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Urinary biomarkers for the early diagnosis of retinopathy and nephropathy in type 1 diabetes mellitus: a “steady stream” of information using proteomics

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Type 1 diabetes mellitus (T1D) results from autoimmune destruction of the pancreatic β cell, leading to absolute insulin deficiency and chronic hyperglycemia.¹ T1D affects nearly 3 million individuals in the United States and, for reasons that remain unclear, the worldwide incidence is increasing at a rate of approximately 3% per year.² Currently the third most common chronic health condition among American youth, T1D generates an estimated annual cost of more than \$14 billion, with much of this cost related to the expense of caring for macrovascular and microvascular disease-related complications.³

Microvascular diabetic complications, including retinopathy, nephropathy, and neuropathy, occur as a result of damage to small blood vessels and are among the most devastating consequences of longstanding T1D.⁴ Diabetic retinopathy (DR) arises from damage to the retinal and glial cells that occurs secondary to endothelial dysfunction, inflammation, oxidative stress, hypoxia, and signaling through advanced glycation end products. The most advanced form of retinopathy is described as proliferative DR and is characterized by neovascularization within the retina, with leakage and hemorrhage from these newly formed but structurally inadequate vessels. DR is the leading cause of blindness in persons between the ages of 20 years and 74 years.^{4,5} Diabetic nephropathy (DN) is equally devastating and ranks as the most common cause of end-stage renal disease in the United States. DN results from structural changes within the renal microvasculature and glomeruli. These changes include expansion of the extracellular matrix and basement membrane, mesangial thickening, and fibrosis that occur secondary to increased glomerular capillary pressure and activation of the renin-angiotensin-aldosterone system as well as other inflammatory pathways.⁶⁻⁸

The American Diabetes Association (ADA) recommends annual screening for DR and DN beginning 5 years after diagnosis. Screening for nephropathy is then performed annually by measuring the albumin-to-creatinine ratio in a randomly collected urine sample. DN is defined by proteinuria of more than 300 mg in 24 hours, and the presence of this level of proteinuria is referred to as severely increased albuminuria (previously known as macroalbuminuria). Moderately increased albuminuria, previously known as microalbuminuria, is defined as albumin excretion of 30–299 mg in 24 hours. Severely

increased albuminuria is thought to represent a disease continuum preceded by moderately increased albuminuria.

Although current screening paradigms for nephropathy are relatively inexpensive and easy to perform, there are a number of problems associated with this approach. The level of albumin excretion can vary widely in an individual based on blood pressure, hydration status, recent exercise, fever, and infection, impacting the assay's sensitivity and specificity, as well as interindividual variability.^{9,10} Furthermore, although albuminuria may serve as an indicator of nephropathy, it does not function as a robust disease predictor; there can often be a high degree of glomerular damage by the time albumin excretion is found to be clinically increased.¹⁰

Screening for retinopathy can be even more complicated. Beginning 5 years after diagnosis, the American Diabetes Association recommends annual performance of a dilated clinical eye examination by an ophthalmologist or optometrist. Difficulties with this screening strategy include access to qualified professionals, resulting in long referral turnaround times. Furthermore, there are numerous patient-perceived barriers related to the dilated fundoscopic examination, such as procedure-related anxiety and inconvenience of mydriasis.^{11,12} Screening techniques can also have variable outcomes, with direct ophthalmoscopy limited by sensitivity and subjectivity of interpretation, and retinal photography limited by artifacts and misinterpretation of images.¹³ Because of these challenges, even large-scale campaigns initiated to improve retinopathy screening among individuals with diabetes report compliance rates of as low as 50%.¹⁴

