to guide or self-sort the gel images into the nephropathy phenotypes. By using machine learning methods, groups of spots were identified whose collective expression vectors were able to differentiate or distinguish sample origin. A remarkable aspect of this work is that all of the proteins that allowed for differentiation of the nephropathy phenotype were common serum proteins; mainly glycoproteins that exist in multiple charge forms resulting from differential post-translational processing. These proteins included albumin, α-1-antitrypsin (AAT), α-1 microglobulin, complement factor B Ba fragment, haptoglobin, hemopexin, orosomucoid, plasma retinol binding protein, transferrin, transthyretin, vitamin D binding protein, and zinc-α2-glycoprotein. Although urine contains proteins derived from renal cells, the major urinary protein component originates from plasma filtered by the renal glomerulus. This work did not distinguish whether the protein charge forms were present in the plasma or generated in the urine. This work excellently establishes the danger in ascribing protein expressional differences to diseases versus disease complications. Further, these data support the position that protein expressional differences validated by orthogonal methods should include not only normal controls but disease complication controls if the goal is identification of a sensitive and specific disease biomarker.

Other 2DE methods have been used to study DN urinary proteomics and one with promise is the differential in-gel electrophoresis (DIGE method). DIGE is a method for higher throughput analyses of sample because of the ability to mix 2 samples (ie, case and control) and coelectrophoresis proteins, thereby reducing variability when making between gel comparisons. Three cyanine dyes that are chemically reactive toward lysine side chains and N-terminal α-amino groups are available for use in this method. These cyanine dyes are referred to commercially as Cy-2, Cy-3, and Cy-5. Typically, Cy-3 and Cy-5 dyes are used to label pairs of case and control protein samples. Cy-2 dyes typically are used to label an internal standard derived from equivalent mass aliquots of all samples. The DIGE experiment is achieved by mixing equal masses of labeled protein, yielding a single sample that then is used for a 2DE experiment. The gel is imaged sequentially by a fluorescence scanner using combinations of excitation and emission wavelengths specific to the Cy-2, -3, or -5 dyes. The fluorescence volumes (per spot or total) for all Cy-3 or Cy-5 scans are normalized to the internal standard Cy-2 scans, thus allowing direct comparison of case and control protein expression levels. Examples of the DIGE analyses are provided in

