Kidney Medicine

Circulating Protein and Metabolite Correlates of Histologically Confirmed Diabetic Kidney Disease

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Rationale & Objective: Diabetic kidney disease (DKD) is one of the leading causes of end-stage kidney disease globally. We aim to identify proteomic and metabolomic correlates of histologically confirmed DKD that may improve our understanding of its pathophysiology.

Study Design: A cross-sectional study.

Setting & Participants: A total of 434 Boston Kidney Biopsy Cohort participants.

Predictors: Histopathological diagnosis of DKD on biopsy.

Outcomes: Proteins and metabolites associated with DKD.

Analytical Approach: We performed linear regression to identify circulating proteins and metabolites associated with a histopathological diagnosis of DKD (n = 81) compared with normal or thin basement membrane (n = 27), and other kidney diseases without diabetes (n = 279). Pathway enrichment analysis was used to explore biological pathways enriched in DKD. Identified proteins were assessed for their discriminative ability in cases of DKD versus a distinct set of 48 patients with diabetes but other kidney diseases.

Results: After adjusting for age, sex, estimated glomerular filtration, and albuminuria levels, there were 8 proteins and 1 metabolite that differed between DKD and normal/thin basement membrane, and 84 proteins and 11 metabolites that differed between DKD and other kidney diseases without diabetes. Five proteins were significant in both comparisons: C-type mannose receptor 2, plexin-A1, plexin-D1, renin, and transmembrane glycoprotein NMB. The addition of these proteins improved discrimination over clinical variables alone of a histopathological diagnosis of DKD on biopsy among patients with diabetes (change in area under the curve 0.126; $P = 0.008$).

Limitations: A cross-sectional approach and lack of an external validation cohort.

Conclusions: Distinct proteins and biological pathways are correlated with a histopathological diagnosis of DKD.

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Kidney Med. 6(12):100920. Published online October 16, 2024.

doi: 10.1016/j.xkme.2024.100920

Diabetic kidney disease (DKD) is one of the leading causes of end-stage kidney disease globally.[1] Associated with a combination of metabolic and hemodynamic derangements, DKD is typically treated by promoting tight glycemic control, inhibiting the renin-angiotensin-aldosterone system (RAAS), and, more recently, through the use of sodium-glucose cotransporter 2 inhibitors. Despite these interventions, DKD continues to be a leading source of morbidity and mortality. Omics approaches may improve our understanding of the pathophysiology of the condition, identify targets in causal pathways, and ultimately guide future treatment strategies.

Proteomic and metabolomic profiling technologies have evolved to now quantify thousands of molecules in a small amount of blood, providing a comprehensive view into metabolic alterations associated with disease. Previous studies identified several serum biomarkers for prognosis and risk stratification among patients with diabetes and kidney disease.[2-4] However, few of these studies used histologically confirmed cases of DKD, instead relying on the presence of albuminuria or decreased estimated glomerular filtration rate (eGFR), which are imperfect markers of DKD.[5,6] Integrating biopsy data along with omics approaches may allow for more specific investigation of disease pathology.

In this study, we conducted a multiomics analysis to investigate biological pathways associated with DKD using histologically confirmed cases from the Boston Kidney Biopsy Cohort (BKBC). To do this, we performed proteomic and metabolomic profiling on plasma samples collected at the time of biopsy and then evaluated the association between circulating proteins and metabolites with the presence or absence of DKD on biopsy, independent of kidney function. We next performed gene set enrichment analysis to identify the underlying dysregulated biological pathways. Finally, we tested whether adding circulating biomarkers to a clinical model improved discrimination of a histopathological diagnosis of DKD among patients with a history of diabetes.

METHODS

Study Population

The BKBC is a prospective cohort study of 676 adult patients (>18 years old) who underwent a native kidney biopsy between September 2006 and June 2016 at 3 tertiary care hospitals in Boston, Massachusetts. Study protocol and design have been previously described in detail.[7] Blood and urine samples were provided on the day of the biopsy. Inability to provide consent, enrollment in competing studies, severe anemia, and pregnancy excluded individuals from participation. Our study population consisted of 434 participants with proteomic data available. All individuals

PLAIN-LANGUAGE SUMMARY

In the following study, we aimed to identify proteins, metabolites, and biological pathways that are associated with a diagnosis of diabetic kidney disease on biopsy. After adjusting for demographic characteristics and baseline renal function, we identified 5 proteins that were significantly associated with diabetic kidney disease, both in comparison to individuals without kidney disease and those with nondiabetic kidney disease: C-type mannose receptor 2, plexin-A1, plexin-D1, renin, and transmembrane glycoprotein NMB. We also found that these proteins may enhance our ability to distinguish between diabetic kidney disease and other causes of kidney disease in a group of patients with diabetes.

provided consent, and the study protocol was approved by the partners human research committee (the Brigham and Women's hospital institutional review board).

Covariates

Participants' information, including demographics, medical history, medication lists, and relevant laboratory data, was collected during the biopsy visit. To calculate eGFR, the creatinine-based chronic kidney disease epidemiology collaboration 2021 equation was utilized.[8] Serum creatinine values were obtained from the electronic medical record on the biopsy day. If not available, blood samples collected on the biopsy day were used to measure serum creatinine. Spot urine protein-to-creatinine ratio or urine albumin-to-creatinine ratio (ACR) from the date of kidney biopsy up to 3 months before biopsy were retrieved from the electronic medical record. For participants without these values, the urine albumin-to-creatinine ratio was measured from urine collected on the biopsy day. Serum and urine creatinine were measured using a Jaffe-based method, whereas urine albumin was measured using an immunoturbidometric method.