Clinical data suggest that aggressive reductions in hyperglycemia and early treatment are paramount in preventing and limiting progression of microvascular diabetic complications.^{15,16} The seminal Diabetes Control and Complications Trial provided crucial insight into the relationship between hyperglycemia and microvascular complications, and follow-up of these individuals in the Epidemiology of Diabetes Interventions and Complications study showed this protection could extend even beyond the circumscribed period of improved glycemic control, suggesting the presence of “metabolic memory” that impacts continued susceptibility to diabetic complications.¹⁷⁻¹⁹ Further research has also implicated other factors in the pathophysiology and predisposition toward microvascular complications. These include several modifiable risk factors such as dyslipidemia, hypertension, and smoking.^{10,20} However, discrepancies in the predicted severity and prevalence of T1D-associated complications and the presence or absence of these traditional risk factors implicates the existence of other patient-intrinsic as well as patient-extrinsic features that modify disease progression.^{4,21} Such factors have yet to be elucidated completely, making identification of the highest risk individuals a challenging endeavor.²² Furthermore, it should be noted that intense glycemic control is typically complicated by hypoglycemia, which may lead to increased morbidity and mortality for some individuals.²³⁻²⁶ Therefore, given the inadequacy of current screening paradigms, as well as the inability to predict complications based on traditional assessments, a high priority in the field is the discovery of new diagnostic assays or biomarkers. Such biomarkers may serve to identify more accurately those with the highest risk of microvascular complications at earlier time points, with the goal of targeting these individuals for more aggressive treatment strategies.

In this issue of *Translational Research*, Caseiro et al²⁷ have performed a set of exploratory experiments to address this critical need. The authors used a proteomics-based approach to analyze urine samples from a small cohort of 15 individuals with T1D, all of whom had a disease duration of at least 15 years. The diagnosis of T1D was based on the presence of circulating anti-islet cell, anti-insulin, or antiglutamic acid decarboxylase antibodies. In 5

subjects, T1D was complicated by retinopathy and nephropathy, whereas 5 subjects had retinopathy only. The remaining 5 individuals with T1D were free of chronic complications. Urine from 5 healthy control subjects was analyzed in parallel, and all subjects were matched by age and gender.

Midstream urine samples were collected and proteins were separated by sodium dodecylsulfate Polyacrylamide gel electrophoresis. Bands were then subjected to in-gel digestion with trypsin followed by liquid chromatography and tandem mass spectrometry to identify all peptides present in the urine samples. The abundance of identified proteins was then estimated by calculating an exponentially modified protein abundance index, which was used to analyze differences between groups. Last, a bioinformatics approach, referred to as protein-protein interaction analysis, was performed to identify relevant biologic pathways represented by group-specific changes in urinary protein levels.

A number of observations were made and are detailed in the accompanying article. Notably, of 219 unique proteins identified in the cohort, only 8 proteins were common to all subjects with diabetes. Bioinformatic analysis assigned these proteins to functional classes associated with wound healing and coagulation. Interestingly, a higher number of unique proteins were identified in the urine of subjects with T1D plus retinopathy. The 59 proteins identified in the urine of these subjects were found to be involved in ganglioside metabolism and glycosphingolipid catabolic processes. A total of 9 unique proteins were identified in the urine of subjects with T1D complicated by retinopathy and nephropathy. Pathway analysis suggested these proteins were involved in platelet degranulation and wound healing.

Discovery “omics” analysis of tissue and bodily fluids is used increasingly for biomarker development for a variety of disease states. The article by Caseiro et al²⁷ provides a cross-sectional analysis of the excreted urine proteome of a small cohort of individuals with T1D. Studied individuals were either free of microvascular complications, had retinopathy alone, or had retinopathy plus nephropathy. This study provides some insight into the nature of diabetic complications and represents a valid starting point for the development of novel urinary biomarkers to predict or detect T1D microvascular complications. However, it is important to note that the pathway from a discovery “omics” study to a clinically relevant and usable screening or diagnostic test is arduous. The walk along this pathway involves a number of considerations and refinements. First, a successful predictive biomarker must offer adequate sensitivity, but it must also be fairly specific to a particular disease state. Without assurances of specificity, such an assay would have an unacceptable rate of false-positive results when used in the clinical setting. This is particularly challenging when studying disease states associated with generalized inflammation like autoimmune conditions, cancer, or cardiovascular disease. For example, gelsolin, which was elevated here in all subjects with T1D, has been found to be elevated in the serum of individuals with a wide variety of disorders, including ischemic stroke, cervical cancer, and multiple sclerosis.²⁸⁻³⁰