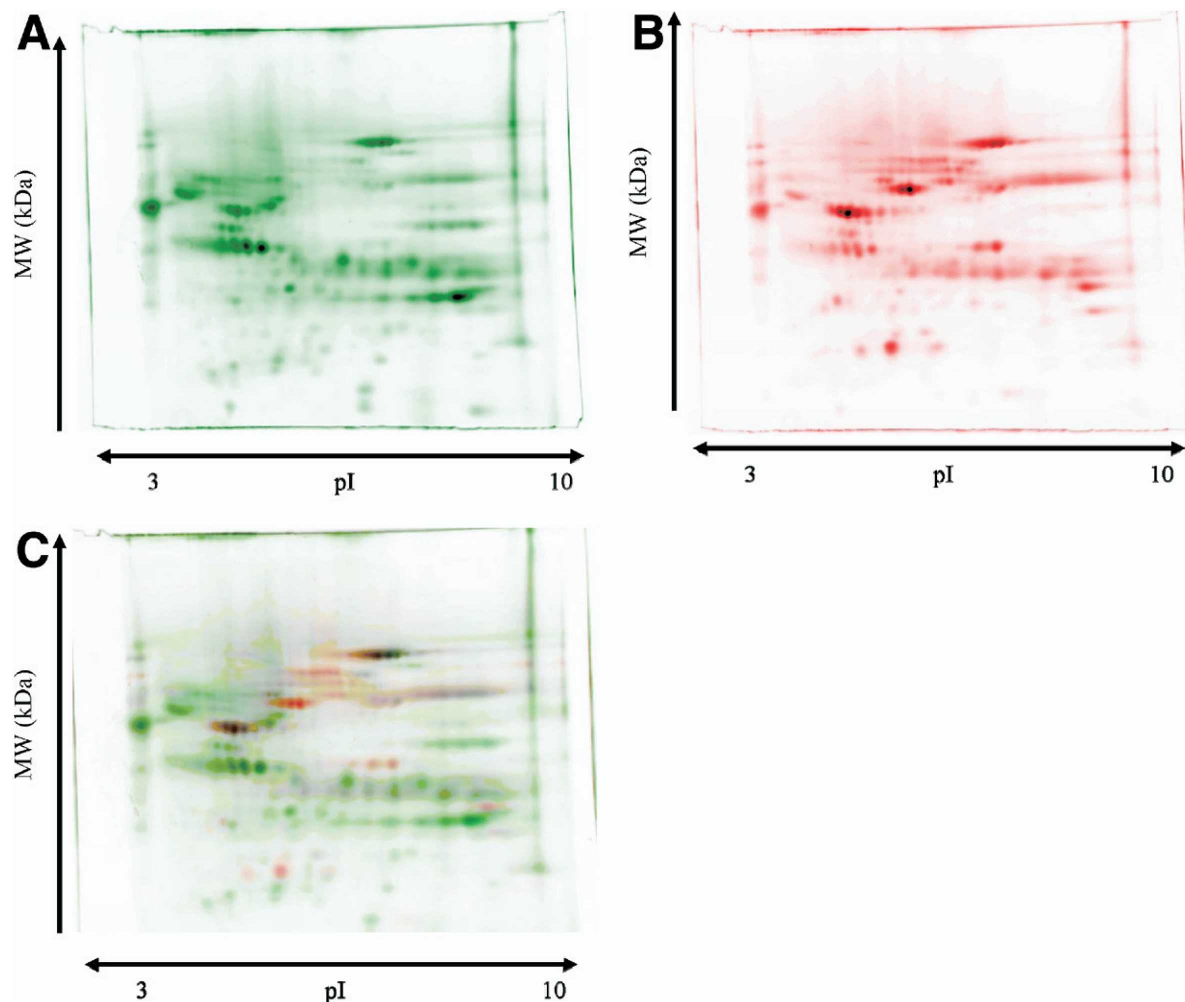

Figure 1. Urine from T1DM patients with and without significant renal function decline (RFD+ and RFD−) was depleted of albumin using an immunodepletion resin (VivaScience, Sartorius, Edgewood, NY) by the manufacturer's guidelines. The immunodepleted urine was concentrated and desalted using trichloroacetic acid (TCA) precipitation. A 100-μg protein aliquot was redissolved into a urea/thiourea/3-[(3-cholamidopropyldimethylammonio]-1-propane sulfonate (CHAPS)/Tris HCl pH 8.5 buffer to completely denature urinary proteins. The protein samples were labeled using the provided Cy-dyes according to the manufacturer's guidelines (GEHealthcare, Amersham Biosciences, Piscataway, NJ); T1DM RFD (−) labeled with Cy-3 and T1DM RFD (+) labeled with Cy-5. An internal standard was created by pooling 50 μg albumin immunodepleted T1DM RFD (−) urine protein and 50 μg albumin immunodepleted T1DM RFD (+) and labeled with Cy-2 dye. Aliquots (25 μg) of each sample ad-mixed and rehydrated into a 7-cm 3–10 pH immobilized pH gradient (IPG) strip and protein mixture co-focused for 2,000 volt-hours (Vh). The strip was subsequently reduced with two incubations of a dithiothreitol (DTT)-based equilibration buffer and alkylated with one wash of an iodoacetamide-based equilibration buffer. The proteins were transferred into a 4% to 12% gradient NuPAGE (Invitrogen, Carlsbad, CA) gel using 200 V/200 W/200 A electrophoresis profile. At each stage of the experiment the dye and the label proteins were protected from light to avoid photo bleaching of the fluorophore. Fluorescence imaging for the Cy-2, Cy-3 (Fig 1A) and Cy-5 (Fig 1B) was achieved sequentially using appropriate excitation/emission wavelength combinations on a Perkin Elmer ProXpress (Waltham, MA). Overlay or composite image (Fig 1C) development with the Cy-3 and Cy-5 images was achieved following fluorescence normalization and spot matching using the Cy-2-labeled internal standard protein.