Evaluation of Histopathology

The evaluation of histopathology in BKBC has been previously described in detail.[7] Briefly, kidney biopsies were examined under light microscopy by 2 kidney pathologists who assigned histopathological scores. During joint pathology review sessions, cases would be discussed to reach a consensus if there was initial disagreement. Scoring criteria for histopathological lesions were based on the proposal by Sethi et al.[9] Patients' charts were reviewed alongside the final histopathological diagnosis to identify primary and secondary clinicopathologic diagnoses. Our primary exposure of interest was a histopathological diagnosis of DKD.

Proteomic and Metabolomic Profiling

Participants' blood samples were collected on the day of the biopsy, aliquoted, and stored at −80 °C. A slow off-rate modified aptamer-based capture array was used to quantify the relative concentrations of 6,592 plasma proteins or protein complexes in 434 participants. Briefly, 250 μL of plasma stored at −80 °C was sent to SomaLogic (Boulder, CO) for identification and quantification of plasma proteins using SomaScan, which utilizes chemically modified oligonucleotides as binding agents for proteins and protein complexes. Plasma samples were analyzed in 2023 using the V4.1 SomaScan platform. The mean coefficient of variation on 12 quality control samples was 4.2%, and in 8 blind duplicate pairs it was 4.7%. Metabolite profiling was performed on 444 participants (418 overlapping with those included in the proteomic measures) with untargeted mass spectrometry per standard protocols at Metabolon, Inc in the HD4 Platform. For inclusion in analyses, endogenous metabolites had to be present in at least 80% of participants. The mean coefficient of variation on 11 quality control samples was 17%, and in 9 blind duplicate pairs it was 15%. Protein and metabolite measurements were reported in relative fluorescence units before log2 transformation due to skewed distributions. Metabolites were scaled to the median value before log2 transformation. For both proteins and metabolites, values beyond 5 standard deviations on the log2 scale were winsorized.

Statistical Analyses

Covariates were summarized using frequency and percentage, mean and standard deviation, or median and interquartile range if not normally distributed. In the first part of the study, our main comparator groups were individuals with a diagnosis of normal or thin basement membrane (TBM) (grouped to form our healthy kidney set) and individuals without diabetes but with other kidney diseases (patients with a histopathological diagnosis other than normal, TBM, or DKD). Linear regression was used to evaluate the association of each protein or metabolite with a diagnosis of healthy kidney versus DKD, and other kidney diseases versus DKD. Models were adjusted for age, sex, eGFR, and log-transformed ACR. A prespecified α level of 7.59×10^{-6} set by Bonferroni correction (0.05/6,592 proteins) was used to determine statistical significance in linear regression analyses of proteins, with a corresponding value of 4.72×10^{-5} ($P < 0.05/1,060$ metabolites) in metabolomic analyses. β coefficients from linear regressions were interpreted as a relative measure of protein/metabolite level (ie, positive β values signify higher levels of a protein or metabolite in normal/TBM or other kidney disease vs DKD, and vice versa). Proteins that significantly different between DKD and both comparison groups were selected for evaluation in the second part of the study, which used a new comparison group, patients with diabetes but other kidney diseases. We then compared the area under the curve (AUC) of a logistic regression model incorporating age, sex, eGFR, and

log-transformed ACR, with one additionally incorporating identified proteins, for discrimination of DKD versus other histopathological diagnoses among patients with diabetes. Fig 1 summarizes our study design.

Pathway Enrichment Analysis

Gene ontology, Kyoto Encyclopedia of Genes and Genomes (KEGG), and Reactome pathway enrichment analyses were used to further characterize sets of biological processes (ie, pathways) related to analyzed proteins. Gene ontology is a computational analysis tool designed to represent functional knowledge of genes and gene products. It encompasses 3 key aspects of gene annotations, namely molecular function, cellular component, and biological process.[10] The KEGG encodes current knowledge on cellular processes and standardizes gene annotations, linking genomic information with higher-order functional information.[11] Reactome is an open-source database that organizes entities involved in reactions into a network of biological interactions and categorizes them into pathways.[12] The normalized enrichment score was used as an index for relative expression of a pathway.[13] The core genes accounting for a pathway's enrichment score were identified in the leading edge subset analysis.[13] The significance level was set at 0.05 after the Benjamin-Hochberg adjustment for the false discovery rate in our study.

RESULTS

Baseline Characteristics

There were 434 BKBC patients with available proteomic profiling included in the study. The mean age was 54 years; 48% of participants were women, and 9% identified as Hispanic. The mean eGFR was 51 mL/min/1.73m^2, and median baseline ACR was 1,011 mg/g. Most patients had a history of hypertension (59%), and 29% had a history of diabetes (type 2 diabetes mellitus [24%], and type 1 diabetes mellitus [5%]). A total of 81 patients had evidence of DKD on histopathology. Our first comparator group consisted of 26 patients with healthy kidneys: 11 individuals with normal biopsy samples and 15 individuals with TBM. Our second comparator group consisted of 279 individuals with no history of diabetes and with a histopathological diagnosis other than normal, TBM, or DKD (other kidney diseases). Our last subgroup consisted of 48 patients with a history of diabetes and a histopathological diagnosis other than DKD (Table 1). The indications for biopsy and the grading of histopathological lesions for each of these groups are presented in Table S1 and Table S2, respectively.