A number of creative approaches have been used to help overcome challenges related to specificity. A recent elegant example involved an in-depth proteomic assessment of plasma that was performed to identify potential biomarkers of lung adenocarcinoma. The beauty of this study was that it involved analysis of multiple complementary model systems. To determine the unique proteomic signature of lung cancer above a background of cancer-related and/or generalized inflammation, the authors analyzed plasma from 4 distinct mouse models of lung cancer; models of pancreatic, ovarian, colon, prostate, and breast cancer; as well as 2 models of generalized inflammation. A number of potential proteins of interest were identified, and the next step was then to determine whether these proteins emanated directly from the lung cancer cells. To test this question, conditioned media were analyzed

from 21 independent adenocarcinoma cell lines and 5 proteins were identified as bona fide biomarkers, which were validated subsequently using human samples.³¹

A related but equally important question in biomarker development is whether changes observed in the urine have the capacity to reflect changes occurring at the level of the retina or kidney. Associating changes in urinary protein with progression of DN is certainly plausible and a rational approach. At face value, however, linking a measurable change in urinary protein to molecular events occurring within the retina in T1D is slightly more tenuous. Caseiro et al²⁷ identified several proteins involved in ganglioside metabolism and glycosphingolipid catabolic processes in the urine of subjects with T1D complicated by retinopathy. Gangliosides are lipid species that serve as a major glycolipid component of neuronal plasma membranes.³² Intriguingly, a previous study found that certain ganglioside species serve as mediators of retinal pericyte injury in response to advanced glycation end product signaling, offering an interesting biologic plausibility to these findings.³³ In addition, there is precedent for the investigation of urinary biomarkers for other ophthalmologic conditions such as macular degeneration.³⁴

A final but important consideration for the development of a successful biomarker is the assay's ability to predict early pathophysiology before the onset of clinically evident disease. For example, in the case of DN, this would be at a time when structural damage is minimal or more amenable to treatment and reversal strategies, and before the onset of severely increased albuminuria. The subjects studied by Caseiro et al²⁷ had already progressed to severely increased albuminuria, and they were studied in a cross-sectional approach. A next logical step would then be the examination of larger and clinically well-characterized cohorts monitored over longitudinal periods of time. Such an approach would allow changes in promising biomarkers to be assessed at multiple time points before the onset of DN. In a recent article, Zurbig et al³⁵ performed such an analysis and tested longitudinally collected urine samples from subjects with type 1 and type 2 diabetes. Samples were analyzed using a proteomics-based panel of 273 urinary peptides called the CKD273, which had been shown previously to have high sensitivity and specificity in the prediction of chronic kidney disease. Within this cohort of persons with diabetes, the authors found evidence for decreased renal collagen excretion that preceded the development of DN by 5 years.^{35,36} As a proof-of-principal application of this approach, a placebo-controlled intervention study is currently underway in Europe to test the effect of spironolactone on the progression to DN in subjects classified as high risk using the CKD273 panel.³⁷

Between 2001 and 2009, the prevalence of T1D rose 23% among children in the United States.^{38,39} As the prevalence of this disease increases, so too will diabetes-associated morbidity and mortality, and these statistics underscore the need for new and improved screening methods that allow for the earlier detection of potentially devastating microvascular complications. In this exploratory study, Caseiro et al²⁷ have described the urinary proteome of a small cohort of individuals with T1D complicated by retinopathy or retinopathy and nephropathy. Although the findings presented are preliminary, they are nonetheless hypothesis generating and represent a valid starting point for efforts aimed at biomarker development. Moreover, these findings illustrate the “steady stream” of biologic and pathophysiological information potentially available from a simple and painlessly procured urine sample.

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