Figure 1. These studies, performed in our laboratory, compared urine from microalbuminuric T1DM patients with and without progressive renal function decline. The urine was immunodepleted using the VivaScience Albumin Depletion Kit (Sartorius, Inc, Edgewood, NY). Proteins were concentrated and desalted by precipitation. Aliquots of individual urine pro-

tein samples (50 μg) were redissolved in a pH 8.5 urea-thiourea–based system and labeled with Cy-dye reagents (per Amersham guidelines [GE Healthcare, Amersham Biosciences, Piscataway, NY]). The total protein mass used for the 2DE experiment was 75 μg, with 25 μg Cy-2, 25 μg Cy-3, and 25 μg Cy-5–labeled protein material. Figure 1A represents Cy-3 (assigned as green) imaging of the resulting 2DE gel. Figure 1B represents Cy-5 (assigned as magenta) imaging of the same 2DE gel. Figure 1C represents software-guided overlay of Cy-3 and Cy-5 images. Perfectly overlaying peaks of equal protein loading appear as black. The composite gel spot image derived from variation of protein expression appears as a color combination of green and red. Expressionally, unique gel spots appear as red or gel within the composite image.

Sharma et al[27] used DIGE to study the urine of a diverse group of DN patients (n = 3) and observed the differential expression of a prominent protein AAT. AAT was up-regulated in the urine of diabetics. AAT expression subsequently was confirmed by enzyme-linked immunosorbent assay in the urine of several type 1 (n = 6) and type 2 (n = 13) diabetics. The majority of these individuals were on angiotensin converting enzyme inhibitor (ACEi) therapy and hydroxymethylglutaryl coenzyme A (HMGCoA) reductase inhibitor therapy. Immunohistochemistry analyses of human kidney sections (n = 15) showed increased staining of AAT in fibrotic areas including glomerular, interstitial, perivascular, and luminal proximal tubular regions. Although AAT has been identified previously to increase in the urine of individuals with diabetes, this study nonetheless showed the potential of DIGE to increase the throughput of urinary proteomic analyses. A larger, well-matched, DIGE-based study of control urine (n = 9), T2DM urine from individuals with normoalbuminuria (NA) (n = 10), microalbuminuria (MA) (n = 13), and microalbuminuria (n = 10) was conducted by Rao et al.[28] The analyses identified 195 protein spots representing 62 unique proteins of predominantly plasma origin. This observation and the observation that most of these proteins are glycoproteins is consistent with the previous data (Varghese et al[26]), suggesting that many of the differentially regulated urine proteins will be serum proteins wherein the DN phenotype is reflected by the posttranslation processing and not the specific protein identity.

DIRECT ANALYSIS OF THE DN URINARY PROTEOME USING MS

Electrophoretic methods of proteome analyses often are thought to be competitive with methods that directly analyze the proteome with MS methods. However, our perspective is that each method has a complementary place in proteomic discovery efforts. One-dimensional electrophoresis and 2DE have higher protein mass load capacities and low-abundant proteins can be detected by increasing the mass load into the electrophoretic system. MS methods such as those employing high sensitivity ion counting detectors have the advantage of sensitivity to femtomoles (or attomoles) of analyte, direct sample analysis, and the significant decrease in sample loss during the analysis. 2DE methods have the benefit of observing protein isoforms and distinguishing specific per-spot data that include isoelectric point, mass, and spot volume. Obtaining data with liquid chromatography–mass spectrometry (LC-MS) requires sophisticated computer-assisted data analyses platforms that might not be available to all laboratories. One area in which MS (eg, CE-MS, MALDI-TOF-MS, or SELDI-MS) excels over electrophoresis is in biomarker discovery. As previously referenced, Wittke et al[16] used CE-MS to identify a pattern of 247 prevalent urinary polypeptides in normal human urine. Fiedler et al[17] used reversed-phase resin-coated magnetic beads to capture urinary polypeptides, profiling 427 urinary polypeptides by MALDI-TOF MS. Otu et al[29] used SELDI to develop a list of putative biomarker masses from the Pima Indian Study wherein the urine was collected years before the onset of nephropathy. As evidenced in this study, the lack of mass accuracy of the SELDI technique does not result in the identification of specific protein or peptide masses but rather low-resolution mass windows. A few groups in collaboration, however, have set new limits of urinary peptide biomar-

ker discovery using CE-MS, including discovery of biomarkers of DN and also response to angiotensin II receptor blocker (ARB) therapy.