Proteins and Metabolites Associated With DKD

A total of 6,592 proteins and 1,060 metabolites were included in the analysis. After adjusting for demographic characteristics (age and sex), eGFR, and urine ACR levels,

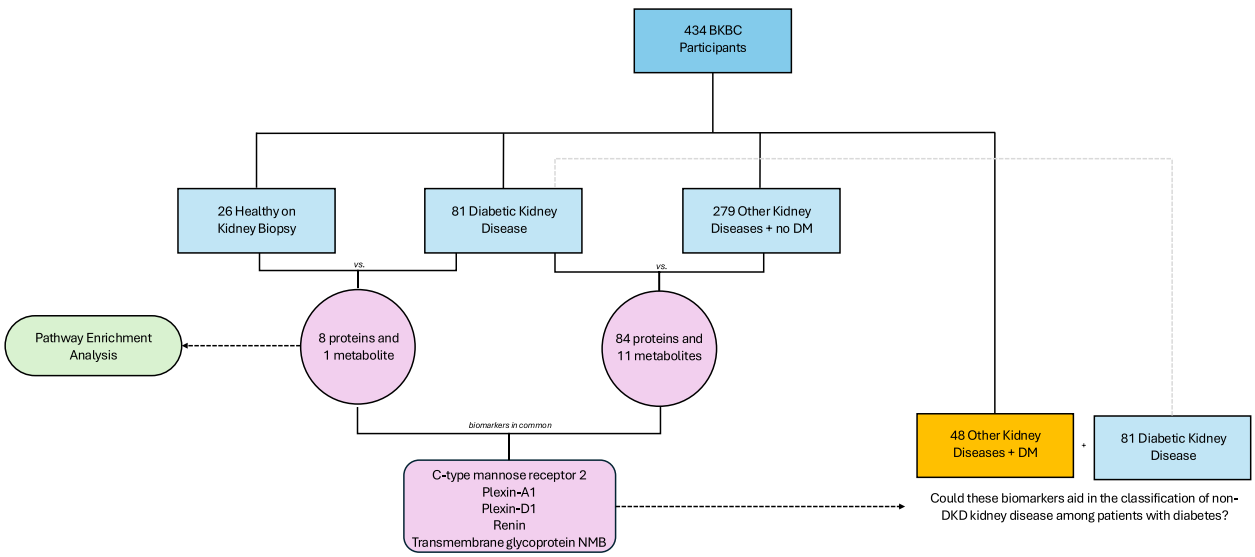

Figure 1. Summary of the study design. The study population of 434 Boston Kidney Biopsy Cohort participants were divided into 4 groups: 26 patients with normal/thin basement membrane (TBM) on kidney biopsy (healthy kidneys), 81 patients with diabetic kidney disease (DKD), 279 patients with other kidney pathologies on biopsy and no medical history of diabetes, and 48 patients with other kidney pathologies on biopsy and a medical history of diabetes. The latter 48 patients were set aside during the first analyses, in which linear regressions were used to identify proteins and metabolites associated with normal/TBM and other kidney diseases/no diabetes versus DKD (single reference; 3 category variable). *P* values from normal/TBM versus DKD were used for pathway enrichment analysis. A subset of 5 proteins that had significant associations in both regressions were identified. Logistic regression was then used to evaluate if these 5 proteins could improve of prediction of DKD versus non-DKD kidney pathologies among 129 patients with diabetes (81 of the original patients with DKD and 48 of the set aside patients with other kidney pathologies and a medical history of diabetes).

Table 1. Baseline Characteristics of the Boston Kidney Biopsy Cohort

Characteristic	Overall	DKD	Normal or TBM	Other Kidney Disease (No DM)	Other Kidney Disease (DM)[a]
N	434	81	26	279	48
Age, y	54.4 (16.2)	58.7 (14.3)	39.3 (12.8)	53.6 (16.4)	60.0 (13.8)
Women	206 (47.5%)	42 (51.9%)	19 (73.1%)	124 (44.4%)	21 (43.8%)
Hispanic	40 (9.3%)	11 (13.8%)	6 (23.1%)	17 (6.1%)	6 (12.5%)
Serum creatinine, mg/dL	2.2 (1.9)	2.4 (1.6)	0.9 (0.5)	2.2 (2.0)	2.7 (1.9)
eGFR, mL/min/1.73 m^2	50.9 (33.2)	37.8 (23.6)	98.1 (27.1)	52.4 (32.7)	38.2 (27.4)
ACR, mg/g	1,018.3 (224.2-3,012.0)	3,122.6 (1,017.1-4,755.1)	74.7 (5.0-315.1)	926.9 (196.0-2,529.6)	973.6 (183.9-3,891.5)
Hypertension	256 (59.0%)	68 (84.0%)	5 (19.2%)	145 (52.0%)	38 (79.2%)
DM type 1	21 (4.8%)	16 (19.8%)	0 (0.0%)	0 (0.0%)	5 (10.4%)
DM type 2	105 (24.2%)	62 (76.5%)	0 (0.0%)	0 (0.0%)	43 (89.6%)
CKD	157 (36.2%)	49 (60.5%)	2 (7.7%)	85 (30.5%)	21 (43.8%)
Use of ACE inhibitor	132 (30.4%)	32 (39.5%)	2 (7.7%)	81 (29.0%)	17 (35.4%)
Use of ARB	70 (16.1%)	20 (24.7%)	1 (3.8%)	37 (13.3%)	12 (25.0%)
Use of MRA	11 (2.5%)	6 (7.4%)	0 (0.0%)	3 (1.1%)	2 (4.2%)
Use of corticosteroids	68 (15.7%)	6 (7.4%)	5 (19.2%)	49 (17.6%)	8 (16.7%)
Use of CCB	120 (27.6%)	42 (51.9%)	0 (0.0%)	60 (21.5%)	18 (37.5%)
Use of β blocker	156 (35.9%)	45 (55.6%)	2 (7.7%)	84 (30.1%)	25 (52.1%)

Note: Data are presented as mean (standard deviation), frequency (%), and median (interquartile range).
Abbreviations: DKD, diabetic kidney disease; TMB, thin basement membrane; DM, diabetes mellitus; GFR, glomerular filtration rate; ACR, albumin-to-creatinine ratio; CKD, chronic kidney disease; ACE, angiotensin converting enzyme; ARB, angiotensin-receptor blocker; MRA, mineralocorticoid receptor antagonist; CCB, calcium channel blocker.
[a]Evaluated in the second part of the analysis only.

there were 8 unique proteins (identified by 10 SomaScan aptamers) and one metabolite that were significantly associated ($P < 7.59 \times 10^{-6}$ for proteins and $P < 4.72 \times 10^{-5}$ for metabolites) with a histopathological diagnosis of DKD, when compared with individuals with healthy kidneys (normal/TBM). These included semaphorin-5A, C-type mannose receptor 2, plexin-A1, plexin-D1, angiotensinogen, renin, transmembrane glycoprotein NMB, and trem-like transcript 1 protein. If multiple aptamers of the same protein were identified, the aptamer with the lowest *P* value was presented and used in subsequent analyses (Fig 2; Tables S3 and S4).

After adjusting for the same covariates, we identified 84 unique proteins (identified by 94 SomaScan aptamers) and 11 metabolites that were significantly associated ($P < 7.59 \times 10^{-6}$ for proteins and $P < 4.72 \times 10^{-5}$ for metabolites) with DKD, when compared with individuals without diabetes and with other kidney diseases on histopathology. A total of 5 proteins were significant in both sets of analyses: renin, plexin-A1 (PLXA1), plexin-D1 (PLXD1), transmembrane glycoprotein NMB: extracellular domain (GPNMB:ECD), and C-type mannose receptor 2, all of which had higher levels in DKD compared to both the normal/TBM and the other kidney diseases + no diabetes subgroups (Table 2 and Fig 3).

Pathway Enrichment Analysis

Enrichment analysis performed using the associations between circulating proteins and the presence of DKD (vs normal/TBM) revealed 6 pathways that were differentially expressed among patients with DKD, compared with those with healthy kidneys. These included pathways on collagen-containing extracellular matrix, axon guidance nervous system development, cell-substrate junction, and focal adhesion (Fig 4). In leading edge analyses, where we identified the core genes accounting for a pathway's enrichment score, several of the 8 proteins identified in the normal/TBM versus DKD analyses were found to play an important role in upregulated biological pathways, including angiotensinogen in the collagen-containing extracellular matrix pathway, semaphorin-5A and plexin-A1 in axonal guidance, and C-type mannose receptor 2 in cell-substrate junction and focal adhesion.