Urine peptide patterns distinct to DN for both T1DM and T2DM as compared with normal urine were developed using CE-MS by Meier et al[30] and Mischak et al.[31] T1DM polypeptide profiles were established using T1DM spot urine samples (n = 44) and 9 healthy age-matched control urines. The CE-MS samples were sorted into 3 subgroups for analyses (group I, normoalbuminuria, <2.5 mg albumin/mmol creatinine; group II, intermittent microalbuminuria, 2.5-3.5 mg albumin/mmol creatinine; and group III, microalbuminuria, >35 mg albumin/mmol creatinine). Routinely, greater than 800 ions per CE-MS urine analysis were observed and T1DM urine could be distinguished from normal urine by 54 separated ions. Further, binary differences (ie, peptide presence or absence) of 88 different peptides distinguished microalbuminuric-diabetic urine from normo-albuminuria-diabetic or intermittent-albuminuria-diabetic urine. Empiric formulas were developed that allowed for evaluation of polypeptide ion lists in a urine sample and assignment of that sample to the 4 urine groups (1 normal and 3 diabetic groups). Mischak et al[31] screened T2DM (n = 112) urine against normal (n = 39) urine samples. The comparison of polypeptide patterns indicated that the data sorted the samples in a fashion that accounted for renal damage (degree of albuminuria). Here, the polypeptide pattern was observed in individuals with albumin excretion rates of greater than 100 mg albumin/L, to a lesser degree in individuals with lower levels of urinary albumin.

CE-MS has been shown to have diagnostic as well as prognostic capacities. CE-MS biomarker profiling has been applied toward the study of DN response to ARB therapy.[32] This well-designed study used age-, sex-, and diabetes duration–matched urine samples from T2DM patients with the following (1) NA (n = 20), (2) diabetic retinopathy (DR) (n = 20), (3) diabetic DR and MA (n = 20), and (4) DR and macroalbuminuria (n = 18). The changes in urinary polypeptide patterns of patients with macroalbuminuria were followed up through a 2-month, randomized, double-blinded, crossover trial treatment with placebo, or 8, 16, and 32 mg of ARB (candesartan). The urinary peptide profile of NA and MA T2DM patients were identical regardless of DR. However, discretely different urinary peptide patterns could be observed for NA versus MA. The urinary peptidome as profiled by CE-MS encompassed a total of 4,551 polypeptides. Of these, 113 peptides were observed to distinguish between NA and MA T2DM patients. Extending their studies beyond biomarker profiling, Rossing et al[32] attempted to identify amino acid sequences or sequence tags for these proteins using tandem mass spectrometry. The peptides were shown to be sources from common plasma proteins and an extracellular matrix protein. A total of 11 peptides were sequence tagged: albumin (6 peptides), collagen (2 peptides), uromodulin (2 peptide), and immunoglobulin heavy chain (1 peptide). Fifteen of the 113 peptides were significantly differentially expressed after ARB, but not placebo treatment. The expression change of these peptides was from the microalbuminuria expression level toward levels more closely associated with NA. In a discovery sense, these data suggested that CE-MS is a robust peptide profiling approach. Further and mechanistically, these data suggest that the proteases that are involved in proximal tubular protein metabolism are affected differentially by ARB treatment and the angiotensin II signaling pathway.

PROTEOMIC ANALYSES OF RENAL PROTEINS IN ANIMAL MODELS OF DIABETES

The *db/db* mouse is an animal model of T2DM. In this model, relabeled as the *lepr^db^* model, the animals carry an autosomal-recessive mutation in the leptin receptor gene. Leptin is a small (~16 kd) protein hormone expressed by adipose tissue. Normally, leptin binding to leptin receptor long form regulates satiety and metabolism through a signal transducer and activator of transcription 3 (STAT3)-dependent signaling pathway. *Db/db* mice overeat, gain weight, become hyperinsulinemic, hyperglycemic, and hyperlipidemic. Tilton et al[33] used 2DE to study the renal cortex proteins whose expression was dysregulated in the *db/db* model of T2DM.