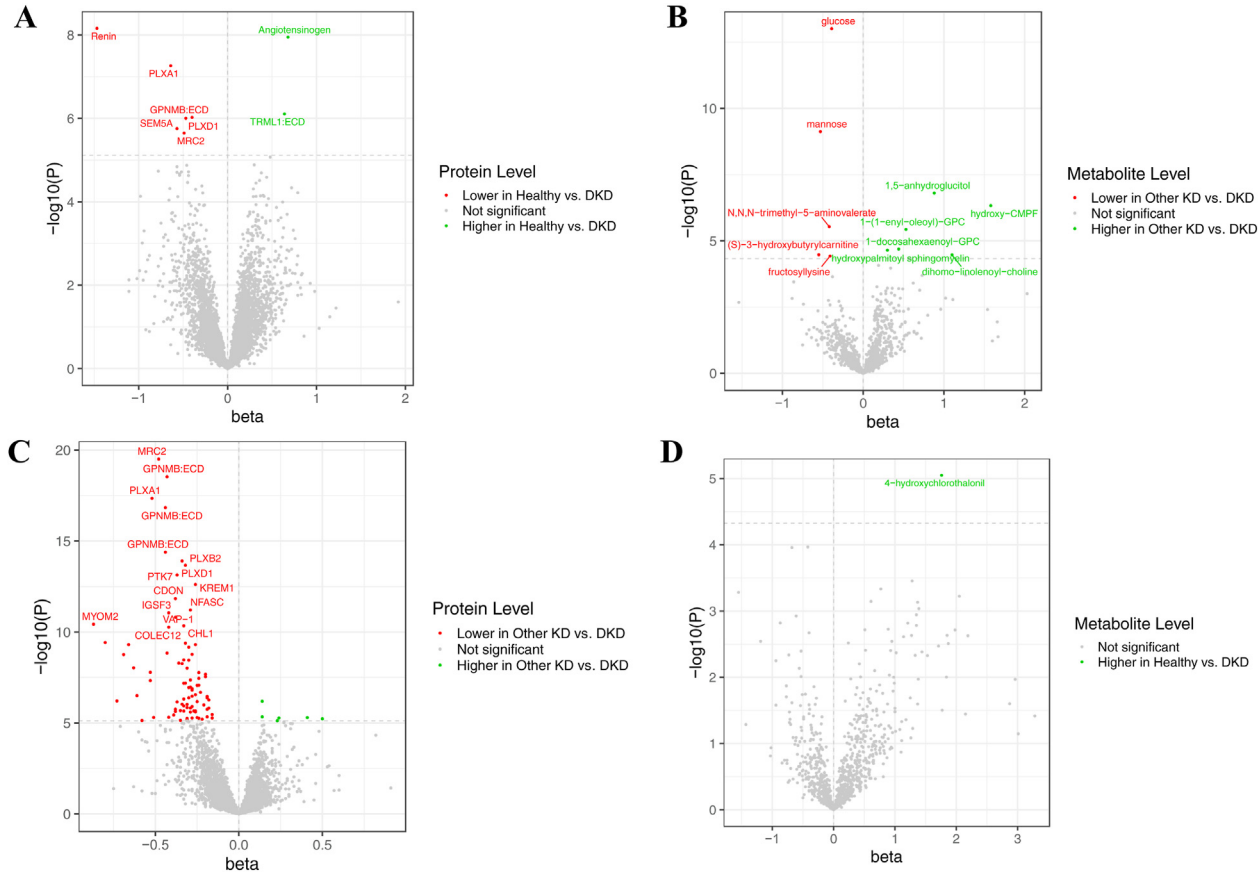

Figure 2. Volcano plots of identified molecules, after adjusting for demographics, baseline estimated glomerular filtration, and baseline urine albumin-to-creatinine ratio. (A) Proteins associated with normal/thin basement membrane (TBM) versus diabetic kidney disease (DKD). (B) Metabolites associated with normal/TBM versus DKD. (C) Proteins associated with other kidney disease (among patients without diabetes) versus DKD. Some proteins not labeled in this plot for legibility. See Table S3 for full list of identified proteins. (D) Metabolites associated with other kidney disease (among patients without diabetes) versus DKD. The horizontal dotted lines mark the Bonferroni-corrected threshold of significance.

Table 2. Biomarkers Identified in Linear Regressions of Normal/TBM Versus DKD (reference) and Other Kidney Disease (no diabetes) Versus DKD (reference), adjusted for demographic characteristics (age, sex), baseline eGFR, and urine ACR

Protein	Symbol	β (95% CI): Normal/TBM vs DKD	*P* Value: Normal/TBM vs DKD	β (95% CI): Other KD (no DM) vs DKD	*P* Value: Other KD (no DM) vs DKD
Renin	Renin	−1.47 (−1.96 to −0.97)	6.93E-09	−0.80 (−1.06 to −0.55)	3.77E-10
Plexin-A1	PLXA1	−0.64 (−0.87 to −0.41)	5.46E-08	−0.52 (−0.64 to −0.40)	4.52E-18
Plexin-D1	PLXD1	−0.40 (−0.56 to −0.24)	9.43E-07	−0.32 (−0.40 to −0.24)	2.14E-14
Transmembrane glycoprotein NMB: extracellular domain	GPNMB:ECD	−0.47 (−0.65 to −0.28)	9.95E-07	−0.43 (−0.53 to −0.34)	2.94E-19
C-type mannose receptor 2	MRC2	−0.49 (−0.69 to −0.29)	2.25E-06	−0.48 (−0.58 to −0.38)	3.15E-20

Note: β coefficients from linear regressions were interpreted as a relative measure of protein/metabolite level (ie, negative β values signify lower levels of a protein or metabolite in normal/TBM or other kidney disease vs DKD).
Abbreviations: DM, diabetes mellitus; DKD, diabetic kidney disease; eGFR, estimated glomerular filtration rate; KD, kidney disease; TBM, thin basement membrane.

Use of Circulating Molecules to Predict the Presence of DKD

Finally, we aimed to determine whether the identified circulating proteins could aid in prediction of DKD versus non-DKD kidney pathologies among patients with diabetes. We built a logistic regression model to predict the presence of DKD versus all other histopathological diagnoses, using clinical characteristics alone. For these analyses, we used the 81 individuals with DKD and the 48 as-yet unanalyzed individuals with a history of diabetes and non-DKD diagnoses on histopathology. Among these 129 patients, the AUC of a model using demographic characteristics, eGFR, and ACR to distinguish DKD versus other kidney disease without DKD was 0.663. After addition of the 5 proteins of interest (C-type mannose receptor 2, plexin-A1, plexin-D1, renin, and transmembrane glycoprotein NMB), the AUC increased to 0.789 (change in AUC 0.126, P = 0.008) (Fig 5). At a histopathological level, the specific lesions that were most strongly associated with DKD versus other kidney diseases among patients with diabetes, included tubular atrophy/interstitial fibrosis, arterial sclerosis, arteriolar hyalinosis/sclerosis, mesangial matrix expansion, and mesangial hypercellularity (Table S5).