A problematic issue associated with the 2DE method is variability in gel images and resulting difficulties during image matching. This problem was addressed using pooled renal cortex extracts with incorporation of an internal standard. Case (*db/db*) and control (*db/m*) renal cortex protein extract (1 mg) from 5 animals was pooled. The samples were adjusted to contain 0.5% internal standard protein (apomyoglobin), which would be used during gel image matching and spot volume normalization. The experiment was repeated using a second set of animals and 6 replicate gels were run for each pooled sample per experiment. The cumulative number of protein spots detected varied as follows: experiment 1: 743 total and 204 differentially ($P < .05$); experiment 2: 643 total and 273 differentially ($P < .05$). Proteins whose expression difference was higher than 2-fold were selected for identification using MALDI-TOF MS and LC-ESI-MS methods.

A finding of this study is the congruence of observed and expected protein expression differences. Fifty-five percent of all proteins dysregulated were involved with some aspect of metabolism and more specifically mitochondrial metabolic pathways including lipid and fatty acid metabolism enzymes. Considering only the differentially expressed mitochondrial proteins using advanced bioinformatics algorithms (Ingenuity Pathways Analysis, Redwood City, CA), including expressional values as inputs, indicated that the most prominent biological network affected in this T2DN model was that of lipid metabolism. Moreover, the Ingenuity Pathways Analysis discerned network-contained peroxisome proliferator activated receptor α (PPARα) as a central regulatory axis of this network. These data are consistent with recent reports of PPARα agonists' renoprotective properties in experimental models of diabetes. In addition, these data generated in an unbiased fashion provide more than 140 molecular targets with which to follow the action of PPARα agonists in selective follow-up studies.

To date, 3 animal models of T1DM have been examined with proteomic methods. These models include one spontaneous model and 2 chemical models. The spontaneous T1DM OVE26 mouse model arises from a calmodulin transgene driven by the insulin promoter. Overexpression of calmodulin occurs selectively in the β-cell and results in β-cell death. The 2 chemical models are alloxan and streptozotocin, which result in β-cell necrosis. Of these models, the OVE26 model is the closest to resembling the human DN phenotype. Diao et al[34] used the alloxan model to study the serum, liver, and kidney proteome before and after electroporation of the insulin gene into the sural muscle. The mice were injected (intraperitoneally) with 175 mg/kg alloxan after a 48-hour fast. At day 7, mice with fasting blood glucose levels greater than 20 mmol/L were used for gene transfer or vector transfer. At day 28, mice receiving gene transfer had blood glucose levels statistically equivalent to the nondiabetic alloxan mice. Protein lysate was isolated from kidneys and liver. Serum protein was analyzed without depletion of overabundant proteins. All protein was separated and compared using 2DE and protein staining. Diao et al[34] observed and identified, using MS and bioinformatics analyses, 14 liver proteins, 15 renal proteins, and 14 serum proteins whose expression changed after diabetes induction. With subsequent rescue by gene transfer 5 liver proteins had significant expressional inversions (ie, reversal of protein expression from a decrease to an increase or vice versa) including up-regulation of regulcalcin (gi|6677739), haao protein (gi|15277547), and glyeraldehyde-3-dehydrogenase (gi|34785735), and down-regulation of serine hydroxymethyl transferase I (gi|6677943) and methionine adenosyltransferase (gi|476917).

After rescue of the diabetic phenotype, 5 renal proteins had significant expressional inversions including up-regulation of 14-3-3 zeta (gi|1841387), acetyl-CoA dehydrogenase (gi|20071667), a mitofilin homologue (gi|26328849), heat shock protein-90/glucose regulated protein-94 (gi|14714615), and down-regulation of pyruvate carboxylase (gi|32822907). Seven serum proteins were regulated and included up-regulation of plasminogen (gi|31982113) and down-regulation of complement factor I (gi|13959319), α-fetoprotein (gi|191675), albumin (gi|26341396), apolipoprotein (Apo)A-IV (gi|29477189), ApoA-I (2145139), and haptoglobin (gi|41019124). Expression regulation of ApoA-I was confirmed

visually by immunoblot analyses without support of statistical analyses on replicate data. Although the majority of the serum proteins are abundant proteins, several were identified from spots focusing and migrating at masses different from expected (lower isoelectric mass or altered isoelectric point), suggesting some aspect of posttranslational modification developed in the rescued phenotype. Perhaps additional and more interesting data could have been developed using proteomic prefractionation of the cell lysates (cytoplasmic, mitochondrial, nuclear, ER, and so forth) or immunosubtraction of overabundant proteins. These data represent a future application of proteomics in a cocktail fashion to bioinformatically profile multiple organ or biofluid systems and develop hypotheses on the effects of disease treatment or response to treatment.