DISCUSSION

In this study, we identified a set of 5 circulating proteins that were associated with biopsy-confirmed cases of DKD when compared with individuals with healthy kidneys and those with non-DKD kidney diseases. Pathway enrichment analysis identified putative pathways that were differentially expressed in DKD. Our results support the notion that biological processes in collagen and extracellular matrix

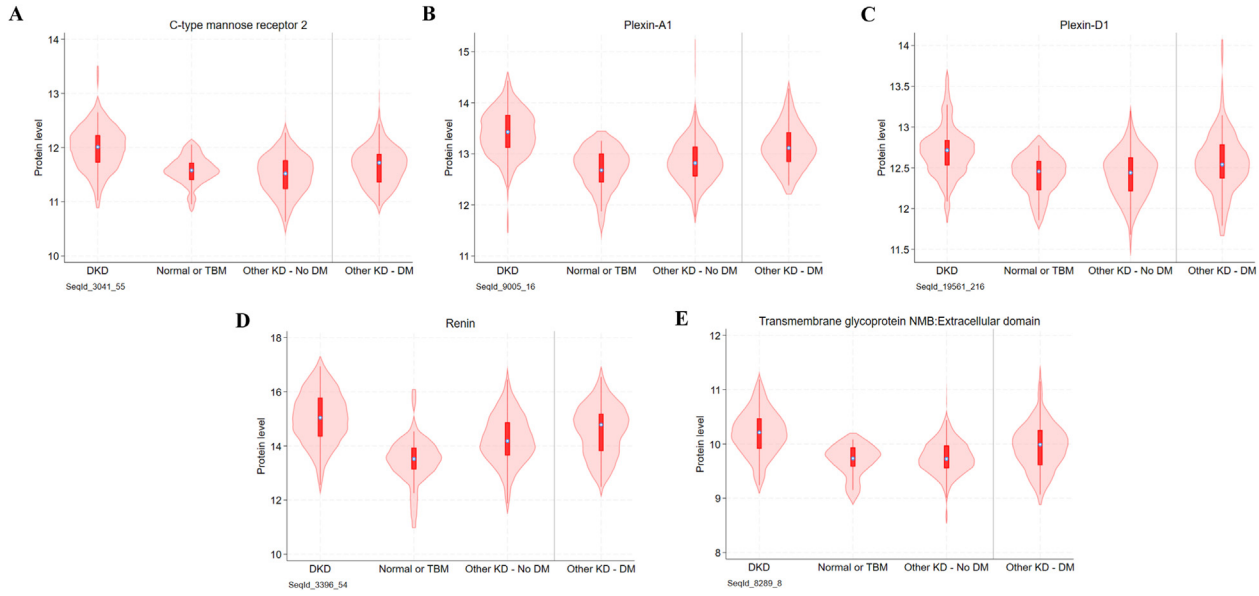

Figure 3. Protein levels (reported as relative fluoresence units) of C-type mannose receptor 2, plexin-A1, plexin-D1, renin, and transmembrane glycoprotein NMB in each of our 4 study subgroups: DKD, normal/thin basement membrane, other kidney disease + no history of diabetes mellitus, and other kidney disease + history of diabetes mellitus.

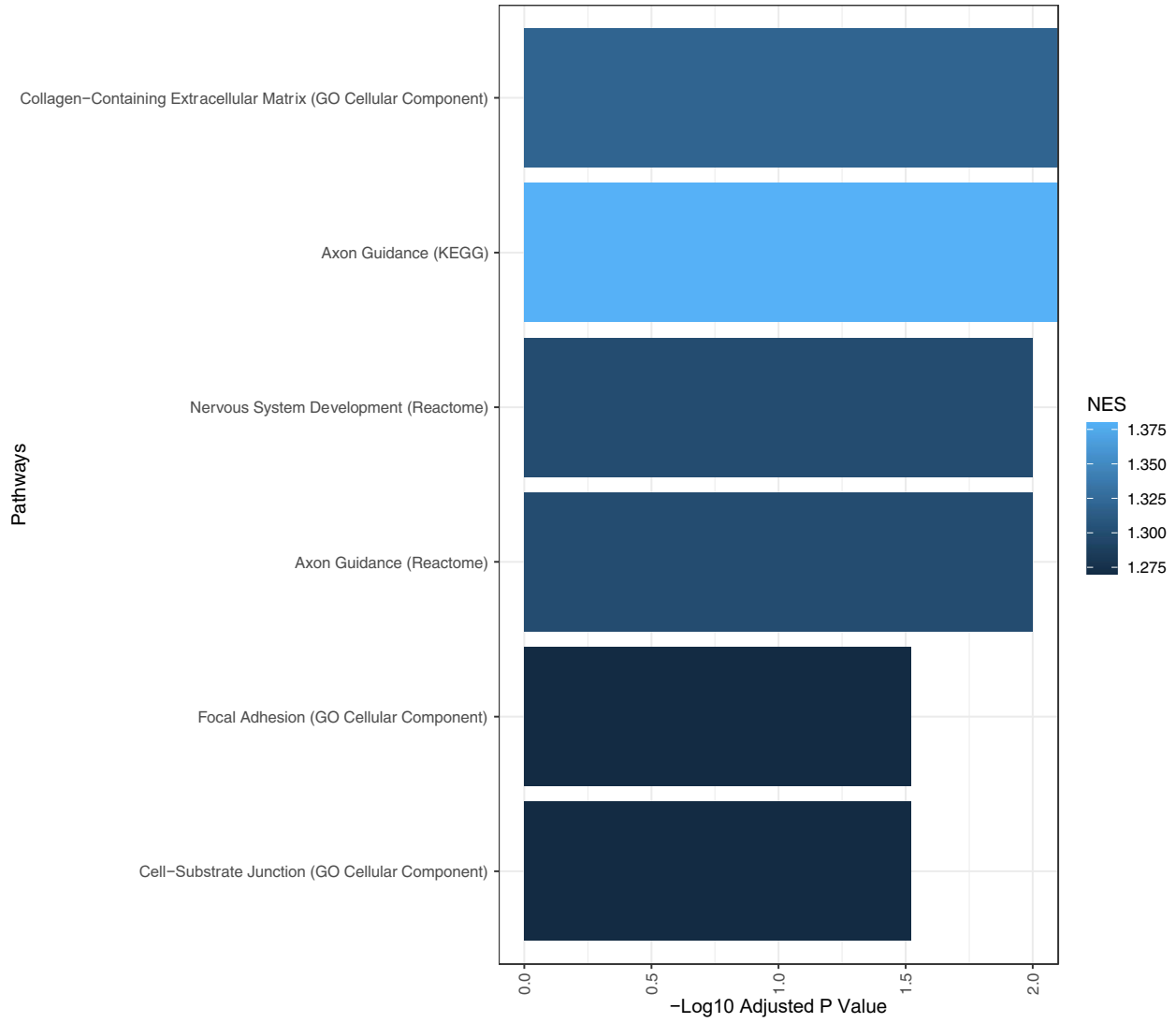

Figure 4. Pathway enrichment analysis for diabetic kidney disease. Biological pathways associated with diabetic kidney disease versus +normal/TBM. NES, normalized enrichment score.

structure and function play an important role in the pathophysiology of DKD. In addition, we found that inclusion of specific circulating proteins resulted in a significant improvement in the classification of DKD versus non-DKD kidney pathologies among patients with diabetes. With validation, these findings may help determine which patients with diabetes and kidney disease may benefit from kidney biopsy, such as those who are likely to have a nondiabetic cause of disease, or potentially inform strategies of treatment.

Our study builds upon previous work, which identified changes in collagen expression and extracellular matrix structure early in the development of DKD.[14,15] In our study, semaphorin-5A, plexin-A1, and C-type mannose receptor 2, emerged as key proteins in biological pathways involved in axonal guidance, cell-substrate junction, and focal adhesion. Sempahorin-5A is a member of the Semaphorin family and is known to play an important role in neural and cardiac development, axonal guidance, and angiogenesis.[16] Semaphorin 5 and other Semaphorin family members have been identified in transcriptomic analyses of DKD, and their gene expression has been found to be dysregulated in later stages of the disease in animal models.[17,18] In addition, plexin-A1 is a known coreceptor of the semaphorin family of proteins, and it is necessary for signaling of class 3 semaphorins and subsequent cytoskeleton remodeling, axonal guidance, and cell migration.[19] Notably, perturbations in the Semaphorin class 3 proteins or their receptors (including plexin-A1) have been associated with severe obesity, thought to occur due to perturbations in the hypothalamic melanocortin circuits involved in energy homeostasis.[20] Finally, the link between diabetic nephropathy and C-type mannose receptor 2, which plays a role in extracellular matrix remodeling and collagen degradation, needs further elucidation. Although some studies have found that C-type

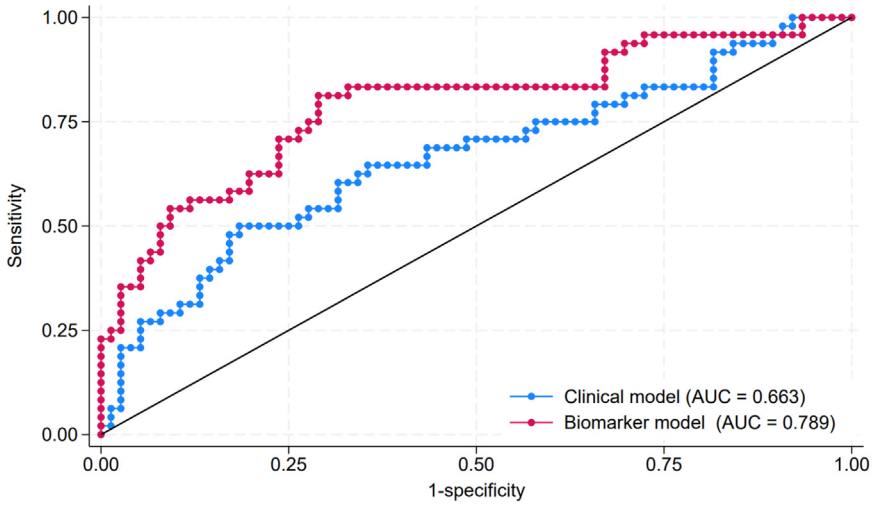

Figure 5. Area under the curve for a clinical model (adjusting for age, sex, baseline estimated glomerular filtration, and urine albumin-to-creatinine ratio), and a biomarker model (clinical model + renin, PLXA1, PLXD1, GPNMB:ECD, and MRC2) for discrimination of diabetic kidney disease versus nondiabetic kidney diseases, among patients with diabetes.

mannose receptor 2 promotes cell proliferation and inhibits apoptosis in DKD,[21] others have found that higher levels of circulating C-type mannose receptor 2 are associated with a lower risk of incident type 2 diabetes.[22] Taken together, our findings support the role of extracellular matrix gene expression in DKD while also identifying novel targets in these pathways.