Other work with serum profiling of the continuum of T2DN renal failure has been reported by Kim et al.[35] Notably this study was powered by high replicate numbers with T2DM patients without MA (n = 30), with MA (n = 29), and with chronic renal failure (CRF) (n = 31). High abundant serum proteins were removed by immunosubtraction using the Agilent Multiple Affinity Removal System (MARS) column (Santa Clara, CA). The Agilent Multiple Affinity Removal System column is effectively a LC affinity depletion column in which antibodies to the 7 most abundant plasma proteins have been immobilized. Serum is passed across the column. Specific high-abundant proteins are removed and the serum proteins typically have been enriched 10-fold. A weak point of this study is the use of silver staining for protein detection. Although silver stain is a sensitive stain, it is a problematic stain to work with for purposes of quantification. Other protein visualization approaches such as the use of dyes that bind to proteins in an equilibrium fashion include colloidal Coomassie Blue and Sypro Ruby. Silver stain is chemically deposited or plated onto the gel surface at sites of exposed protein in a catalytic fashion. Because of the importance of time and protein concentration effects on silver deposition it often is difficult or impossible to reproduce the silver staining and developing process across large sets of gels. Therefore, proteins having a small differential expression between conditions are difficult to discern reliably. However, proteins having a large differential expression can be detected with this method. A total of 17 proteins were regulated differentially between T2DM NA versus MA and a total of 18 proteins were regulated differentially between T2DM NA versus CRF. Twenty-six protein spots representing 17 gene products were identified by MS methods with statistical confidence. Expressional differences for extracellular glutathione peroxidase and ApoE were confirmed by immunoblot analyses. Five gene products were upregulated in the MA and CRF T2DM state and this list of identified proteins included adiponectin. Adiponectin is a regulator of insulin sensitivity, tissue inflammation, and plays a role in the complications of diabetes-related obesity. Adiponectin exists in circulation as varying molecular weight isoforms (ie, varying oligomerization states) and is known to decrease in circulating concentration with diabetes. Further exploration as to the nature of this protein spot could yield information relevant to a key axis of diabetic complications. Here proteomic methods have developed 2 possible plasma biomarkers of DN (extracellular glutathione peroxidase and ApoE) as well as provided other unexpected protein isoforms for study whose biology is mechanistically relevant to the T2DM state.

CONCLUSION: TODAY'S EXPECTATIONS FOR TOMORROW'S REALITY

Diabetes is a complex disease with substantial complexity derived from the variable complications of diabetes such as DN. Proteomic approaches have evolved to deal with disease complexity using such prefractionation methods as used by Sleat et al[21] or Castanaga et al,[18] higher-throughput separation methods such as the DIGE method or higher sensitivity MS methods such as that of Adachi et al.[24] We have seen that likely urine-based biomarkers of renal diseases involving proteinuria will be composed of complex protein-charge-form patterns of urinary-resident serum proteins. Further investigation into the enzymatic pathways producing these biomarker patterns perhaps can yield more relevant mechanistic information into re-

nal glomerular and/or tubular pathophysiology. The variability within the human urinary proteome requires that higher numbers of well-matched replicate samples be compared. Higher numbers of replicates necessitate higher-throughput, higher-sensitivity proteomic methods and will result in the research workflow changing from PAGE-based methods into true LC-MS methods. Because the LC-MS method presently is unable to differentiate between charge-train isoforms of a single protein, PAGE methods will continue to be needed. Nonetheless, because label-free MS methods of protein quantification permeate general proteomic research fields, we should begin to see these methods applied successfully toward the study of DN.[25]

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