Other key proteins identified in our study were renin, plexin-D1, and transmembrane glycoprotein NMB, all of which, alongside plexin-A1 and C-type mannose receptor 2, were associated with DKD, when compared with both normal/TBM and other kidney diseases. Renin has well described roles in the renin-angiotensin-aldosterone axis, which was implicated in the pathogenesis of DKD as early as the 1980s, where an outsized increase in efferent compared with afferent arteriolar constriction led to increased glomerular pressure and development of lesions similar to DKD in streptozotocin-treated rats.[23-25] More recent studies have found that plasma renin activity tends to be low in diabetes, despite increased intrarenal RAAS activation and the nephroprotective effects of RAAS-inhibitors in DKD.[26] We note that our findings reflect renin levels, not activity, and the 2 are not perfectly correlated.[27] Plexin-D1, in addition to belonging to the plexin family of Semaphorin receptors which we discussed previously, has been described as an important mechanosensory receptor in endothelial cells, influencing the development of atherosclerotic lesions through its response to blood flow.[28] Taken together with our findings, this suggests plexin-D1 may either mediate or serve as a biomarker of the microvascular complications of diabetes, including DKD. Finally, glycoprotein NMB, a melanosome-associated glycoprotein, is widely expressed in a variety of tissues, with known functions in cell adhesion,[29] extracellular matrix remodeling,[30] bone differentiation,[31] and invasion of cancers.[32] Recent studies have identified a link between liver-secreted glycoprotein NMB and the development of insulin resistance and lipogenesis.[33] The protein has also been recently associated with renal decline and cataract formation in diabetes.[34,35] From a metabolite perspective, some of the strongest associations were observed for glucose and mannose, which were higher in DKD when compared with our other kidney pathologies + no diabetes subgroup. Although not a surprising finding, it provides a measure of face validity to our approach.

Our study has some notable strengths. By using biopsy specimens to identify patients with DKD, we were able to more directly study associations of specific biomarkers and molecular pathways with disease pathology, instead of relying on less-accurate markers such as the presence of albuminuria.[7,8] This approach also allowed us to distinguish patients with a histopathological diagnosis of DKD versus other causes of kidney disease among individuals with diabetes. In addition, a large-scale omics approach provided a panel of over 6,000 proteins to explore perturbations of many biological processes. Limitations were also present. SomaScan, the aptamer-based technology we used to quantify proteins, can have inconsistent correlations with more validated immunoassays,[36] warranting additional validation of our findings in enzyme-linked immunosorbent assay studies. Similarly, our results reflect a single cohort. We lacked a control group with diabetes and without kidney disease. And, although our study provides robust evidence for associations of specific circulating proteins and pathways with DKD, our approach was cross-sectional and cannot support inferences of causality nor assessment of temporality. This is especially relevant when studying DKD, an entity in which pathophysiological mechanisms evolve and change significantly over time, from inciting metabolic

factors to glomerular hyperfiltration, renal hypertrophy, and eventually, glomerulosclerosis and interstitial fibrosis.[37] Finally, there is heterogeneity of DKD on biopsy, with variations in glomerular lesions and tubulointerstitial patterns; future studies should explore whether the identified proteins, metabolites, and pathways apply across different histopathologic lesions.

In summary, this study nominates several known and novel proteins and pathways associated with DKD. These findings provide valuable insights for future investigations into potential targets for therapy and opportunities for non-invasive diagnosis.

SUPPLEMENTARY MATERIALS

Supplementary File (PDF)

Table S1: Indication for biopsy among BKBC participants and subgroups. Data presented as frequency of individuals (%).

Table S2: Mean (standard deviation) of histopathological scores by lesion type in study subgroups. Scoring criteria for histopathological lesions were based on the proposal by Sethi et al. (2017).

Table S3: Linear regression of proteins associated with normal/TBM vs DKD and other kidney disease (among patients without diabetes) vs DKD, after adjusting for demographic characteristics (age, sex), baseline estimated GFR, and baseline urine ACR.

Table S4: Linear regression of metabolites associated with normal/TBM vs DKD and other kidney disease (among patients without diabetes) vs DKD, after adjusting for demographic characteristics (age, sex), baseline estimated GFR, and baseline urine ACR.

Table S5: Mean (standard deviation) of histopathological scores in DKD vs other kidney diseases + diabetes mellitus. *P* values for statistically significant differences in mean scores between groups.

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Authors' Contributions: MEG is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. MEG, IES, EPR, SSW, and CL-S conceptualized the study. IES, AnS, RP, IES, EPR, and SSW generated data. AdS was responsible for formal analysis. AdS and CL-S were responsible for data visualization. MEG was responsible for securing funding. All authors interpreted results. Each author contributed important intellectual content during manuscript drafting or revision and accepts accountability for the overall work by ensuring that questions pertaining to the accuracy or integrity of any portion of the work are appropriately investigated and resolved.

Support: This study was funded by R01DK108803 and K24HL155861 (MEG). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Financial Disclosure: The authors declare that they have no relevant financial interests.

Peer Review: Received May 29, 2025, as a submission to the expedited consideration track with 4 external peer reviews, including a statistical editor. Direct editorial input from the Editor-in-Chief. Accepted in revised form July 8, 2024.

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